IN VIVO INTERACTIONS BETWEEN SIGMA RECEPTORS AND NPY- & CGRP-RELATED PEPTIDES IN THE BRAIN

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TABLE OF CONTENTS

CHAPTER V

CHAPTER VI
General Discussion and Conclusion

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ABSTRACT

The existence of sigma (σ) receptors have been proposed by Martin and co-workers (1976) as a new class of opioid receptors to explain certain "psychotomimetic-like" behaviors induced by some benzomorphans (*N*-allylnormetazocine, pentazocine) in the chronic spinal dog. Later on, the observation that the typical opioid antagonist, naloxone, failed to antagonize some of these behavioral effects led to a reconsideration of the nature of σ receptors. It was determined that although several σ ligands displayed reasonable affinity for the phencyclidine (PCP) receptor and that their activation could mimic PCP "psychotomimetic-like" effects in animals, σ receptors were a different identity and shared no commonality with opioid or PCP receptors. Since then, σ receptors have generated a great deal of interest mostly in relation to their possible implication in psychosis like schizophrenia. More recently, σ sites have been associated with various other functions such as the modulation of posture and movement, neuroprotection, anxiety and depression, pain control, drug abuse, regulation of endocrine and immune functions, and cognitive behaviors.

Although evidence for the existence of an endogenous σ ligand have been reported, the full characterization of such putative endogenous ligand(s) has yet to be achieved. Roman and co-workers (1989) proposed that neuropeptide Y (NPY) and peptide YY (PYY) could act as endogenous σ ligands since both NPY and PYY competed with high affinity (nM) for [^{3}H](+)SKF 10,047 binding sites in rat brain membrane homogenates. However, various laboratories failed to replicate these *in vitro* findings. In order to clarify this apparent discrepancy, the present thesis investigates the possibility of the existence of *in vivo* interaction(s) between σ receptors and various NPY-related peptides. We first demonstrated that NPY could interact with σ sites *in vivo*, since certain NPY-related peptides could, to some extent, compete for *in vivo* [^{3}H](+)SKF 10,047/σ binding in the mouse hippocampal formation (Chapter 2). In the course of a specificity study, we found that the calcitonin gene-related peptide (CGRP)

also potently competed for *in vivo* [^{3}H](+)SKF 10,047/σ binding in the mouse hippocampus. We thus included CGRP-related peptides in subsequent studies and found that certain CGRP-related peptides significantly competed for [^{3}H](+)SKF 10,047/σ binding in the mouse hippocampus, in a manner similar to NPY (Chapter 3). Using an *ex vivo* autoradiographic approach, we further demonstrated that NPY- and CGRP-related peptides could interact with σ receptors not only in the hippocampus but in most, if not all, brain areas enriched with σ sites (Chapter 4). Finally, the behavioral relevance of these interactions was demonstrated. We found that various NPY- and CGRP-related peptides attenuated learning impairments induced by the systemic administration of the non-competitive NMDA-receptor antagonist MK-801. These effects were blocked by the purported σ antagonist BMY-14802, supporting the involvement of σ receptors in certain NMDA-related cognitive processes (Chapter 5). Taken together, our results suggest that selected NPY- and CGRP-related peptides could act as neuromodulators of σ receptor functions *in vivo*, and that these modulatory effects are behaviorally relevant.

RESUME

L'existence des récepteurs sigma (σ) fut avancée par Martin et coll. (1976) lors d'études visant le développement de nouveaux analgésiques. Etant donné la nature des effects "psychotiques" provoqués par divers composés se liant à ce nouveau site, notamment les benzomorphanes et leurs derivés (*N*-allylnormétazocine et pentazocine), celui-ci fut d'abord classé parmi les récepteurs opiacés. Par la suite, ayant constaté que chez le chien "spinal chronique", certains effets comportementaux induits par ces benzomorphanes n'étaient pas bloqués par un antagoniste typique des récepteurs opiacés, le naloxone, le récepteur σ fut confondu avec le récepteur de la phencyclidine (PCP) dont l'activation produit des effets comportementaux rappelant ceux induits par les ligands σ de la classe des benzomorphanes. Ce n'est que quelques années plus tard que les sites σ furent distingués des récepteurs à la phencyclidine et qu'une identité propre et unique leur fut attribuée. Depuis, les récepteurs σ ont engendré énormément d'intérêt attribuable, en partie, à leur implication possible dans les psychoses. Plus récemment, les sites σ ont été associés à diverses fonctions telles la modulation de la posture et du mouvement, la neuroprotection, l'anxiété et la dépression, le contrôle de la douleur, l'abus de drogues, la régulation de diverses fonctions endocriniennes et immunitaires ainsi que de certaines fonctions cognitives.

Quoique l'existence d'un ligand endogène des récepteurs σ fût suggérée, la caractérisation de cette molécule endogène, sinon de ces molécules, demeure à être établie. Roman et collaborateurs (1989) ont suggéré que le neuropeptide Y (NPY) et le peptide YY (PYY) pourraient agir en tant que ligands σ endogènes, puisque ces deux peptides purent inhiber (nM) la liaison d'un ligand σ, le [^{3}H](+)SKF 10047 (*N*-allylnormétazocine), dans des homogénats de cerveaux de rats. Par contre, d'autres laboratoires n'ont pas réussi à reproduire ces résultats *in vitro*. Dans le but de résoudre cette contradiction apparente, la présente thèse explore la possibilité d'interaction(s) *in vivo*, entre le récepteur σ et les peptides de la famille du NPY. Nous avons d'abord

démontré que le NPY pouvait interagir avec les sites σ, *in vivo*, puisque certains fragments et analogues du NPY purent, dans une certaine mesure, déplacer la liaison *in vivo* du [^{3}H](+)SKF 10047 mesurée dans des homogénats d'hippocampe de souris (Chapitre 2). Lors d'une série d'expériences visant à démontrer la spécificité des effets obtenus avec le NPY, nous avons observé que le peptide lié au gène de la calcitonine (CGRP) pouvait aussi déplacer la liaison, *in vivo*, du [^{3}H](+)SKF 10047 dans des homogénats d'hippocampe de souris et ce, de manière semblable au NPY. La seconde étape de cette thèse consiste donc en l'étude de l'interaction *in vivo*, du CGRP avec le récepteur σ dans l'hippocampe (Chapitre 3). Par la suite, en utilisant une technique d'autoradiographie *ex vivo*, nous avons démontré que les interactions observées entre le récepteur σ et le NPY et le CGRP ne se limitaient pas à la formation hippocampale, mais étaient présentes dans la plupart, sinon toutes, les régions du cerveau enrichies en récepteurs σ (Chapitre 4). Finalement, nous avons démontré que ces interactions étaient significatives d'un point de vue comportemental. En utilisant une technique d'évitement passif chez la souris, nous avons montré que divers peptides de la famille du NPY et du CGRP pouvaient atténuer des problèmes d'apprentissage suite à l'administration systémique d'un antagoniste non-compétitif du récepteur NMDA, le MK-801. Les effets de ces peptides sont, de plus, bloqués par l'administration concomitante d'un antagoniste σ, le BMY-14802, suggérant la participation des récepteurs σ dans certains processus cognitifs reliés à l'activation des récepteurs au NMDA (Chapitre 5). Dans l'ensemble, les résultats rapportés dans la présente thèse suggèrent que certains peptides de la famille du NPY et du CGRP peuvent agir en tant que neuromodulateurs de diverses fonctions associées aux sites σ, *in vivo*, et que ces effets modulateurs sont significatifs d'un point de vue comportemental.

MANUSCRIPTS and AUTHORSHIP

As stated in the *Guidelines Concerning Thesis Preparation*, 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 litterature to the subject of the thesis, when this review is appropriate, and (5) a final overall Discussion and/or Summary.

Additional material (procedural 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; supervisor 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 in the candidate's interest to make perfectly clear the responsabilities of the different authors of co-authored papers".

CONTRIBUTION OF THE AUTHORS ON CO-AUTHORED PAPERS

1) ***Rémi Quirion:*** As my supervisor, Dr. Rémi Quirion is co-author on all manuscripts. As such, Dr. Quirion was closely involved in the overall research project and the writing of the manuscripts.

2) ***Alain Fournier and Serge St-Pierre:*** Drs. A. Fournier and S. St-Pierre are co-authors on some manuscripts as they performed synthesis of all the peptides used for the present thesis.

3) ***François Roman and Jean-Louis Junien:*** Drs. F. Roman and J-L. Junien are co-authors on three manuscripts as they were involved in the elaboration of the reseach project leading to the present thesis.

First manuscript

"Evidence for *in vivo* interactions between neuropeptide Y-related peptides and σ receptors in the mouse hippocampal formation" by P. Bouchard, Y. Dumont, A. Fournier, S. St-Pierre and R. Quirion. *The Journal of Neuroscience*, 1993, 13(9): 3926-3931.

Yvan Dumont: Dr. Y. Dumont showed me the *in vivo* binding technique. He was also involved in the statistical analysis of the results and provided help for the writing of this first manuscript.

Second manuscript

"*In vivo* modulation of sigma receptor sites by calcitonin gene-related peptide in the mouse and rat hippocampal formation: Radioligand binding and electrophysiological studies" by P. Bouchard, F. Monnet, R. Bergeron, F. Roman, J-L. Junien, C. de Montigny, G. Debonnel and R. Quirion. *European Journal of Neuroscience*, 1995, 7:1952-1962.

François Monnet: Dr. F. Monnet performed most of the electrophysiological experiments included in this second manuscript.

Richard Bergeron: Dr. R. Bergeron participated in some of the electophysiological recordings.

Fourth manuscript

"Neuropeptide Y and calcitonin gene-related peptide attenuate memory impairments induced by MK-801 likely via a sigma receptor-related mechanism" by P. Bouchard, T. Maurice, S. St-Pierre, A. Privat and R. Quirion. *The Journal of Neuroscience* (submitted)

Tanguy Maurice: Dr. T. Maurice showed me the behavioral technique used in this fourth manuscript. In addition, we performed the experiments and analysis of the results together.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

As stated in the *Guidelines Concerning Thesis Preparation*, Faculty of Graduate Studies and Research, McGill University:

> "Elements in the thesis that are to be considered as contributions to original knowledge must be clearly indicated in the preface or by a separate statement at the beginning or at the end of the thesis. This requirement is mandatory for Ph.D. Thesis."

Overall, the present thesis examined the potential role of various peptides as neuromodulators of σ receptor systems, *in vivo*. The original aim of this reseach project was to investigate the mode of interaction of various NPY-related peptides with σ sites, *in vivo*. The focus on NPY and related peptides originated from the observation that NPY and PYY could compete, with nanomolar affinity, with the prototypical σ ligand, [^{3}H](+)SKF 10047, in rat brain membrane homogenates *in vitro*, suggesting that NPY and PYY could act as endogenous σ ligands (Roman et al., 1989). This finding generated a great deal of interest since the endogenous ligand(s) for the σ site was yet unidentified. However, attempts to replicate these *in vitro* findings remained unsuccessful (Quirion et al., 1991; Tam et al., 1991) and doubts were raised as to the genuine existence of NPY/σ interactions. Accordingly, we undertook a series of experiments in order to investigate the possibility of interactions between NPY-related peptides and σ sites, *in vivo*, and to examine the potential role of NPY as endogenous σ ligand.

Data reported in our first manuscript (Chapter 2) confirmed the existence of these interactions using *in vivo* membrane binding assays. We demonstrated that certain NPY-related peptides were able to significantly inhibit [^{3}H](+)SKF 10047/σ binding in the mouse hippocampal formation, *in vivo*, and corroborated previous reports by Monnet and colleagues (1990, 1992) who showed that certain NPY-related peptides interacted with σ sites *in vivo*, as they were able to modulate in a haloperidol-sensitive manner, the NMDA-induced activation of rat CA_3 dorsal hippocampal neurons.

While determining the specificity of the effects of NPY-related peptides on *in vivo* [^{3}H](+)SKF 10047/σ binding, we found that calcitonin gene-related peptide (CGRP), used first as control peptide in our paradigm, also interacted with σ sites *in vivo*. Accordingly, the aim of the second manuscript (Chapter 3) was to investigate the *in vivo* interactions between σ sites and various CGRP-related peptides in the hippocampal formation, using both *in vivo* binding assays and electrophysiological recordings. This second paper describes the effects of selected CGRP-related peptides on *in vivo* σ binding in the mouse hippocampal formation, and on NMDA-induced activation of CA_3 pyramidal cells of the rat hippocampus. This manuscript is a contribution to original knowledge as it reported, for the very first time, the existence of interactions between CGRP and σ sites.

The third manuscript (Chapter 4) is a logical extension of the first two papers. In an attempt to shed more light on the nature of the relationship between σ sites and NPY- and CGRP-related peptides, we investigated the neuro-anatomical profile of these interactions using an *ex vivo* autoradiographic approach. We reported that NPY- and CGRP-related peptides interacted, *in vivo*, with σ sites in most, if not all, brain

regions enriched with σ receptors. This manuscript represents the first evidence that NPY and CGRP could interact with σ receptors throughout the brain.

Finally, the aim of the fourth series of experiments (Chapter V) was to examine the potential behavioral relevance of the observed *in vivo* interactions of NPY and CGRP with σ receptor sites. Following a report by Maurice and colleagues (1994) that certain σ ligands were able to attenuate MK-801 -induced learning impairments in the mouse, we decided to investigate the effect of various NPY and CGRP-related peptides in that regard. The results reported in this fourth manuscript (Chapter 5) describe the ability of selected NPY- and CGRP-related peptides to attenuate MK-801 -induced learning impairments, and constitute the first report that the *in vivo* interactions observed between σ sites and NPY and CGRP are behaviorally relevant.

The fifth paper (Appendix I; Bouchard and Quirion, *Neuroscience*, 1996) describes the distributional profile of σ receptor subtypes (σ_1 and σ_2) in the rat brain using an *in vitro* autoradiographic approach. This work represents the first and unique clear anatomical localization of σ receptor subtypes in the rat brain. The demonstration of a selective distributional profile of σ receptor subtypes in the rat brain may assist in determining the functional role(s) of these receptors.

Chapter I

GENERAL INTRODUCTION

SIGMA RECEPTORS

1.0 Historical background

1.1 The sigma/opioid receptor

The notion of sigma receptor has emerged in the mid-seventies from investigations of opiate derivatives. Attempts to develop less addicting opiate analgesics led to the extensive study of many benzomorphans (Deutsch et al., 1988) such as *N*-allylnormetazocine (SKF 10,047), pentazocine and cyclazocine. Although some of these potent analgesic compounds did not display the morphine-like induced euphoria generally associated with the addictive properties of opiates, their usefulness was restricted by unpleasant subjective side effects. The racemic mixture of the benzomorphan drugs, often designated sigma (σ) opiates ("sigma" for the effects induced by the prototypical σ ligand, SKF 10,047), have been reported to produce a variety of aversive effects such as sedation, drowsiness, grogginess, drunkenness, dysphoria, irritability, insomnia, delusions and hallucinations (Haertzen, 1970; Keats and Telford, 1964; Martin and Sloan, 1994).

The existence of sigma receptors was first proposed by Martin and co-workers (1976) following a series of experiments using the "chronic spinal dog" to investigate the role of some benzomorphans in analgesia, and their potential addictive properties (Gilbert and Martin, 1976; Martin et al., 1976). In the dog, the effects induced by the racemic mixture of SKF 10047 included mydriasis, tachycardia, tachypnea and manic-like hyperactivity identified as "canine delirium" (Martin et al., 1976; Vaupel, 1983). In

an attempt to classify the various receptor types associated with the observed physiological effects, Martin and co-workers (Gilbert and Martin, 1976; Martin et al., 1976) attributed the analgesic effects induced by racemic benzomorphans to the the mu (μ) receptor (for morphine), the sedative effect to the kappa (κ) receptor (for ketocyclazocine) and the term sigma (σ; SKF 10047 or *N*-allylnormetazocine) was used to identify the receptor mediating the psychotomimetic (manic-like) symptoms. The σ receptor as originally defined was included in the opioid family since early experiments on "σ/opioid" receptors were performed with the racemic mixture of benzomorphan compounds. Therefore many effects induced by these σ ligands including analgesia and sedation were antagonized by naloxone and/or naltrexone (Gilbert and Martin, 1976; Martin et al., 1976). It is not until stereoselectivity studies were performed that the σ receptor was associated with the dextrorotatory enantiomers of "opioid" benzomorphans. Then, it was determined that the σ site was non-opioid since some of the effects induced by (+)benzomorphan σ ligands were not antagonized by naloxone or naltrexone. Accordingly, in the mid-eighties, the σ receptor was defined as a non-opioid receptor with high affinity for (+)benzomorphans (Quirion et al., 1992a), and the purported psychotomimetic properties of benzomorphan σ ligands were attributed to the dextrorotatory enantiomers of these compounds (for review see Musacchio, 1990).

1.2 The σ/PCP receptor

Phencyclidine (PCP) is an arylcyclohexylamine compound that was introduced into medical practice in 1958 as a potent anesthetic drug (Chen et al., 1959). As for anesthetic benzomorphans such as SKF 10047 and cyclazocine (Haertzen, 1970; Keats and Telford, 1964), the medical usefulness of PCP was limited by the important subjective side effects experienced by patients recovering from anesthesia.

Hallucinations, maniacal excitement, drunkenness and severe agitation were common side effects seen after the medical use of PCP (Meyer et al., 1959; Sonders et al., 1988). As for racemic benzomorphan analgesics, PCP, known as "Angel Dust" among users, became a very popular illicit drug in North America. Its abuse in humans produces symptoms resembling schizophrenia including memory impairments, anxiety, disorganized though processes, distorded body image, delusions and hallucinations (Allen and Young, 1978; Showalter et al., 1977). Because PCP induces symptoms resembling schizophrenia, this drug was found useful to study psychotomimetic-like behaviors in animals (Domino and Luby, 1981).

1.2A σ *vs PCP binding profiles*

In the mid-eighties, the σ receptor was though to share commonality with the phencyclidine (PCP) site associated with the *N*-methyl-*D*-aspartate (NMDA) ion channel, mostly on the basis of the competition of [^{3}H]PCP binding by the prototypical σ ligand (+)SKF 10047. Conversely, [^{3}H](+)SKF 10047 was shown to bind PCP receptors (Largent et al., 1986b; Mendelsohn et al., 1985; Sircar et al., 1986). However, the respective drug selectivity patterns of σ and PCP receptors display several differences. Eventhough PCP and σ sites bind [^{3}H]PCP and the σ ligand [^{3}H](+)SKF 10047 with submicromolar affinity, PCP has a much higher affinity for PCP sites while (+)SKF 10047 binds with higher affinity to σ sites (Largent et al., 1984; 1986b; Sircar et al., 1986; Su, 1982; Tam, 1985; Weber et al., 1986). The σ receptor is highly sensitive to several neuroleptics, notably haloperidol, whereas the PCP site is insensitive to antipsychotic drugs (Itzhak, 1988; Largent et al., 1984; 1986b; Mendelsohn et al., 1984; Sircar et al., 1986; Su, 1982; Tam, 1983; 1985; Tam and Cook, 1984). Conversely, PCP is weak against [^{3}H]haloperidol binding (Itzhak,

1987; Tam and Cook, 1984). σ receptors bind preferentially [^{3}H]DTG (Weber et al., 1986), [^{3}H](+)3-PPP (Gundlach et al., 1985; 1986a; Largent et al., 1984) and [^{3}H]dextromethorphan (Musacchio et al., 1988; 1989a,b) and display high stereoselectivity for benzomorphans such as [^{3}H](+)pentazocine (Bowen et al., 1990; 1993; De Costa et al., 1989) and [^{3}H](+)SKF 10047 (Contreras et al., 1986b; Gundlach et al., 1985; Tam, 1985). In comparison, PCP receptors bind selectively [^{3}H]PCP and a thienyl derivative of PCP, [^{3}H]TCP, (Gundlach et al., 1986; Vignon et al., 1986), and display very poor stereoselectivity for SKF 10047 (Tam, 1985). Finally, in contrast with [^{3}H]SKF 10047, [^{3}H]PCP binding was shown to be sensitive to sodium ions (Tam, 1985).

1.2B *Anatomical distribution of σ vs PCP receptors*

Differences in the respective brain distributional profile of σ and PCP receptors have been well demonstrated using receptor autoradiography (Largent et al., 1986b). σ and PCP receptors were found to bind different cell types. For example, σ receptor labeling is often associated with cell bodies of large pyramidal cells as seen in the hippocampal formation (Largent et al.,1984; 1986b; Quirion et al., 1981; Sircar et al., 1986; Su, 1982; Tam, 1985; Weber et al., 1986) whereas pyramidal cells are almost devoid of PCP labeling (Contreras et al., 1986; Maragos et al., 1988; Reid et al., 1990; Rothman et al., 1989; Vignon et al., 1986; Zukin and Zukin, 1979). Moreover, high densities of σ receptors are present within the Purkinje cell layer of the cerebellum in contrast with the moderate amounts of PCP sites (Contreras et al., 1987b; Gundlach et al., 1986a,b; Maragos et al., 1988; McLean and Weber, 1988). Subcellular fractionation studies have demonstrated that σ receptors were mainly concentrated in the microsomal fraction (Craviso and Musacchio, 1983a; Knight et al., 1991b; McCann

et al., 1989; 1994; McCann and Su; 1990) whereas PCP sites associated with the NMDA receptor are found in the synaptic membrane fraction (Fagg and Matus, 1984; Knight et al., 1991b). Finally, in contrast with the abundance of σ receptors in various endocrine and immune tissues (Wolfe et al., 1989; Wolfe and DeSouza, 1992; 1994) very little or no PCP sites were found in a variety of endocrine or immune cells as shown by the lack of [^{3}H]TCP (Contreras et al., 1986; Gundlach et al., 1986b) or [^{3}H]MK-801 (Bowery et al., 1988; Loo et al., 1987) labeling in the rat pituitary, adrenal, testis or ovary (for reviews see Wolfe and DeSouza, 1992; 1994).

1.2C *Functional studies*

Behavioral drug discrimination studies using pigeons, rodents and primates added to the confusion between σ and PCP receptors. Rats trained to discriminate between (+)SKF 10047 and saline fully generalize with PCP (Steinfels et al., 1987). Conversely, squirel monkeys and rats trained to discriminate PCP from saline generalize to (+)SKF 10047 (Brady et al., 1982). However, this effect seems to be selective for the σ benzomorphan (+)SKF 10047 since animals trained to recognize (+)pentazocine do not generalize to PCP and congeners (Shannon, 1983; Steinfels et al., 1987; 1988). This situation might be partly explained by the affinity of (+)SKF 10047 for the PCP receptor. This hypothesis is supported by the finding that the relative potencies of various PCP-like compounds to substitute for (+)SKF 10047-induced effects in the pigeon correlate well with their relative affinity for PCP, but not (+)SKF 10047 receptor sites (Picker, 1991). Behavioral experiments in rodents have shown that specific ligands for σ and PCP sites can induce similar psychotomimetic-like behaviors. MK-801, a selective PCP receptor ligand with no affinity for the σ receptor (Loo et al., 1987; Wong et al., 1988), and the σ ligand DTG (Weber et al., 1986)

which displays very low affinity for the PCP site, were found to elicit sniffing, rearing, circling, backpedaling and weaving behaviors (Contreras et al., 1988) indicating that they represent two distinct receptors that can be both involved in mediating stereotyped behaviors in the rat (Contreras et al., 1988).

1.3 The σ/haloperidol receptor

The finding that the antipsychotic haloperidol was among the most potent inhibitors of [^{3}H](+)SKF 10047 binding, with a K_i of about 4 nM (Itzhak, 1988; Tam and Cook, 1984), led to the redefinition of the σ/PCP receptor as the σ/haloperidol receptor. The fact that several dopamine D_2 receptor antagonists such as haloperidol and other phenothiazines potently interact with σ sites might suggest that σ and dopamine receptors share some commonality. However, beside the differential distributional profiles of σ and dopamine receptors, at least two lines of evidence clearly demonstrate that this is not the case. First, σ receptors are insensitive to dopamine (Weber et al., 1986) and second, σ receptors are stereoselective for the (-) isomer of the neuroleptic butaclamol whereas D_2 receptors display stereoselectivity for (+)butaclamol (Largent et al., 1984; Su, 1982; Tam and Cook, 1984; Weber et al., 1986). Accordingly, the σ receptor does not belong to the dopamine receptor family.

2.0 Current nomenclature of sigma receptor subtypes

Two subtypes of σ receptor sites (σ_1 and σ_2) have been rather well characterized thus far (Bowen et al., 1989a; Hellewell and Bowen, 1990; Itzhak and Stein, 1991a,b; Itzhak et al., 1991a; Rothman, 1991; Su et al., 1991; Vilner and Bowen, 1992; Zhou and Musacchio, 1991; for reviews see Itzhak, 1994; Quirion et al., 1992; Walker et al.,

1990). A nomenclature committee, held during the "*Third United States-French-sponsored International Seminar on Phencyclidine and Sigma Drugs*" (La Grande Motte, Montpellier, France, September 1991) proposed a general classification for the σ_1 and σ_2 receptor subtypes (Quirion et al., 1992; table 1). Accordingly, both σ_1 and σ_2 sites have similar affinity for the σ ligands [^{3}H]haloperidol and [^{3}H]DTG while dextrorotatory benzomorphans such as (+)SKF 10047 and (+)pentazocine preferentially label σ_1 sites (Hellewell and Bowen., 1990; Itzhak and Stein, 1991a,b; Itzhak et al., 1991a; Su et al., 1991; Vilner and Bowen, 1992). The levorotatory enantiomers of benzomorphans display only low to moderate affinity for σ sites and do not distinguish between subtypes. However, the σ_2 binding site displays reverse stereoselectivity in regard to benzomorphans as levorotatory isomers have higher affinity than dextrorotatory counterparts for this subtype (Di Paolo et al., 1991; Hellewell and Bowen, 1990; Quirion et al., 1992).

Other σ ligands such as the antitussive drug dextromethorphan (Craviso and Musacchio; 1983a,b; Klein et al., 1988; Klein and Musacchio; 1989; Musacchio et al., 1989a; Zhou and Musacchio, 1991) and the anticonvulsant drug carbetapentane (Rothman et al., 1991), can also distinguish between σ_1 and σ_2 receptor subtypes since both compounds were reported to behave as selective σ_1 ligands. Additionally, phenytoin can allosterically modulate the σ_1 receptor apparently without affecting the σ_2 site (McCann and Su, 1992; Klein et al., 1990; Musacchio et al., 1988; 1989a,b; Zhou and Musacchio, 1991). Moreover, σ_1 and σ_2 receptors are differently regulated by G-protein modifying agents. The σ_1 subtype is reportedly sensitive to the modulatory effects of GTP and Gpp(NHp) (Itzhak, 1989; Itzhak et al., 1991b; Itzhak and Khouri, 1988; Itzhak and Stein, 1991b; Musacchio et al, 1989b) while the σ_2 subtype is not (Itzhak and Stein, 1991b). In addition, binding parameters at σ_1 and σ_2

sites are differently affected by a sub-chronic treatment with haloperidol (Bailey and Karbon, 1993; Itzhak and Stein, 1991b).

Table 1.

A proposal for the classification of σ binding sites

Quirion et al. (1992) Trends in Pharmacological Sciences, 13, 85-86.

Ligands	Affinities for σ receptor subtypes σ_1	σ_2
Selective ligands		
(+)Pentazocine	high	low
(+)SKF 10,047	moderate to high	very low
Carbetapentane	high	very low
Dextromethorphan	high	very low
Non-selective ligands		
Haloperidol	high	high
DTG	high	high
(+)-3-PPP	high	moderate to high
(-)Pentazocine	low to moderate	low to moderate
(-)SKF 10,047	low	low
Naloxone	inactive	inactive
Other characteristics		
* Phenytoin sensitivity	yes	no
* GTP sensitivity	yes	no
* Sensitivity to subchronic treatment with Haloperidol	yes	no

3. Putative $\sigma_3$and σ_4 receptor subtypes

Recently, two new σ receptor subtypes termed σ_3 and σ_4, were identified and partially characterized (Booth et al., 1993; Bowen et al., 1995; Myers et al., 1994). Both σ_3 and σ_4 subtypes display distinct pharmacological profiles in comparison to the already well characterized σ_1 and σ_2 sites, the key differentiating feature being their low affinity for DTG. The putative σ_3 receptor subtype displays preferential afinity (nM) for a series of PATs-related compounds [(1-phenyl-3-amino-1,2,3,4-tetrahydronaphtalenes (1-phenyl-3-aminotetralins)] as well as for haloperidol and spiperone, and a diminished sensitivity to DTG and (+)pentazocine in comparison to the σ_1 and σ_2 receptor subtypes. Moreover, σ_3 receptor sites have been proposed to be involved in the modulation of tyrosine hydroxylase activity and dopamine synthesis in the striatum (Booth et al., 1993; Myers et al., 1994). On the other hand, Bowen et al. (1995) described a putative σ_4 receptor subtype present in the guinea pig brain and C6 glioma cells. This σ_4 site displays high affinity for a series of aryl ethylene diamine-related compounds such as BD-737, BD-738, BD-1008 and BD-1063, low micromolar affinity for haloperidol, moderate affinity for reduced haloperidol and has low to negligible affinity for DTG and (+)pentazocine.

Additional studies will be required in order to confirm if these new sites are genuine members of the σ receptor family.

4.0 Physical characteristics of sigma receptors

4.1 σ receptors as proteins

At least four lines of evidence demonstrate the proteinic nature of σ receptors (see Walker et al., 1990). σ receptor binding sites are sensitive to 1) Temperature: the binding, to guinea pig brain membranes, of [^{3}H]SKF 10047 (Su, 1982) and [^{3}H]dextromethorphan (DM) (Craviso and Musacchio, 1983a) was significantly reduced following heat treatment; 2) pH: it was observed that the optimum pH for both [^{3}H](+)-3-PPP and [^{3}H]DM binding was between 8.0-8.9 (Craviso and Musacchio, 1983a; Largent et al., 1987). The specific binding of [^{3}H]DM in guinea pig brain was reported to decrease below pH 8.0 (Craviso and Musacchio, 1983a), while specific [^{3}H](+)-3-PPP binding in rat brain increases over the pH range of 7.0 to 8.9 (Largent et al., 1987); 3) σ receptor binding parameters are also influenced by protein-modifying agents (Craviso and Musacchio, 1983a,b; Itzhak, 1989; Itzhak and Khouri, 1988; Itzhak and Stein, 1991b; Su, 1982;). It was shown, using guinea pig brain membranes, that proteases such as trypsin and α-chymotrypsin, decrease the binding of both [^{3}H]SKF 10047 (Su, 1982) and [^{3}H]DM (Craviso and Musacchio, 1983a,b); 4) and finally, irradiation of brain membranes with ultraviolet light, a treatment which modifies protein structure, was shown to modulate σ binding parameters (Bowen et al., 1989a).

4.2 Molecular weight determination

Partial purification and isolation of σ receptors supports the existence of multiple σ receptor subtypes. Two different techniques have been used to determine the molecular weight(s) (MW) of σ sites. The molecular sizing technique led to an estimation of MWs ranging from 150 to 669 kDa in guinea pig brain membranes and in

rat and bovine cerebellum (Kavanaugh et al., 1988; 1989; Schuster et al., 1995) and a 450 kDa σ-like molecule in the rat liver (McCann and Su, 1991). Studies using photoaffinity labeling technique with [^{3}H]AzDTG (azido-DTG) as probe, led to the partial purification of protein-like molecules of 29 kDa in the guinea pig brain and rat and bovine cerebellum (Kavanaugh et al., 1988; Schuster et al., 1995), two protein-like molecules of 18 and 21 kDa in PC12 cell line (Hellewell and Bowen, 1990) and a 29 kDa molecule in NCB-20 cells (Adams et al., 1987). In addition, using [^{3}H]IACoc (Iodoazidococaine) as probe, a peptide of 26 kDa was identified in rat brain and liver as well as in human placenta (Kahoun et Ruoho, 1992). The relative rank order of potency using various σ ligands as well as the observed enantioselectivity for the binding of SKF 10047 suggest that the 29 kDa protein found in guinea pig and rat brain corresponds to the σ_1 sites (Schuster et al., 1995) whereas the lower MW proteins (18-21 kDa) found in PC12 cells represent σ_2 sites (Hellewell and Bowen, 1990). The much higher molecular masses identified using the sizing technique (150-669 kDa) suggests that the lower MW proteins (18-29 kDa) are part of a larger protein complex and that σ receptors may represent a subunit of this complex (Schuster et al., 1995; Su and Junien, 1994; Walker et al., 1990).

4.3 Structure-activity relationships

σ receptors bind several classes of compounds of various chemical structures. These include butyrophenones (haloperidol; Tam and Cook, 1984), dextrorotatory benzomorphans ((+)pentazocine and (+)SKF 10047; Su, 1982) and (+)morphinans (dextromethorphan, dextrallorphan; Musacchio et al., 1988; 1989a,b), arylcyclohexylamines (PCP-like compounds; Tam, 1985), guanidines (DTG; Weber et al., 1986), phenylpiperidines ((+)-3-PPP; Largent et al., 1984; 1986b), piperazines

(BMY-14802, rimcazole; Ferris et al., 1986; Taylor and Dekleva, 1987) and steroids (progesterone; Su et al., 1988). Other classes of compounds that bind σ receptors include cytochrome P-450 inhibitors (proadifen and lobeline; Klein et al., 1991), various antidepressants (Ferris et al., 1991a,b; Schmidt et al., 1989) including monoamine oxydase inhibitors (Itzhak et al., 1991a,b; Itzhak and Kassim, 1990), anticonvulsants (Klein and Musacchio, 1989) and certain histamine ligands (Gray et al., 1990). This high degree of heterogeneity in chemical structures that have moderate to high affinity for σ receptors hampered the finding of highly specific ligands for these sites and raised doubt about their potential physiological significance. However, it seems likely that these chemically different compounds may share a common structural property enabling them to bind σ sites (for review see DeCosta and He, 1994).

5. Distribution of σ receptors

5.1 Central nervous system

Membrane receptor binding and *in vitro* and *in vivo* autoradiography using various radioligands ([^{3}H]haloperidol, [^{3}H]DTG, [^{3}H](+)SKF 10047, [^{3}H](+)pentazocine, [^{3}H](+)-3-PPP, [^{3}H]dextromethorphan, [^{3}H]ifenprodil and [^{3}H]DuP 734) demonstrated the widespread distribution of σ sites throughout the brain of various species including mouse (Benavides et al., 1992; Compton et al., 1987; Ferris et al., 1991c; Koe et al., 1989; Martin et al., 1984; Sircar et al., 1986; Weissman et al., 1990), rat (Bouchard and Quirion., 1996; Gundlach et al., 1986a; Itzhak, 1987; Sircar et al., 1986), guinea-pig (Basile et al., 1992; Gundlach et al., 1986a; McLean and Weber, 1988; Su, 1982), cat (Graybiel et al., 1989), monkey (Mash and Zabetian,

1992) and human (Barnes et al., 1992; Jansen et al., 1991a,b; Simpson et al., 1992; Tam and Zhang, 1988; Weissman et al., 1988; Zabetian et al., 1994). Specific σ receptor labeling is found to be widely, but discretely distributed in the brain.

In the rodent, highest densities of σ receptors are seen in areas related to motor functions such as the motor nuclei of the cranial nerves, the red nucleus, the substantia nigra pars compacta and mid-layers of primary and secondary motor cortices. Limbic structures including the dentate gyrus and the pyramidal cell layer of the hippocampal formation, midbrain regions such as the dorsal raphe, the central gray, and hindbrain regions such as the locus coeruleus are very rich in σ labeling. The rat pineal gland was also reported to contain high levels of σ receptors (Jansen et al., 1989). Lower, but still significant amounts of specific σ labeling are found in various other structures including most hypothalamic nuclei and the Purkinje cell layer of the cerebellum, and lower amounts are usually found in regions such as the thalamus and basal ganglia (Bouchard et Quirion; 1996; Contreras et al., 1987b; Culp et al., 1992; Graybiel et al., 1989; Gundlach et al., 1986a; Largent et al., 1986b; McLean and Weber, 1988; Walker et al., 1992). Furthermore, σ receptors are abundant in the spinal cord, particularly in the ventral horn and dorsal root ganglia (Gundlach et al., 1986a).

In the monkey brain, σ receptors are highly concentrated in cortical areas such as the orbitofrontal, cingulate, insular, parahippocampal and temporal gyri, as well as in the amygdala, hippocampus, hypothalamus and autonomic relay nuclei of the brainstem (Mash and Zabetian, 1992). This anatomical distribution stongly suggests that σ receptors may play a role in various functions involving the limbic system. In human brain, σ receptors are particularly abundant in the cerebellum, orbitofrontal cortex, laminae II-IVA of the neocortex, nucleus accumbens, substantia nigra pars compacta,

dentate granular cells of the hippocampus and pineal gland (Jansen et al., 1991a,b; Mash and Zabetian, 1992; Weissman et al., 1988).

It is thus obvious that the distributional profile of σ receptor binding displays species differences. This likely accounts for the different effects induced by σ ligands among species and may be critical to assess the role of σ sites in humans.

σ_1 and σ_2 receptor subtypes in the brain

In the rodent brain, the distributional profile of σ_1 and σ_2 receptor subtypes as labeled with [^{3}H]DTG, [^{3}H](+)SKF 10047 or [^{3}H](+)pentazocine is rather similar. Autoradiography studies revealed that the anatomical ditribution of specific [^{3}H]DTG and [^{3}H](+)pentazocine labeling is overlapping in the rat brain (Bouchard and Quirion, 1996; Appendix 1; Figure 2), which is consistent with an earlier study performed in the guinea-pig brain (Walker et al., 1992). However, the ratio of σ_1 to σ_2 receptor sites vary among species, the concentration of σ_2 sites being apparently superior in the rat brain (Leitner et al., 1994). Concentrations of σ receptor subtypes also vary in different brain structures. In both rat and guinea-pig brains, the amount of σ_2 sites is apparently higher in regions related to motor functions, whereas regions such as the hippocampal formation and various thalamic and hypothalamic nuclei are mostly enriched with σ_1 sites (Bouchard and Quirion, 1996; Bowen et al., 1993; Walker et al., 1992) although McCann et al. (1994) observed significant higher amounts of σ_2 sites in the rat hippocampal formation. The precise distributional profile of σ_1 vs σ_2 sites will await the development of specific probes for the σ_2 subtype. In that regard, the development of such σ_2-selective ligand has been documented ([^{3}H]BIMU-1; Bonhaus et al., 1993), and its use should allow for a better characterization of σ receptor subtypes.

5.2 Subcellular localization

Fractionation studies using rat or guinea pig brain tissues demonstrated that high affinity σ receptor sites labeled with either [^{3}H]haloperidol, [^{3}H]DTG, [^{3}H](+)SKF 10047, [^{3}H]dextromethorphan or [^{3}H](+)-3-PPP were mostly concentrated in the microsomal fraction, whith much lower levels of binding occuring in synaptosomal, mitochondrial or myelin fractions (Craviso and Musacchio, 1983a; Knight et al., 1991b; McCann et al., 1989; 1994; McCann and Su; 1990). The subcellular distributional profile of σ sites was found to be very similar to that reported for endoplasmic reticulum markers (McCann et al., 1989; 1994; McCann and Su, 1990). Although it is conceivable that these intracellular σ sites represent internalized or nascent receptors on their way to cell surface (Itzhak, 1994; Knight et al., 1991b), it appears likely that σ sites may be involved in some intracellular function(s) (Knight et al., 1991b).

σ_1 vs σ_2 receptor subtypes

In the C57BL/6 mouse brain, major differences in the ratio of the binding to different subcellular fractions (microsomal, mitochondrial and synaptosomal) were reported for [^{3}H]DTG, [^{3}H](+)SKF 10047 and [^{3}H](+)-3-PPP. The mitochondrial fraction was reported to be twice as rich in [^{3}H](+)-3-PPP binding sites in comparison with [^{3}H]DTG binding, while the pattern of the subcellular distribution of [^{3}H](+)SKF 10047 binding was similar to that of [^{3}H](+)-3-PPP (Itzhak et al., 1991a). Interestingly, fractionation studies using σ_2 containing cell lines including C6 glioma, NG108-15 or N1E-115 indicated that σ/[^{3}H]DTG binding sites associated with these cells were mostly concentrated in the crude plasma membrane/mitochondrial P_2 fraction (Vilner and Bowen, 1992) in contrast to the microsomal P_3 fraction where rodent brain σ sites are primarily located. These studies demonstrate that σ sites are not preferentially

concentrated in the same cellular fraction in all cell types (Vilner and Bowen, 1992) and that this localization may differ among species or, alternatively, this may reflect the existence of heterogenous patterns of subcellular distribution for different σ receptor subtypes.

5.3 σ receptors in peripheral tissues

σ receptors are not confined to the central nervous system. They are also abundantly found in peripheral organs such as the vas deferens, gastrointestinal tract (DeHaven-Hudkins et al., 1991; Roman et al., 1988; 1989a; Su and Wu, 1990; Vaupel and Su, 1987) and heart (Dumont and Lemaire, 1991), as well as in various endocrine and immune tissues incuding pituitary, adrenal gland, testis, ovary, spleen (Wolfe et al., 1989; Wolfe and De Souza, 1992; 1994), peripheral blood leukocytes (Wolfe et al., 1988; Wolfe and DeSouza, 1992; 1994), seminal vesicle, prostate, epididymis (Vu et al., 1991; 1993), liver (Hellewell et al., 1994; McCann and Su, 1991; Samovilova et al., 1988), kidney (Bowen et al., 1992) and adrenal medulla (Matheson et al., 1991; Rogers and Lemaire, 1989; 1990) (for reviews see Su, 1991; Su and Junien, 1994; Wolfe and DeSouza, 1992; 1994). σ receptors were also found on human and rodent T and B lymphocytes (Carr et al., 1991; Fudenberg et al., 1984; Wolfe et al., 1988; Wolfe and De Souza, 1992) and on murine thymocytes (Carr et al., 1991). The widespread distribution of σ receptors in the nervous system and in peripheral organs involved in various immune and endocrine functions suggests that σ sites may be implicated in the integration between nervous, immune and endocrine systems.

5.4 Sigma receptors in various cell lines

5.4A The PC12 σ site

The existence of multiple σ receptor types was evidenced using PC12 cell membranes (Hellewell and Bowen, 1990). PC12 cells, which are interestingly devoid of PCP binding sites, have been found to bind [^{3}H]DTG and [^{3}H](+)-3-PPP with high affinity (Hellewell and Bowen, 1990; Yang et al., 1989). Bowen and co-workers (Hellewell and Bowen, 1990) found that the σ receptors associated with PC12 cells were different from the σ sites present in guinea pig brain. In comparison with the latter tissue, PC12 σ sites have substantially lower affinity for dextrorotatory enantiomers of morphinans and benzomorphans and display higher affinity for their levorotatory counterpart (Hellewell and Bowen, 1990), indicative of a σ_2 nature.

σ_2 receptors have also been identified in other clonal cell lines of neuronal origin including NB41A3, S-20Y, N1E-115 and on glial cells-derived clones such as the C6 glioma and the neuroblastoma x glioma hybrid NG108-15 cell line (Vilner and Bowen, 1992).

5.4B The NCB-20 cell low affinity σ site

In addition to contain high-affinity binding sites for [^{3}H](+)-3-PPP and [^{3}H](+)SKF 10047 (σ_1; Kushner et al., 1988; Largent et al., 1986a) low affinity non-σ_1, non-σ_2 /σ receptors were identified in NCB-20 cells. Most σ ligands were found to bind this "unusual" σ site, but with an affinity in the μM range. Additionally, this low affinity σ site displayed reversed stereoselectivity for benzomorphan drugs (Wu et al., 1991). Interestingly, the rank order of potencies of various σ ligands at this site highly correlated with their potencies to block a tonic potassium channel, and it has been

proposed that this σ site may be a tonic potassium channel or, altenatively, a site that can allosterically modulate potassium channels (Wu et al., 1991).

6. Sigma receptors and the cytochrome P-450

The high density of σ sites in the liver (Hellewell et al., 1994; McCann and Su, 1991; Samovilova et al., 1988) and their important concentration within the microsomal fraction of the rodent brain (Craviso and Musacchio, 1983a; McCann et al., 1989; 1994; McCann and Su; 1990; Knight et al., 1991b) prompted some investigators to propose that σ receptors could be associated with the drug-metabolizing enzyme complex, cytochrome P-450 (Klein et al., 1991; McCann et al., 1989; Ross, 1990; 1991).

6.1 Binding studies

Support for this hypothesis arose from the finding that various inhibitors of the liver cytochrome P-450 substrates like proadifen (SKF-525A) and L-lobeline inhibited, with nanomolar affinity, the binding of various σ ligands such as [^{3}H]dextromethorphan, [^{3}H](+)-3-PPP and [^{3}H]DTG in the rodent brain and liver (Klein et al., 1991; Ross, 1991). However, several findings make this hypothesis doubtful. In contrast to earlier reports (Klein et al., 1991; Ross, 1991), Basile et al. (1992a) showed that proadifen and piperonyl butoxide inhibited [^{3}H]DTG and [^{3}H](+)pentazocine binding in rat brain and liver homogenates with only low potency (IC_{50}: 740nM - 10μM). In addition, induction of cytochrome P-450 enzymes with either 3-methylcholanthrene or phenobarbital did not alter the affinity or density of

[^{3}H]DTG and [^{3}H](+)pentazocine binding sites in rat brain, but significantly increased the density of σ/[^{3}H]DTG binding sites in the liver (Basile et al., 1992a). Moreover, liver, but not brain microsomal preparations, were found to metabolize σ sites labeled with [^{3}H]dextromethorphan (Craviso and Musacchio, 1983a). Therefore, it seems unlikely that brain σ sites are associated with the cytochrome P-450 complex. However, a possible association or interaction of σ sites with cytochrome P-450 enzymes in the liver has not been fully excluded yet.

6.2 Electrophysiological study

It is well established that several σ ligands are able to potentiate, in a haloperidol-sensitive manner, the NMDA-induced firing of CA_3 hippocampal neurons of the rat brain, *in vivo* (Monnet et al., 1990a; 1992b). Using this electrophysiological paradigm, Monnet et al. (1992c) have shown that complete inactivation of cytochrome P-450 enzymes with proadifen and piperonyl butoxide did not alter the potentiating effect of DTG on NMDA-induced activation of CA_3 dorsal hippocampus pyramidal neurons, suggesting a lack of involvement of cytochrome P-450 in the modulation of the NMDA response by σ ligands (Monnet et al., 1992c). This study provides additional support for a differentiation between σ sites and cytochrome P-450 enzymes in brain tissue.

7. σ receptor regulation

7.1 Haloperidol treatment

In vivo subchronic treatments of rats and mice with various σ ligands provides further evidence for the heterogeneity of σ binding sites. Itzhak and Alerhand (1989)

were the first to report on the reduction in σ/[^{3}H](+)SKF 10047 binding following haloperidol treatment. Using [^{3}H](+)pentazocine as probe, Matsumoto et al. (1989) observed a long-lasting decrease in σ binding sites following a 5 to 60-day treatment with haloperidol. Likewise, it was reported that subchronic treatment with haloperidol or its metabolite, reduced haloperidol, decreased σ/[^{3}H](+)-3-PPP binding in rat and guinea pig brain membranes (Ericson and Ross, 1992; Musacchio and Klein, 1992; Riva and Creese, 1990). Itzhak and Stein (1991b) observed a 75% reduction of σ/[^{3}H](+)-3-PPP binding sites and a reduced responsiveness to guanine nucleotides in rat brain membranes following repeated administration of haloperidol. These changes were transient since a complete recovery in the number of [^{3}H](+)-3-PPP binding sites and responsiveness to guanine nucleotides was observed 28 days after termination of the treatment. Likewise, Riva and Creese (1990) observed a significant decrease in the B_{max} of [^{3}H](+)-3-PPP in mid/hind brain membranes and cerebellum as well as an increased K_D for mid/hind brain membranes after a chronic treatment (21 days) with haloperidol. In comparison, repeated haloperidol administration did not modify [^{3}H]DTG binding parameters, suggesting a different mode of regulation for (+)-3-PPP vs DTG /σ binding sites. Since DTG is a non-selective σ ligand while (+)-3-PPP acts primarily on σ_1 sites (Hellewell and Bowen, 1990; Quirion et al., 1992), these results were interpreted as a preferential down-regulation of the σ_1 receptor subtype following subchronic treatment with haloperidol (Bowen, 1994). However, reduction in σ receptor binding labeled with [^{3}H]DTG was observed following a 10-day haloperidol treatment in the rat (Matsumoto et al., 1989b) and in post-mortem human brain tissue obtained from long-term haloperidol-medicated patients (Reynolds et al., 1991), suggesting that σ_2 receptors could also be down-regulated following haloperidol treatment.

7.2 DTG, (+)pentazocine, remoxipride, rimcazole and clozapine

In contrast to what was observed following haloperidol treatment, Beart et al. (1989) reported that subchronic treatment with DTG or rimcazole increased the number of σ/[^{3}H](+)-3-PPP binding sites in rat brain membranes. In comparison, a 28-day treatment with (+)pentazocine did not affect binding parameters of σ sites labeled with [^{3}H]haloperidol (Weissman and DeSouza, 1991) whereas treatments with clozapine (21 days; Riva and Creese, 1991) and remoxipride (14 days; Ericson and Ross, 1992) did not modulate [^{3}H](+)-3-PPP binding (Riva and Creese, 1991).

It thus appears that the regulation of σ binding sites following chronic drug treatment is still poorly understood and additional studies will be necessary in order to elucidate the mechanisms underlying this phenomenon.

8. Signal transduction mechanisms

8.1 Coupling of σ receptors to guanine nucleotide binding proteins

An increasing body of evidence suggests that σ receptors may be coupled to guanine nucleotide binding proteins, notably the σ_1 subtype (for review see Bowen, 1994; Quirion et al., 1992). This assumption is supported by several binding, electrophysiological and functional studies. However, conflicting results have been reported and additional studies will be necessary to solve this issue.

8.1A Binding studies

There is evidence that at least one subtype of σ receptors is coupled with guanine nucleotide binding proteins (G-proteins). Pertussis and cholera toxin treatments known

to modify the coupling of Gi/o and Gs respectively, modulate the binding of various σ ligands. Pertussis and cholera toxins decreased the binding of [^{3}H](+)-3-PPP in rat and guinea pig brain membranes (Basile et al., 1992b; Itzhak, 1989). Other agents known to affect the coupling of receptors with G-proteins such as GTP, Gpp(NH)p and N-ethylmaleimide (NEM; a non-selective agent) were also shown to modulate σ ligand binding (Basile et al., 1992; Beart et al., 1989; Itzhak, 1989; Itzhak and Khouri, 1988). GTP and Gpp(NH)p decrease the binding of [^{3}H](+)-3-PPP and [^{3}H](+)SKF 10047 to rat brain membranes (Itzhak and Khouri, 1988). Competition studies have shown a biphasic displacement of [^{3}H](+)-3-PPP by (+)-3-PPP and the dextrorotatory benzomorphan σ ligands SKF 10047, pentazocine and cyclazocine (Itzhak, 1989; Itzhak and Khouri, 1988). Gpp(NH)p eliminated the high affinity component of the curve suggesting that the high affinity site was G-protein -coupled (Itzhak, 1989; Itzhak and Khouri, 1988). The decrease of [^{3}H](+)-3-PPP binding induced by GTP was shown to be due to a decrease in binding affinity, with no change in B_{max} (Beart et al., 1989), adding further support to the likely coupling of the σ/[^{3}H](+)-3-PPP binding sites to a G-protein. In comparison, GTP did not affect the displacement of [^{3}H](+)-3-PPP by non-benzomorphan σ ligands such as haloperidol, chlorpromazine, DTG and rimcazole (Beart et al., 1989; Itzhak, 1989), suggesting that these ligands interacted with a σ site different than that labeled by (+)benzomorphans and (+)3-PPP. Accordingly, it was proposed that σ_1 and not σ_2 receptor sites were associated with guanine nucleotide binding proteins (Quirion et al., 1992). On the other hand, Connick and colleagues (1992b) found that [^{3}H]DTG binding parameters were modulated by guanine nucleotide binding proteins in guinea-pig brain membranes. Gpp(NH)p decreased by about 37% [^{3}H]DTG binding and eliminated the high affinity component of the biphasic displacement by (+)-3-PPP, (+)SKF 10047 and dextromethorphan, but

not that of DTG and haloperidol (Connick et al., 1992b), providing further support for the differential interaction of σ_1 and σ_2 receptor subtypes with G-protein(s).

8.1B Functional studies

Few functional studies also support the contention that σ receptors might be G-protein -coupled. It has been shown that NMDA-mediated [^{3}H]noradrenaline (NE) release from rat hippocampal slices was modulated by σ ligands (Monnet et al., 1992a; Roman et al., 1991b). Monnet et al., (1992a) demonstrated that the haloperidol-sensitive modulation by JO-1784 (a σ_1 ligand; Roman et al., 1990) and (+)-3-PPP was abolished by an *in vivo* pretreatment with pertussis toxin, and *in vitro* slice treatment with NEM, supporting the coupling of σ sites with at least Gi/Go types of guanine nucleotide binding proteins. Electrophysiological recordings in the CA_3 region of the rat hippocampal formation also support the G-protein coupling of σ receptors. Recently, Monnet et al., (1994) demonstrated that the potentiating effect of DTG and JO-1784, but not that of (+)pentazocine, on NMDA-evoked firing of CA_3 pyramidal neurons, was prevented by inactivation of Gi/o proteins induced by a previous local (*in vivo*) administration of pertussis toxin. These experiments argue for the existence of multiple σ receptor subtypes, at least one being coupled to G-proteins. However, the ineffectiveness of pertussis toxin treatment on (+)pentazocine activity in this paradigm (Monnet et al., 1994) is at odd with the likely coupling of σ_1 receptors with G-proteins (Quirion et al., 1992), since this molecule is rather selective for σ_1 sites (Bowen et al., 1990; 1993; DeCosta et al., 1989; Quirion et al., 1992). This apparent discrepancy may be explained by the recent proposal on the possible heterogeneity of σ_1 sites in the CA_3 region of the dorsal hippocampus (Debonnel et al., 1996). In keeping with the results of Monnet et al. (1994), it was suggested that, in the hippocampus, the σ_1 subtype

activated by the σ_1 selective ligand JO-1784 may be G-protein -coupled whereas the (+)pentazocine-activated σ_1 site is not (Debonnel et al., 1996). Alternatively, a possible interaction of (+)pentazocine with more than one σ receptor subtype, or with a yet to be characterized non-σ receptor, should also be considered. Support for this hypothesis is provided by the bell-shape effect induced by various σ ligands, including (+)pentazocine, on the NMDA-induced activation of the rat CA_3 dorsal hippocampal neurons (Bergeron et al., 1993; Monnet et al., 1990a, 1992b). The potentiating effect of (+)pentazocine (and other σ ligands) on NMDA-induced firing (see section 11.1) was shown to be significantly reduced over a certain concentration range. Since it was demonstrated that this phenomenon did not relate to σ receptor desensitization, this suggests that at higher concentrations, (+)pentazocine may bind different receptor subtypes (Bergeron et al., 1993). In that regard, the modulatory effect of (+)pentazocine on NMDA-induced [^{3}H]NE release from rat hippocampal slices (Gonzales-Alvear and Werling, 1995b) support the heterogeneity of σ_1 receptor subtypes in the hippocampus, and the contention that (+)pentazocine could interact with both σ_1 and σ_2 sites in the hippocampal formation (see section 11.3). Moreover, a recent report from Walker and co-workers (Walker et al., 1996) suggests that (+)pentazocine could, in addition to the σ_1 and σ_2 receptors, interact with a non-σ receptor, *in vivo* (see section 12.1)

Further studies will be necessary in order to determine the genuine nature of (+)pentazocine binding with respect to σ receptor subtypes, and the possibility of its interaction with a yet to be defined non-σ receptor. This may help to elucidate the issue concerning the possible association of the σ_1 receptor with guanine nucleotide binding proteins.

8.1C Discrepancies

Some data do not support the coupling of σ receptors with guanine nucleotide binding proteins. In the guinea-pig or rat brain membranes GTP, Gpp(NH)p, GTPγs and pertussis toxin do not modulate, or modulate only slightly, the binding of [^{3}H]DTG, [^{3}H]SKF 10047 and [^{3}H](+)pentazocine (Basile et al., 1992b; Beart et al., 1989; DeHaven-Hudkins et al., 1992; Itzhak and Stein, 1992). Additionally, [^{3}H]haloperidol binding was shown to be insensitive to guanine nucleotides (Beart et al., 1989; Itzhak, 1989). It has been proposed that the effect (or lack of effect) of G-protein -modifying agents on different σ ligands may reflect their agonist vs antagonist property for a given subtype (Su and Junien, 1994). Alternatively, the possibility of a "tight coupling" between the σ receptor and its G-protein as been suggested to explain the low potencies of guanine nucleotides to modulate binding parameters of various σ ligands (Bowen, 1994). Conversely, a loose coupling between the σ receptor and its G-protein could also explain the weak (or absence of) effect of G-protein modifying agents.

A second argument against the likely coupling of σ sites with guanine nucleotide binding proteins resides in the molecular weights determined for both σ_1 (25-29 kDa; Hellewell and Bowen, 1990; Kavanaugh et al., 1988; 1989) and σ_2 (18-21 kDa; Hellewell and Bowen, 1990; Hellewell et al., 1994) sites, that are too low to allow for the seven transmembrane domain normally observed for G-protein -coupled receptors (64-120 kDa; Strosberg, 1991; Bowen; 1994). Finally, the reported microsomal localization of σ receptors (Craviso and Musacchio, 1983; Knight et al., 1991b; McCann et al., 1989; McCann and Su, 1990) does not support their association with G-proteins.

The type of interaction between σ receptors with guanine nucleotide binding proteins remains to be elucidated, and the existence of multiple σ receptor subtypes may underly some of the discrepancies observed.

8.2 Calcium-regulatory mechanisms and potassium channels

8.2A Binding studies

A related mechanism, possibly associated to the activation of G-proteins, concerns the modulation of neuronal Ca^{2+} fluxes. It was shown that Ca^{2+} modulators can interact with σ receptors (Klein et al., 1985; Rothman et al., 1991). The binding of dextromethorphan to guinea pig brain membranes was found to be inhibited, in the nanomolar range, by Ca^{2+} antagonists such as prenylamine, cinnarizine and hydroxyzine (Klein et al., 1985). Ca^{2+} channel modulators also differently affected [^{3}H]DTG binding to σ_1 and σ_2 receptor subtypes suggesting that σ sites may bear an allosteric binding domain which could regulate Ca^{2+} fluxes (Rothman et al., 1991). Moreover, inorganic Ca^{2+} channel blockers such as Cd^{2+} were shown to increase the dissociation rate of the σ ligand [^{3}H]DTG from the purported σ_2 receptor subtype, suggesting a likely association of this binding site with Ca^{2+} channels (Rothman et al., 1991). The σ_1 receptor subtype was also postulated to be associated with a cation channel. Basile et al. (1992b) have demonstrated that the binding of the selective σ_1 ligand, [^{3}H](+)pentazocine, to guinea pig cerebellum was modulated by several cations, including Ca^{2+}. Moreover, inhibitors of intracellular Ca^{2+} mobilization (TMB-8 and cinnarizine) as well as nonselective cation channel blockers (hydroxyzine, tetracaine, prenylamine, amiodarone and proadifen) potently inhibited [^{3}H](+)pentazocine binding

in the guinea pig cerebellum with a rank order of potency consistent with an involvement of σ sites. These authors concluded that the potent inhibition of [^{3}H](+)pentazocine binding by divalent and trivalent cations may reflect the association of the σ_1 (and possibly σ_2) receptor with a cation channel (Basile et al., 1992b).

8.2B Functional studies

Functional studies also suggested associations between σ sites and Ca^{2+} regulatory mechanism(s) in nerve cells. Several antipsychotic drugs, including σ ligands, were shown to block voltage dependent Ca^{2+} channels (Fletcher et al., 1994; Quirion et al., 1985). The two σ ligands, dextromethorphan and dextrorphan, can inhibit K^+ - stimulated Ca^{2+} uptake into rat brain synaptosomes and PC-12 cells, apparently via N-type and L-type Ca^{2+} channels, respectively (Carpenter et al., 1988). Voltage clamp experiments on NCB-20 cells revealed that haloperidol, (+)-3-PPP and (+)pentazocine blocked, at μM concentrations, a tonic outward K^+ current with a rank order of potency consistent with an involvement of the σ_2 receptor subtype (Wu et al., 1991). Ela and co-workers (1994) demonstrated that low μM concentrations of (+)-3-PPP, haloperidol and (+)pentazocine induced specific changes in contractility, Ca^{2+} currents and beating rates in cultured cardiomyocytes. The increase in Ca^{2+} currents appeared to be mediated by corresponding changes in Ca^{2+} influx (Ela et al., 1994). The effects of the σ ligands on cardiomyocytes were not affected by pertussis toxin pretreatment, indicating that these effects were unlikely mediated via direct interaction with pertussis toxin-sensitive G-proteins. This suggests that, although purportedly coupled to G-proteins, some σ receptors could be directly associated with cation channels such as Ca^{2+} or K^+ channels, and that σ ligands may exert some of their effects directly by acting through those cation channels. Interestingly, several studies support the

existence of significant interactions between σ sites and K^+ channels. σ ligands were reported to modify K^+ conductance in various tissues from different species (Bobker et al., 1989; Fletcher et al., 1989; Kennedy and Henderson, 1990; Neumaier and Chavkin, 1989). The possible involvement of σ receptor sites in the modulation of K^+ channels activity is strengthened further by the finding that the antipsychotic drug chlorpromazine, which displays reasonable affinity for σ receptors (Tam and Cook, 1984), inhibits various types of K^+ channels and K^+ currents in neuronal and non-neuronal tissues (Dinan et al., 1987; Kon et al., 1994). Interestingly, binding in rat cortex of the σ ligand (+)-3-PPP was shown to be inhibited in the low nM range by various class III antiarrhytmic drugs which block voltage-dependent K^+ channels (amiodarone, clofilium tosylate and RP 58866) (Jeanjean et al., 1993). These authors suggested that rat brain (+)-3-PPP binding sites shared properties of the K^+ channels (which are the targets of class III antiarrhytmic drugs) and proposed that the σ_2 receptor subtype may be a K^+ channel. More recently, using a whole-cell voltage-clamp technique in rat and mouse cultured hippocampal pyramidal neurons, Church and Fletcher (1995) demonstrated that multiple high voltage-activated Ca^{2+} channels currents were reduced by various σ ligands. Furthermore, these σ ligands attenuated K^+-evoked rises in intracellular free Ca^{2+} concentrations in rat hippocampal neurons. However, the effects observed were not attributed to a modulatory action through high-affinity σ receptors, since μM concentrations of σ ligands were needed, and there was no correlation between the rank-order of potency of the compounds tested as Ca^{2+} channel blockers and their affinities for σ receptors. These latter effects could therefore be mediated via an interaction with the low affinity σ receptor described in NCB-20 cells (Wu et al., 1991). However, the mechanism(s) whereby σ compounds modulate Ca^{2+} channels activity may be multiple and remain to be elucidated.

8.3 Modulation of phosphoinositide turnover

σ receptor activation can modulate the function of some receptors coupled to phosphoinositide stimulation (for review see Bowen et al., 1994). At concentrations up to 100 μM, σ ligands do not directly modulate phosphoinositide turnover by themselves. However, at concentrations above 100 μM, σ receptor ligands such as DTG, (+)pentazocine, haloperidol and dextrallorphan dose-dependently attenuate the ability of the cholinergic agonists, carbachol and oxotremorine-M, to stimulate inositol phosphate production (PPI turnover) in synaptoneurosomes preparations from rat brain (Bowen et al., 1988; 1989b; 1992; Tolentino and Bowen, 1989; Bowen, 1994), with a rank-order of potency correlating with their ability to inhibit [^{3}H](+)-3-PPP/σ binding from guinea-pig brain membrane preparations (Bowen et al., 1988; 1992).

σ ligands have been found to bind muscarinic receptors at μM concentrations (Bowen et al., 1989b; 1992; 1994; Brog and Beinfeld, 1990; Candura et al., 1990; DeHaven-Hudkins and Hudkins, 1991; Hudkins and DeHaven-Hudkins, 1991; Vargas and Peshnick, 1991). However, it seems unlikely that direct binding of σ ligands to these receptors would be responsible for their effects on phosphoinositide turnover since σ ligands such as DTG and (+)pentazocine are competitive inhibitors of muscarinic ligands such as [^{3}H]oxotremorine-M and [^{3}H]pirenzepine while they inhibit the muscarinic phosphoinositide response non-competitively (Bowen et al., 1989b; 1992; Ferris et al., 1991b). However, one study by Candura et al. (1990) does not support this view. Using rat cerebral cortical slices, these authors reported a competitive inhibition of the carbachol-stimulated phosphoinositide turnover by (+)pentazocine and DTG. Accordingly, they attributed the modulatory effects of σ ligands on phosphoinositide metabolism to the affinity of σ ligands for muscarinic

receptors (Candura et al., 1990). Further studies will be needed in order to resolve this issue.

9. Allosteric interactions

The existence of allosteric interactions between benzomorphan and non-benzomorphan σ ligands have been suggested. This model proposes that (certain) σ receptors may consist of a macromolecule bearing at least two linked binding sites interacting with each other to change the conformational state of the receptor, one that would bind dextrorotatory benzomorphans such as (+)SKF 10047 and (+)pentazocine while the other would bind non-benzomorphan σ ligands such as haloperidol, DTG and (+)3-PPP (Bowen et al. 1989a). An allosteric binding domain for non-opioid antitussive and anticonvulsant drugs may also be present on a putative σ macromolecule (Musacchio et al., 1988; 1989b).

9.1 Benzomorphan vs non-benzomorphan binding domains

The more striking evidence for an allosteric interaction between benzomorphan and non-benzomorphan binding domains on a σ macromolecule stems from the effects induced by ultraviolet (UV) light irradiation of rat brain membranes (Bowen et al., 1989a). Irradiation of membranes with a 254 nm wavelength decreased the binding of [^{3}H]DTG and [^{3}H](+)-3-PPP, while enhancing that of [^{3}H](+)SKF 10047. UV light irradiation also diminished the potency of DTG and (+)-3-PPP, but not that of (+)pentazocine and (+)SKF 10047, to compete for [^{3}H](+)SKF 10047 binding sites (Bowen et al., 1989a). It appears likely that UV light can disrupt the non-benzomorphan binding domain whereas having no effect on the benzomorphan one

(Bowen et al., 1989a; Walker et al., 1990). Furthermore, in non-irradiated rat brain membranes, benzomorphan and non-benzomorphan σ ligands were shown to exert different effects on [^{3}H](+)-3-PPP binding parameters in crude synaptosomal membrane preparations. DTG and haloperidol were shown to increase the K_D, whereas (+)pentazocine and (+)SKF 10047 decreased both the K_D and B_{max} of [^{3}H](+)-3-PPP binding. This suggests that DTG and haloperidol are competitive inhibitors of [^{3}H](+)-3-PPP binding whereas (+)SKF 10047 and (+)pentazocine are uncompetitive inhibitors, and strengthens the hypothesis that benzomorphan and non-benzomorphan σ ligands may have different binding domains on the same σ macromolecule, allowing for allosteric interactions (Bowen et al., 1989a).

This model is further supported by the effects induced by metaphit on σ ligand binding parameters. Metaphit is an isothiocyanate ligand shown to irreversibly bind PCP (Contreras et al., 1986) and σ receptors (Bluth et al., 1989) in the rodent brain. Metaphit has different effects on the binding of various radiolabeled σ ligands. Binding parameters of [^{3}H]DTG and [^{3}H](+)-3-PPP were found to be more sensitive to acylation by metaphit than that of [^{3}H](+)SKF 10047. Treatment of rat brain membranes with metaphit produced a dose-dependent decrease of various σ ligands, but the degree of sensitivity to acylation by metaphit varied depending on the σ ligand, [^{3}H]DTG being the most sensitive (Bluth et al., 1989). For example, a 50% decrease in σ/[^{3}H]DTG binding sites was obtained after an *in vitro* pretreatment with 1 μM metaphit while the same decrease in σ/[^{3}H](+)SKF 10047 binding required 50 μM of this compound. These findings support the hypothesis that benzomorphan and non-benzomorphan σ compounds interact differently with σ receptors (Bluth et al., 1989; Bowen et al., 1989a; Walker et al., 1990).

9.2 Dextromethorphan binding domain

Musacchio and co-workers (Musachhio et al., 1988; 1989b) proposed that σ/dextromethorphan (DM) receptors may bear an allosteric domain for antitussive and anticonvulsant drugs, an hypothesis that was suggested by the effects induced by phenytoin and ropizine on [^{3}H]DM binding parameters (Craviso and Musacchio, 1983b; Musachhio et al., 1988). These compounds were found to increase, in a concentration-dependent manner, [^{3}H]DM binding to guinea-pig brain membranes, due to an increased affinity. It was also shown that [^{3}H](+)-3-PPP (Karbon et al.,1991; Musacchio et al., 1989b) and [^{3}H](+)SKF 10047 (Karbon et al., 1991) binding were enhanced by phenytoin, in contrast with [^{3}H]DTG or [^{3}H]haloperidol binding (Karbon et al., 1991). These studies suggest that σ receptors may possess an allosteric binding domain for drugs such as phenytoin and ropizine allowing for an allosteric modulation of the binding of selective σ ligands to their own binding domain.

10. Endogenous sigma ligands

- For reviews see Debonnel et al., 1994; Patterson et al., 1994

10.1 Isolation and partial purification of putative endogenous σ ligands

Evidence has accumulated supporting the existence of endogenous σ ligands. In 1987, Su and co-workers reported on the isolation and partial purification of endogenous substances from the guinea pig brain. Two compounds termed "sigmaphins" were isolated, and both were able to inhibit the binding of [^{3}H](+)SKF 10047 in standard competition assays. These unidentified substances were most likely peptidergic as pretreatment of the extracts with trypsin resulted in a partial inhibition of [^{3}H](+)SKF 10047 binding (Su et al., 1987). Likewise, Sonders et al. (1986) reported

on the isolation and partial purification of low molecular weight proteins from bovine brain extracts, which competitively and reversibly inhibited [^{3}H]DTG binding (Sonders et al., 1986). Contreras et al. (1987a) performed a partial purification of a protein extracted from porcine brain, using inhibition of [^{3}H](+)SKF 10047 binding as indicative of σ activity. This procedure resulted in the purification of an unknown substance whose σ binding was eliminated by incubation with pronase (Contreras et al., 1987a). Zhang et al (1988) performed a partial purification of a potential endogenous σ compound from the human brain. The σ activity was followed by inhibition of [^{3}H](+)SKF 10047 binding in a standard binding assay. The partial purification of this endogenous peptide-like substance resulted in activity at both σ and PCP receptors (Zhang et al., 1988). An endogenous substance isolated from porcine liver was shown to inhibit [^{3}H](+)SKF 10047 binding. The partially purified endogenous substance was found to be resistant to pronase digestion, thermostable and was soluble in water and organic sovents (Nagornaia et al., 1988).

Taken together, these findings strongly argue for the existence of an endogenous σ ligand.

10.2 Indirect evidence for endogenous σ ligand(s)

Using rat hippocampal slice preparations, Neumaier and Chavkin (1989) have shown that a substance can be released, upon depolarization of the hippocampal slice (either by focal electrical stimulation of the perforant path or the mossy fibres), which inhibited the binding of [^{3}H]DTG and [^{3}H](+)-3-PPP (Connor and Chavkin, 1991; 1992a,b; Neumaier and Chavkin, 1989). The reduction in σ receptor binding was time- and Ca^{2+} -dependent (Connor and Chavkin, 1991) suggesting that the activation of the perforant path or the mossy fibres caused the release of an endogenous σ ligand

(Connor and Chavkin, 1991). Using membrane binding assays, further examination of potential endogenous substances mediating the effects observed showed that ionic zinc (Zn^{2+}) completely displaced [^{3}H]DTG binding with an IC_{50} of 130 μM. Zn^{2+} had much lower potency against the σ_1-selective ligand (+)pentazocine (IC_{50}: 2 mM), suggesting that Zn^{2+} may act as endogenous ligand or neuromodulator for the σ_2 receptor (Connor and Chavkin, 1992a).

10.3 Neuroactive steroids

10.3A Binding studies

Some steroids including progesterone, testosterone and pregnenolone sulfate were shown to inhibit the binding of [^{3}H](+)SKF 10047 and [^{3}H]haloperidol in guinea-pig spleen and brain membranes with K_is between 268 and 4074 nM (Su et al., 1988). However, controversies still remain as to the relevance of these *in vitro* findings, given the relatively low potency of these steroids, including progesterone, to compete for σ sites. The likelyhood that progesterone act as an endogenous σ ligand has also been challenged on the basis that normal plasma concentrations of progesterone would not allow for sufficient levels of this substance to be present in the CNS (about 2 % of plasma level) and hence to make it a potential endogenous σ ligand (Schwartz et al., 1990).

10.3B Functional studies

Selected σ ligands were shown to modulate, in a haloperidol-sensitive manner, NMDA-evoked [^{3}H]norepinephrine (NE) release from preloaded hippocampal slices (Monnet et al. 1992a). Similar modulatory effects were recently reported with some

steroids including dehydroepiandrosterone sulfate (DHEA S), pregnenolone sulfate (PREG S) and progesterone (Monnet et al., 1995). While none of the steroids tested affected the spontaneous [^{3}H]NE release by themselves, nM concentrations of DHEA S increased while PREG S decreased, the NMDA-induced stimulation of [^{3}H]NE efflux from rat hippocampal slices. These modulatory effects were suppressed by σ ligands such as haloperidol and BD-1063, as well as by progesterone suggesting the involvement of σ receptors (Monnet et al., 1995). Furthermore, pertussis toxin pretreatment of hippocampal slices prevented the effects of DHEA S and PREG S, suggesting the involvement of σ_1 receptors in the NMDA-induced [^{3}H]NE release paradigm (Monnet et al., 1995).

In vivo electrophysiological recordings of hippocampal CA_3 pyramidal cells in the rat confirmed the σ-mediated modulatory potencies of DHEA and progesterone on NMDA-induced activation. Bergeron et al. (1996) recently demonstrated that low doses (μg/kg range) of DHEA selectively and dose-dependently potentiated NMDA-induced activation of CA_3 pyramidal neurons in the rat hippocampus, this potentiation being reversed by the putative σ antagonists NE-100 and haloperidol. Likewise, progesterone, which had no effect by itself, reversed the NMDA-evoked potentiation induced by DHEA and other σ ligands including DTG, JO-1784 and (+)pentazocine, providing further evidence for the involvement of σ sites in the modulatory effects of these steroids on NMDA-induced response. Furthermore, *in vivo* pertussis toxin pretreatment abolished the potentiating effect of DHEA, suggesting that σ_1 receptors were involved. Interestingly, Bergeron et al. (1996) also showed that ovariectomy in female rats increased the potency of DTG on the NMDA-induced response. This suggests that decreases in steroid concentrations seen following ovariectomy freed a

significant amount of σ sites allowing higher concentrations of [^{3}H]DTG binding at σ sites. These experiments provide support for the hypothesis that neurosteroids such as DHEA and progestrone act as potential endogenous σ ligands.

10.4 NPY-related peptides

Roman and colleagues (1989b) suggested that peptides of the pancreatic polypeptide family, namely neuropeptide Y (NPY) and polypeptide YY (PYY), could act as endogenous ligands for σ receptors since these peptides were shown to compete with high affinity for *in vitro* [^{3}H](+)SKF 10,047/σ binding sites in rat brain membrane homogenates. This finding generated tremendous interest. Nevertheless, these data were not replicated using well established *in vitro* membrane binding assays (Quirion et al., 1991; Tam and Mitchell, 1991).

On the other hand, recent studies strongly suggest the existence of such interactions, *in vivo*. Monnet et al. (1990a,c; 1992b) demonstrated that, in a manner similar to certain σ ligands (section 12.4), iontophoretic application of certain NPY-related peptides selectively and dose-dependently potentiated NMDA-induced firing in the pyramidal CA_3 sub-field of the rat hippocampus (Monnet et al., 1990a; 1992b). This potentiation was prevented by the intravenous administration of low doses (20 μg/kg) of the purported σ antagonists haloperidol and BMY-14802, supporting the involvement of σ receptors in these NPY-related effects. Additionally NPY, in a manner similar to certain σ ligands, modulated NMDA-evoked [^{3}H]noradrenaline release from preloaded rat hippocampal slices, this potentiation being blocked by haloperidol (Roman et al., 1991a,b) providing further support for the involvement of σ receptors.

NPY was also shown to interact with σ receptors in the periphery. NPY, in a manner similar to the selective σ ligand JO-1784, was reported to decrease intestinal ion transport and to reverse inhibition of gastric acid secretion induced by corticotrophin-releasing factor. These NPY-modulatory effects likely involved σ sites since they were suppressed by haloperidol and/or BMY-14802 (Gué et al., 1992a,b; Rivière et al., 1990).

11. Interactions between σ receptors and various neurotransmitter systems

11.1 Glutamatergic transmission

Several studies demonstrated the enhancing properties of various σ ligands on NMDA-mediated glutamatergic neurotransmission in the hippocampal formation, *in vivo* (Bergeron et al., 1993; Debonnel et al., 1996; Monnet et al., 1990a; 1992b). Intravenous administration of low doses of σ ligands including DTG, (+)pentazocine, JO-1784, BD-737 and antidepressant drugs with affinity for σ sites such as sertraline and clorgyline, potentiate (from 2- to 10-fold) selectively and dose-dependently, the neuronal firing of the CA_3 region of the rat dorsal hippocampus upon microiontophoretically application of NMDA. In keeping with their potentiating effect on NMDA-induced response, these ligands were classified as σ agonists (Bergeron et al., 1993; Monnet et al., 1990a; 1992b). On the other hand, σ ligands such as haloperidol, (+)-3-PPP, BMY-14802 and NE-100 do not modulate the NMDA-induced neuronal activation but suppress σ agonists -induced potentiating effect, suggesting

that these ligands act as σ antagonists (Bergeron et al., 1993; Monnet et al., 1990a; 1992b), at least in this experimental paradigm.

Debonnel et al. (1996) identified the anatomical location of different σ receptor subtypes involved in the potentiating effects of σ ligands on the NMDA response in the rat hippocampal formation. A presynaptic σ site, located on terminals of the mossy fiber system where σ ligands such as DTG and JO-1784 potentiate NMDA-induced neuronal firing, and a second site likely located post-synaptically in both CA_1 and CA_3 regions where (+)pentazocine exerts its effects. It thus appears, from these experiments, that the selective σ_1 ligands JO-1784 and (+)pentazocine could act on different σ_1 receptor subtypes in the hippocampal formation (Debonnel et al., 1996).

The potentiating effect of σ ligands on NMDA-induced neuronal activation is in contrast with studies where an inhibitory effect of σ ligands on NMDA-induced activation was found. Connick et al. (1992a) reported a non-selective decrease in NMDA- and QUIS-evoked response following microiontophoretically applied DTG to neurons of rat hippocampal slice preparation or to cortical and hippocampal neurons, *in vivo*. Moreover, haloperidol did not antagonize the modulatory effects of DTG, suggesting the non-involvement of σ sites (Connick et al., 1992a). These results are in keeping with earlier studies that reported a reduction of NMDA-induced excitation of rat spinal neurons by (+)pentazocine, *in vivo* (Lodge et al., 1988), and the attenuating effect of cyclazocine, SKF 10047 and (+)pentazocine on NMDA-induced depolarization of neurons from rat cortical slices (Aram et al., 1989). It may be that the attenuating effects of σ ligands on NMDA-induced neuronal activation was due to an interaction with PCP rather than σ sites. Some recent findings may support this contention. It was demonstrated that structurally diverse σ ligands attenuated NMDA

(but not kainate or AMPA) -evoked currents in mouse cultured hippocampal pyramidal neurons under whole-cell voltage- clamp (Fletcher et al., 1995). However, micromolar concentrations of the σ compounds were needed in order to induce these attenuating effects. The rank-order of potency, the non-competitive mode of action, the lack of interaction with the polyamine binding sites and the voltage- and use-dependence of the block of NMDA-evoked currents by some σ ligands including DTG, (+)pentazocine, (+)-3-PPP, dextromethorphan, caramiphen and carbetapentane, was interpreted as an antagonistic action mediated through the NMDA receptor ionophore (Fletcher et al., 1995). Therefore, the observed enhancing effects of various σ ligands on NMDA-evoked neuronal activation in the CA_3 region of the rat hippocampus *in vivo* (Bergeron et al., 1993; Debonnel et al., 1996; Monnet et al., 1990a; 1992b), may relate to the low doses used, allowing for a specific interaction with high-affinity σ receptors in certain subfields of the hippocampus.

The mechanism of interaction between σ ligands and the NMDA receptor remains to be elucidated, but a direct action of σ-related compounds with the NMDA receptor complex as well as a molecular coupling between the two sites is unlikely considering the overall different distributional profiles of these two receptor sites (section 1.2B). Therefore an indirect relationship between σ and NMDA systems appears more likely at this point.

11.2 Dopaminergic transmission

A great deal of data have been gathered which are supportive of a role for σ receptors in the modulation of dopaminergic transmission in the central nervous system. Evidence for the existence of interactions between σ and dopaminergic systems

are derived from anatomical, behavioral, neurochemical and electrophysiological experiments.

11.2A Anatomical evidence

The high levels of σ receptors in regions of the brain containing terminals and cell bodies of nigrostriatal dopamine neurons such as the substantia nigra and striatum (Bouchard et al., 1996; Graybiel et al., 1989; Gundlach et al., 1986a; Largent et al., 1986b; Leitner et al., 1994; McLean and Weber, 1988; Walker et al., 1990; 1992) suggested the possibility that σ and dopaminergic systems may interact in these brain regions. The observation that destruction of nigrostriatal dopaminergic neurons with 6-hydroxydopamine reduced the levels of σ receptors in the substantia nigra supports this hypothesis (Gundlach et al., 1986a).

11.2B Behavioral evidence

It has been demonstrated that σ ligands can modulate behaviors known to be under dopaminergic control. Various σ ligands were shown to mimic certain aspects of the behavioral effects induced by the dopamine agonist amphetamine since intracerebroventricular or systemic administration of DTG or (+)SKF 10047 induced stereotypic behaviors and hyperlocomotion in the rat (Contreras et al., 1988). BMY-14802, rimcazole and DuP 734 were able to block the locomotor stimulant effect of cocaine in mice, at doses that do not produce significant behavioral effects (Cook et al., 1992; Menkel et al., 1991). In the rat, intranigral administration of DTG or (+)pentazocine elicits contralateral circling (Walker et al., 1988; Weiser et al., 1995) that is suppressed by the destruction of dopaminergic neurons with 6-hydroxydopamine (Goldstein et al., 1989), stongly suggesting that σ ligands-induced turning behavior is

mediated via dopamine release in the striatum (Goldstein et al., 1989; Walker et al., 1988). A synergistic interaction between σ receptors and dopaminergic systems is further suggested by the observation that administration of the dopamine reuptake inhibitor amphetamine potentiates the circling behavior induced by microiontophoretic administration of (+)pentazocine or DTG (Goldstein et al., 1989).

11.2C Electrophysiological effects

σ ligands have been widely studied in relation to their effects on the firing activity of neurons from various brain areas. A clear outcome from these electrophysiological experiments is that many structurally dissimilar compounds with high affinities for σ receptors are able to modulate the firing pattern of neurons located in brain regions contributing to dopaminergic transmission such as neurons of the nigrostriatal pathway (Clark et al., 1985; Meltzer et al., 1992; Piontek and Wang, 1986; Steinfels et al., 1989; Steinfels and Tam; 1989; Watchel and White, 1988; Zhang et al., 1992; 1993). However, despite compelling evidence for the modulatory activity of certain σ compounds on the activity of dopaminergic neurons, doubts remain as to the genuine involvement of σ sites in mediating these effects (Meltzer et al., 1992). Conflicting data have been reported in that σ ligand-induced changes (inhibition vs potentiation) in the firing rate of dopaminergic neurons are not consistent between studies and that modulatory effects are not always observed (Meltzer et al., 1992; Zhang et al., 1992). The lack of selectivity of several σ ligands as well as the existence of multiple σ receptor subtypes add to the hurdle of getting consistent results between studies. Consequently, it was pointed out that the modulatory property of some σ ligands on the firing activity of dopamine neurons might actually be accounted for their affinity at other, non-σ, receptors (Walker et al., 1994).

Effects of σ ligands on midbrain dopamine neurons

Single unit activity of dopaminergic neurons of the substantia nigra pars compacta has been shown to be modulated in a dose-related fashion by σ ligands. Intravenous administration of (+)-3-PPP in rats dose-dependently inhibited the firing of A9 and A10 dopaminergic neurons, this effect being completely abolished by haloperidol (Clark et al., 1985). Similarly, intravenous administration of DTG, (+)pentazocine and (+)-3-PPP were shown to decrease single unit activity in the subtantia nigra pars compacta (Steinfels et al., 1989) while the purported σ antagonists haloperidol and BMY-14802 increased it (Matthews et al., 1986; Steinfels et al., 1989; Wachtel and White, 1988) and reversed the inhibition induced by (+)-3-PPP (Steinfels and Tam, 1989). On the other hand, Zhang et al. (1992) failed to obtain any modulation of the firing rate pattern of dopamine neurons after i.v. administration of DTG and JO-1784, while they observed that (+)pentazocine increased the firing of nigrostriatal and mesoaccumbal dopamine neurons (Zhang et al., 1992). The atypical antipsychotic rimcazole, which displays significant affinity for σ sites (Ferris et al., 1986), has also been shown to modulate the electrical activity of midbrain dopaminergic neurons since acute and chronic administration of rimcazole altered the spontaneous firing rate of A10 but not that of A9 cells (Piontek and Wang, 1986). Rimcazole also antagonized the modulatory effect of (+)SKF 10047 on A10 neurons (Ceci et al., 1988). Intriguingly, Meltzer et al. (1992) failed to observe any significant modulatory effect on the firing activity of A9 neurons upon i.p. administration of DTG and haloperidol. The potentiating effect of (+)pentazocine and (+)SKF 10047 were attributed to their affinity at the PCP site while that of BMY-14802 was argued to be consistent with its potency at serotonergic (5-HT_{1A}) receptors rather than at σ sites (Meltzer et al., 1992). On the basis of their results, Meltzer et al. (1992) concluded that activity at σ receptors was

not responsible for the modulation of dopaminergic neurons by σ ligands. They found support for this statement from data that have shown only a modest effects of DTG and (+)pentazocine on dopamine neuronal activity (French and Ceci, 1990; Zhang et al., 1992). This is in sharp contrast with the complete inhibition of dopamine cell firing activity observed with these ligands (Steinfels et al., 1989). However, it was pointed that the kind of anesthetic used during electrophysiological recordings might account for some of the discrepancies observed (Gudelsky, 1995; Weiser et al., 1995). Some anesthetic compounds were shown to affect drug-induced (including DTG and haloperidol) nigrostriatal neuron firing rate (Hamilton et al., 1992; Kelland et al., 1989) and tyrosine hydroxylase activity (Mereu et al., 1984; Weiser et al., 1995). Consequently, electrophysiological data should be interpreted with caution.

Electrophysiological effects in acute vs chronic situation

Acute vs chronic administration of various σ ligands may have different effects on the firing rate and pattern of individual dopamine neurons. This possibility was examined by Zhang and colleagues (1992) when they compared the effect of acute vs chronic administration of DTG and JO-1784 on the firing activity of A9 and A10 dopaminergic cells. Interestingly, they found that while DTG and JO-1784 had no effect on midbrain dopamine activity when given acutely, chronic treatment with (+)pentazocine and DTG increased the number of spontaneously firing A10 cells while chronic administration of JO-1784 decreased the number of active A9 cells and increased the firing rate of A10 cells.

11.2D Neurochemical effects

Various σ ligands have been shown to modulate the release and synthesis of dopamine in the rodent brain. Using *in vivo* microdialysis studies, it was demonstrated that intraperitoneal injections of various σ ligands such as DTG, (+)pentazocine, (+)SKF 10047, DuP 734, BMY-14802, remoxipride and (-)butaclamol were followed by an increase in the extracellular concentrations of dopamine in the striatum (Gudelsky, 1995; Gudelsky and Nash, 1992; Patrick et al., 1993). The σ receptor subtype involved in this effect is controversial. Patrick et al. (1993) suggested the involvement of σ_2 sites since lower doses of DTG than (+)pentazocine were needed to produce the increase in extracellular dopamine. On the other hand, Gudelsky (1995) argued for an involvement of σ_1 sites because (+)benzomorphans had higher potency than their (-) counterparts in inducing the same effect (Gudelsky, 1995). In any case, none of these sudies allowed to rule out the involvement of one particular σ receptor subtype, and it is conceivable that both sites can mediate the obseved increase in extracellular concentrations of dopamine in the striatum. In addition, it was demonstrated that σ ligands -facilitating effect on dopamine release from nigrostiatal neurons was not mediated via a dopamine transporter-related mechanism since GBR 12909, a dopamine uptake inhibitor, did not mask the stimulatory effect of (+)pentazocine on extracellular striatal dopamine levels (Gudelsky, 1995). This is consistent with the finding that σ_1-selective ligands such as (+)pentazocine and (+)SKF 10047 did not compete for [^{3}H]WIN 35,428, a cocaine analog with high affinity for the dopamine transporter, in rat caudate-putamen homogenates (Izenwasser et al., 1993). However, various σ ligands including haloperidol, (+)-3-PPP, rimcazole, ifenprodil, carbetapentane, caramiphen, and PRE-084 were shown to inhibit, in a concentration-related manner, the binding of [^{3}H]WIN 35,428 in rat stiatum homogenates

(Izenwasser et al., 1993) and to inhibit [^{3}H]dopamine uptake in rat striatal slices or synaptosomes (Ferris et al., 1986; Izenwasser et al., 1993, Woodward and Harms, 1992). The low potencies (K_i:100 nM-5 μM in competition assays; IC_{50} > 4 μM in [^{3}H]DA uptake inhibition assays) of these σ ligands and the lack of effect of DTG and (+)benzomorphans exclude the σ_1 and σ_2 receptor subtypes in mediating the interaction with the dopamine transporter. Therefore, an interaction with a yet to be characterized σ subtype, possibly of low affinity, is possible but seems rather unlikely to be involved in the facilitating effects of σ ligands on DA release in the striatum.

Consistent with a stimulating effect of σ ligands on dopamine synthesis in the striatum is the increase in dopamine metabolites such as 3-methoxytyramine (3-MT), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the rat striatum following peripheral or central adiministration of the σ_1 ligands (+)pentazocine and (+)SKF 10047 (Iyengar et al., 1990). Haloperidol, clozapine and BMY-14802 were also shown to increase basal DOPAC levels and to reverse amphetamine-induced decline in DOPAC in rat striatum (Pierce and Rebec, 1992). Tyrosine hydroxylase activity, as an indication of dopamine synthesis, was also found to be stimulated by σ ligands. (+)Pentazocine increased the activity of tyrosine hydroxylase in rat striatal minces (Booth and Baldessarini, 1991) while DTG, injected directly into the rat substantia nigra, produced an increase in tyrosine hydroxylase activity in the stiatum (Weiser et al., 1995). This later study constitutes a direct demonstration that activation of σ receptors at the level of the substantia nigra can modulate dopamine formation in the striatum (Weiser et al., 1995).

In contrast to the direct stimulatory effect of σ ligands on dopamine synthesis and release, NMDA-stimulated dopamine release was found to be inhibited by various σ compounds (Gonzales-Alvear and Werling, 1994; 1995c). σ agonists including DTG, BD 737 and (+)pentazocine inhibited, in a concentration-dependent manner, NMDA-stimulated dopamine release from rat striatal slices (Gonzales-Alvear and Werling, 1994; 1995c). The non-selective σ ligands haloperidol, DTG and BD 1008 as well as the purported σ_1 antagonist DuP 734 (Culp et al., 1992; Tam et al., 1992), reversed the inhibitory effect of (+)pentazocine and BD 737, suggesting a prominent involvement of σ_1 sites (Gonzales-Alvear and Werling, 1994; 1995c). It was further demonstrated that the σ_1 receptors regulating striatal DA release were located on (striatal) dopaminergic nerve terminals since (+)pentazocine and BD 737 still inhibited NMDA-induced DA release in the presence of tetrodotoxin treatment (Gonzales-Alvear and Werling, 1995c). This location is consistent with the observed (+)pentazocine -induced increase in DA synthesis in rat striatal slices (Booth and Baldessarini, 1991). These studies demonstrate that σ ligands (at least of the σ_1 type) can induce their effects by acting directly on dopaminergic nerve terminals.

11.3 Noradrenergic transmission

σ receptors are highly concentrated in the locus coeruleus (Gundlach et al., 1986a; McLean and Weber, 1988), one of the major cluster of noradrenergic cell bodies. Locus coeruleus noradrenergic cells innervate all cortical areas, the hippocampus, specific thalamic and hypothalamic nuclei, the olfactory bulb, the cerebellum and the spinal cord. Consequently, interactions between σ and noradrenergic systems would be of important functional relevance.

In the cerebral cortex, hippocampus and cerebellum, activation of noradrenergic pathways classically produce inhibition of spontaneous neuronal discharge. Accordingly, various σ ligands including DTG, (+)pentazocine, dextrallorphan and BD-1008 were shown to inhibit the frequency of Purkinje cell firing in the cerebellum whereas (+)-3-PPP exerted an opposite effect (Kim and Bickford, 1992). Involvement of the noradrenergic system in this phenomenon is further supported by the attenuation of the inhibitory effect of DTG following destruction of noradrenergic neurons (Kim and Bickford, 1992). σ ligands were also shown to modulate [^{3}H]norepinephrine (NE) release stimulated by NMDA in rat hippocampal (Gonzales-Alvear and Werling, 1995b; Monnet et al., 1992a; 1995) and cerebellar (Gonzales-Alvear et al., 1995a) slices. DTG and the σ_1 compound JO-1784, were reported to inhibit and potentiate respectively, NMDA-evoked [^{3}H]NE release from preloaded rat hippocampal slices, in a concentration-dependent manner (Monnet et al., 1992a; 1995). The involvement of σ receptors in this phenomenon was shown by the blocking effect of haloperidol on DTG- and JO-1784 -induced effects. Moreover, pertussis toxin treatment prevented DTG-induced decrease of NMDA-evoked [^{3}H]NE release suggesting that the σ receptor involved in the modulatory effect of DTG is coupled to a guanine nucleotide binding protein, likely the σ_1 site (Monnet et al., 1992a). On the other hand, Gonzales-Alvear and Werling (1995b) observed an inhibitory effect of BD-737 as well as (+)pentazocine, on NMDA-induced [^{3}H]NE release from rat hippocampal slices, while DTG was inactive. The complete reversal of BD-737 -induced effects by DuP 734 (a selective σ_1 ligand) confirmed the involvement of σ_1 sites in the modulatory effects of σ ligands on NMDA-stimulated [^{3}H]NE release. However, although DTG completely reversed (+)pentazocine mediated inhibition of NMDA-stimulated [^{3}H]NE release, DuP 734 and haloperidol only partially reversed the effect induced by higher concentrations

of (+)pentazocine (>100 nM) whereas a complete reversal was observed when both compounds were combined. This led the authors to hypothesise the involvement of σ_2 receptors in the modulatory effect of (+)pentazocine on NMDA-evoked [^{3}H]NE release, since higher concentrations of (+)pentazocine could bind σ_2 receptors (K_i of (+)pentazocine for σ_2 sites: 440 nM; Connor and Chavkin, 1992). Interestingly, haloperidol prevented the modulatory effects induced by (+)pentazocine but not that of BD-737, further suggesting that a haloperidol-insensitive type of σ_1 receptor was possibly involved in the modulatory effects observed (Gonzales-Alvear and Werling, 1995b). The rat cerebellum was also reported to be sensitive to the modulatory effects of σ ligands on NMDA-stimulated [^{3}H]NE release, *in vitro*. In preloaded rat cerebellar slices, (+)pentazocine and BD 737 inhibited NMDA-stimulated [^{3}H]NE release in a concentration-dependent and monophasic manner. The σ_1-selective antagonist DuP 734 (Culp et al., 1992; Tam et al., 1992), significantly reversed (+)pentazocine- and BD 737-induced effects indicating the involvement of σ_1 sites (Gonzales-Alvear et al, 1995b). These later observations point out the complex interactions of σ systems with NE neurotransmission and the need for a better delineation of σ receptor subtypes classification.

Recently, the σ_1 probe (+)SKF 10047 was shown to potentiate, *in vivo*, isoproterenol (a potent adrenergic agent) -induced *N*-acetyltransferase activity in the rat pineal gland. The potentiating activity of (+)SKF 10047 was prevented by pretreatment with rimcazole, suggesting that σ_1 and noradrenergic systems may interact in the pineal gland to modulate *N*-acetyltransferase activity (Steardo et al., 1995).

In the adrenal medulla, σ ligands block the release of catecholamine including noradrenaline, but at concentrations much higher (1000 fold) than their affinity for σ receptors (Paul et al., 1992; 1993). σ receptor activation have also been reported to enhance some effects induced by NE in the periphery. DTG, haloperidol and BMY-14802 potentiated in a concentration-dependent manner, NE-induced contractions of the rat vas deferens and rat tail artery, likely via blockade of NE uptake (Kennedy and Henderson, 1989; Massamari and PiperDuckles, 1989, 1991). NE uptake blockade has also been observed using rat brain synaptosomal preparations (Rogers and Lemaire, 1991). Taken together, these data indicate that σ drugs can modulate a broad variety of NE-sensitive effects likely via σ receptor activation.

11.4 Cholinergic transmission

In *in vitro* membrane binding assays, σ ligands such as DTG, (+)pentazocine and (+)-3-PPP display very low (μM range) or no affinity for muscarinic receptors (Bowen et al., 1989b; 1992; 1994; Brog and Beinfeld, 1990; Candura et al., 1990; DeHaven-Hudkins and Hudkins, 1991; Hudkins and DeHaven-Hudkins, 1991; Vargas and Peshnick, 1991). Conversely, in guinea pig brain membrane preparations, muscarinic ligands including atropine, pirenzepine, QNB, muscarine, carbachol or oxotremorine exhibit no affinity for σ sites labeled using either [^{3}H](+)pentazocine or [^{3}H]DTG (DeHaven-Hudkins and Fleissner, 1992; DeHaven-Hudkins and Hudkins, 1991). These studies argue for a lack of direct interaction between σ and cholinergic systems. However, a growing body of evidence suggests the existence of indirect interactions between σ and cholinergic systems, including both muscarinic and nicotinic receptors.

Several σ ligands were found to attenuate muscarinic agonists-stimulated phosphoinositide metabolism in rat brain synaptoneurosomes (Bowen et al., 1988a; 1989b; 1992; 1994; Candura et al., 1990; Vargas and Pechnick, 1991; see section 8.3). DTG, (+)SKF 10047, (+)pentazocine and haloperidol inhibit acetylcholine-induced depolarization of guinea pig myenteric neurons (Galligan et al., 1989). Racemic and dextrorotatory SKF 10047 and JO-1784 potentiate, whereas DTG decrease, electrical stimulation and KCl-evoked acetylcholine release from rat (Junien et al., 1991) and guinea pig (Siniscalchi et al., 1987) cerebral slices. JO-1784 was also shown to potentiate KCl-evoked high-affinity [^{3}H]choline uptake from mouse hippocampal synaptosomes *in vivo* (Junien et al., 1991). These effects most likely involved σ sites since the modulatory effects of σ ligands were stereoselective and reversed by haloperidol (Junien et al., 1991). Likewise, (+)SKF 10047, racemic pentazocine, DTG and (+)-3-PPP dose-dependently increased extracellular acetylcholine levels in rat frontal cortex, *in vivo* (Matsuno et al., 1992; 1993a). (+)SKF 10047 was reported to increase, in a haloperidol-sensitive manner, rat hippocampal acetylcholine levels as well (Matsuno et al., 1995). The mechanisms involved in (+)SKF 10047-induced potentiation of acetylcholine release are controversial. Siniscalchi et al. (1987) suggested that (+)SKF 10047 induced its effects via muscarinic receptors since it was antagonized by atropine whereas Junien et al. (1991) proposed that (+)SKF 10047-induced response was mediated via σ receptors since it was antagonized by haloperidol. It seems therefore likely that (+)SKF 10047 can potentiate [^{3}H]acetylcholine release by interacting at both sites. Finally, behavioral studies of cognitive performance in the rodent suggest an interaction between σ and muscarinic systems (Matsuno et al., 1993b; 1994; Maurice et al., 1994a; section 12.3).

DTG and (+)pentazocine were also reported to inhibit, in a concentration-dependent and non-competitive manner, nicotine-stimulated catecholamine release in adrenal chromaffin cells. The rank order of potency to inhibit nicotine-stimulated catecholamine release correlated well with that obtained for several σ ligands to compete for [^{3}H](+)pentazocine (but not for [^{3}H]DTG binding in the presence of 10μM dextrallorphan), in homogenates from rat adrenal medulla, suggesting a selective interaction between σ_1 and nicotinic receptors (Paul et al., 1993). Furthermore, nicotine significantly enhanced the association rate of [^{3}H](+)pentazocine, but not that of [^{3}H]DTG, in adrenal medulla homogenates, suggesting the existence of allosteric interactions between σ_1 and nicotinic receptors in adrenal chromaffin cells. The authors (Paul et al., 1993) suggested that the σ_1 receptor could reside within the nicotinic receptor-gated ion channel in chromaffin cells, arguing that this location would be consistent by the observed inhibition, by (+)pentazocine, of nicotine-stimulated Ca^{2+} influx in these cells. Further studies will be required to confirm and extend these preliminary findings.

12. Postulated functions and clinical implications

12.1 Posture and movement

As revealed by autoradiographic studies (Bouchard et al., 1996; Gundlach et al., 1986a; Largent et al., 1984; 1986b; McLean and Weber, 1988), the discrete anatomical distribution of σ receptors in several areas of the brain involved in motor control argues for their role in motor functions. In that regard, Walker and co-workers (for reviews see Walker et al., 1990; 1994) generated a great deal of data supporting this hypothesis.

12.1A Effects induced by σ ligands on the rubro-cerebellar system

Microiontophoretic application of σ ligands including DTG, dextrallorphan and (+)pentazocine into the red nucleus of the rat brain, dose-dependently inhibits the firing activity of its neurons (Matsumoto and Walker, 1988a; 1992). Similar effects were obtained in the cerebellum where systemic and local administration of DTG decreased Purkinje cell activity (Kim and Bickford, 1992).The electrophysiological effects of σ ligands on rubral neurons are most likely mediated through σ receptors because the red nucleus is highly enriched in σ sites and contains very low densities of other receptors (dopaminergic, serotonergic, cholinergic and PCP) that bind σ ligands with significant affinity (Walker et al., 1990).

Microinjections of various σ ligands ((+)SKF 10047, (+)pentazocine, DTG, dextrallorphan and haloperidol) in the red nucleus of the rat brain were found to induce dystonia characterized by a marked deviation of the head angle (torticolli). The σ_2 receptor subtype was suggested to be particularly involved in the induction of dystonia since DTG was more potent than (+)pentazocine. No such correlation was observed in the guinea pig, purportedly because σ_1 receptors are preponderant in this specie (Walker et al., 1992). More recently, Matsumoto et al. (1995) provided additional evidence for the involvement of σ_2 sites in the control of posture and movement. Using two new σ ligands, BD1047 and BD1063, they observed that BD1047, which displays a 10-fold higher affinity than BD1063 for the σ_2 subtype, had a higher potency to antagonize the dystonic posture induced by DTG, suggesting a prominent role for the σ_2 subtype in σ ligand-induced dystonia. These behavioral effects seem truly mediated via σ receptors since a variety of ligands for other receptors including PCP (a non-

competitive NMDA receptor antagonist), sulpiride and clozapine (two dopamine receptors antagonists) as well as 8-OH-DPAT (a 5-HT_{1A} agonist) failed to produce dystonia (Matsumoto et al., 1990; Walker et al., 1988). Moreover, a significant correlation ($r = 0.94$) between the degree of torticolli induced by σ ligands and their affinity for [^{3}H]DTG binding in the rat brain supports the involvement of σ receptors (Matsumoto et al., 1990; Walker et al., 1988).

Developmental studies of σ binding parameters demonstrated that brains of young adult rats (2-3 months) had [^{3}H]DTG binding sites of higher density and affinity in comparison with older rats (5-6 months) (Matsumoto et al., 1989a). Walker and colleagues undertook a series of experiments to determine if these binding parameters were behaviorally relevant in relation to posture and motor control. They found that young adult rats had a greater alteration in head angle following microinjection of DTG into the red nucleus and a greater circling response following microinjection into the substantia nigra (Matsumoto et al., 1989). These results were substantiated by the high correlation found ($r=0.87$) between σ binding at various ages in 30 to 150 day-old rats, and the potency of DTG in producing dystonia when injected into the red nucleus (Hemstreet et al., 1993). These studies provide evidence that ontogenic changes in σ binding parameters are behaviorally relevant.

A recent study by Matsumoto et al. (1996) provided strong support for the selective involvement of σ_2 receptors in posture and movement. This study demonstrated that, whereas [^{3}H](+)pentazocine binding was of lower density and affinity in middle aged rats (5-6 months old) in comparison with younger rats (2-3 months old), there was no difference between these two groups with respect to the extent of the circling behavior and dystonia following microinjection of (+)pentazocine

in the rat substantia nigra or red nucleus. Likewise, the significant decrease in [^{3}H](+)pentazocine binding following chronic administration of haloperidol did not affect the dystonic posture induced by intrarubral administration of (+)pentazocine, suggesting that the σ_1 receptor subtype is not involved in the motor effects (i.e. contralateral circling and dystonia) produced by σ ligands (Matsumoto et al., 1996). Surprisingly, the postural changes induced by (+)pentazocine could not be antagonized or attenuated with the purported σ antagonist, BD 1047, suggesting the involvement of a yet unidentified receptor. This study supports the hypothesis that (+)pentazocine may act on multiple receptor subtypes (Debonnel et al., 1996, Gonzales-Alvear and Werling, 1995b; Monnet et al., 1994).

12.1B Effects induced by σ ligands on the nigrostiatal dopaminergic system

- The reader is refered to section 11.2

12.1C Clinical implications

The high levels of σ receptors in brain areas associated with the control of posture and movement, the modulatory effects of several σ ligands on cell activity of various pathways involved in motor control in addition to their motor behavioral effects in rodents suggest that σ system(s) may be involved in various pathologies related to motor functions. Consequently, σ receptor ligands could be useful for treating posture and/or movement disorders. Support for this hypothesis is derived from clinical observations in humans and functional data in animals.

Dystonia

Lesions of areas highly enriched in σ labeling such as the red nucleus, cerebellum and locus coeruleus can cause dystonia in rodents, as well as in human and non-human primates. Direct support for a role of σ receptors in the pathogenesis of idiopathic dystonia (Walker et al., 1990) stems from the effects of certain σ ligands upon their injection into the red nucleus (Walker et al., 1988; Matsumoto et al., 1990), and by the observation of an important decrease in affinity (500%) and increase in number of [^{3}H]DTG/σ sites (200%) in genetically dystonic rats (Bowen et al., 1988). However, these findings were not replicated (Weissman et al. 1993) and further studies will be required to settle this issue.

Tardive dyskinesia

The high affinity of several neuroleptics for σ sites (Su, 1982; 1991; Ferris et al., 1986; 1991a; Tam and Cook, 1984) raises the possibility of their involvement in the development of tardive dyskinesia, since long-term treatment with various typical neuroleptics such as haloperidol are known to induce tardive dyskinesia in humans. Interestingly, new "atypical" neuroleptics (i.e. clozapine and sulpiride) that do not produce catalepsy in rats (and thus with only small liability to induce dystonia in humans; Friedman et al., 1987; Lindstrom, 1988; Walker et al., 1990) display only moderate affinity for σ receptors (Walker et al., 1990). The possible involvement of σ receptors in tardive dyskinesia is supported by the observation that an haloperidol metabolite (reduced haloperidol), which have been shown to accumulate in the brain of patients chronically treated with this drug (Korpi et al., 1984), has high affinity for σ receptors but only moderate affinity for dopamine D_2 receptors. It is thus tempting to speculate that the development of tardive dyskinesia may be initiated or potentiated by

an accumulation of reduced haloperidol and its binding to σ sites in the brain of chronically-treated patients. Interestingly, DTG and (+)pentazocine were found to induce vacuous chewing movement in the rat (Patrick et al., 1993), a phenomenon commonly observed in long-term neuroleptic-treated patients.

12.2 Neuroprotection vs neurotoxicity

Several σ ligands have been found to be neuroprotective *in vivo* and *in vitro*. However, given the affinity of many σ neuroprotective agents for the PCP/NMDA receptor complex including (+)SKF 10047 (Lysko et al., 1990; 1992a,b), dextromethorphan (O'Neill et al., 1994), ifenprodil (Gotti et al., 1988; Pontecorvo et al., 1991), eliprodil (Poignet et al., 1992) and opipramol (Rao et al., 1990c), it was proposed that σ ligands induced their neuroprotective effects via their antagonistic property at the NMDA receptor. Nevertheless, many σ ligands with no affinity for the PCP/NMDA receptor complex were also found to be neuroprotective.

12.2A Neuroprotective activity of σ-selective compounds

A strong case supporting the involvement of σ receptor sites in neuroprotection is made from the fact that BMY-14802, a selective σ ligand with no significant affinity for NMDA receptors (Taylor et al., 1990; Taylor and Dekleva, 1987) is neuroprotective in various paradigms. Preadministration of BMY-14802 protected rats against death induced by 1 min exposure to nitrogen (Taylor et al., 1990; 1992); significantly decreased the potency of NMDA in producing convulsions/seizures in mice (Kaiser et al., 1992; Taylor et al., 1990; 1992); protected against ischaemia-induced neuronal damage in the gerbil (Contreras et al., 1991; Moon et al., 1990; O'Neill et al., 1994) and reversed harmaline and *D*-serine -induced elevations in

cerebellar cyclic GMP levels (Rao et al., 1990; 1991). JO-1784, another selective σ ligand without significant affinity for PCP/NMDA receptors (Roman et al, 1990), also exhibited neuroprotective properties since this compound was shown to significantly attenuate ischaemia-induced neuronal cell death in the CA_1 region of the gerbil hippocampus and ischaemia-induced increase of nitric oxyde synthase activity in several brain regions (O'Neill et al., 1995a). Haloperidol and DTG, two "classical" σ ligands devoid of affinity for PCP/NMDA receptors, prevented ischemia-evoked release of glutamate *in vitro* (Keana et al., 1989; Lobner and Lipton, 1990) and attenuated NMDA-induced glutamate release from rat striatal slices (Ryan et al., 1990). Recently, DeCoster et al. (1995) demonstrated the *in vitro* neuroprotective activity of various σ ligands including DTG, (+)SKF 10047, (+)pentazocine, haloperidol, dextromethorphan and carbetapentane. These σ ligands all prevented (with IC_{50}s from 0.81-46.3 μM) glutamate-induced morphological changes and increase in lactate dehydrogenase release in mature cultured rat cortical neurons, with a rank-order of potency correlating with activity at σ_1 sites (DeCoster et al., 1995).

These studies suggest that activating σ receptor *per se* may mediate the neuroprotective effects of certain σ ligands (Taylor et al., 1992). However, this does not preclude the possibility that the neuroprotective effects observed using selective "σ/non-NMDA" receptor ligands were indirectly modulated via an interaction between σ and NMDA receptor systems.

σ-mediated neuroprotection could reflect the effects of various σ ligands on calcium dynamics in brain cells (see section 8.2). Direct support for this hypothesis is the recent finding that maximally neuroprotective concentrations of various σ ligands including the dextrorotatory benzomorphans SKF 10047, pentazocine and cyclazocine,

as well as haloperidol, DTG, carbetapentane and dextromethorphan all modulated glutamate- and/or KCl-induced calcium fluxes in rat cultured cortical neurons (Klette et al., 1995). The effects observed suggest that modulation of neuronal calcium concentrations either through receptor and/or voltage-gated calcium channels, likely contribute to σ ligand-mediated neuroprotection (Klette et al., 1995).

12.2B σ ligand-mediated neurotoxicity

The demonstrated neuroprotective effects of various σ compounds both *in vivo* and *in vitro* is in sharp contrast with the recently reported *in vitro* and *in vivo* neurotoxic activities of some σ compounds. Reduced haloperidol, which has high affinity for σ sites, was shown to induce long-lasting abnormal postural changes when injected into the rat red nucleus. This motor effect was most likely related to the observed gliosis and loss of magnocellular neurons in and around the injection site (Bowen et al., 1990b). Neuroleptics with σ receptor affinity, applied on C6 glioma cells, were reported to cause marked morphological changes resulting in cell death, the rank order of potency of agents causing this effect correlated with their affinity for σ sites (Vilner and Bowen, 1993). More recently, Vilner et al. (1995) reported on the neurotoxic activity of several stucturally unrelated σ ligands on several cultured cell lines. In C6 glioma cells, certain σ ligands induced concentration-dependent changes in cellular morphology such as loss of processes as well as cessation of cell division, within a period as short as 3 to 6 hr. These changes were apparently reversible after cessation of treatment with the σ drug, but cell death occured after longer incubation time (Vilner et al., 1995). However, despite their nanomolar affinity for σ sites, micromolar concentrations ($\geq$30 μM) of σ ligands were required to induced the neurotoxic effects. It was suggested that this discrepancy could result from the reduced

affinity of σ ligands under cell culture conditions (Vilner et al., 1995). Nevertheless, DeCoster et al. (1995) failed to induce neurotoxicity in rat primary cortical cells with high micromolar concentrations of various σ ligands. However, the treatment periods were relatively brief (5 min to 24 hrs) and the experiments were carried out with younger neurons (DeCoster et al., 1995). This latter parameter could be important since it was shown that cultured primary neurons become more sensitive to glutamate-induced toxicity with age (Frandsen and Schousboe, 1990). Additionally, it is likely that σ ligands may be neuroprotective at low doses whereas being neurotoxic at higher doses, or upon longer administration periods. Although it was demonstrated that σ receptors *per se* were involved in σ ligand-induced neurotoxicity, *in vitro*, the mechanisms whereby this occurs remains to be established (Vilner et al., 1995). As it was pointed out, since σ receptors have been identified in a variety of neuronal and non-neuronal human tumors (Bem et al., 1991; Vilner et al., 1995), the cytotoxic property of certain σ compounds could be found useful for treatment of tumors (Vilner et al., 1995).

12.3 Cognition

A possible involvement of σ receptors in learning processes has recently been suggested (Earley et al., 1991; Matsuno et al., 1994; Maurice et al., 1994a,b,c). Using short- and long-term memory paradigms, it was shown that σ ligands that did not affect memory tasks by themselves such as DTG (10-100 μg/kg s.c.; bell-shaped effect), (+)SKF 10047 (100-300 μg/kg), (+)pentazocine (100-300 μg/kg) and PRE-084, attenuated learning impairments induced by systemic injection of the non-competitive NMDA receptor antagonist, MK-801 (100 μg/kg s.c.), in the mouse. This σ-mediated attenuation of learning impairment was prevented by the administration of purported σ

receptor blockers such as NE-100 (1 mg/kg i.p.) or BMY-14802 (10 mg/kg i.p.) (Maurice et al., 1994a,c), as well as by a sub-chronic treatment with haloperidol (4 mg/kg/day for 7 days) for JO-1784 -induced modulatory effects (Maurice et al., 1994c). The modulation of cognitive performance induced by these σ ligands has been attributed to the σ_1 receptor subtype (Maurice et al., 1994a) since the (+) isomers of the benzomorphans SKF 10047 and pentazocine were more potent than the (-) isomers. However, the non selective σ ligand DTG was the most effective drug in antagonizing the memory impairments induced by MK-801 (Maurice et al., 1994a). Thus, the potential involvement of the σ_2 subtype cannot be fully excluded, and the use of selective σ_2 ligands will be necessary in order to determine if this receptor subtype is involved in cognitive processes such as learning and memory. Nevertheless, these series of experiments provided behavioral functional evidence for a modulatory role of σ ligands on NMDA-dependent cognitive processes.

A similar modulation of cognitive performances using short- and long-term memory paradigms was observed on cholinergic nicotinic-dependent memory processes since DTG attenuated mecamylamine (10 mg/kg i.p.) but not scopolamine (1 mg/kg i.p.) -induced amnesia in the mouse (Maurice et al., 1994a). On the other hand, and in contrast to what was observed in mice, pre-administration of DTG (0.25-8 mg/kg), (+)-3-PPP (0.25-4 mg/kg) and JO-1784 (0.25-16 mg/kg), dose-dependently attenuated scopolamine-induced amnesia while rimcazole and (+)SKF 10047 were without significant effect as shown using a passive avoidance paradigm in the rat (Earley et al., 1991). These results suggest that σ ligands may interact with the nicotinic and muscarinic cholinergic systems to modulate cognitive processes in the rodent, and that σ drugs may be useful to enhance cholinergic functions and be of therapeutic value for

treating illnesses with cognitive components associated with cholinergic deficits such as Alzheimer's disease.

Ameliorating effects of σ ligands on memory impairments induced by systemic administration of the 5-HT releaser, p-chloroamphetamine, were also reported (Matsuno et al., 1993b; 1994). DTG, (+)SKF 10047, (+)-3-PPP and racemic pentazocine, significantly reduced p-chloroamphetamine -induced impairment of passive avoidance tasks in mice (Matsuno et al., 1993b; 1994). Furthermore, the ameliorating effect of the selective σ_1 ligand (+)SKF 10047 on p-chloroamphetamine -induced amnesia was blocked by concomitant administration of either scopolamine (a muscarinic receptor antagonist) or hemicholinium-3 (an inhibitor of the Na^+-dependent high-affinity choline uptake site). The authors interpreted these data as an involvement of (+)SKF 10047 with both central σ and cholinergic systems (Matsuno et al., 1994). However, it was not examined if the effects induced by DTG, (+)SKF 10047, (+)-3-PPP and (±) pentazocine were antagonized by purported σ antagonists such as NE-100 or BMY-14802. These additional data would give further support for the possible involvement of σ receptors in p-chloroamphetamine -induced cognitive deficits. However, Matsuno et al. (1995) recently reported data supporting this hypothesis since they have shown that (+)SKF 10047 (5 mg/kg; but not (+)SKF 10047) exerted an anti-amnesic effect against scopolamine (0.75 mg/kg, prior training session) -induced memory impairments in the rat, in addition to elicit a significant increase in hippocampal extracellular acetylcholine level. Furthermore, both effects were reversed by the σ antagonist haloperidol (0.25 mg/kg) (Matsuno et al., 1995) suggesting the existence of interaction(s) between σ (at least σ_1 subtype) receptors and the central cholinergic system(s), *in vivo*.

σ receptor ligands such as DTG and (+)SKF 10047 (μg/kg s.c. dose range) were also shown to reverse carbon monoxide-induced amnesia in the mouse. This phenomenon likely involved σ receptors since concomitant administration of the σ antagonist BMY-14802, which did not modulate cognitive performances by itself, blocked the modulatory effect induced by DTG on carbon monoxyde -induced cognitive impairments (Maurice et al., 1994b). Although difficult to interpret, these data provide additional evidence for the involvement of σ receptors in the modulatory effects induced by various σ ligands in cognitive behaviors.

12.4 Affective disorders

It was proposed that σ receptors may play a role in anxiety and depression (Debonnel et al., 1993; Tam, 1994). Many antidepressant drugs bind σ receptor sites with high affinity. Such drugs include sertraline, opipramol, deprenyl and clorgyline (Debonnel, 1993; Itzhak and Kassim, 1990; Rao et al., 1990c; Schmidt et al., 1989). As it was previously demonstrated for other σ ligands (Monnet et al., 1990a; 1992b), sertraline and clorgyline have also been shown to modulate NMDA-induced neuronal activation of the CA_3 region of the rat hippocampus. The effects induced by both antidepressant drugs were antagonized by haloperidol, supporting the involvement of σ receptors (Bergeron et al., 1993). Furthermore, few functional and behavioral studies argue in favor of the involvement of σ sites in anxiety and depression. In the rat, low doses of BMY- 14802 were found to inhibit stress-related increase in plasma corticosterone levels (Matheson et al., 1991). These studies suggest that certain σ ligands might have potent anxiolytic and antidepressant properties (Tam, 1994).

12.5 Psychosis

σ receptor sites have generated tremendous interest since their identification (Martin et al., 1976) likely because of their postulated involvement in psychiatric disorders such as schizophrenia, and the associated possibility for the development of selective antipsychotic σ compounds (for reviews see Chavkin, 1990; Debonnel, 1993; Deutsch et al., 1988; Ferris et al., 1991a; Itzhak and Stein, 1991a; Junien and Leonard, 1989; Monnet, 1993; Musacchio, 1990; Snyder and Largent, 1989; Su, 1991; 1993; Walker et al., 1990). The postulated role for σ sites in psychosis originally stems from observations that certain benzomorphans with high affinity for σ receptors such as *N*-allylnormetazocine and pentazocine could induce hallucinations in man (Keats and Telford, 1964) and delirium in chronic spinal dog (Martin et al., 1976; Vaupel, 1983). However, early investigations used racemic mixtures of benzomorphan σ ligands raising the possibility that their (-) isomers, which bind opioid but not σ sites (Quirion et al., 1992), were in fact responsible for the psychotomimetic symptoms (Musacchio, 1990). This hypothesis is supported by the observation that the levorotatory isomer of SKF 10047, the prototypical σ ligand widely used in early σ studies, has high affinity for kappa receptors (Pfeiffer et al., 1982), which activation can mediate psychotomimetic-like effects in animals and humans (Pfeiffer et al., 1986). Furthermore, many clinical data concluded that the (-) enantiomer of benzomorphans were responsible for the subjective effects induced by the racemic mixture of these compounds (Belleville and Forrest, 1968; Forrest et al., 1969; Jasinski et al., 1968; 1970; for review see Musacchio, 1990). Moreover, (+)SKF 10047 has significant affinity for the PCP/NMDA receptor in addition to σ sites. Therefore, since PCP receptors were found to mediate various psychotomimetic-like behaviors in animals and humans as well as to exacerbate psychotic symptoms in schizophrenic patients (Allen

and Young, 1978; Domino and Luby, 1981; Showalter et al., 1977; for review see Meltzer, 1991), it appears likely that psychotomimetic symptoms observed in several behavioral studies using this drug were, indeed, mediated via PCP sites. Nevertheless DTG, which displays no apparent affinity for either kappa or PCP sites (Weber et al., 1986), was also found to induce psychotomimetic-like behaviors in the rodent (Contreras et al., 1988). However, the propensity of DTG to induce psychotomimetic-like behaviors have recently been challenged, since it was observed that DTG had motor depressant effects instead of inducing stereotypic behaviors (Ruckert and Schmidt, 1993).

On the other hand, several lines of evidence suggest the involvement of σ sites in psychosis. High densities of σ binding sites are found in various limbic structures recognized for their involvement in psychotic disorders, including the frontal cortex, hippocampus and basal ganglia (Gundlach et al., 1986a; Contreras et al., 1987b; McLean and Weber, 1988). More convincing of a role for σ sites in psychosis is their affinity for some classical (eg. haloperidol and chlorpromazine) and potentially atypical (remoxipride, rimcazole, umespirone, Dup 734) neuroleptics (Ferris et al., 1986; Largent et al., 1988; Tam and Cook, 1984; Snyder and Largent, 1989; Su, 1982; 1986; 1991). In addition, subchronic treatments with haloperidol have been found to decrease σ receptor binding in rodent (Itzhak et Alerhand, 1989; Karbon et Enna, 1991; Kizu et al., 1991; Matsumoto et al., 1989b; 1990; Reynolds et al., 1991) and post-mortem human brain tissues from schizophrenic patients were shown to contain lower levels of σ receptor sites in the cortex and cerebellum (Shibuya et al., 1992; Weissman et al., 1991). A role for σ receptors in psychosis is further suggested by the effects of various σ ligands on acute and chronic administrations of psychostimulants such as cocaine and metamphetamine. For example, BMY-14802 and rimcazole significantly attenuated the

hyperlocomotion induced by cocaine in mice (Menkel et al., 1991) and prevented the development of sensitization to cocaine and metamphetamine in a dose-related manner (Ujike et al., 1992a; 1996). Furthermore, sensitization to cocaine or metamphetamine induced a supersensitivity to (+)-3-PPP administration (Ujike et al., 1992b,c) possibly by modulating σ systems (Ujike et al., 1992b,c).

Thus, in spite of numerous indications in favor of the involvement of σ receptors in psychotic disorders, the genuine σ nature of these effects remains to be fully established (Musacchio; 1990; Walker et al., 1990). Nevertheless, the fact that σ receptors may not be involved in the etiology of psychosis does not necessarily preclude the usefulness of certain σ ligands as potential therapeutic agents (Musacchio, 1990).

GENERAL OBJECTIVES

Over the last decade, our knowledge about σ receptors has greatly improved. However, the identification of the endogenous σ ligand(s) is still yet to be accomplished. In 1989, it was suggested that NPY and PYY could act as endogenous σ ligands since Roman and colleagues observed that these peptides could potently inhibit the binding of the prototypical σ ligand, [^{3}H](+)SKF 10047, in rat brain membrane homogenates, *in vitro*. These findings generated tremendous interest, but attempts to replicate these data remained unsuccessful (see section 10.4). Accordingly, the primary objective of the present thesis was to investigate the potential interaction(s) between NPY-related peptides and σ receptors *in vivo*. This goal was accomplished using *in vivo* binding assays (Chapter II) and autoradiography (Chapter III) in the mouse brain. In addition, CGRP-related peptides were included in the thesis since we observed, during a specificity study, that CGRP could interact with σ receptors in a manner similar to NPY. We thus investigated the potential interaction between σ receptors and various CGRP-related peptides using *in vivo* binding assays and autoradiography, and electrophysiological experiments (Chapters III and IV). Finally, we examined the behavioral relevance of these *in vivo* interactions by investigating the effects of various peptides of the NPY and CGRP families on MK-801 -induced learning impairments in the mouse (Chapter V). Taken together, these various *in vivo* approaches allowed us to demonstrate the existence of interactions between σ receptors and NPY- and CGRP-related peptides in the brain, as well as to identify a "peptide-selectivity profile" for these interactions.

Chapter II

EVIDENCE FOR *IN VIVO* INTERACTIONS BETWEEN NEUROPEPTIDE Y-RELATED PEPTIDES AND SIGMA RECEPTORS IN THE MOUSE HIPPOCAMPAL FORMATION

Preface to chapter 2.

This first manuscript address the question of the possibility of the existence of interaction(s) between neuropeptide Y (NPY)-related peptides and σ receptors in the brain. Although evidence for the existence of endogenous σ ligand(s) has accumulated, its identification is yet to be reported. NPY and PYY were suggested as potential endogenous σ ligands since they were reported to have nanomolar affinity for [^{3}H](+)SKF 10047/σ sites in crude rat brain membrane homogenates (Roman et al., 1989). However, several attempts to replicate these *in vitro* findings by independant laboratories remained unsuccessful (Quirion et al., 1991; Tam et al., 1991). In order to investigate the possibility of interaction between σ receptors and peptides of the NPY family we undertook a series of *in vivo* binding experiments. Using this approach, we were able to confirm the ability of selected NPY-related peptides to interact with σ receptor sites *in vivo* since NPY, PYY, [Leu^{31}Pro^{34}]NPY and NPY$_{2\text{-}36}$ significantly and selectively competed for [^{3}H](+)SKF 10047/σ binding sites in the mouse hippocampal formation.

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EVIDENCE FOR *IN VIVO* INTERACTIONS BETWEEN NEUROPEPTIDE Y-RELATED PEPTIDES AND SIGMA RECEPTORS IN THE MOUSE HIPPOCAMPAL FORMATION

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ABSTRACT

Recently, it was proposed that neuropeptide Y (NPY) and peptide YY (PYY) could act as endogenous ligands for sigma (σ) binding sites, as both NPY and PYY competed with high affinity (nM) for [^{3}H](+)SKF 10,047 binding sites in rat brain membrane homogenates (Roman et al., Eur. J. Pharmacol., 174, 301-302, 1989). However, various laboratories failed to replicate these *in vitro* findings. In order to clarify this apparent discrepancy and investigate further possible interactions between NPY and σ related sites, we evaluated the effects of NPY, PYY and homologues, as well as non-related peptides, on *in vivo* [^{3}H](+)SKF 10,047 binding parameters in the mouse hippocampal formation. As expected, haloperidol (2mg/kg), a prototypical σ receptor ligand, competed for 90% of *in vivo* hippocampal labelling observed following a peripheral intravenous injection of [^{3}H](+)SKF 10,047. Intracerebroventricular (i.c.v.) injections of 300 to 3000 pmoles of either NPY, PYY, NPY_{2-36} and the Y_1 agonist [Leu^{31},Pro^{34}]-NPY, inhibited significant proportions (17-35%) of haloperidol-sensitive *in vivo* [^{3}H](+)SKF 10,047 hippocampal labeling. However, a Y_2 receptor agonist, NPY_{13-36}, and non-related peptides such as neurotensin and VIP, as well as adrenalin, failed to alter *in vivo* [^{3}H](+)SKF 10,047 hippocampal binding. It thus appears that NPY, PYY and a selective Y_1 agonist can interact in a concentration-dependant manner, with *in vivo* [^{3}H](+)SKF 10,047 labelling in the mouse hippocampal formation. This effect demonstrates selectivity as a Y_2 agonist, unrelated peptides and adrenalin failed to alter *in vivo* σ labelling. This *in vivo* interaction may be relevant to some of the respective biological actions of NPY and σ-related molecules.

INTRODUCTION

The existence of sigma (σ) receptor binding sites in the brain has generated much interest over the past few years likely because of their possible involvement in psychosis-related behaviors (for recent reviews see Deutsch et al., 1988; Junien and Leonard, 1989; Snyder and Largent, 1989; Chavkin, 1990; Musacchio, 1990; Walker et al., 1990; Ferris et al., 1991; Itzhak and Stein, 1991a; Su, 1991). This assumption is mostly based on the fact that high densities of σ binding sites are found in various limbic structures, including the hippocampus (Gundlach et al., 1986; Contreras et al., 1987b; McLean and Weber, 1988), and to the high affinity of some neuroleptics (eg. haloperidol and remoxipride, but not sulpiride) for σ sites (Su, 1982; 1991; Tam and Cook, 1984; Taylor and Dekleva, 1987). This suggested the possible development of a new class of σ-related antipsychotic drugs devoid of dopaminergic activity (Su, 1986; Taylor and Dekleva, 1987; Snyder and Largent, 1989). Additional support for this hypothesis came from observations suggesting that certain σ drugs such as N-allylnormetazocine (NANM or SKF 10,047) induced hallucinations in man (Keats and Telford, 1964) and delirium in chronic spinal dog (Martin et al., 1976; Vaupel, 1983); although the genuine σ nature of these effects remains to be fully established (Quirion et al., 1987; Walker et al., 1990).

The abundance and discrete distribution of σ sites in the mammalian brain may also reveal the possible existence of endogenous σ-related ligands. Early on, few groups (Quirion et al., 1984; Su et al., 1986; Contreras et al., 1987a; Su and Vaupel, 1988; Zhang et al., 1988; Connor and Chavkin, 1991; 1992) reported on the possible isolation of such molecules altough their purification to homogeneity still remains to be

accomplished. Thus, the report by Roman et al. (1989) suggesting that peptides of the pancreatic polypeptide family, namely neuropeptide Y and polypeptide YY, were able to compete with high affinities for *in vitro* [^{3}H](+)SKF 10,047/σ binding sites generated tremendous interests. However, it has been difficult to replicate these findings using well established *in vitro* membrane binding assays (Quirion et al., 1991; Tam and Mitchell, 1991).

On the other hand, evidence has recently accumulated supporting the existence of interactions between NPY and σ-related systems, *in vivo*. For exemple, Monnet et al., (1990a; 1990b; 1992a; 1992b; 1992c) have shown that both NPY and σ agonists, in a haloperidol-sensitive manner, are able to potentiate N-methyl-D-aspartate (NMDA)-induced firing in the pyramidal CA_3 sub-field of the hippocampal formation (but see McQuiston and Colmers, 1991; Connick et al., 1992). Additionally, NPY and a selective σ ligand, JO 1784, can decrease intestinal transport apparently via haloperidol-sensitive σ receptors (Rivière et al., 1990). Moroever, it appears that NPY and homologues, as well as various σ ligands, are able to potentiate NMDA-induced [^{3}H]noradrenaline release in rat hippocampal slices (Monnet et al., 1991; Roman et al., 1991). Accordingly, we decided to investigate further possible interactions between NPY-related peptides and σ receptor sites in the mouse hippocampus using an *in vivo* binding approach; the hippocampal formation being chosen as this region is most enriched with both σ (Gundlach et al., 1986; Contreras et al., 1987b; McLean and Weber, 1988; Weissman et al., 1988; 1990) and NPY (Martel et al., 1986; 1990a; Lynch et al., 1989; Dumont et al., 1990; 1992a; 1992b) receptors. Our results suggest that NPY, PYY and a selective agonist of the Y_1 receptor sub-type, can interact with a certain proportion of *in vivo* [^{3}H](+)SKF 10,047 labelling in the mouse hippocampus.

MATERIALS & METHODS

Materials:

[^{3}H](+)SKF 10,047 (53 Ci/mmol; Commission Energie Atomique, Saclay, France) was provided by the Institut de Recherches Jouveinal, Fresnes, France. All NPY-related peptides as well as neurotensin and vasoactive intestinal polypeptide (VIP) were synthesized in our laboratories as described earlier (St-Pierre et al., 1981; Martel et al., 1990b). Adrenalin, haloperidol, trizma base (Tris), NaCl and polyethyleneimine (PEI) were purchased from Sigma Chemicals (St-Louis, MI, USA). MK-801 was from Research Biochemicals Inc. (Natick, MA, USA), Ether and GF/B Whatman filters were obtained from Fisher (Montréal, Canada) and Ecolite TM+ was purchased from ICN Biochemicals (Montreal, Canada).

Animals:

Male CD-1 mice (20-24 g.) were purchased from Charles River (St-Constant, Québec, Canada). Animals were housed in a temperature and humidity controlled room on a 12 hour light/dark cycle, and were fed *ad libitum* with standard laboratory chow and tap water. They were kept under these conditions for at least 24 hours prior to the experiment. Animal care was according to protocols and guidelines approved by McGill University and the Canadian Council for Animal Care.

Binding experiments:

For *in vivo* binding experiments, mice were injected at time (t)=0 with 2 mg/kg haloperidol (100 µl i.p.), MK-801 (0.05, 0.1, 0.2 or 0.5 mg/kg 100 µl i.p.) or saline

(0.9% NaCl, 100 µl i.p.) in order to determine the proportion of *in vivo* [^{3}H](+)SKF 10,047 labelling which can be related to σ sites. At t=15 min., under light ether anesthesia, animals received either a peptide injection (3 µl i.c.v.) or saline (3 µl i.c.v.). Fifteen minutes later (t=30 min.), animals were injected in the tail vein with 5 µCi [^{3}H](+)SKF 10,047 (200 µl i.v.). Animals were sacrificed by dislocation at t=60 min. Brains were then rapidly removed and hippocampi dissected on ice and immediately homogenized in 1 ml ice-cold buffer (Tris-HCl 5 mM, pH 7.4 at 4°C) using a teflon/glass Potter-Elvehjem probes (12 strokes). Immediately thereafter, 1 ml of ice-cold Tris-HCl buffer was added to this homogenate before filtration of 1.5 ml of this preparation through three GF/B glass filters (pretreated for at least 24 hours in buffer containing 0.05% PEI) under vacuum using a twelve holes Millipore CAT apparatus. Filters were then washed twice with 5 ml ice-cold incubation buffer and then placed in vials containing 5 ml Ecolite TM+ scintillation cocktail. Radioactivity was determined using a Beckman LS1800 counter with 40% efficiency. The remaining 0.5 ml of the homogenate was kept for protein determination (Lowry et al., 1951). Statistical analyses were performed using the Student-t test; $p<0.05$ being considered statistically significant.

RESULTS

In preliminary experiments we failed to obtain evidence for *in vitro* interactions between NPY, PYY and [^{3}H](+)SKF 10,047/σ sites as the two peptides, in concentrations ranging from 10^{-11} to 10^{-6} M, did not compete for *in vitro* [^{3}H](+)SKF 10,047 binding in mouse brain membrane preparations. These findings confirmed data obtained earlier in the rat brain (Quirion et al., 1991; Tam and Mitchell, 1991).

In vivo [^{3}H](+)SKF 10,047 binding in the mouse hippocampus:

An equivalent of approximately 15 fmol/mg of protein was detected in the mouse hippocampus following an injection in the tail vein of 5 μCi of the σ ligand, [^{3}H](+)SKF 10,047. In the presence of haloperidol (2mg/kg i.p.) residual [^{3}H](+)SKF 10,047 hippocampal binding accounted for only 1.48 fmol/mg of protein, revealing that approximately 90% (13.52 fmol/mg protein) of total labelling was sensitive to the prototypical σ ligand (Fig. 1). The NMDA/PCP receptor ligand MK-801 did not inhibit the specific [^{3}H](+)SKF 10,047 labelling in the mouse hippocampal formation at doses of 0.05, 0.1, 0.2 and 0.5 mg/kg i.p. (n=26; data not shown).

***Competition by NPY-related peptides*:**

As shown in Figure 3, NPY, NPY_{2-36}, [Leu^{31},Pro^{34}]-NPY (a purported Y_1 agonist; Fuhlendorff et al., 1990) and PYY were able to displace approximately one-third of specific *in vivo* [^{3}H](+)SKF 10,047 labelling in the mouse hippocampus. The fragment NPY_{13-36} (a Y_2 agonist; Wahlestedt et al., 1986) and the analogue desamido-NPY (NPY-OH) were inactive. As shown in Fig. 2, various doses of NPY (300, 600,

1000, 1500 and 3000 pmol, i.c.v.) demonstrated that this peptide inhibited the *in vivo* hippocampal labelling of [^{3}H](+)SKF 10,047 in a dose-related manner. The minimal effective dose appears to be in the range of 600 pmol while a maximal effect was observed at 1500 pmol; a higher dose of NPY (3000 pmol) failing to produce a greater effect (Fig. 2).

The respective potency of various NPY-related peptides to inhibit *in vivo* [^{3}H](+)SKF 10,047 labelling in the mouse hippocampus is shown in Fig. 3. At doses of 1500 and 3000 pmol, the homologue PYY most potently inhibited specific [^{3}H](+)SKF 10,047 labelling, its effects being apparently even greater than that of NPY. In contrast, NPY-OH failed to inhibit [^{3}H](+)SKF 10,047 labelling (Fig. 3a) as expected for this biologically inactive analogue (Wahlestedt et al., 1986).

The fragment NPY_{2-36} was able to mimic the effect of the full NPY molecule on [^{3}H](+)SKF 10,047 labelling (Fig. 3b). However a shorter C-terminal fragment, NPY_{13-36}, a purported Y_2 agonist (Wahlestedt et al., 1986) failed to alter [^{3}H](+)SKF 10,047 labelling at 1500 pmol (Fig. 3b). On the other hand, the Y_1 agonist [Leu^{31},Pro^{34}]-NPY, was the most potent competitor of *in vivo* [^{3}H](+)SKF 10,047 labelling tested in this study with a profile similar to that of NPY, namely a dose of 1500 pmol inducing a greater effect than that of 3000 pmol (Fig. 2 and 3b).

Specificity of NPY for in vivo hippocampal [^{3}H](+)SKF 10,047 labelling:

As a mean to assess the specificity of the effect of NPY and related analogues for *in vivo* [^{3}H](+)SKF 10,047 labelling, we investigated next the effects of two other classes of peptides, namely neurotensin (1500 pmol) and VIP (1500 pmol), and of the well known vasoconstrictor/neurotransmitter, adrenalin (1500 and 3000 pmol). These three molecules failed to alter *in vivo* [^{3}H](+)SKF 10,047 labelling in the mouse hippocampus at doses tested here (Fig. 4).

DISCUSSION

The present study demonstrates that 1) NPY and its homologue PYY dose-dependently inhibited the *in vivo* binding of the prototypical σ ligand [^{3}H](+)SKF 10,047 in the mouse hippocampal formation, 2) a similar effect is also seen with a Y_1 ([Leu^{31},Pro^{34}]-NPY) but not a Y_2 (NPY_{13-36}) receptor agonist suggesting receptor sub-type specificity, 3) a biologically inactive NPY analogue, NPY-OH, failed to alter *in vivo* [^{3}H](+)SKF 10,047 labelling and 4) other biologically active polypeptides such as neurotensin and VIP, which are known to induce various cerebrovascular and neuronal effects (Rioux et al., 1981; Wilson et al., 1981), and adrenalin failed to modulate *in vivo* [^{3}H](+)SKF 10,047 labelling, demonstrating the specificity of the interactions between certain NPY-related peptides and *in vivo* [^{3}H](+)SKF 10,047 binding in the mouse hippocampus.

The hippocampal labelling observed following an intravenous injection of [^{3}H](+)SKF 10,047 is very likely representing binding to σ sites which are most abundant in this region (Largent et al., 1986; Contreras et al., 1987b; Weissman et al., 1990). While (+)SKF 10,047 can bind with relatively high affinities to both phencyclidine (PCP) and σ sites (Su, 1982; Largent et al., 1986; Quirion et al., 1987; McCann et al., 1989), only the σ component is sensitive to haloperidol (Su, 1982; Tam and Cook, 1984). Moroever, four different doses (0.05, 0.1, 0.2 and 0.5 mg/kg i.p., n=26) of the high affinity NMDA/PCP receptor ligand, MK-801 (Loo et al., 1987), did not reduce the specific *in vivo* [^{3}H](+)SKF 10,047 labelling in the mouse hippocampus. Thus, the high sensitivity of *in vivo* hippocampal [^{3}H](+)SKF 10,047 labelling to haloperidol together with the ineffectiveness of MK-801 to inhibit the specific [^{3}H](+)SKF 10,047 labelling in the mouse hippocampus *in vivo*, confirms the σ nature

of the related sites. Our results also extent previously reported *in vivo* [^{3}H](+)SKF 10,047/σ-related binding data (Ferris et al., 1988; Tam et al., 1988; Weissman et al., 1990). However, it is unclear if, under the present assay conditions, *in vivo* [^{3}H](+)SKF 10,047 labelling represents binding to more than one sub-type of σ sites. Recently, evidence has been obtained to support the existence of at least two classes (σ_1 and σ_2) of σ sites (Hellewell and Bowen, 1990; Itzhak and Stein, 1991a; 1991b; Itzhak et al., 1991; Su et al., 1991; Quirion et al., 1992; Vilner and Bowen, 1992). These two putative sub-types demonstrate similar affinity for haloperidol, while (+)SKF 10,047 apparently preferentially labels the σ_1 sites (Hellewell and Bowen., 1990; Itzhak and Stein, 1991b; Itzhak et al., 1991; Su et al., 1991; Quirion et al., 1992; Vilner and Bowen, 1992), although it can also possibly bind to the σ_2 site at concentrations comparable to that used in the present study (5 μCi i.v.). Additional experiments using other σ-related radioligands such as [^{3}H](+)pentazocine (σ_1) and [^{3}H]DTG (in presence of a saturating concentration of dextrallorphan) for σ_2 sites (DiPaolo et al., 1991; Bowen et al., 1992) will be required to clarify this issue. Already, in a series of preliminary experiments, we attempted to investigate the *in vivo* labelling of the mouse hippocampus following intravenous injections of either [^{3}H]DTG or [^{3}H](+)pentazocine. However, in contrast to [^{3}H](+)SKF 10,047, the amounts of radioactivity recovered in the hippocampal formation using either probes were too low to permit adequate pharmacological characterization (150 to 500 DPM per 5 μCi). Moreover, *in vivo* [^{3}H]DTG labelling was apparently mostly restricted to ventricular epithelia as revealed by *in vivo* autoradiography (Bouchard et al., unpublished results). Thus, it appears that other radioligands will have to be developed in order to adequately investigate *in vivo* binding profiles of σ_1 and σ_2 sites in the mouse brain.

The extent of the competition seen between [^{3}H](+)SKF 10,047 and NPY-related peptides following their respective *in vivo* injections also supports the possible labelling by the radioligand of two classes of σ sites. While haloperidol competed for up to 90% of [^{3}H](+)SKF 10,047 labelling in the mouse hippocampus, i.c.v. injections of NPY and its homologues inhibited approximately one-third of the labelling suggesting that [^{3}H](+)SKF 10,047 could interact with sub-types of σ sites; only one being accessible to NPY and related peptides.

The precise nature of *in vivo* interactions between [^{3}H](+)SKF 10,047 labelling and NPY remains speculative. It clearly displays specificity as two other families of well known peptides, neurotensin and VIP, failed to alter *in vivo* [^{3}H](+)SKF 10,047 binding under our assay conditions. Similarly, the effects of NPY-related peptides are unlikely to be only related to changes in cerebral blood flow as adrenalin, a potent constrictor of the cerebral vasculature, as well as neurotensin and VIP, two peptides with potent cerebrovascular activites (Rioux et al., 1981; Wilson et al., 1981), failed to modify *in vivo* [^{3}H](+)SKF 10,047 labelling of the mouse hippocampus.

We (Quirion et al., 1991) and others (Tam and Mitchell, 1991) were unable to obtain clear evidence for the existence of *in vitro* receptor binding interactions between a variety of σ and NPY-related molecules. Interestingly, various peptidase inhibitors are generally used in *in vitro* binding assays to insure the integrity of NPY peptides used either as radioligands and/or competitors (Lynch et al., 1989; Martel et al., 1990a; 1990b; Quirion et al., 1991). Could it be that an endogenously-generated NPY metabolite is able to act as a σ ligand *in vivo*? While clear evidence are currently lacking to support this hypothesis, Contreras et al. (1987a) proposed the existence of endogenous σ ligands of peptidergic nature. It is thus of interest that only certain NPY-related peptides were able to modulate the *in vivo* labelling of [^{3}H](+)SKF 10,047 in the

mouse hippocampus. Both desamido-NPY (NPY-OH) and the relatively selective Y_2 agonist, NPY_{13-36} (Wahlestedt et al., 1986) failed to demonstrate any activity in this assay. In contrast NPY, its homologue PYY, the fragment NPY_{2-36} and the Y_1 agonist [Leu^{31},Pro^{34}]-NPY (Fuhlendorf et al., 1990) behaved as potent competitors. Taken together, these data may suggest that if a metabolite(s) of NPY is responsible for the inhibition of [^{3}H](+)SKF 10,047/σ labelling, it could be a N-terminal fragment likely containing amino-acid residue located between position 2 and 13. Alternatively, it could be that NPY and some of it's related peptides can induce the release of a yet to be characterized endogenous σ ligand which could then inhibit the *in vivo* binding of [^{3}H](+)SKF 10,047. Recent data by Connor and Chavkin (1991, 1992) are especially interesting in that regard as they provide evidence for the existence of endogenous σ ligands in the hippocampus. It is also possible that NPY-related peptides, by modulating signal transduction activity, could alter *in vivo* [^{3}H](+)SKF 10,047/σ labelling. These various possibilities are currently under investigation.

The ligand selectivity profile observed here for NPY and its homologues is most reminescent of a Y_1-like profile (Wahlestedt et al., 1986; 1990; Fuhlendorff et al., 1990; Quirion et al., 1990; Dumont et al., 1992a) on the basis of the potent activity of the Y_1 analogue [Leu^{31},Pro^{34}]-NPY and the ineffectiveness of NPY_{13-36}, a Y_2 agonist (Wahlestedt et al., 1986), to modulate hippocampal *in vivo* [^{3}H](+)SKF 10,047 labelling. The high potency of PYY in this assay would also exclude an interaction with the newly characterized Y_3 receptor sub-type as PYY was reported to be inactive on this receptor class (Balasubramanian et al., 1990; Grundemar et al., 1991; Wahlestedt et al., 1992). While it is certainly too early to claim the existence of possible structural homologies between NPY and σ receptor classes, the pattern of *in vivo* interactions observed in the present study, and those of others (Monnet et al., 1990a, 1990b) raises

interesting possibilities as the various NPY receptor sub-types (Rimland et al., 1991; Herzog et al., 1992; Larhammar et al., 1992) and likely the σ_1 sites (Itzhak and Stein, 1991b; Itzak et al., 1991; Bowen at al., 1992; Quirion et al., 1992) could all belong to the G-protein coupled rhodopsin receptor super family.

In summary, the present study revealed the existence of *in vivo* interactions between certain NPY-related peptides and [^{3}H](+)SKF 10,047/σ labelling in the mouse hippocampus. Further studies are currently in progress to determine the mechanism(s) responsible for these interactions and the possible functional significance of these findings.

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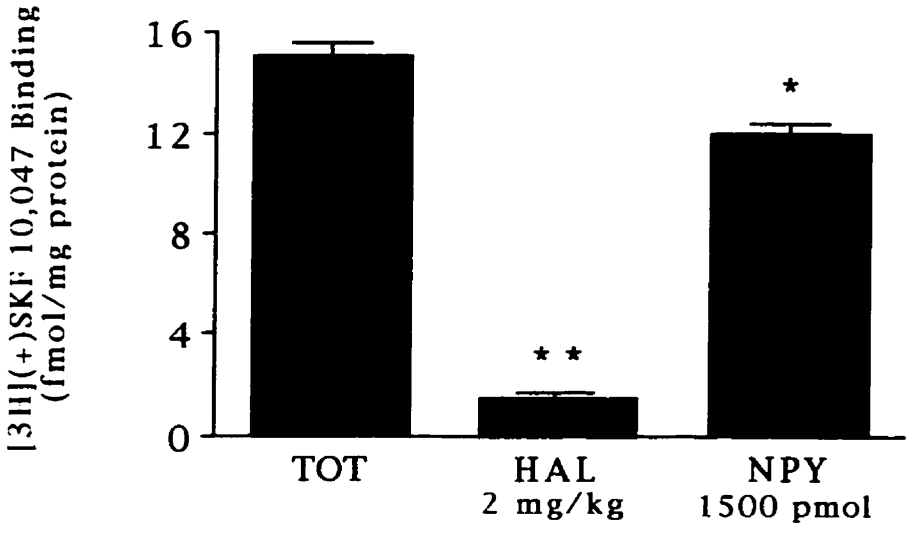

Figure 1. [^{3}H](+)SKF 10,047 related radioactive material present in the mouse hippocampal formation following a peripheral i.v. injection (5 μCi per 200 μl, tail vein, Total, n=51). [^{3}H](+)SKF 10,047 binding is most likely related to σ sites as haloperidol (2 mg/kg i.p., n=11) almost completely inhibited (90%) labeling in the mouse hippocampus. Similarly, although to a lesser extent, i.c.v. injections of NPY (1500 pmol, n=23) significantly inhibited hippocampal [^{3}H](+)SKF 10,047 labeling. *$p<0.005$ **$p<0.001$

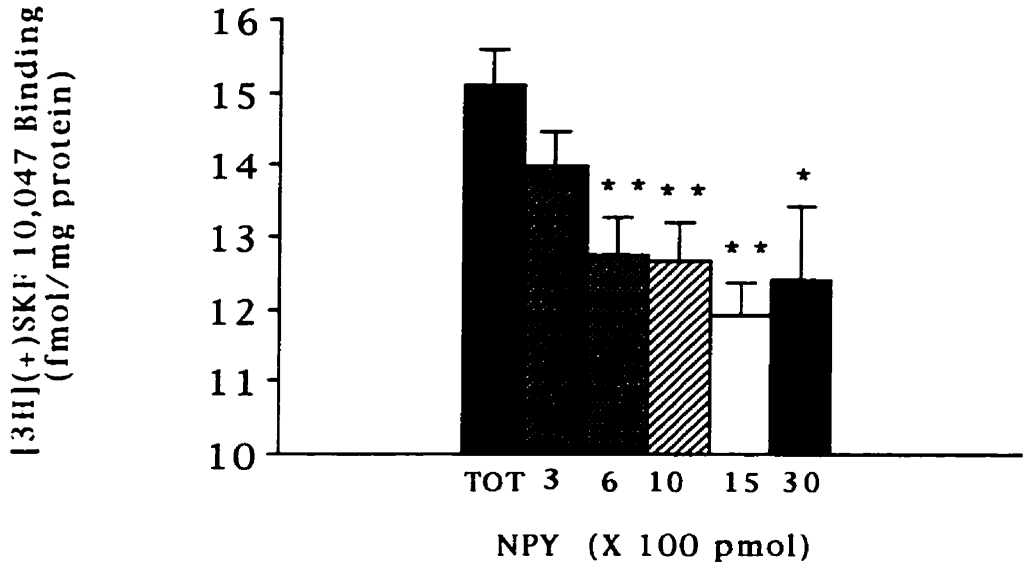

Figure 2. Dose-dependancy demonstration of the effect of NPY on *in vivo* [^{3}H](+)SKF 10,047 labeling of the mouse hippocampal formation. I.c.v. injections of increasing doses of NPY significantly inhibited *in vivo* [^{3}H](+)SKF 10,047 labeling (Total, n=51) with a minimally effective dose in the range of 600 pmol (n=23), a lower dose (300 pmol, n=25) inhibited binding to a non-significant extent. 1000 pmol (n=23) produced a greater inhibition than that of 600 pmol while maximal effectiveness was observed at 1500 pmol (n=23); 3000 pmol (n=11) being somewhat less effective. *$p<0.05$ **$p<0.005$

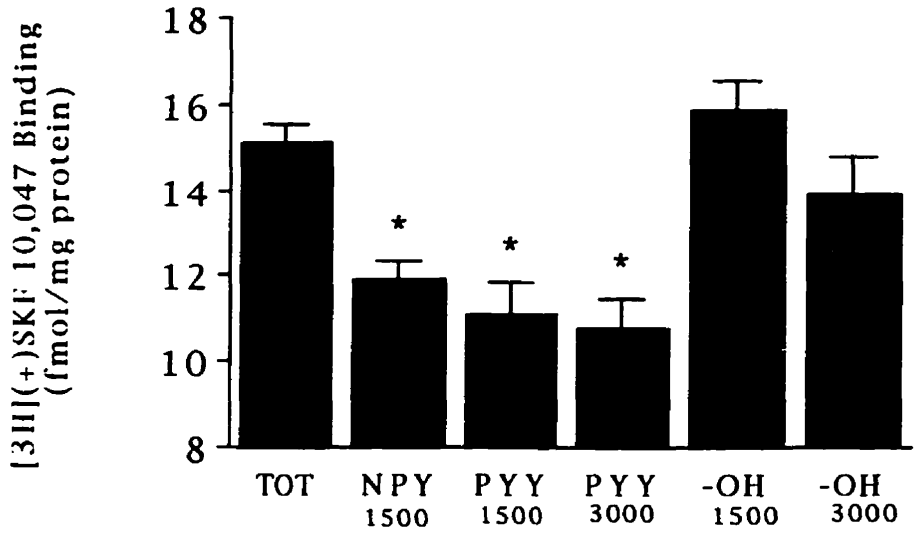

Figure 3A. Comparative potencies of NPY, PYY and desamido-NPY (NPY-OH) to inhibit *in vivo* [^{3}H](+)SKF 10,047 labeling (Total, n=51) in the mouse hippocampal formation. PYY at 1500 pmol (n=13) and 3000 pmol (n=8) was slightly more potent than NPY at 1500 pmol (n=23), to inhibit labeling while NPY-OH at 1500 pmol (n=7) and 3000 pmol (n=13) failed to produce any significant effect. *$p<0.005$

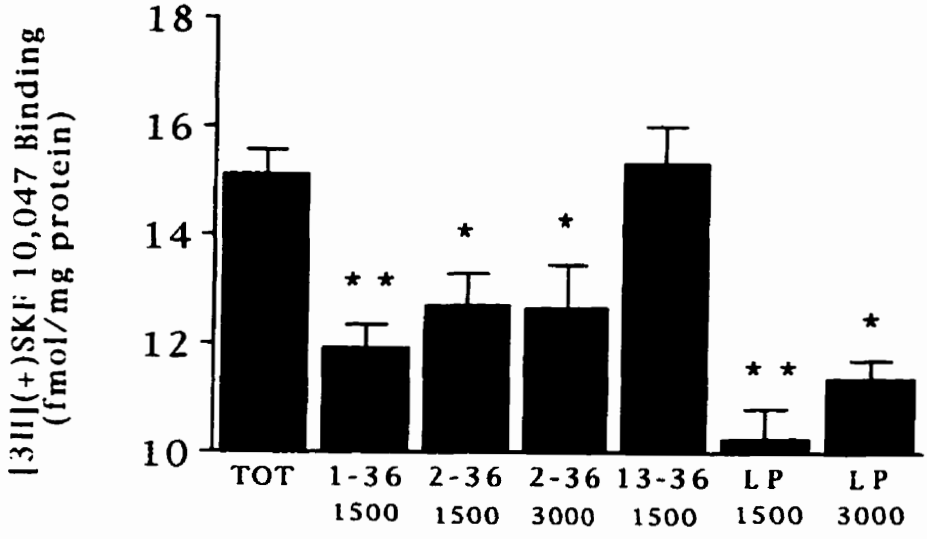

Figure 3B. Comparative potencies of NPY_{1-36}, C-terminal fragments (NPY_{2-36} and NPY_{13-36}) and the analogue [$Leu^{31}Pro^{34}$]-NPY (LP) to inhibit *in vivo* [^{3}H](+)SKF 10,047 labeling (Total, n=51) in the mouse hippocampal formation. NPY_{2-36} at 1500 pmol (n=20) and 3000 pmol (n=10) significantly inhibited [^{3}H](+)SKF 10,047 binding albeit to a lower extent than the full peptide, at 1500 pmol (n=23). A prototypical Y_2 fragment, NPY_{13-36} (1500 pmol, n=14), failed to exert any effect on [^{3}H](+)SKF 10,047 labeling, while the Y_1 agonist [$Leu^{31}Pro^{34}$]-NPY potently competed for labeling at 1500 pmol (n=14) and 3000 pmol (n=7). *$p<0.05$ **$p<0.005$

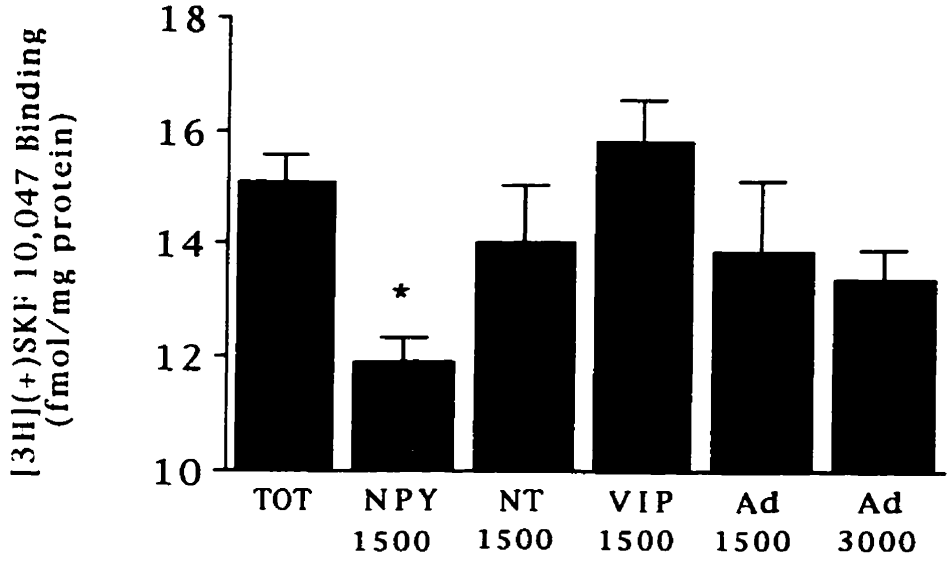

Figure 4. Comparative effects of NPY (n=23), neurotensin (NT; 1500 pmol, n=9) and vasoactive intestinal polypeptide (VIP; 1500 pmol, n=17), and the catecholamine adrenalin (Ad; 1500 pmol, n=7 and 3000 pmol, n=11) on *in vivo* [^{3}H](+)SKF 10,047 labeling (Total, n=51) in the mouse hippocampal formation. At this concentration, it is evident that only NPY significantly interacted with *in vivo* [^{3}H](+)SKF 10,047 labeling. *p<0.005

Chapter III

IN VIVO MODULATION OF SIGMA RECEPTOR SITES BY CALCITONIN GENE-RELATED PEPTIDE IN THE MOUSE AND RAT HIPPOCAMPAL FORMATION: RADIOLIGAND BINDING AND ELECTROPHYSIOLOGICAL STUDIES

Preface to chapter 3.

In our first paper we demonstrated that selected NPY-related peptides could interact with σ receptors as they competed for *in vivo* [^{3}H](+)SKF 10047/σ binding in the mouse hippocampal formation. While determining the specificity of these effects, we observed that calcitonin gene-related peptide (rCGRPβ), which was first used as a control in our paradigm, also significantly competed for *in vivo* [^{3}H](+)SKF 10047/σ binding. Consequently, the aim of this second series of experiments was to investigate the possibility of *in vivo* interactions between σ receptors and various CGRP-related peptides using membrane binding assays and electrophysiological recordings. This second paper describes the effects of selected CGRP-related peptides on *in vivo* [^{3}H](+)SKF 10047/σ binding in the mouse hippocampal formation and on NMDA-induced activation of CA_3 pyramidal cells of the rat hippocampus. The present manuscript reported, for the first time, the existence of interactions between σ receptors and certain peptides of the CGRP family.

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IN VIVO MODULATION OF SIGMA RECEPTOR SITES BY CALCITONIN GENE-RELATED PEPTIDE IN THE MOUSE AND RAT HIPPOCAMPAL FORMATION: RADIOLIGAND BINDING AND ELECTROPHYSIOLOGICAL STUDIES

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ABSTRACT

Possible interactions between sigma (σ) receptor sites and calcitonin gene-related peptides (CGRP) were investigated using receptor subtypes-related analogues and fragments on *in vivo* [^{3}H](+)SKF 10,047/σ binding in the hippocampus, and electrophysiological recording of the NMDA-induced activation of CA_3 pyramidal neurons, two well-established σ assays (Monnet et al., 1992a; Bouchard et al., 1993). In both paradigms, CGRP and the agonist [Cys(ACM)^{2,7}]hCGRPα, modulated σ systems. *In vivo* binding experiments demonstrated that CGRP and [Cys(ACM)^{2,7}]hCGRPα inhibited 25% to 40% of specific [^{3}H](+)SKF 10,047 labeling in the mouse hippocampal formation while the purported antagonist, $hCGRP_{8-37}$ was inactive. The specificity of this modulation was demonstrated further by the lack of effect of other vasoactive peptides including the atrial natriuretic peptide, substance P and its N-terminal fragment, substance P_{1-7}. In the CA_3 subfield of the rat dorsal hippocampus, hCGRPα decreased (up to 61%) the NMDA-induced activation of the pyramidal neurons. Conversely, the linear analogue [Cys(ACM)^{2,7}]hCGRPα enhanced (by 85%) the NMDA-induced activation of CA_3 pyramidal neurons while the antagonistic fragment $hCGRP_{8-37}$ had no effect. Haloperidol, a high affinity σ receptor ligand, inhibited by 90% the *in vivo* [^{3}H](+)SKF 10,047 labeling, and prevented the modulation of the NMDA-induced activation by hCGRPα and [Cys(ACM)^{2,7}]hCGRPα. It thus appears that CGRP can modulate σ-related systems in the hippocampal formation.

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide generated from the alternate tissue-specific splicing of the calcitonin gene (Amara et al., 1982). The widespread distribution of CGRP in the central and peripheral nervous systems has been demonstrated using immunohistochemical approaches and *in situ* hybridization (for reviews: Ishida-Yamamoto and Toyama, 1989; Poyner, 1993). The existence of high affinity CGRP binding sites has been demonstrated in various brain areas using both membrane binding assays and autoradiographic techniques (for reviews: Quirion et al., 1992b; Poyner, 1993). At least two CGRP receptor subtypes have been identified on the basis of the differential potencies of agonists and antagonists in a variety of *in vitro* and *in vivo* functional assays (Dennis et al., 1989; Quirion et al., 1992b; Poyner, 1993). CGRP and its homologues have been reported to induce vasodilation and cardiac acceleration, as well as to modulate nociceptive parameters, to decrease food intake and spontaneous motor activity (Ishida-Yamamoto and Toyama, 1989; Jolicoeur et al., 1992). Neurochemically, CGRP has been shown to potently modulate dopaminergic neurotransmission in limbic structures likely involved in various neurological and psychiatric disorders (Drumheller et al., 1992).

Sigma (σ) ligands, by acting on specific receptor sites, represent another class of molecules that have been shown to modulate dopaminergic neurotransmission and possibly play a role in psychiatric disorders such as schizophrenia (for reviews see Deutsch et al., 1988; Junien and Leonard, 1989; Snyder and Largent, 1989; Walker et al., 1990; Itzhak and Stein, 1991a; Debonnel, 1993; Su 1993). Interestingly, there is evidence suggesting the existence of interactions between σ receptors and neuropeptide

Y (NPY)-related peptides (Monnet et al., 1992a-c; 1994; Rivière et al., 1990; Roman et al.,1993; Bouchard et al.,1993).

In order to assess the specificity of these interactions, various other peptides were tested (Bouchard et al., 1993). While most were inactive, we observed that intracerebroventricular injections of CGRP potently inhibited *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus. Furthermore, CGRP-related peptides apparently failed to directly compete for *in vitro* σ binding site, (see Table 1). This prompted us to investigate the existence of possible interactions between CGRP and σ receptor sites using two complementary approaches, namely *in vivo* receptor labeling and unitary recording in the hippocampus, a limbic region enriched with σ receptor sites (Gundlach et al., 1986; Contreras et al., 1987b; McLean and Weber, 1988; Weissman et al., 1990).

MATERIALS & METHODS

Materials:

[^{3}H](+)SKF 10,047 (53 Ci/mmol; Commissariat à l'Energie Atomique, Saclay, France) was provided by the Institut de Recherche Jouveinal, Fresnes, France, while [^{3}H]DTG (38 Ci/mmol) was from NEN. [^{125}I]hCGRPα was purchased from Amersham Canada (Toronto, Ontario). All CGRP-related peptides, amylin, atrial natriuretic peptide (ANF) and substance P (SP) were synthesized in the laboratories using solid-phase synthesis (Dennis et al., 1989) or purchased from Peninsula Lab (Palo Alto, CA). The purity of the peptides was evaluated by analytical high-performance liquid chromatography, elemental and amino acid analyses, and was over 95% in all cases. Remaining minor impurities have not been characterized. Stock solutions of these peptides were dissolved at 10^{-3} M in HPLC grade distilled water, aliquoted and stored at -80 ^{o}C for less than two weeks. Haloperidol was obtained from Sigma Chemicals (St-Louis, MI, USA) or McNeil Laboratories (Stouffville, Canada), while bovine serum albumin (BSA), bacitracin, leupeptin, Trizma base (Tris), sodium chloride (NaCl), N-methyl-D-aspartate (NMDA), polyethyleneimine (PEI) and SP_{1-7} were obtained from Sigma Chemicals. Ether and GF/B Whatman filters were obtained from Fisher (Montréal, Canada). Ecolite TM+ was obtained from ICN Biochemicals (Montréal, Canada) and quisqualate (QUIS) from Tocris Neuramin (Buckhurst Hill, Essex, U.K).

Animals:

Male CD-1 mice (20-24 g.) and Sprague-Dawley rats (200-250 g.) were purchased from Charles River (St-Constant, Québec, Canada). Animals were housed in a temperature

and humidity controlled room on a 12 hr light/dark cycle, and were fed *ad libitum* with standard laboratory chow and tap water. They were kept under these conditions for at least 24 hr prior to the experiments. Animal care was according to protocols and guidelines approved by McGill University and the Canadian Council for Animal Care.

In vitro *binding experiments:*

For all binding assays, mice were sacrificed by decapitation. The brain (minus cerebellum) was rapidly removed on ice and processed as described below. The results of *in vitro* binding assays are based on at least two experiments, each performed in triplicate.

[^{3}H]DTG and [^{3}H](+)SKF 10,047 binding

Membrane preparation:

Brains minus cerebellum were first homogenized in 25 vol 50 mM Tris-HCl buffer (pH 7.4) at 4°C using a Brinkmann polytron (setting 6, 30 sec), and centrifuged at 48 000 x g for 20 min. This procedure was repeated twice to wash crude membranes. Following the second centrifugation, pellets were suspended in 10 vol 50 mM Tris-HCl buffer (pH 7.4), homogenized with the polytron (setting 6, 30 sec), and pre-incubated for 30 min at 37°C in order to remove possible endogenous ligands. At the end of the pre-incubation period, the homogenate was re-centrifuged for 20 min (48 000 x g, 4°C) and the pellet suspended in 10 vol of 50 mM Tris-HCl buffer (pH 7.4, 4°C). Binding experiments were performed at final protein concentrations of 0.5 to 1.0 mg/ml. Protein concentrations were determined according to Lowry et al. (1951).

Receptor binding assays:

For both [^{3}H]DTG (3 nM) and [^{3}H](+)pentazocine (5 nM) binding assays, membranes were incubated for 1 hr at room temperature, in 5 mM Tris-HCl buffer (pH 7.4) containing 0.05% bacitracin and 0.1% BSA to avoid degradation of the peptides, along with increasing concentrations (10^{-12} to 10^{-5}M) of unlabeled rCGRPβ, [Cys(ACM)$^{2-7}$]hCGRPα and $CGRP_{8-37}$. Non-specific binding was determined using 10 μM haloperidol. The incubation was performed in a final vol of 0.5 ml of the following composition: 250 μl of incubation buffer, 100 μl of radioligand diluted in the incubation buffer, 50 μl of peptides or haloperidol (stock solutions diluted in distilled water), and 100 μl of membrane homogenate. At the end of incubation period, free ligand was separated from the membrane-bound 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% PEI solution. Samples were washed three times with 5 ml of 5 mM Tris-HCl buffer (pH 7.4 at 4°C). Filters were then transfered to vials with 5 ml Ecolite TM$^+$ scintillation cocktail. The radioactivity was then assessed 24 hrs later, using a Beckman LS1800 counter with 40% efficiency.

[^{125}I]hCGRPα binding

Membrane preparation:

Mouse brain tisues were homogenized in 20 vol of ice-cold 25 mM Tris-HCl buffer plus 50 mM NaCl (pH 7.4) using a Brinkmann polytron (setting 6, 30 sec), and centrifuged at 48 000 x g for 20 min at 4°C. This procedure was repeated twice to wash

crude membranes. After the second centrifugation, the pellets were homogenized using the polytron in 10 vol of this same buffer. The homogenate was then pre-incubated for 30 min at 37°C in order to destroy putative endogenous ligands. At the end of pre-incubation period, pellets were re-centrifuged at 48 000 x g for 20 min. Final pellets were resuspended in 10 vol 50 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.4). Protein concentrations were determined according to Lowry et al. (1951).

Receptor binding assays:

Membranes (500-600 µg/ml) were incubated with [^{125}I]hCGRPα (40pM) at room temperature for 1 hr. in the following incubation buffer: 50 mM Tris-HCl, 100 mM NaCl, 0.2% BSA, 0.5% leupeptin, 0.4 mM bacitracin, 5 mM $MgCl_2$, with increasing concentrations of unlabeled DTG, (+)SKF 10,047 and (+)pentazocine ranging from 10^{-12} to 10^{-5} M. Non-specific binding was defined by adding 1 µM unlabeled hCGRPα to the incubation media. The incubation was performed in a final vol of 0.5 ml of the following composition: 250 µl of incubation buffer, 100 µl of radioligand diluted in the incubation buffer, 50 µl of competing drugs or unlabeled hCGRPα, and 100 µl of membrane homogenate. At the end of the incubation, bound iodinated hCGRPα 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 4 hrs in 0.1% PEI solution. Tubes were rinsed three times with 5 ml of ice-cold buffer (25 mM Tris-HCl, 50 mM NaCl, pH 7.4). Radioactivity on individual filters was then measured using a gamma counter (1282 Compugamma, LKB, Rockville, MD).

In vivo *binding experiments:*

These experiments were performed according to a protocol described in detail elsewhere (Bouchard et al., 1993). Mice were injected at time (t)=0 with 2 mg/kg of haloperidol (100 µl, i.p.) or saline (0.9% NaCl; 100 µl, i.p.) in order to determine the proportion of *in vivo* [^{3}H](+)SKF 10,047 labeling related to σ sites. At t=15 min. under light ether anesthesia, animals received either a peptide injection (3 µl, i.c.v., stock solution dissolved in saline) or saline. The i.c.v. injection of the peptides at this time point has been chosen to allow for the proprer penetration by the peptides of brain parenchyma before the injection of the radioligand. Fifteen min later (t=30 min), animals were injected in the tail vein with 5 µCi of [^{3}H](+)SKF 10,047 (200 µl, i.v.). Animals were sacrificed by dislocation at t=60 min. Brains were then rapidly removed and hippocampi dissected on ice and immediately homogenized in 1 ml ice-cold buffer (Tris-HCl 5 mM, pH 7.4 at 4°C) using a teflon/glass Potter-Elvehjem probes (12 strokes). Immediately thereafter, 1 ml of ice-cold Tris-HCl buffer was added to this homogenate before filtration of 1.5 ml of this preparation through three GF/B glass filters (pretreated for at least 24 hours in buffer containing 0.05% PEI) under vacuum using a twelve holes Millipore CAT apparatus. Filters were then washed twice with 5 ml ice-cold incubation buffer and then placed in vials containing 5 ml Ecolite TM+ scintillation cocktail. Radioactivity was determined using a Beckman LS1800 counter with 40% efficiency. The remaining 0.5 ml of the homogenate was kept for protein determination (Lowry et al., 1951).

Electrophysiological recordings from the CA_3 dorsal hippocampus pyramidal neurons:

These experiments were performed as described in detail elsewhere (Monnet et al., 1992a-c). Animals were anesthetized with urethane (1.25 g/kg, i.p.) and mounted in a stereotaxic apparatus. Body temperature was maintained at 37°C throughout the experiments. Five-barrelled glass micropipettes, preloaded with fibreglass strands in order to promote capillary filling, were pulled in the conventional manner (Haigler and Aghajanian, 1974) and their tips broken back to 8-12 μm under microscopic control. The central barrel, used for extracellular unitary recording of CA_3 dorsal hippocampus pyramidal neurons, was filled with a 2 M NaCl solution. The impedance of the central barrel was typically between 2 and 5 MΩ. One side barrel, filled with 2 M NaCl, was used for current balancing. The other side barrels, used for microiontophoresis, were filled with NMDA (10 mM in 200 mM NaCl, pH 8), QUIS (1.5 mM in 400 mM NaCl, pH 8) and a solution of either hCGRPα, $hCGRP_{8-37}$ or $[Cys(ACM)^{2,7}]$hCGRPα (0.1 mM in 150 mM NaCl and BSA 0.1%, pH 4). A solution of BSA (0.1% in 150 mM NaCl, pH 4) was used as control. The walls of each barrel which were to contain CGRP or its related peptides were coated with BSA 0.1% prior to filling. The solutions of NMDA and QUIS were stored at -20°C, and those of hCGRPα, $hCGRP_{8-37}$, $[Cys(ACM)^{2,7}]$hCGRPα at -70°C, in individual plastic tubes, precoated with BSA.

After removal of the dura mater, the micropipette was lowered into the CA_3 region of the dorsal hippocampus (L: 4.2 mm and A: 4.2 mm, at a depth of 3.5 to 4.5 mm from the cortical surface; Paxinos and Watson, 1982). Action potentials were amplified and passed through a differential amplitude discriminator generating square pulses which were fed to a computer and to a counter from which integrated firing rate

histograms were generated and displayed on a Gould paper chart recorder (model RS 3200). Pyramidal neurons were identified according to their long duration (0.8-1.5 ms) and large amplitude (0.5-2 mV) action potentials and by the presence of characteristic "complex spike" discharges alternating with simple spike activity (Kandel and Spencer, 1961).

The durations of the microiontophoretic applications of the excitatory amino acids, NMDA and QUIS, were kept constant at 50 sec. The currents used for ejecting NMDA ranged from -12 to -30 nA and from -2 to -8 nA for QUIS. For a given neuron, the currents of NMDA and QUIS were adjusted to obtain a firing frequency between 7 and 15 Hz and thereafter maintained constant for the remainder of the experiment. The duration of the microiontophoretic applications and the intensity of the currents used were stored in a computer, permitting the calculation of the total number of spikes generated/nanoCoulomb (nC; 1 nC being the charge generated by 1 nA applied for 1 sec.). Alternative applications of NMDA and QUIS were carried out for several minutes to insure a stable neuronal response before the beginning of the applications of the peptides.

The effects of the microiontophoretic applications of the peptides were assessed by determining the number of spikes generated/nC of NMDA and QUIS before and during the microiontophoretic application of the peptides. Each value was calculated by the computer as the mean of three consecutive applications chosen when the peptides had reached their maximal effects. In order to rule out any possible artifactual effects of the microiontophoretic applications, ejections of Na^+ or Cl^- with currents similar to those used to apply the peptides were carried out through the balance barrel containing

a 2 M NaCl solution. In no instances did such applications mimic the effects of the peptides studied.

Statistical Analyses:

For the *in vivo* binding experiments, statistical analyses were performed using a one way ANOVA for independent samples followed by a post-hoc comparison with the Newman-Keuls test. For the electrophysiological recordings, all results are expressed as the mean ± SEM of the number of spikes generated/nC of NMDA or QUIS, *n* being the number of neurons tested. Statistical significance was assessed using the paired Student's *t*-test with the Dunnett's correction for multiple comparisons. Probability values smaller than 0.05 were considered as significant.

RESULTS

In vitro *CGRP/σ receptor interactions:*

As shown in Table 1, no evidence for *in vitro* receptor binding interactions were observed between CGRP and σ binding sites as exemplified by the lack of affinity of CGRP-like peptides for σ sites, and vice versa.

In vivo *[^{3}H](+)SKF 10,047 binding:*

An equivalent of approximately 15.3 fmol/mg protein labeling was detected in the mouse hippocampus following an injection in the tail vein of 5 μCi of the σ ligand [^{3}H](+)SKF 10,047. In the presence of haloperidol (2mg/kg, i.p.), residual [^{3}H](+)SKF 10,047 hippocampal binding was of 1.48 fmol/mg protein, revealing that approximately 90% (13.8 fmol/mg protein) of total [^{3}H](+)SKF 10,047 labeling was haloperidol-sensitive (Fig. 1), confirming the σ nature of the *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse hippocampal formation.

Effects of CGRP-related peptides on* in vivo *[^{3}H](+)SKF 10,047 labeling:

Rat CGRPβ (rCGRPβ) (Fig. 2) and human CGRPα (hCGRPα) (Fig. 3) were equipotent at a dose of 1500 pmol (3 μl, i.c.v.) as they inhibited approximately 25% of specific [^{3}H](+)SKF 10,047 labeling.

Figure 2 shows the dose-response histogram obtained with rCGRPβ at doses ranging from 300 to 5000 pmol. The dose of 300 pmol (n=9) of rCGRPβ did not significantly inhibit *in vivo* [^{3}H](+)SKF 10,047 binding to the mouse hippocampal

formation. The minimally effective dose required to significantly inhibit specific [^{3}H](+)SKF 10,047/σ labeling was of approximately 1000 pmol (n=7), with maximal effectiveness reached at 3000 pmol (n=5). Increasing the dose to 5000 pmol (n=6) did not enhance the inhibitory effect.

Figure 3 depicts the effects of a series of CGRP-related peptides on *in vivo* [^{3}H](+)SKF 10,047 binding. At a dose of 1500 pmol (n=8), the fragment hCGRP$_{8-37}$, a purported CGRP$_1$ antagonist (Dennis et al., 1990), failed to inhibit [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus. In contrast, the analogue [Cys(ACM)^{2,7}]hCGRPα (n=15), a purported CGRP$_2$ agonist (Dennis et al., 1989), inhibited 23% of specific [^{3}H](+)SKF 10,047 labeling at a dose of 1500 pmol. Amylin (1500 pmol; n=15), a peptide that shares 46% sequence homology with CGRP as well as many of its biological actions (Cooper et al., 1987) inhibited 29% of specific [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus.

Effects of other peptides on* in vivo *[^{3}H]SKF 10,047 labeling:

We have previously demonstrated that other vasoactive peptides including vasoactive intestinal polypeptide (VIP) and neurotensin, as well as the neurotransmitter adrenaline, do not modulate *in vivo* [^{3}H](+)SKF 10,047 binding in the mouse hippocampus (Bouchard et al., 1993). To further assess specificity, other peptides were evaluated in this paradigm. Atrial natriuretic peptide (ANF; 1500 pmol; n=9; specific binding: 14.5 fmol/mg protein) did not alter *in vivo* [^{3}H](+)SKF 10,047 binding. Moreover, as the neurokinin substance P (SP) is often colocalized with CGRP in various areas of the peripheral and central nervous systems (Wiesenfeld-Hallin et al., 1984), we investigated next the effects of SP (1500 pmol) and of its N-terminal fragment SP$_{1-7}$

(1500 pmol), as this later molecule has been reported to modulate both phencyclidine (PCP) (Larson and Sun, 1993) and σ (Mousseau et al., 1992) receptor binding. Neither SP (n=14; specific binding: 13.25 fmol/mg protein) nor its fragment SP_{1-7} (n=8; specific binding: 12.38 fmol/mg protein) inhibited specific [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus *in vivo*.

Effect of concomitant administration of CGRP and NPY on in vivo ***[^{3}H](+)SKF 10,047 labeling:***

We have previously shown that NPY-related peptides can modulate *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse hippocampal formation (Bouchard et al., 1993). To evaluate the existence of possible interaction between CGRP- and NPY-related peptides on *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus, rCGRPβ (1500 pmol) and NPY, PYY or [Leu^{31}, Pro^{34}]-NPY (1500 pmol) were injected simultaneously to mice (3 µl, i.c.v.; n=5 to 7 for each combination). No evidence of additive or inhibitory action was detected as the combination of the two peptides at their most effective doses (1500 pmol) failed to induce a greater effect on *in vivo* [^{3}H](+)SKF 10,047 labeling, than either peptide alone (Fig.4).

Electrophysiological recordings of the rat CA_3 dorsal hippocampal neurons:

Under urethane anesthesia, the CA_3 dorsal hippocampus pyramidal neurons discharged at a frequency below 1 Hz. The microiontophoretic application of the dicarboxylic excitatory amino acid receptor agonists, NMDA and QUIS, increased the firing activity of all CA_3 dorsal hippocampus pyramidal neurons studied. Neither of the CGRP-related peptides applied locally by microiontophoresis with a current intensity

up to 20 nA, nor the high affinity σ ligand haloperidol administered intravenously at a dose active on σ sites (Monnet et al., 1992a-c), affected significantly their spontaneous firing rate. In addition, control solutions of NaCl (150 mM) and BSA (0.1%) applied microiontophoretically (20 nA) did not modify the neuronal firing activity.

Effects of hCGRPα:

Superimposed to 50 sec applications of NMDA or QUIS, hCGRPα was applied microiontophoretically for three consecutive periods of 15 to 20 min with currents of 5, 10 and 20 nA. hCGRPα current-dependently decreased both NMDA- and QUIS-induced activations of CA_3 hippocampal pyramidal neurons (Fig. 5 and 6). The microiontophoretic application of hCGRPα with a current of 5 nA produced a minimal reduction of the NMDA and QUIS responses (Fig. 6). When a 10 nA current of hCGRPα was applied, the effects of NMDA and QUIS were reduced by 35% and 27%, respectively. Raising the ejecting current to 20 nA resulted in a 61% reduction of the NMDA response and a 45% reduction of the QUIS response. The maximal effects of each of the currents used (5, 10 or 20 nA) on both NMDA and QUIS responses were obtained within the initial 10 min of the microiontophoretic application of hCGRPα and vanished progressively over a period of 10 to 15 min following the cessation of its application. Haloperidol, at a low dose (20 μg/kg, i.v.) which by itself has no effect on NMDA- or QUIS-induced activations but was shown to suppress the potentiation of the NMDA response induced by σ ligands (Church and Lodge, 1990; Monnet et al., 1992b), abolished the suppressant effect of the microiontophoretic application of hCGRPα on NMDA-induced activation of CA_3 pyramidal neurons (Figs. 5 and 6). This suppression appeared within a few min after the injection of the butyrophenone. In contrast, the same dose of haloperidol did not reverse the

suppressant effects of hCGRPα on the QUIS response (Fig. 6B). Spiperone, another butyrophenone which possesses a binding profile similar to that of haloperidol except for its low affinity for the σ receptors was also tested. The intravenous administration of 20 μg/kg of spiperone did not modify the effects of CGRPα on QUIS and NMDA-induced activations (Figs 6C and 6D).

Effect of [Cys(ACM)^{2,7}]hCGRPα:

Superimposed to applications of NMDA or QUIS, [Cys(ACM)^{2,7}]hCGRPα was applied microiontophoretically for 15 to 20 min with a 20 nA current. In these conditions, [Cys(ACM)^{2,7}]hCGRPα potentiated by 85% the NMDA response, whereas it reduced by 25% the QUIS-induced activation of CA_3 pyramidal neurons (Figs. 7 and 8). These effects were observed within the initial 5 min of microiontophoretic application of the analogue and vanished progressively over a period of 10 to 15 min following the cessation the microiontophoretic application of [Cys(ACM)^{2,7}]hCGRPα. Haloperidol, at the low dose of 20 μg/kg, i.v., abolished the potentiating effect of [Cys(ACM)^{2,7}]hCGRPα on NMDA-induced activation of CA_3 pyramidal neurons (Figs. 7 and 8A). The same dose of spiperone (20 μg/kg, i.v.) did not modify the potentiating effect of [Cys(ACM)^{2,7}]hCGRPα on the NMDA response (Figs 8C and 8D).

Effects of $hCGRP_{8-37}$:

In the same model, $hCGRP_{8-37}$ applied microiontophoretically with currents of 5, 10 and 20 nA had no effect on the activation of CA_3 pyramidal neurons induced by NMDA (Fig. 9A). Furthermore, when applied concomitantly with hCGRPα,

hCGRP$_{8-37}$ (20 nA) failed to antagonize the suppressant effect of hCGRPα on NMDA-induced activation of CA_3 pyramidal neurons. It is noteworthy that following the cessation of the concurrent microiontophoretic applications of hCGRPα and hCGRP$_{8-37}$, the neuronal response to NMDA recovered (Fig. 9C).

DISCUSSION

The results obtained in the present studies, namely *in vivo* (but not *in vitro*) binding in the mouse hippocampus and *in vivo* unitary recording of CA_3 pyramidal neurons of the rat hippocampus, are in agreement. In both paradigms, CGRP (hCGRPα or rCGRPβ) and the linear agonist, [Cys(ACM)^{2,7}]hCGRPα (Dennis et al.,1989), modulated σ systems while no such interaction was observed with $hCGRP_{8-37}$, a purported antagonist (Dennis et al., 1990). In the mouse hippocampus, hCGRPα, rCGRPβ, [Cys(ACM)^{2,7}]hCGRPα and amylin significantly inhibited the labeling of the σ ligand [^{3}H](+)SKF 10,047. In the rat, hCGRPα current-dependently decreased the NMDA-induced activation of CA_3 hippocampal pyramidal neurons, whereas [Cys(ACM)^{2,7}]hCGRPα exerted the opposite effect. The current-dependency character of the effect induced by hCGRPα is in agreement with a receptor-mediated effect of the peptide. $hCGRP_{8-37}$ had no effect on *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus, nor on the activation of CA_3 pyramidal neurons by NMDA. Moreover, when applied concomitantly with hCGRPα, $hCGRP_{8-37}$ failed to antagonize the inhibitory effect of hCGRPα on NMDA-induced activation of CA_3 pyramidal neurons of the rat hippocampus. This could suggest that the modulatory effects exerted *in vivo* by CGRP-related peptides on σ receptor binding and electrophysiological activation are likely mediated by an atypical class of sites insensitive to $CGRP_{8-37}$. This hypothesis is supported by the finding that amylin was a potent competitor of *in vivo* [^{3}H]SKF-10,047 labelling, in contrast to its affinity for $CGRP_1$ and $CGRP_2$ receptors (van Rossum *et al*, 1994). Further studies will be

required to precisely determine which receptor subtype mediated the inhibition of *in vivo* [^{3}H](+)SKF 10,047 labeling and the σ-modulation of NMDA-induced cell firing in the CA_3 hippocampal subfield.

The present data also revealed the specificity of the effects observed, as only certain peptides such as CGRP (hCGRPα and rCGRPβ), [Cys(ACM)^{2,7}]hCGRPα and amylin were found to be active in *in vivo* labeling study and as the electrophysiological activity of the hippocampal pyramidal neurons was differentially modulated by hCGRPα and [Cys(ACM)^{2,7}]hCGRPα following the application of NMDA or QUIS. Moreover, the effects observed with CGRP, its linear analogue [Cys(ACM)^{2,7}]hCGRPα and amylin are unlikely to be related to significant changes in cerebral blood flow as other vasoactive peptides such as VIP and neurotensin (Bouchard et al., 1993), in addition to ANF, SP and SP_{1-7} failed to modulate *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus.

Most importantly, these studies confirm that the effects observed were mediated via a modulation of σ systems. While the σ ligand, [^{3}H](+)SKF 10,047, can bind with relatively high affinities to both PCP and σ sites (Su, 1982; Largent et al., 1986; Quirion et al., 1987), only the σ component is sensitive to haloperidol (Su, 1982; Tam and Cook, 1984), which blocked 90% of *in vivo* [^{3}H](+)SKF 10,047 labeling. In addition, it has previously been shown that the high affinity NMDA/PCP receptor ligand, MK-801 (Loo et al., 1987), does not reduce the specific *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus (Bouchard et al., 1993). These observations suggest that, *in vivo*, [^{3}H](+)SKF 10,047 mostly labels σ receptor sites, in keeping with previous studies (Ferris et al., 1988; Tam et al., 1988; Weissman et al., 1990;

Bouchard et al.,1993). Hence, the hippocampal [^{3}H](+)SKF 10,047 labeling observed in our study very likely represents binding to σ sites which are known to be most abundant in this region (Largent et al., 1986; Contreras et al., 1987b; Weissman et al., 1990).

Moreover, in the rat hippocampus, haloperidol (20 μg/kg, i.v.), which by itself has no effect on NMDA- and QUIS-induced activation (Church and Lodge, 1990; Monnet et al., 1992a-c), prevented the inhibitory effect of microiontophoretic applications of hCGRPα as well as the potentiating effect of [Cys(ACM)^{2,7}]hCGRPα, on NMDA-induced activation of CA_3 pyramidal neurons. The suppressant effect seen here with haloperidol is very similar to the haloperidol-induced suppression of the potentiation of the NMDA response induced by several σ ligands (Monnet et al., 1992a), suggesting that the effects of hCGRPα and [Cys(ACM)^{2,7}]hCGRPα on NMDA-induced activation are mediated via σ systems. However, haloperidol did not prevent the effect of hCGRPα and [Cys(ACM)^{2,7}]hCGRPα on the QUIS-induced response, indicating that CGRP-related peptides may directly or indirectly modulate various glutamate receptor-related effects, but that only its modulation of the NMDA response may be related with σ-associated mechanisms.

The precise nature of *in vivo* interactions between CGRP-related peptides and σ systems remains to be elucidated. The effects displayed by this peptide family on the *in vivo* [^{3}H](+)SKF 10,047 binding in the mouse hippocampus are apparently selective but not exclusive as we have previously reported similar associations between NPY-related peptides and *in vivo* σ labeling in this tissue (Bouchard et al., 1993; Roman et

al., 1993). This is also fully consistent with the *in vivo* electrophysiological observation that NPY-related peptides can modify, in a haloperidol-sensitive manner, NMDA-induced activation of CA_3 pyramidal neurons of the rat dorsal hippocampus (Monnet et al., 1992a-c).

On the basis of difficulties in obtaining clear evidence, *in vitro*, for the existence of interactions between a variety of tritiated σ ligands and NPY-related molecules (Quirion et al., 1991; Tam and Mitchell, 1991), it was proposed that an endogenously-generated NPY metabolite could act, *in vivo*, as a σ receptor ligand to account for the inhibition of *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus (Bouchard et al., 1993). The same reasoning could also apply to CGRP-related peptides as those molecules failed to compete *in vitro*, for the σ binding sites. Conversely, σ ligands did not demonstrate any affinity for [^{125}I]hCGRPα binding. However, it would seem rather unlikely that structurally related metabolites could be generated from the catabolism of CGRP- and NPY-related peptides, as the primary structures of these two peptides are markedly different. It would appear more likely that CGRP and [Cys(ACM)^{2,7}]hCGRPα modulate, via an indirect mechanism, *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus and the NMDA-induced response in the rat hippocampus. For instance, CGRP-related peptides could induce the release of yet-unidentified endogenous σ receptor ligand(s) that would interfere with [^{3}H](+)SKF 10,047/σ labeling and modulate the NMDA-induced response. Connor and Chavkin (1991; 1992) have recently provided evidence for the existence of an endogenous σ ligand in the hippocampus, while other workers have reported on the partial characterization of σ-like molecules of peptidergic nature (Su et al., 1986; Contreras et al., 1987a; Zhang et al., 1988). Another possibility could be that CGRP and related

peptides exerted their *in vivo* effects by modulating signal transduction pathways associated with σ receptors. In that regard, it is of interest that the σ_1 receptor subtype and CGRP receptors have been proposed to be coupled to G-proteins (Itzhak and Stein, 1991b; Bowen et al., 1992; Quirion et al., 1992a; van Rossum et al., 1993; Monnet et al., 1994).

In summary, the present series of experiments demonstrate the existence of *in vivo* associations between CGRP-related peptides and σ systems in the rat and mouse hippocampal formation. Studies are currently in progress to determine the mechanisms involved and the physiological relevance of these observations.

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Table 1. Lack of direct *in vitro* receptor binding affinity between sigma and CGRP sites in mouse brain membrane preparations

	Radioligands: IC_{50} (nM)		
Competitors	[^{3}H]CGRP	[^{3}H]DTG	[^{3}H](+)pentazocine
rCGRPβ	2.8±0.6	> 1000	> 1000
$hCGRP_{8-37}$	3.6±0.9	> 1000	> 1000
[Cys(ACM)^{2,7}]hCGRPα	4.1±1.2	> 1000	> 1000
DTG	> 1000	14.7±2.9	36.0±4.7
(+)pentazocine	> 1000	21.1±3.7	6.4±1.9
(+)SKF 10,047	> 1000	ND	ND

Data represent the mean ± SEM of at least two experiments, performed in duplicate. The IC_{50} is the concentration of a given competitor required to compete for 50% of specific binding sites ND, not determined.

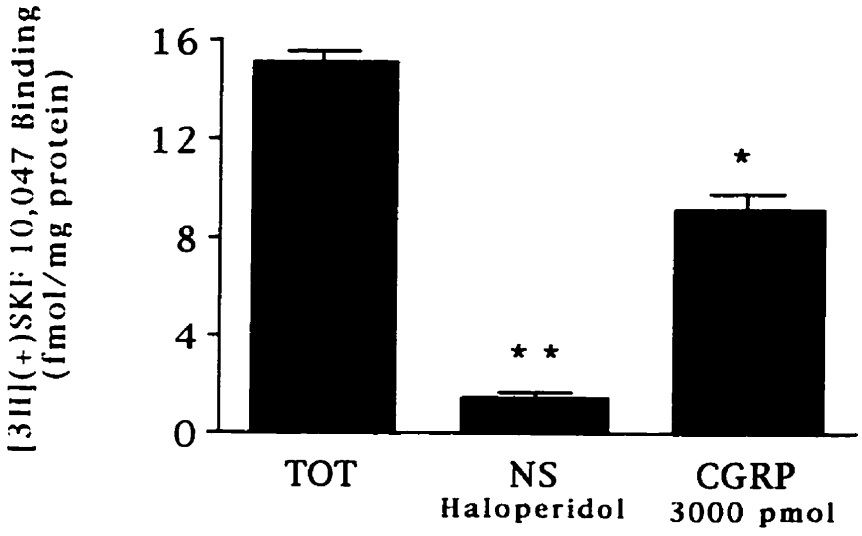

Figure 1. Total (TOT) radioactive labeling in the mouse hippocampus following an injection in the tail vein of the σ ligand [^{3}H](+)SKF 10,047 (5 μCi per 200 μl, n=61). Non-specific binding (NS) as determined by haloperidol (2 mg/kg, i.p., n=15) revealed that 90% of [^{3}H](+)SKF 10,047 binding was related to σ sites in the mouse hippocampus *in vivo*. Although to a lesser extent, i.c.v. injections of rCGRPβ (3000 pmol, n=5) significantly inhibited *in vivo* [^{3}H](+)SKF 10,047 labeling. *$p < 0.05$

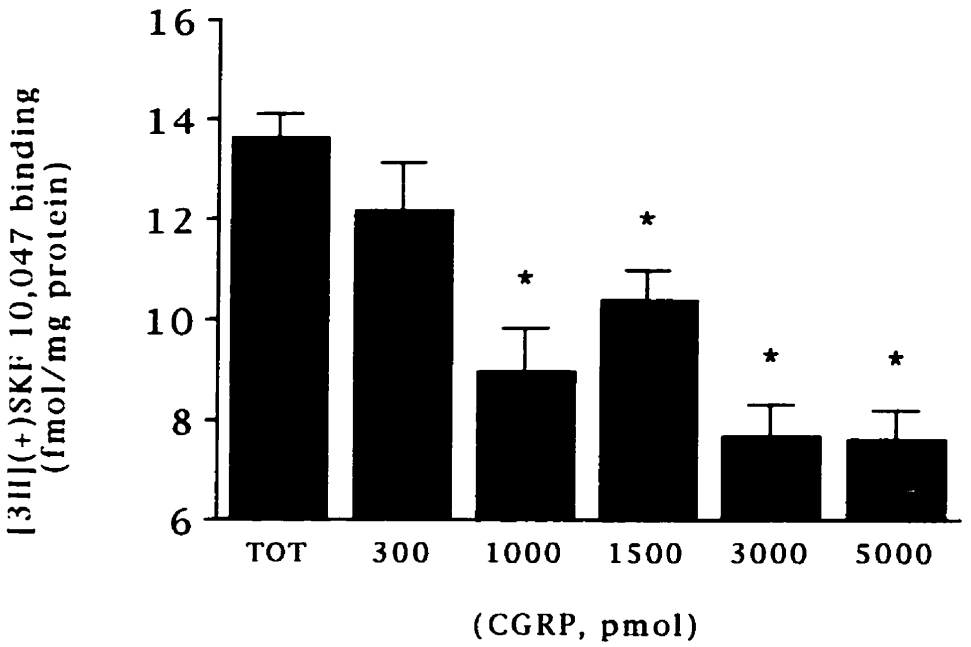

Figure 2. Effects of various doses of rat CGRPβ (rCGRPβ) on the specific *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus. I.c.v. injections of rCGRPβ ranging from 1000 to 5000 pmol significantly inhibited *in vivo* [^{3}H](+)SKF 10,047 labeling while the lower dose tested (300 pmol, n=9) did not inhibit *in vivo* [^{3}H](+)SKF 10,047 labeling to a significant extent. The maximal degree of inhibition was observed at 3000 pmol (n=5). Increasing the dose to 5000 pmol (n=6) did not further enhanced the inhibition of *in vivo* [^{3}H](+)SKF 10,047 labeling. *$p<0.05$

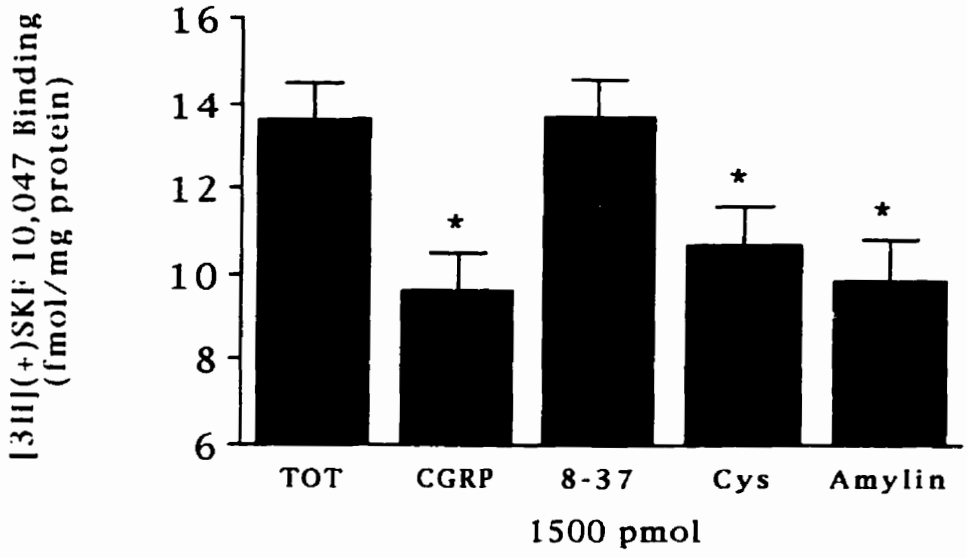

Figure 3. Comparative potencies of various CGRP-related peptides to inhibit the specific *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus. Human CGRPα (CGRP; n=8), its purported $CGRP_2$ receptor subtype agonist [Cys(ACM)^{2,7}]hCGRPα (Cys; n=15) and amylin (n=15) inhibited *in vivo* [^{3}H](+)SKF 10,047 labeling in a similar fashion in the mouse hippocampus. However, the purported $CGRP_1$ receptor subtype antagonist, $hCGRP_{8-37}$ (8-37; n=8), displayed no effect on *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse hippocampus. *$p<0.05$

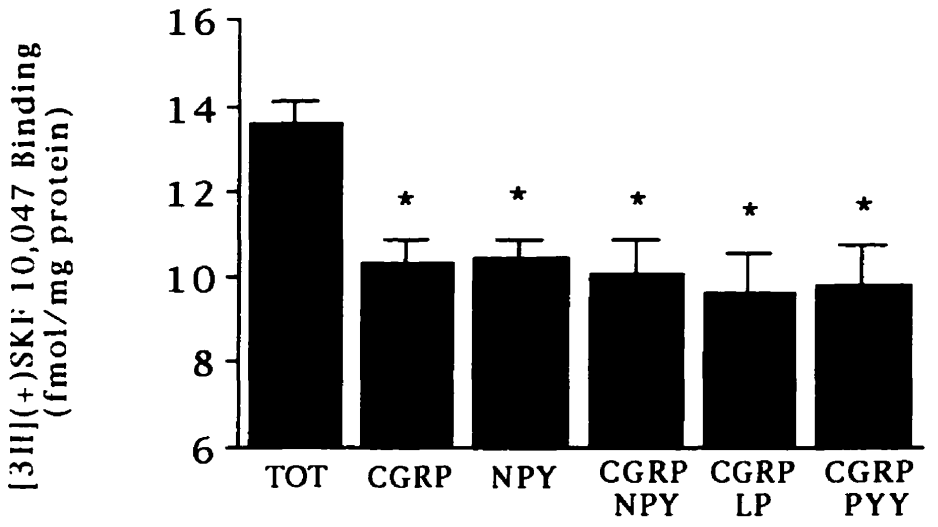

Figure 4. Comparative effect of rCGRPβ (n=17) and NPY (n=13), as well as the combination of rCGRPβ and NPY-related peptides (NPY, [Leu^{31}Pro^{34}]-NPY(LP) and PYY) on *in vivo* [^{3}H](+)SKF 10,047/σ labeling in the mouse hippocampus. No evidence of additive or antagonistic action was detected between rCGRPβ and NPY-related peptides at 1500 pmol (total of 3000 pmol, 3 µl, i.c.v.; n=5-7 for each group), as their combination failed to induce a greater inhibition of *in vivo* [^{3}H](+)SKF 10,047 labeling, than either peptide alone. *p<0.05

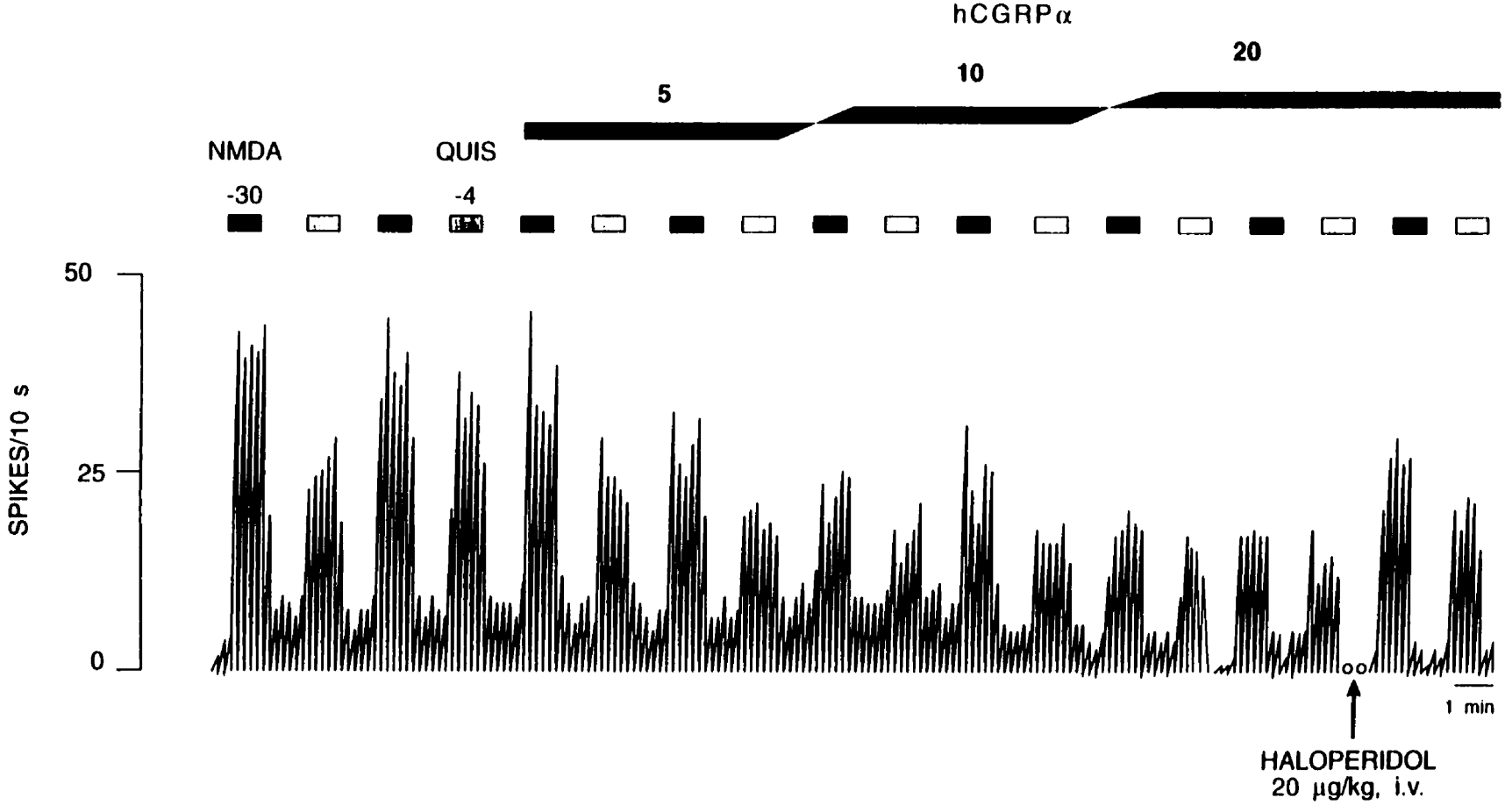

Figure 5. Integrated firing rate histogram of a rat CA_3 dorsal hippocampus pyramidal neuron showing the effects of microiontophoretic applications of NMDA and QUIS before, and during the microiontophoretic application of hCGRPα (5, 10 and 20 nA). Bars indicate the duration of applications for which currents are given in nA. Dots correspond to a 10-15 min interruption of the trace in this and subsequent figures.

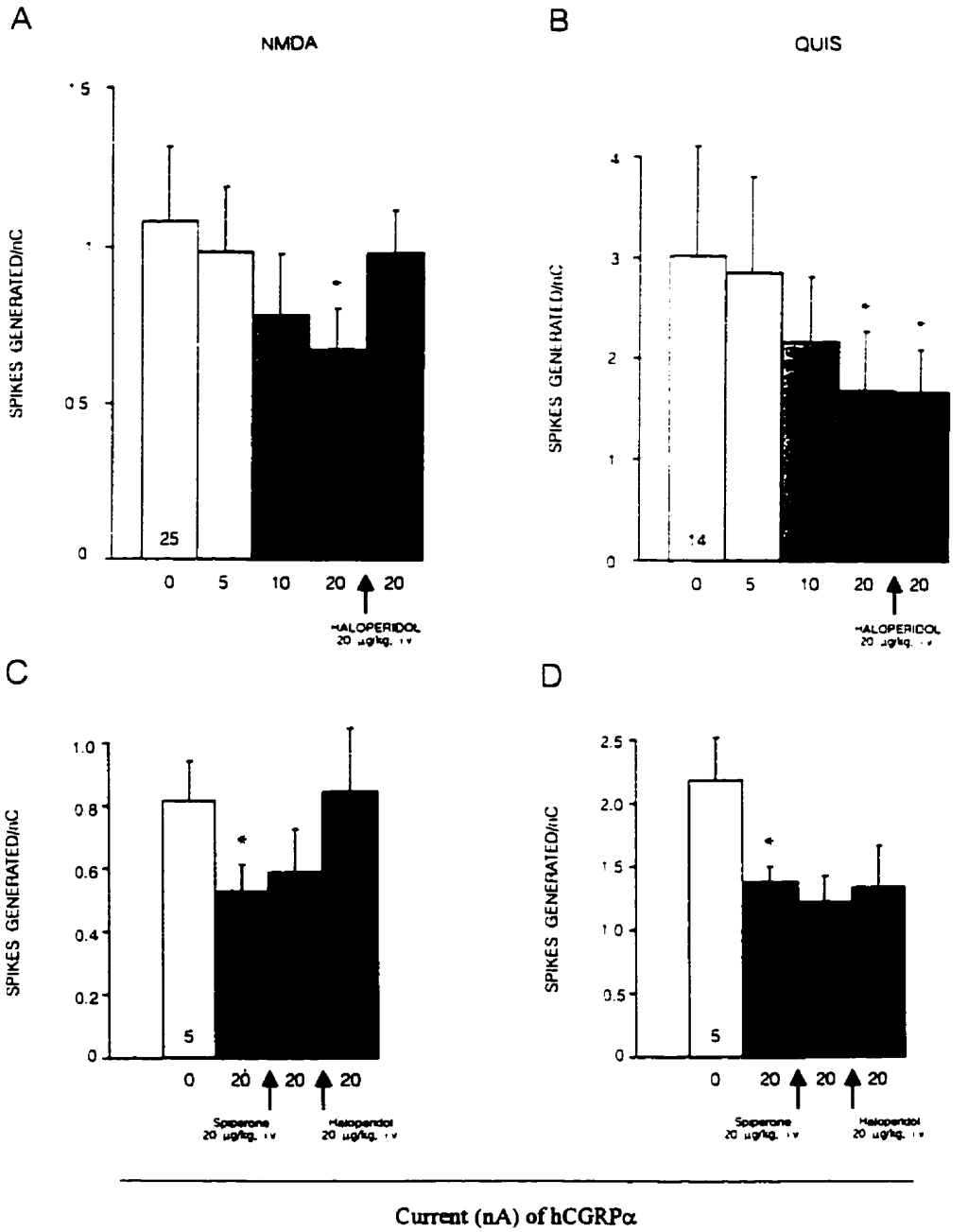

Figure 6. Responsiveness, expressed as the number of spikes generated per nC (mean ± SEM) of CA_3 dorsal hippocampus neurons to microiontophoretic applications of NMDA (A,C) and QUIS (B,D), before (open columns) and during (grey columns) microiontophoretic applications of hCGRPα, and following the intravenous administration of spiperone (20 μg/kg; dark grey columns) (C,D) or haloperidol (20 μg/kg; black columns). The number at the bottom of the first column of each histogram in this and in subsequent figures indicates the number of neurons tested. In all series of experiments, the same neurons were recorded from during the complete sequence. All applications of NMDA or QUIS were of 50 sec. *$p<0.05$

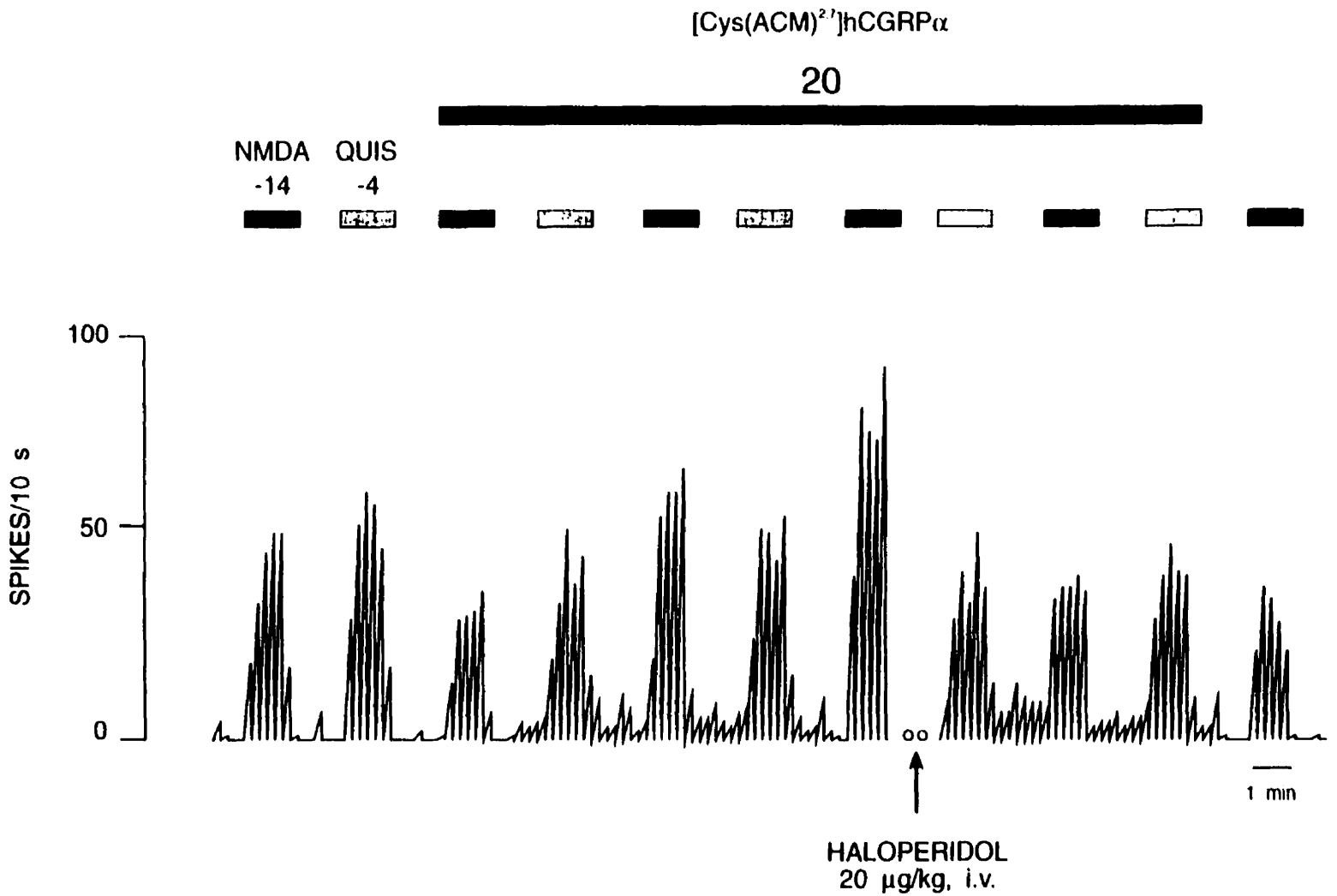

Figure 7. Integrated firing rate histogram showing the response of a CA_3 dorsal hippocampus pyramidal neuron to microiontophoretic applications of NMDA and QUIS before and during the microiontophoretic application of [Cys(ACM)^{2,7}]hCGRPα and following the intravenous administration of haloperidol (20 µg/kg).

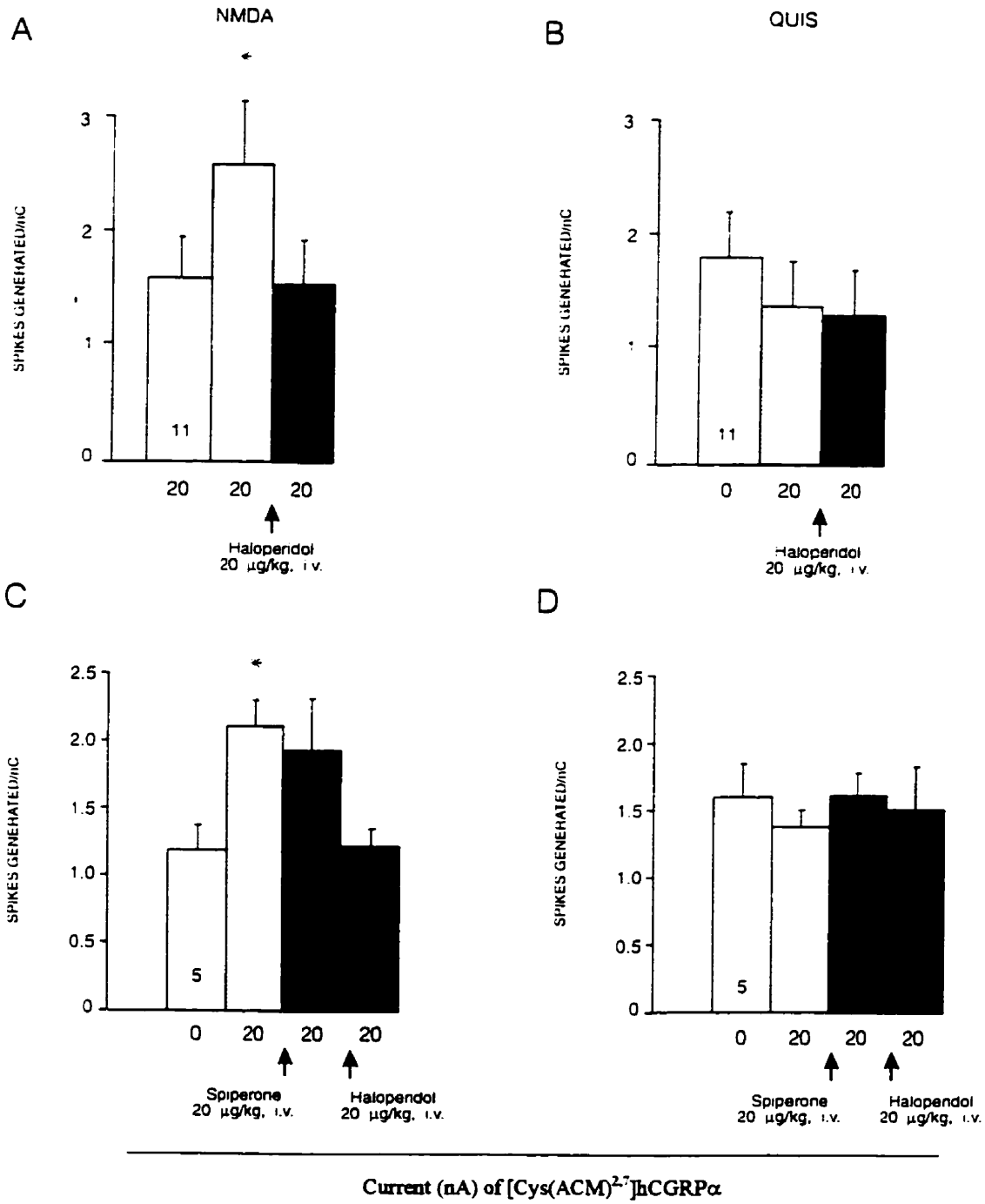

Figure 8. Responsiveness, expressed as the number of spikes generated per nC (mean ± SEM) of CA_3 dorsal hippocampus neurons to microiontophoretic application of NMDA (A,C) and QUIS (B,D), before (open columns) and during (grey columns) microiontophoretic applications of [Cys(ACM)$^{2-7}$]hCGRPα, and following the intravenous administration of spiperone (20 µg/kg; dark grey columns) (C,D) or haloperidol (20 µg/kg; black columns). *$p<0.05$.

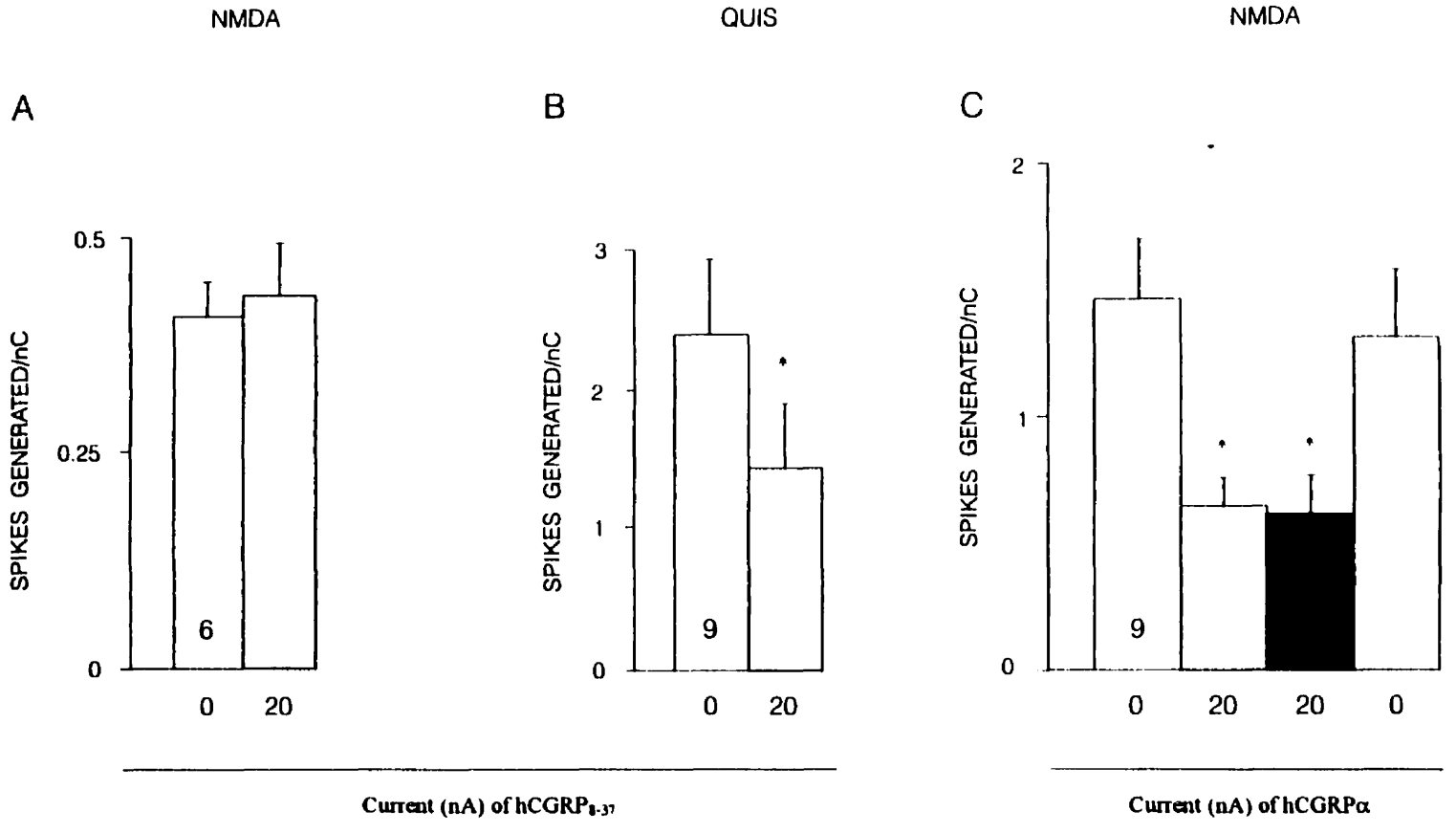

Figure 9. (A) Responsiveness, expressed as the number of spikes generated per nC (mean ± SEM) of CA_3 dorsal hippocampus neurons to microiontophoretic applications of NMDA before (open column) and during (grey column) microiontophoretic applications of $hCGRP_{8-37}$. (B) Responsiveness of CA_3 dorsal hippocampus neurons to microiontophoretic applications of QUIS before (open columns) and during (grey column), the microiontophoretic application of $hCGRP_{8-37}$. (C) Responsiveness of CA_3 dorsal hippocampus neurons to microiontophoretic applications of NMDA before (open column) and after (second open column) the injection of peptides to demonstrate full recovery. In between, hCGRPα (20 nA) was shown to be fully effective in reducing NMDA-induced firing (grey column) while the addition of $CGRP_{8-37}$ (20 nA) failed to alter the inhibitory action of hCGRPα itself (dark column). *$p<0.05$.

Chapter IV

AUTORADIOGRAPHIC EVIDENCE FOR THE MODULATION OF *IN VIVO* SIGMA RECEPTOR LABELING BY NEUROPEPTIDE Y AND CALCITONIN GENE-RELATED PEPTIDE IN THE MOUSE BRAIN

Preface to chapter 4.

This third study is a logical extension of the first and second manuscripts in which NPY- and CGRP-related peptides were shown to interact with hippocampal σ receptors, *in vivo*. In an attempt to shed more light on the nature of the relationship between σ receptors and NPY- and CGRP-related peptides, we investigated the neuro-anatomical profile of these interactions using an *in vivo/ex vivo* autoradiographic approach. In this third manuscript, we report that certain NPY- and CGRP-related peptides can interact, *in vivo*, with σ receptors in all brain areas enriched with σ sites since PYY, [Leu^{31}Pro^{34}]NPY and rCGRPβ significantly competed for [^{3}H](+)SKF 10047/σ labeling throughout the mouse brain.

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AUTORADIOGRAPHIC EVIDENCE FOR THE MODULATION OF *IN VIVO* SIGMA RECEPTOR LABELING BY NEUROPEPTIDE Y AND CALCITONIN GENE-RELATED PEPTIDE IN THE MOUSE BRAIN

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ABSTRACT

Peptides of the neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP) families have been reported to modulate, *in vivo*, σ receptor systems in the mouse and rat hippocampal formation (Monnet et al., 1992b,c; Bouchard et al., 1993; 1995; Roman et al., 1993). In an attempt to determine if these interactions were specific to the hippocampal formation, quantitative *ex vivo* autoradiography was used with [^{3}H](+)SKF 10,047 as σ ligand following intracerebroventricular injections of various NPY and CGRP peptides. High levels of specific [^{3}H](+)SKF 10,047 labeling were concentrated in various cranial nerve nuclei, while lower but still significant amounts of labeling were seen in the cortex, hippocampus, various hypothalamic nuclei, red nucleus, substantia nigra, central gray and cerebellum. In all brain areas enriched with specific [^{3}H](+)SKF 10,047/σ labeling, the Y_1 receptor subtype agonist [Leu^{31}Pro^{34}]-NPY, as well as PYY and rCGRPβ inhibited, to rather similar extent, [^{3}H](+)SKF 10,047 labeling. The Y_2 receptor agonist NPY_{13-36} had no effect in any of the regions studied. These results extend findings obtained in the hippocampal formation and demonstrate the existence of *in vivo* modulatory effects of NPY and CGRP-related peptides on σ sites throughout the mouse brain.

INTRODUCTION

Over the past few years, sigma (σ) receptor sites have generated much interest mostly because of their possible involvement in the pathogenesis of schizophrenia and in neuroleptic-associated motor disturbances (for reviews see Junien and Leonard, 1989; Snyder and Largent, 1989; Walker et al., 1990; Su, 1993). The significant concentrations of σ sites in various limbic structures including the hippocampus (Gundlach et al., 1986; Largent et al., 1986; Contreras et al., 1987b; McLean and Weber, 1988), as well as the high affinity of some purported psychotomimetic opioids (N-allylnormetazocine, pentazocine, cyclazocine) and clinically effective typical (haloperidol, chlorpromazine) and atypical neuroleptics (rimcazole, umespirone) for σ sites supported this contention (Itzhak, 1988; Largent et al., 1988; McCann and Su, 1990). However, the direct involvement of σ receptor sites in psychosis remains to be established, recent data being more supportive of a modulatory role in motor behaviors (Walker et al. 1988; 1990; Matsumoto et al., 1990).

Homogenate binding assays and receptor autoradiography have demonstrated the widespread distribution of σ sites throughout the brain of various species including mouse, rat, guinea pig, monkey and human (for reviews see Walker et al., 1990; Su, 1993). These studies also showed that σ binding sites are distinct entities from opioid, dopaminergic and PCP receptors. Recent data have suggested that σ receptor binding sites are likely heterogenous and the existence of at least two (σ_1 and σ_2) distinct classes has been proposed (for reviews see Walker et al., 1990; Quirion et al., 1992; Su, 1993).

σ receptor labeling has also been studied *in vivo* in the mouse brain using various radioligands including [^{3}H](+)SKF 10,047 (Martin et al., 1984; Compton et al., 1987; Weissman et al., 1990; Ferris et al., 1991), [^{3}H]haloperidol (Weissman et al., 1990), [^{3}H]3-PPP (Koe et al., 1989) and [^{3}H]ifenprodil (Benavides et al., 1992) while *ex vivo* autoradiography was employed to demonstrate the unique distribution of the labeling (Weissman et al., 1990; Benavides et al., 1992). Interestingly, while [^{3}H](+)SKF 10,047 can bind with relatively high affinities to both PCP and σ sites *in vitro* (Su, 1982; Largent et al., 1986; Quirion et al., 1987), various studies suggest that *in vivo*, (+)SKF 10,047 binds with high affinity to σ sites but interacts only weakly with PCP sites (Compton et al., 1987; Weissman et al., 1990; Ferris et al., 1991). It thus appears that [^{3}H](+)SKF 10,047 is a most useful probe for *in vivo/ex vivo* σ receptor investigations.

The presence and unique distribution of σ binding sites in the mammalian brain suggest the possible existence of endogenous ligands/modulators for those sites. Already, various candidates including zinc (Connor and Chavkin, 1991) and neuropeptide Y (NPY; Roman et al., 1989) have been proposed. However, the existence of direct *in vitro* interactions between σ and NPY receptors subsequently failed to be confirmed (Quirion et al., 1991; Tam and Mitchell, 1991). Interestingly, we have shown that in contrast to *in vitro* data, intracerebroventricular (i.c.v.) injections of NPY and, more recently calcitonin gene-related peptide (CGRP), can modulate *in vivo* [^{3}H](+)SKF 10,047/σ labeling in the mouse hippocampus (Bouchard et al., 1993; 1995; Roman et al., 1993). However, it remains to be established if *in vivo* [^{3}H](+)SKF 10,047 labeling can be modulated by these two peptide families in other brain areas. Accordingly, we used an *ex vivo* autoradiography approach to determine if [^{3}H](+)SKF

10,047 labeling is sensitive to i.c.v.-injected NPY or CGRP peptides (or both) in regions of the mouse brain enriched with σ sites.

MATERIALS AND METHODS

Materials:

[^{3}H](+)SKF 10,047 (53 Ci/mmol; Commissariat à l'Energie Atomique, Saclay, France) was provided by the Institut de Recherche Jouveinal, Fresnes, France. NPY-related peptides and CGRP were synthesized and purified as described earlier (Dennis et al., 1989; Dumont et al., 1993). All peptides were originally dissolved at 10^{-3} M in 0.9% saline solution and kept frozen at -40 °C. This solution was again dissolved in 0.9% saline the day of the experiment. Haloperidol, heparin and NaCl were purchased from Sigma Chemicals (St-Louis, MI, USA) and ether was obtained from Fisher (Montréal, Québec, Canada). Tritium-sensitive films and tritium low-activity standards were from Amersham Canada, Montréal.

Animals:

Male CD-1 mice (22-24 g.) were purchased from Charles River (St-Constant, Québec, Canada). Animals were housed in a temperature and humidity controlled room on a 12 hour light/dark cycle, and were fed *ad libitum* with standard laboratory chow and tap water. They were kept under these conditions for at least 24 hours prior to the experiment. Animal care was according to protocols and guidelines approved by McGill University and the Canadian Council for Animal Care.

In vivo *labeling:*

For *in vivo* labeling, mice were injected at time (t)=0 with 2 mg/kg haloperidol (100 µl i.p.) or saline (0.9% NaCl, 100 µl i.p.) in order to determine the proportion of [^{3}H](+)SKF 10,047 labeling which can be related to σ sites (Bouchard et al., 1993). At t=15 min., under light ether anesthesia, animals received either a peptide injection (3 µl i.c.v., 1500 pmol) or saline (3 µl i.c.v.); this dose of peptides was chosen since found to be optimal in earlier studies based on membrane homogenate preparations (Bouchard et al., 1993; 1995). Fifteen minutes later (t=30 min.), animals were injected in the tail vein with 5 µCi of the σ ligand [^{3}H](+)SKF 10,047 (200 µl i.v.). At t=60 min., mice were perfused, under ether anesthesia, with saline (0.9% NaCl) plus heparin (100 units/ml) in order to minimize the non-specific binding. Brains were then rapidly removed and immediately frozen in 2-methylbutane at -40°C. Perfused mice brains were kept frozen at -80°C until sliced.

Ex vivo *quantitative autoradiography:*

Each brain was sliced using a refrigerated cryostat, in 20 µm thick coronal sections mounted on gelatin coated slides, dessicated overnight at 4°C and stored at -80°C prior to film exposure. Slides were then apposed, along with tritium standards, to tritium-sensitive films for nine months before development and quantification as described earlier (Dumont et al., 1993).

Optical densities (expressed in fmol/mg tissue, wet weight) in various brain regions were quantified by computerized densitometry by means of an MCID image analyzer system (Imaging Research Inc., St-Catharines, Ontario, Canada). Densitometric analyses were performed directly on autoradiographic films and standardized using low activity tritium standards. Specific [^{3}H](+)SKF 10,047 labeling for a given area was

obtained by substracting the non-specific labeling calculated from haloperidol-injected mice (i.p.), from the labeling obtained in saline-injected mice (i.p.). The effect of i.c.v.-injected peptide on [^{3}H](+)SKF 10,047 labeling was assessed by substracting the specific labeling quantified in saline- vs peptides-treated mice.

Statistical Analysis:

Statistical analysis was performed using a two-way analysis of variance (ANOVA) for independent samples, followed by post-hoc comparisons using the Neuman-Keuls test. Probability values less than 0.05 were considered significant.

RESULTS

Ex vivo *[^{3}H](+)SKF 10,047 labeling of the mouse brain:*

Throughout the mouse brain, specific binding was determined in the presence of 2 mg/kg haloperidol and represented 50 to 60% of total *in vivo* [^{3}H](+)SKF 10,047 labeling (Fig. 1). Cranial nerve nuclei such as the oculomotor, trigeminal, facial and hypoglossal nuclei were among the most densely labeled regions with specific [^{3}H](+)SKF 10,047 labeling ranging from 16 to 27 fmol/mg of wet tissue (Table 1). Lower, but still important labeling densities were concentrated in mesencephalic structures like the red nucleus, periaqueductal gray matter and substantia nigra (10.5-14 fmol/mg wet weight), as well as in some diencephalic structures including various hypothalamic nuclei, the preoptic area and the zona incerta (11.5-15.5 fmol/mg wet weight) (Table 1). Most cortical areas (5.5-10 fmol/mg wet weight), the pyramidal layer of the CA_3 sub-field and the dentate gyrus of the hippocampal formation (8-9 fmol/mg wet weight), the superior gray layer of the superior colliculi and the cerebellum (9.5-10.7 fmol/mg wet weight) were all moderately enriched with [^{3}H](+)SKF 10,047 labeling (Table 1). Other structures such as the nucleus accumbens, septum, caudate-putamen, amygdaloid body and thalamus were also labeled, although to a lesser extent (5-8.5 fmol/mg wet weight). In contrast, white matter areas such as the corpus callosum, were not enriched with specific [^{3}H](+)SKF 10,047 labeling (less than 2 fmol/mg wet weight).

Modulation of* in vivo *[^{3}H](+)SKF 10,047 labeling by NPY- and CGRP-related peptides:

In the mouse brain, injections of [Leu^{31}Pro^{34}]-NPY, an agonist of the Y_1 receptor subtype (Fuhlendorf et al., 1990), as well as PYY and rCGRPβ significantly ($p<0.001$) inhibited specific *in vivo* [^{3}H](+)SKF 10,047 labeling in the regions studied. In contrast, the fragment NPY_{13-36}, an agonist at the NPY Y_2 receptor subtype (Wahlestedt et al., 1986), failed to modulate *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse brain (Fig. 2).

In the cortex, [Leu^{31}Pro^{34}]-NPY, PYY and rCGRPβ inhibited between 27 and 68% of specific *in vivo* [^{3}H](+)SKF 10,047 labeling (Fig. 2A). At the level of the pyramidal cell layer of the hippocampus, [Leu^{31}Pro^{34}]-NPY and PYY inhibited 40 to 80% of the specific labeling while rCGRPβ completely blocked specific [^{3}H](+)SKF 10,047 labeling in the CA_1 and CA_2 regions (Fig. 2B). [Leu^{31}Pro^{34}]-NPY, PYY and rCGRPβ were also effective in inhibiting 27 to 80% of [^{3}H](+)SKF 10,047 labeling in the nucleus accumbens, caudate-putamen, thalamus and hypothalamus (Fig. 2C). In structures of the mesencephalon such as the superior gray layer of the superior colliculi, the periaqueductal gray matter and the red nucleus, the tested peptides inhibited 30 to 60% of the specific labeling (Fig. 2D); similar data being obtained in various cranial nerve nuclei (Fig. 2E).

Statistical analysis did not reveal any significant difference between the respective potency of the peptides in their inhibition of *in vivo* [^{3}H](+)SKF 10,047 labeling in any of the regions studied (two-way ANOVA for unrelated samples; interaction "peptide X region"= $p<0.9$).

DISCUSSION

The present study examined the discrete autoradiographic distribution of σ receptor binding sites in the mouse brain *in vivo*, using [^{3}H](+)SKF 10,047 as radioligand. Specific [^{3}H](+)SKF 10,047/σ labeling represented about 50% of total binding, as defined using 2 mg/kg haloperidol (i.p.) and according to a well established protocol (Compton et al., 1987; Ferris et al., 1991). The highest levels of [^{3}H](+)SKF 10,047 labeling were found in cranial nerve nuclei, while lower levels were seen in the red nucleus, periaqueductal gray matter and substantia nigra, the hypothalamus and the preoptic area. Most cortical areas, the hippocampus and the cerebellum were moderatly enriched with [^{3}H](+)SKF 10,047 binding, whereas regions such as the nucleus accumbens, septum, caudate-putamen and thalamus were only weakly labeled. The observed pattern of *in vivo* [^{3}H](+)SKF 10,047 labeling corresponds rather well to that previously reported by others using *in vivo* autoradiography (Compton et al., 1987; Weissman et al., 1990) and membrane binding assays (Compton et al., 1987). The present study also demonstrates that the NPY homologue PYY, the Y_1 receptor agonist [Leu^{31}Pro^{34}]-NPY and the CGRP homologue rCGRPβ, significantly inhibited *in vivo* [^{3}H](+)SKF 10,047 binding throughout the mouse brain, while a Y_2 receptor agonist, NPY_{13-36}, had no effect. These data extend previous results that demonstrated the inhibition of *in vivo* hippocampal [^{3}H](+)SKF 10,047/σ labeling by NPY (Bouchard et al., 1993) and CGRP (Bouchard et al., 1995) -related peptides, and support the hypothesis that certain, but not all, NPY- and CGRP-related peptides could act as modulators of σ receptor systems *in vivo* (Monnet et al., 1992 b,c; Bouchard et al., 1993; 1995; Roman et al., 1993). This unique property is not shared by various other

peptides such as substance P, neurotensin, atrial natriuretic factor and vasoactive intestinal peptide, and the neurotransmitter adrenaline, or some NPY (NPY_{13-36} and NPY-COOH) (Bouchard et al., 1993) and CGRP ($CGRP_{8-37}$) analogues (Bouchard et al., 1995), demonstrating its specificity.

Although it is well known that [^{3}H](+)SKF 10,047 can bind, *in vitro*, with high affinity to both PCP and σ sites (Su, 1982; Largent et al., 1986; Quirion et al., 1987), its use in trace amounts for *in vivo* labeling apparently uniquely recognizes σ sites (Weissman et al., 1990; Ferris et al., 1991; Bouchard et al., 1993; 1995). This assumption is supported further by the fact that haloperidol, a well-established high affinity σ ligand with very weak affinity for PCP sites (Su, 1982; Tam and Cook, 1984), can block up to 90% of *in vivo* [^{3}H](+)SKF 10,047 binding in the mouse hippocampal formation (Bouchard et al., 1993; 1995). Taken together, these results strongly suggest that specific *in vivo* [^{3}H](+)SKF 10,047 labeling, as determined using haloperidol, represents binding to σ receptor sites. The use of more selective radioligands such as [^{3}H]DTG or [^{3}H](+)pentazocine could be preferable to [^{3}H](+)SKF 10,047 for the *in vivo* visualization of σ sites. Unfortunately, our early experiments with these probes were unsuccessful as amounts of radioactivity recovered in brain homogenates following i.v. injections of various doses of those ligands were too low to allow for adequate pharmacological studies. As [^{3}H](+)SKF 10,047 binds with higher affinity to the σ_1 than the σ_2 receptor subtype (see Quirion et al., 1992), it is likely that the *in vivo* labeling observed here mostly but not exclusively represents σ_1 sites.

There is now increasing evidence to support the existence of *in vivo* interactions between σ sites and NPY- and CGRP-related peptides (Monnet et al., 1992b,c; Bouchard et al., 1993; 1995; Roman et al., 1993). For example, it has been shown that NPY-related peptides can compete, in a dose-dependent manner, for *in vivo* [^{3}H](+)SKF 10,047/σ receptor labeling in the mouse hippocampal formation (Bouchard et al., 1993). The observed inhibition was specific as certain NPY analogues (NPY_{13-36}, NPY-COOH) as well as other neuroactive peptides (VIP, neurotensin) had no effect (Bouchard et al., 1993; Roman et al., 1993). Furthermore, Monnet et al. (1992b,c) demonstrated that, in a fashion similar to highly selective σ drugs including DTG and JO-1784, various NPY-related peptides modulated NMDA-induced firing activity of pyramidal CA_3 dorsal hippocampal neurons in the rat. As for σ drugs, the effects of NPY were antagonized by purported σ ligands such as haloperidol and BMY-14802, but not by a dopamine receptor blocker devoid of affinity for σ receptors, like spiperone. It has also been observed that the stimulatory effect of NMDA on [^{3}H]noradrenaline release is potentiated, in a haloperidol-sensitive manner, by σ ligands (JO-1784 and (+)3-PPP) and NPY-related peptides (Roman et al., 1991; Monnet et al., 1992a). Taken together, these results are strongly sugestive of the existence of *in vivo* interaction between NPY-related peptides and σ receptor systems.

While in our previous studies various neuropeptides failed to alter *in vivo* [^{3}H](+)SKF 10,047 labeling, we noted that CGRP-related peptides inhibited *in vivo* [^{3}H](+)SKF 10,047 binding in the mouse hippocampus, and modulated, in a haloperidol-sensitive manner, NMDA-induced firing activity in the rat CA_3 sub-field (Bouchard et al., 1995). Hence, it would appear that in addition to NPY, peptides of the CGRP family can act, *in vivo,* as modulators of hippocampal σ systems. Data from the present study

now clearly demonstrate that [^{3}H](+)SKF 10,047/σ labeling is significantly inhibited by NPY-related peptides (PYY and [Leu^{31}Pro^{34}]-NPY) and rCGRPβ throughout the mouse brain. Furthermore, no clear difference was observed between NPY-related peptides and CGRP in their potencies to inhibit [^{3}H](+)SKF 10,047/σ labeling throughout the mouse brain, except in the CA_3 subfield of the hippocampus in which CGRP was relatively more potent. This corresponds rather well with previous *in vivo* binding and electrophysiological studies that have shown that the relative potencies of NPY- and CGRP-related peptides were comparable (Monnet et al., 1992b,c; Bouchard et al., 1993; 1995).

The precise mechanism(s) responsible for the modulatory effects of NPY and CGRP on *in vivo* [^{3}H](+)SKF 10,047/σ labeling in the mouse brain remains to be established. However, it does not appear to relate to a direct action of either peptide families on σ receptors as direct *in vitro* membrane binding assays failed to demonstrate any affinity of CGRP and NPY for σ sites (Quirion et al., 1991; Tam and Mitchell, 1991; Bouchard et al., 1995). Hence, indirect mechanism(s) are most likely and an intermediary step common to both NPY and CGRP is likely as no additive or antagonistic effect between the two peptide families were observed on *in vivo* [^{3}H](+)SKF 10,047 labeling (Bouchard et al., 1995).

One possible hypothesis may be that NPY- and CGRP-related peptides could induce the release of yet-unidentified endogenous σ receptor ligand(s) that would then interfere with *in vivo* [^{3}H](+)SKF 10,047/σ labeling. Connor and Chavkin (1991) have recently provided evidence for the existence of an endogenous σ ligand in the hippocampus which can be released upon nerve stimulation, while other groups reported

the partial characterization of σ-like molecules of peptidic and steroidal nature (Contreras et al., 1987a; Su et al., 1987; Zhang et al., 1988). However, the full characterization of the putative endogenous σ ligands has yet to be reported.

Another interesting possibility may be that NPY and CGRP-related peptides could exert their actions by modulating signal transduction pathways associated with σ receptors. Accumulated evidence suggests that NPY (Herzog et al., 1992), CGRP (van Rossum et al., 1993) and σ (Itzhak, 1989; Itzhak and Stein, 1991; Connick et al., 1992; Monnet et al., 1992c; 1994) receptors are likely coupled to various G-protein subtypes. For example, the σ_1 receptor subtype may be coupled to the Gi/Go and Gs subtypes, as pertussis and cholera toxins purportedly modulate binding parameters and functional effects of σ drugs (Itzhak, 1989; Basile et al., 1992; Monnet et al., 1992a).Therefore, the actions of NPY- and CGRP-related peptides on their respective classes of G-protein -coupled receptors (Gi/o and Gs), could alter the integrity of the σ/G-protein receptor complex leading to decrements in *in vivo* [^{3}H](+)SKF 10,047 binding.

A related mechanism, possibly associated to the activation of G-proteins, concerns the modulation of neuronal Ca^{2+} fluxes. While rather well established for NPY and CGRP receptors (for reviews see Dumont et al., 1992; Poyner, 1992), arguments in favor of possible associations between σ receptors and cellular Ca^{2+} levels are derived from binding and functional studies. For example, it was shown that Ca^{2+} modulators can interact with σ receptors (Klein et al., 1985; Rothman et al., 1991). The σ_1 receptor subtype was also postulated to be associated with a cation channel. Basile et al. (1992) have demonstrated that the binding of the selective σ_1 ligand, [^{3}H](+)pentazocine, to guinea pig cerebellum was modulated by several cations, including Ca^{2+}. Moreover,

inhibitors of intracellular Ca^{2+} mobilization (TMB-8 and cinnarizine) as well as nonselective cation channel blockers (hydroxyzine, tetracaine, prenylamine, amiodarone and proadifen) potently inhibited [^{3}H](+)pentazocine binding in the guinea pig cerebellum with a rank order of potency consistent with an involvement of σ sites.

Functional studies also suggested associations between σ sites and calcium regulatory mechanism(s) in nerve cells. Several antipsychotic drugs, including σ ligands, were shown to block voltage dependent Ca^{2+} channels (Quirion et al., 1985; Fletcher et al., 1994). The two σ ligands, dextromethorphan and dextrorphan, can inhibit K^+ - stimulated Ca^{2+} uptake into rat brain synaptosomes and PC-12 cells, apparently via N-type and L-type Ca^{2+} channels, respectively (Carpenter et al., 1988). Voltage clamp experiments on NCB-20 cells revealed that haloperidol, (+)3-PPP and (+)pentazocine blocked, at μM concentrations, a tonic outward K^+ current with a rank order of potency consistent with an involvement of the σ_2 receptor subtype (Wu et al., 1991). σ ligands were also reported to modify K^+ conductance in various tissues from different species (Bobker et al., 1989; Fletcher et al., 1989; Neumaier and Chavkin, 1989; Kennedy and Henderson, 1990). The possible involvement of σ receptor sites in the modulation of K^+ channels activity is strengthened further by the finding that the antipsychotic chlorpromazine, which displays reasonable affinity for σ receptors (Tam and Cook, 1984), inhibits various types of K^+ channels and K^+ currents in neuronal and non-neuronal tissues (Dinan et al., 1987; Kon et al., 1994). Interestingly, the binding in the rat cortex of the σ ligand (+)3-PPP was shown to be inhibited in low nM range by various class III antiarrhytmic drugs which block voltage-dependent K^+ channels (amiodarone, clofilium tosylate and RP 58866) (Jeanjean et al., 1993). These authors suggested that rat brain (+)3-PPP binding sites shared properties of the K^+ channels

(which are the targets of class III antiarrhytmic drugs) and proposed that the σ_2 receptor subtype may be a K^+ channel. Taken together, these results suggest that σ receptor ligands may exert their effects on Ca^{2+} fluxes directly through Ca^{2+} channels, or via K^+ channels. An association of σ receptors with cation channels could also explain the wide range of effects reported thus far for σ ligands.

NPY and CGRP receptors have also been shown to modulate Ca^{2+} fluxes through either Ca^{2+} or K^+ channels. For example, NPY can inhibit Ca^{2+} currents in sympathetic neurons and hippocampus (Klapstein and Colmers, 1992; Foucart et al., 1993) and reduces, via a pertussis toxin-sensitive mechanism, voltage-dependent Ca^{2+} currents in dorsal root ganglion neurons (Ewald et al., 1988), nodose ganglion neurons (Wiley et al., 1990) and myenteric neurons (Hirning et al., 1990). Interestingly, the activation of the Y_1 vs the Y_2 NPY receptor subtype respectively enhanced and reduced transient high-threshold Ca^{2+} currents via a pertussis toxin-sensitive pathway (Wiley et al., 1993). Hence, NPY receptor subtypes may modulate Ca^{2+} channel(s) via their differential coupling with various G-proteins. Such effects could be involved in the indirect modulation of σ receptor systems as reported in the present study.

CGRP has also been shown to modulate intracellular Ca^{2+} levels either via cAMP-related mechanisms or directly through receptor-operated Ca^{2+} channel(s). For example, CGRP increased Ca^{2+} currents in rat dorsal root ganglion neurons and enhanced excitatory synaptic transmission in the spinal cord (Ryu et al., 1988). CGRP potentiated L-type Ca^{2+} channel currents in amphibian (Ono et al., 1989) and guinea pig (Nakajima et al., 1991) atrial cells, likely via a G-protein (Gs)-dependent adenylate cyclase stimulation (Ono et al., 1989, Nakajima et al., 1991). In the rat nodose neurons, CGRP

can modulate L-type Ca^{2+} channels via a PTX-sensitive mechanism (Wiley et al., 1992). CGRP can also affect K^+ channels as it suppressed K^+ currents in smooth muscle cells of the rat vas deferens (Nakazawa et al., 1992) and in cultured rat cortical neurons (Zona et al., 1991). The effects of CGRP can therefore be mediated via a direct coupling of its G-protein receptor complex to a Ca^{2+} channel or by an indirect mechanism involving cAMP and/or K^+ channels. Accordingly, as for NPY-related peptides, a variety of intracellular pathways could be involved in the modulatory action of CGRP on *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse brain.

In summary, *in vivo* [^{3}H](+)SKF 10,047 labeling in the mouse brain is altered by the i.c.v. injections of peptides of the NPY and CGRP families. These effects are not shared by other neuroactive peptides such as neurotensin, substance P and atrial natriuretic factor. The precise mechanism(s) mediating the inhibitory action of NPY and CGRP on *in vivo* [^{3}H](+)SKF 10,047 labeling remains to be established but is not related to a direct effect on the σ receptor complex. Intracellular pathways involving either G-proteins or the modulation of Ca^{2+} levels (or both) are likely targets to explain the modulatory relationships between σ, and NPY and CGRP systems.

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Table 1.

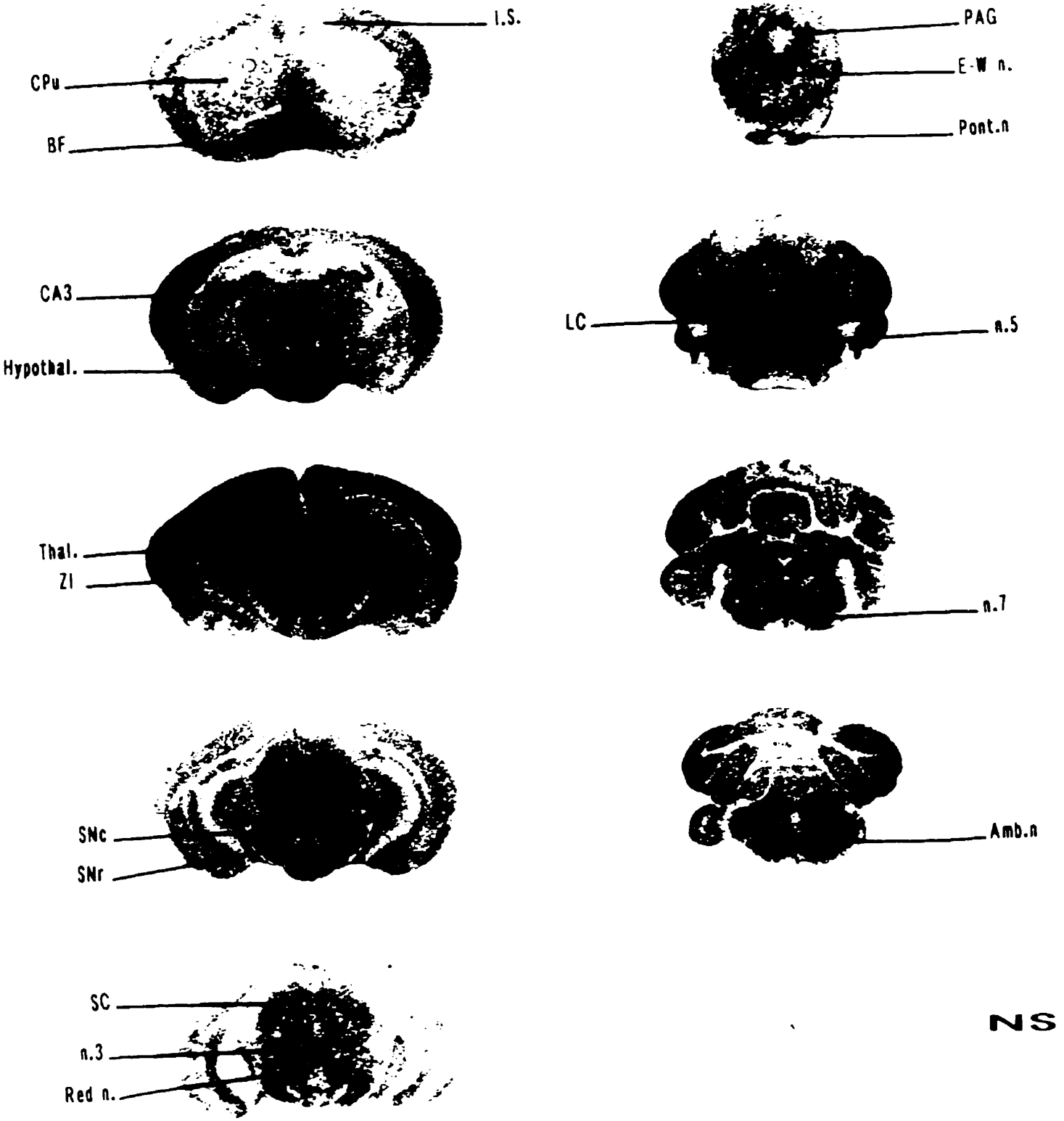
REGIONS	[^{3}H](+)SKF 10,047 specific labeling of the mouse brain, *ex vivo* (fmol/mg tissue)
Cortex	
Frontal	7.3±0.85
Parietal	8.6±0.74
Temporal	7.5±1.73
Occipital	8.8±0.96
Cingulate	7.3±0.93
Piriform	10.1±1.91
Retrosplenial	7.1±0.39
Entorhinal	5.3±0.96
Basal ganglia	
Caudate-Putamen	5.0±0.53
Accumbens	6.6±0.91
Limbic system	
Hippocampus	
CA1 pyramidal	3.8±1.09
CA2 pyramidal	5.1±1.45
CA3 pyramidal	9.3±1.48
Dendate Gyrus	8.2±2.0
Non-pyramidal layers 2.2±0.75	
Limbic system, other	
Septum	8.7±0.67
Amygdala	5.5±0.49
Diencephalon	
Thalamus	8.0±0.65
Hypothalamus	12.1±0.47
Zona incerta	15.5±0.90
Preoptic area	11.6±1.48
Mesencephalon	
Superior colliculi (superior gray layer)	9.5±0.4
Periaqueductal gray matter	11.4±0.77
Red nucleus	13.8±0.66
Substantia nigra compacta	10.6±1.0
Metencephalon	
Cerebellum	10.7±0.73
Pontine nucleus	8.8±0.41
Cranial nerve nuclei	
Oculomotor	16.2±0.99
Trigeminal	27.0±0.78
Facial	30.2±4.93
Hypoglossal	28.0±1.41
Other	
Corpus callosum	2.2±0.54

Autoradiographic distribution of specific [^{3}H](+)SKF 10,047 in the mouse brain, *ex vivo*. Values are expressed as the mean ± S.E.M. of the quantitative analysis performed on four mice, and are expressed in fmol/mg of wet tissue.

Legend to figure 1.

Ex vivo autoradiography of [^{3}H](+) SKF 10.047/σ labeling in the mouse brain. It is evident that labeling is densely distributed with concentrations revealed by darker staining. NS: non-specific labeling determined in presence of 10μM haloperidol. Abbreviations used: Amb.n.: ambiguous nucleus; BF: basal forebrain; CA3: area CA_3 of the hippocampus, pyramidal layer; CPu: caudate-putamen; E-W n.: Edinger-Westphal nucleus; Hypothal.: hypothalamus; I.S.: injection site; LC: locus coeruleus; n.3: occulomotor nucleus; n.5: trigeminal nucleus; n.7: facial nucleus; PAG: periaqueductal gray; Pont.n.: pontine nucleus; SC: superior colliculi; SNc: substantia nigra compacta; SNr: substantia nigra reticulata; Thal.: Thalamus; ZI: zona incerta.

Figure 1.


I.S.
CPu
BF
PAG
E-W n.
Pont.n
CA3
Hypothal.
LC
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ZI
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SNr
Amb.n
SC
n.3
Red n.
NS

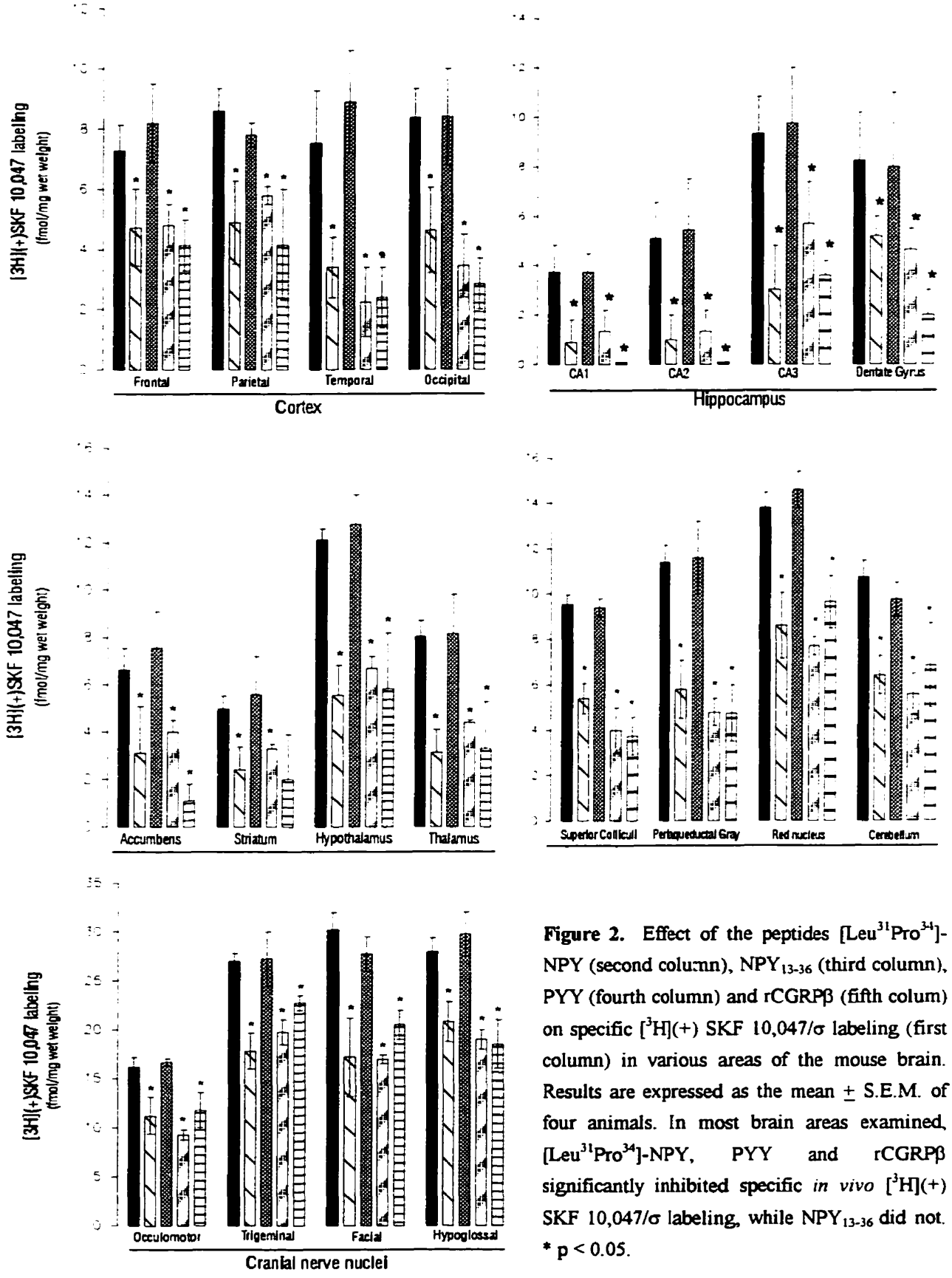

Figure 2. Effect of the peptides [Leu^{31}Pro^{34}]-NPY (second column), NPY$_{13-36}$ (third column), PYY (fourth column) and rCGRPβ (fifth colum) on specific [^{3}H](+) SKF 10,047/σ labeling (first column) in various areas of the mouse brain. Results are expressed as the mean ± S.E.M. of four animals. In most brain areas examined, [Leu^{31}Pro^{34}]-NPY, PYY and rCGRPβ significantly inhibited specific *in vivo* [^{3}H](+) SKF 10,047/σ labeling, while NPY$_{13-36}$ did not. * $p < 0.05$.

Chapter V

NEUROPEPTIDE Y AND CALCITONIN GENE-RELATED PEPTIDE ATTENUATE LEARNING IMPAIRMENTS INDUCED BY MK-801 LIKELY VIA A SIGMA RECEPTOR-RELATED MECHANISM

Preface to chapter 5.

It was previously shown that certain ligands with high affinity for σ receptors such as (+)SKF 10047, (+)pentazocine and DTG significantly attenuated learning impairments induced by the non-competitive NMDA receptor antagonist, MK-801. These attenuating effects were blocked by the co-administration of purported σ antagonists BMY-14802 or NE-100, suggesting the involvement of σ receptors in certain NMDA-mediated cognitive processes. In keeping with our results supporting the existence of *in vivo* interactions between NPY, CGRP and σ receptors (Bouchard et al., 1993; 1995; 1996), the aim of the present series of experiments was to investigate the effects of certain NPY- and CGRP-related peptides on MK-801-induced learning impairments in the mouse, using a step-down passive avoidance paradigm. Administration of MK-801 (i.p.) resulted in major learning deficits that were significantly and selectively attenuated by selected NPY- and CGRP-related peptides. Furthermore, when simultaneoulsy administered with MK-801, the purported σ antagonist BMY-14802 blocked the peptides-induced effects. These results suggest that σ receptors mediated the attenuating effects of NPY- and CGRP-related peptides on learning impairments induced by MK-801 and support the hypothesis that 1) σ receptors can modulate certain cognitive processes associated with NMDA receptor function, and that 2) selected peptides of the NPY and CGRP families can act as neuromodulators of σ receptor systems, *in vivo*.

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NEUROPEPTIDE Y AND THE CALCITONIN GENE-RELATED PEPTIDE ATTENUATE LEARNING IMPAIRMENTS INDUCED BY MK-801 VIA A SIGMA RECEPTOR-RELATED MECHANISM.

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ABSTRACT

It was recently shown that low doses of sigma (σ) receptor ligands like 1,3-di-(2-tolyl)guanidine (DTG), (+)N-allylnormetazocine ((+)SKF 10,047) and (+)pentazocine could antagonize learning impairments induced by dizocilpine (MK-801), a non-competitive antagonist at the *N*-methyl-*D*-aspartate (NMDA) receptor channel. This antagonism was proposed to involve σ receptor sites since it was blocked by the administration of purported σ antagonists such as NE-100 and BMY-14802 (Maurice et al., 1994a). It was also demonstrated that peptides of the neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP) families modulate, *in vivo*, σ labeling and electrophysiological effects in the hippocampal formation (Bouchard et al., 1993; 1995; 1996; Monnet et al., 1992a,b). Accordingly, we investigated here, if NPY- and CGRP-related peptides modulate cognitive processes by interacting with σ sites. In order to test this hypothesis, a step-down passive avoidance task was used. Interestingly, similarly to various σ agonists, NPY, PYY and the Y_1 agonist, [$Leu^{31}Pro^{34}$]NPY (but not NPY_{13-36}, a purported Y_2 agonist), as well as hCGRPα and the purported $CGRP_2$ agonist [$Cys(ACM)^{2-7}$]hCGRPα (but not $CGRP_{8-37}$, a $CGRP_1$ receptor antagonist) significantly attenuated learning impairments induced by MK-801. Furthermore, the effects of NPY, [$Leu^{31}Pro^{34}$]NPY, hCGRPα and [$Cys(ACM)^{2-7}$]hCGRPα were blocked by the administration of the σ antagonist, BMY-14802. Taken together, these data support the hypothesis that NPY- and CGRP-related peptides can interact, *in vivo*, with σ receptors to modulate cognitive processes associated with NMDA receptor function.

INTRODUCTION

Sigma (σ) receptors are widely distributed in the central and peripheral nervous systems as well as in many peripheral organs including endocrine tissues (Su, 1993; Su and Junien, 1994; Walker et al., 1990). The existence of two different classes of σ receptors (σ_1 and σ_2) have been well documented (Quirion et al., 1992; Su, 1993). These subtypes were classified on the basis of their different binding affinities and stereoselectivities for several ligands as well as on their different responses to sub-chronic treatments with haloperidol and G-protein -modifying agents (for details Quirion et al., 1992). Although the endogenous ligand(s) for σ sites has yet to be characterized (Debonnel et al., 1994; Patterson et al., 1994), σ receptors bind several classes of drugs including (+)benzomorphans (SKF 10,047, pentazocine) and morphinans (dextromethorphan and dextrallorphan), guanidines (DTG), piperazine derivatives (BMY-14802), phenothiazines and tricyclic antidepressants (perphenazine, fluphenazine, imipramine, desipramine), as well as monoamine oxydase inhibitors and butyrophenones such as haloperidol (de Costa and He, 1994; Walker et al., 1990). While the high affinity σ ligands DTG and haloperidol do not discriminate between σ_1 and σ_2 receptor subtypes, dextrorotatory benzomorphans such as (+)SKF 10,047, (+)pentazocine and dextromethorphan preferentially interact with the σ_1 site (Bowen et al., 1989; Hellewell and Bowen, 1990; Quirion et al., 1992). Functionally, the σ_1 subtype may be involved in cognitive functions (Maurice et al., 1994a) while it is likely that the σ_2 subtype mediates motor effects observed following the administration of some σ ligands in the red nucleus (Walker et al., 1990).

A possible involvement of σ receptors in learning processes has recently been suggested (Earley et al., 1991; Matsuno et al., 1994; Maurice et al., 1994a,b). σ ligands such as DTG, (−)SKF 10,047, (+)pentazocine and PRE-084 were found to attenuate learning impairments induced by systemic injection of the non-competitive NMDA receptor antagonist MK-801, in the mouse. This attenuation was blocked by the administration of purported σ receptor antagonists such as NE-100 or BMY-14802, as well as by a sub-chronic treatment with haloperidol (Maurice et al., 1994a,b). In a series of electrophysiological experiments, Monnet et al. (1992a,b) demonstrated that high affinity σ ligands including DTG, JO-1784 and (+)pentazocine dose-dependently potentiated NMDA-induced activation of CA_3 pyramidal neurons in the rat hippocampus. The purported σ antagonists haloperidol and BMY-14802 reversed the potentiation of the NMDA response induced by DTG and JO-1784. No such effect was seen for quisqualic acid-induced responses, suggesting that an important role of σ sites could be the modulation of the NMDA receptor complex (Debonnel et al., 1994).

Peptides of the neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP) families have also been shown to modulate NMDA-induced neuronal activation in the rat CA_3 region of the dorsal hippocampus, in a manner highly similar to σ ligands (Bouchard et al., 1995; Monnet et al., 1992a,b). NPY, the Y_1 agonist [$Leu^{31}Pro^{34}$]NPY and the purported Y_2 agonist NPY_{13-36} enhanced the currents induced by iontophoretic application of NMDA, while having no effect on quisqualate-induced currents. This potentiation was reversed by haloperidol, suggesting an interaction between these NPY-related peptides and σ receptors on the modulation of the NMDA receptor complex (Monnet et al., 1992a,b). Similar findings were observed for certain CGRP-related peptides (hCGRPα, rCGRPβ and [$Cys(ACM)^{2-7}$]CGRPα, a purported $CGRP_2$ agonist; Bouchard et al., 1995).

Taken together, these data are strongly supportive of the existence of *in vivo* interactions between σ systems and the NMDA receptor complex, and argue for a role for NPY- and CGRP-related peptides in that regard. In the present study, we investigated if NPY- and CGRP-related peptides could modulate, via a σ-related mechanism, MK-801 -induced memory impairments in the mouse. Our results demonstrate that certain NPY- and CGRP-related peptides can modulate MK-801 -induced memory impairments in the mouse, their effects being blocked by BMY-14802, a σ receptor antagonist.

MATERIALS & METHODS

Animals:

Male Swiss mice (28-33 g) were purchased from Iffa-Credo (l'Arbresles, France). Animals were housed in a temperature and humidity controlled room on a 12 hour light/dark cycle and fed *ad libitum* with standard laboratory chow and tap water. Animals were kept under these conditions for at least three days before being used for behavioral experiments. Experiments were carried out between 10:00 and 18:00 hr in a soundproof and air-regulated laboratory. Animal care was according to protocols and guidelines approved by local authorities.

Drugs:

NPY, CGRP, their analogues and homologues were synthesized in our laboratories as described elsewhere (Dennis et al., 1989; Dumont et al., 1993). Peptides were dissolved in saline solution (0.9% NaCl) and were administered intracerebroventricularly (i.c.v.) as previously described in details (Bouchard et al., 1993; 1995). BMY-14802 (Brystol-Myers, Evansville, Indiana, U.S.A.) was dissolved in a minimal volume of 0.1N HCl and then in saline solution, the pH adjusted to 7 with 1N NaOH. MK-801 (Research Biochemicals Inc., Natick, MA, U.S.A.) was dissolved in a 0.9% saline solution.

Drug treatments:

At time=0, peptides or saline were administered (3µl i.c.v.). At time=10 min MK-801 (0.2 mg/kg i.p.) and/or BMY-14802 (5 mg/kg i.p.) were injected (100 µl/20g of body weight), and the first training session was carried out at time=20 min. Ninety min later,

mice received their second training session and the retention test was given 24 hrs later. No drug treatment was administered for the second training session or the retention test.

Passive avoidance test:

The step-down passive avoidance task, based on negative reinforcement, was used previously to examine cognitive behaviors (Maurice et al., 1994a,b). The apparatus consisted of a transparent acrylic cage (30 x 30 x 40 cm high) with a grid floor inserted in a semi sound proof outer box (35 x 35 x 90 cm high). The cage was illuminated with a 15W lamp during the experimental period. A wooden platform (4 x 4 x 4 cm) was fixed in the center of the grid floor. Electric footshocks (1 Hz, 500 msec, 41.5 V DC) were delivered to the grid floor with an isolated pulse stimulator (Model 2100, A-M Systems, Inc., Everett, WA). The training was carried out in two similar sessions. Each mouse was placed on the platform set in the center of the grid floor. When the mouse stepped down and placed all its paws on the grid floor, footshocks were delivered for 15 sec. Step-down latency (SDL), and the number of flinching reactions and vocalizations were measured. The second session was carried out 90 min after the first, with an upper cut-off time of 60 sec. Animals which did not step down within 60 sec were considered as remembering the task and taken off from the platform. The retention test was carried out 24 hr after the second training. Each mouse was again placed on the platform and the SDL was recorded. The upper cut-off time was 300 sec and no footshock was given.

Data analysis:

Memory performances were evaluated using two parametric measures: 1) SDL, which corresponds to the number of seconds taken by the mouse to quit the platform and place its four paws on the grid floor, and 2) an avoidance criterion defined as SDL>60 sec and higher than three times the SDL showed by the animal during the second training session. The number of animals in each group reaching this criterion was expressed in

percent. SDL could be regarded as a qualitative index of mnesic capacities whereas the avoidance criterion is a quantitative index. The SDL, which did not display a normal distribution since upper cut-off times were set, are expressed as medians and interquartile ranges. Statistical significance was determined using the Kruskal-Wallis' analysis of variances by ranks and post-hoc comparisons were made using the nonparametric Dunn's multiple comparisons test. In all cases, statistical significance was set at $p<0.05$. In addition, sensitivity to footshocks was evaluated in each animal for each training session by summing the numbers of vocalizations and flinching reactions.

RESULTS

Effect of MK-801:

Pre-training administration of MK-801 resulted in highly significant decreases in SDL compared to the control group (Figs. 1, 2 and 3A). The number of animals that reached the avoidance criterion decreased of about 60% compared to the control groups (Fig. 1, 2 and 3B), an index of a poor retention. Additionally, in contrast to MK-801 - treated animals in which an increased sensitivity to footshocks was observed, controls exhibited some habituation to footshocks as shown by a significantly decrease in the number of vocalizations and flinching reactions between the first and second training sessions (Tables 1,2 and 3).

NPY-related peptides:

NPY

Compared to the control group, NPY (750-1500 pmol) did not affect retention, either in term of SDL (Fig. 1A) or in percentage of animals that reached criterion (Fig. 1B). In the MK-801 -treated group, injection of NPY resulted in a dose-dependent and bell-shaped increase in SDL reaching significance at 1000 pmol (Fig.1A), indicating that retention was increased. NPY (750-1500 pmol) did not significantly affect sensitivity to footshocks in comparison to controls, but significantly decreased sensitivity in MK-801 - treated animals during the first and second training sessions (Table 1A).

[Leu^{31}Pro^{34}]NPY

At doses of 1000 and 1500 pmol, the Y_1 agonist, [Leu^{31}Pro^{34}]NPY, significantly decreased retention, both in term of SDL (Fig. 1C) and in the proportion of animals that reached criterion (Fig. 1D). At 1500 pmol, [Leu^{31}Pro^{34}]NPY also significantly antagonized the effect of MK-801 on the passive avoidance task as shown by the SDL (Fig. 1C) while the number of animals that reached criterion was comparable to controls (Fig. 1D). At all doses tested, [Leu^{31}Pro^{34}]NPY did not significantly affect sensitivity to footshocks as compared to controls, neither did it modulate the effect of MK-801 on footshock sensitivity (Table 1B).

NPY$_{13-36}$

Compared to the control group, the Y_2 agonist NPY_{13-36} failed to induce a significant decrease in SDL (Fig. 1E). The proportion of animals that reached criterion was comparable to controls at all doses tested (Fig. 1F). NPY_{13-36} also failed to alter MK-801 -induced learning impairment (Fig. 1E and 1F). NPY_{13-36} (750 pmol) significantly increased sensitivity to footshocks in MK-801 -treated animals (Table 1C).

PYY

The homologue PYY (750-1500 pmol) significantly decreased retention (Figs. 1G and 1H). Moreover, PYY (750-1500 pmol) significantly antagonized the effect of MK-801 in the retention test as shown by an increased SDL (Fig. 1G) and by the proportion of animals that reached criterion (1500 pmol; Fig. 1H). Additionally, PYY (1000-1500 pmol) decreased sensitivity to footshocks when compared to controls and MK-801 -treated animals (Table 1D).

CGRP-related peptides:

hCGRPα

hCGRPα (750-1500 pmol), by itself, did not affect learning performance as compared to the control group (Fig. 2A and 2B). However, in the MK-801 -treated group, hCGRPα induced a dose-dependent increase in SDL (Fig. 2A) and slightly increased the number of animals that reached the avoidance criterion (Fig. 2B). hCGRPα did not affect sensitivity to footshocks in control animals. However, in MK-801 -treated animals, hCGRPα (750 and 1000 pmol) increased sensitivity to footshocks during the second training session (Table 2A).

[Cys(ACM)$^{2-7}$]hCGRPα

The $CGRP_2$ agonist, [Cys(ACM)$^{2-7}$]hCGRPα, had no effect by itself on retention (Fig. 2C and 2D). However, it significantly antagonized the effect of MK-801 at 1500 pmol, both in term of SDL and in the proportion of animals that reached the avoidance criterion (Fig. 2C and 2D). [Cys(ACM)$^{2-7}$] hCGRPα (1000-1500 pmol) increased sensitivity to footshocks in MK-801 -treated animals during both the second training session (Table 2B).

$CGRP_{8-37}$

The purported $CGRP_1$ receptor antagonist $CGRP_{8-37}$ did not significantly alter retention either in term of SDL (Fig. 2E) or in the number of animals that reached the avoidance criterion (Fig. 2F). Furthermore, $CGRP_{8-37}$ had no effect on MK-801 -induced amnesia (Fig. 2E and 2F), nor modified sensitivity to footshocks in control animals. However, in MK-801 -treated groups, $CGRP_{8-37}$ (1500 pmol) increased sensitivity to footshocks during the second training session (Table 2C).

Effect of BMY-14802:

A shown in in Fig. 3, MK-801 (0.2 mg/kg, i.p.) strongly diminished the retention of the task as shown by an important decrease in SDL. BMY-14802 (5 mg/kg, i.p.), did not modify MK-801 -induced amnesia when injected simultaneously (0.2 mg/kg, i.p) (Fig. 3A and 3B).

However, when BMY-14802 was administered along with NPY (1000 pmol), [$Leu^{31}Pro^{34}$]NPY (1500 pmol), hCGRPα (1500 pmol) or [$Cys(ACM)^{2-7}$]hCGRPα (1500 pmol), it significantly blocked the effect of these peptides on MK-801 -induced learning impairments, both in term of SDL (Fig. 3A) or when considering the proportion of animals that reached the avoidance criterion (Fig. 3B). Interestingly, the effect of PYY (1500 pmol) on MK-801 induced amnesia was not prevented by the administration of BMY-14802 (Fig. 3A and 3B).

DISCUSSION

The present study examined if NPY- and CGRP-related peptides could antagonize, possibly via a σ-related mechanism, learning impairments induced by systemic injection of MK-801. Our results show that 1) selected NPY- and CGRP-related peptides attenuated, in a dose-related fashion, learning impairments induced by MK-801 and 2) the effects of both peptide families on MK-801 -induced learning impairments were blocked by BMY-14802, a purported σ receptor antagonist. Taken together, our data suggest that NPY- and CGRP-related peptides can modulate, likely via an action at σ sites, learning processes mediated by the NMDA receptor complex.

Our results are in accordance with earlier studies that have shown *in vivo* interactions between σ markers and NPY- and CGRP-related peptides (Bouchard et al., 1993; 1995; 1996; 1992a,b; Roman et al., 1993). While NPY, PYY and the Y_1 agonist [$Leu^{31}Pro^{34}$]NPY, as well as hCGRPα and the $CGRP_2$ agonist, [$Cys(ACM)^{2-7}$]hCGRPα, did not improve cognitive performance by themselves, they significantly attenuated learning impairments induced by MK-801. In comparison, the Y_2 agonist NPY_{13-36} and the CGRP antagonist $CGRP_{8-37}$, were inactive. Interestingly, these pharmacological profiles correspond rather well with those reported earlier in other models. Using an *in vivo* binding approach, we previously demonstrated that NPY, PYY, [$Leu^{31}Pro^{34}$]NPY, hCGRPα and [$Cys(ACM)^{2-7}$]hCGRPα significantly inhibited [^{3}H](+)SKF 10,047/σ binding in the mouse hippocampus. NPY_{13-36} and $CGRP_{8-37}$ were inactive in that model (Bouchard et al., 1993; 1995; 1996; Roman et al., 1993). Using an electrophysiological paradigm, it was also demonstrated that NPY and [$Leu^{31}Pro^{34}$]NPY potentiated, in a σ/haloperidol-sensitive manner, NMDA-induced neuronal activation in the CA_3 region of the rat

hippocampal formation (Monnet et al., 1992a,b). CGRP-related peptides also modulated, in a σ/haloperidol-sensitive manner, NMDA-induced firing in the CA_3 region of the rat hippocampus (Bouchard et al., 1995). Thus, similarities between *in vivo* binding and electrophysiological data and the present results is highly suggestive of common underlying mechanisms of action.

The modulatory effects of NPY-related peptides on MK-801 -induced learning impairments are unlikely to be related to direct memory-enhancing properties of NPY (Flood and Morley, 1989; Nakajima et al., 1994). It was shown that improved performance in learning behavior and memory tasks induced by NPY are mediated by Y_2 receptors (Flood and Morley, 1989) while the Y_1 subtype was shown to be involved in orexigenic (Flood and Morley, 1989) and locomotor (Heilig et al., 1988) activities. In the present study, the Y_2 agonist NPY_{13-36} was inactive while the Y_1 agonist [$Leu^{31}Pro^{34}$]NPY significantly antagonized MK-801 -induced learning impairments. We failed to observe direct learning and/or memory enhancing properties for any of the tested NPY peptides in our behavioral paradigm. In contrast, a worsening of performance was noticed in mice injected with PYY or [$Leu^{31}Pro^{34}$]NPY. This may be due to a sedative effect induced by the activation of the Y_1 receptor subtype (Heilig et al., 1988). In our procedure, peptides were injected before the first training session while being injected after training in other studies (Flood and Morley, 1989; Nakajima et al., 1994).

hCGRPα, administered i.c.v., induces analgesia (Candeletti and Ferri, 1990; Pecile et al., 1987), anorexia (Krahn et al., 1984) and decreases locomotor activity (Clementi et al., 1992; Jolicoeur et al., 1992). CGRP-related peptides have also been reported to improve learning and memory processes (Kovacs and Telegdy, 1992; 1995). However, the pro-mnesic properties of CGRP cannot explain the modulatory effects of

hCGRPα and [Cys(ACM)$^{2-7}$]hCGRPα on MK-801 -induced learning impairments. Like for NPY, CGRP-related peptides did not improve cognitive performance by themselves. It would thus appears that the antagonistic effect produced by NPY- and CGRP-related peptides on MK-801 -induced learning impairments is dependent upon on a mechanism different from that responsible for the putative, direct cognitive properties of these two peptide families.

Moreover, the effects observed in the present study are unlikely to be due to an action of the studied peptides on pain threshold since peptides active in the MK-801 -induced amnesia paradigm (NPY, PYY, [Leu^{31}Pro^{34}]NPY, hCGRPα, [Cys(ACM)$^{2-7}$]hCGRPα) differentially modulated sensitivity to footshocks. For example, NPY significantly decreased while [Cys(ACM)$^{2-7}$]hCGRPα significantly increased sensitivity to footshocks in spite of a similar inhibition of MK-801 -induced learning impairments. In fact, it is most likely that the observed modulatory actions of NPY- and CGRP-related peptides on MK-801 -induced impairments relate to a σ mechanism. Several σ ligands such as PRE-084 (Maurice et al., 1994b), DTG, (+)SKF 10,047 and (+)pentazocine (Maurice et al., 1994a) produced similar effects on MK-801 -induced learning impairments. More importantly, the effects induced by these σ ligands and those of NPY- and CGRP-related peptides were all blocked by the σ antagonist BMY-14802.

The precise mechanism(s) responsible for *in vivo* interactions between NPY- and CGRP-related peptides and σ systems remains to be established. However, it is very unlikely that these effects are mediated via a direct action of either peptide families on σ receptor sites since earlier studies failed to reveal direct *in vitro* competition by NPY- and/or CGRP-related peptides on σ binding sites labeled using [^{3}H]DTG, [^{3}H](+)pentazocine or [^{3}H](+)SKF 10,047 (Tam and Mitchell, 1991; Bouchard et al.,

1995). Similarly, a direct action of the active peptides at the NMDA receptor complex is not likely since the σ antagonist BMY-14802, which blocked NPY and CGRP effects on MK-801 -induced impairments, display only very low affinity for the NMDA receptor (Largent et al., 1988). Moreover, σ-mediated attenuation of amnesia induced by MK-801 cannot be considered an NMDA-like action since NMDA itself does not reverse MK-801 -induced amnesia (Maurice et al., 1994a; Parada-Turska and Turski, 1990). Accordingly, indirect mechanisms common to NPY and CGRP are most likely involved (Bouchard et al., 1995; 1996). For example, NPY- and CGRP-related peptides could induce the release of yet-unidentified endogenous σ ligand(s) (Patterson et al., 1994) that would then interfere with the NMDA receptor complex via σ sites. Alternatively, NPY and CGRP-related peptides could exert their actions by modulating signal transduction pathways associated with σ systems since NPY (Herzog et al., 1992), CGRP (van Rossum et al., 1993) and σ_1 (Bowen et al., 1994) receptors are all likely associated with G-proteins. A modulatory effect on intracellular Ca^{2+} could also be responsible for the observed effects as it was shown that NPY (Bleakman et al., 1993), CGRP (Poyner, 1992) and σ (Basile et al., 1992; Carpenter et al., 1988; Klein et al., 1985) receptors are involved in calcium regulatory processes in nerve cells.

In summary, the present study demonstrates that selected NPY and CGRP-related peptides can modulate NMDA-dependent learning process as they significantly attenuated MK-801 -induced learning impairments in the mouse. Additionally, this modulation was shown to involve σ sites since the σ antagonist, BMY-14802, blocked the effects of these peptides similarly as observed earlier for various σ ligands. Thus our functional behavioral data support the existence of *in vivo* interactions between NPY- and CGRP-related peptides with σ sites.

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Table 1. Effects of NPY-related peptides (pmol, i.c.v.) and MK-801 (0.2 mg/kg, i.p.) on sensitivity to electric footshocks.

Treatment		n	First Training median/range	Second Training median/range
A) NPY				
saline	saline	25	10.0 [7.0-13.25]	5.0 [3.0-8.0] ##
750	saline	24	7.0 [4.0-11.5]	6.0 [5.0-8.0]
1000	saline	24	9.0 [5.0-13.0]	8.0 [3.0-11.0]
1500	saline	24	6.0 [3.5-11.0]	8.0 [6.25-12.5] #
saline	MK	32	13.0 [9.0-19.0]	10.5 [6.0-15.0] *
750	MK	26	7.0 [4.0-13.0] +	5.0 [3.25-11.25]
1000	MK	32	7.5 [4.0-12.0] +	7.5 [6.0-13.0]
1500	MK	27	8.0 [4.25-11.0] +	6.0 [4.25-9.5]
ANOVA			F=.2229 (p<0.001)	F=17.8 (p<0.05)
B) [Leu^{31}Pro^{34}]NPY				
saline	saline	31	12.0 [8.0-15.0]	8.0 [6.0-12.0] ##
750	saline	16	8.0 [5.5-9.0]	8.5 [7.0-11.5]
1000	saline	28	8.8 [7.0-10.5]	7.0 [5.0-10.0]
1500	saline	31	8.0 [6.0-11.75]	8.5 [6.0-11.0]
saline	MK	28	13.5 [9.0-16.5]	12.0 [10.0-21.5]
750	MK	15	10.0 [6.25-13.5]	12.0 [10.25-13.0]
1000	MK	16	9.5 [7.5-10.0]	8.0 [5.5-10.5]
1500	MK	14	12.5 [10.0-19.0]	10.0 [7.75-14.75]
ANOVA			F=26.3 (p<0.001)	F=32.8 (p<0.001)
C) NPY$_{13-36}$				
saline	saline	13	10.0 [7.75-15.5]	8.0 [6.0-9.0] #
750	saline	10	11.5 [8.0-16.0]	10.5 [10.0-17.0]
1000	saline	18	10.0 [7.0-11.0]	9.0 [7.0-12.5]
1500	saline	10	7.5 [6.0-11.0]	7.0 [6.25-9.0]
saline	MK	16	12.0 [10.0-14.5]	12.0 [8.0-17.5]
750	MK	15	12.0 [9.25-20.75]	17.0 [11.5-25.75] *
1000	MK	18	9.5 [8.0-12.0]	10.0 [8.0-12.0]
1500	MK	18	8.0 [6.0-11.0]	10.0 [5.5-11.5]
ANOVA			F=13.1 (p>0.05)	F=26.0 (p<0.001)

D) PYY

saline	saline	13	10.0 [7.75-15.5]	8.0 [6.0-9.0] #
750	saline	26	6.0 [4.0-7.0] *	6.0 [5.0-9.0]
1000	saline	25	6.0 [3.75-9.0]	5.0 [3.0-6.0]
1500	saline	30	6.0 [2.75-8.0] *	5.0 [4.0-7.0]
saline	MK	16	12.0 [10.0-14.5]	12.0 [8.0-17.5]
750	MK	20	5.0 [3.5-6.5] **↔	6.0 [4.0-8.0] ↔
1000	MK	20	4.5 [2.5-8.0] *↔	7.0 [5.0-8.5]
1500	MK	29	6.0 [4.0-8.75] **↔	7.5 [6.0-11.0]
ANOVA			F=43.7 (p<0.001)	F=26.3 (p<0.001)

Number of vocalizations and flinching reactions of mice that received either NPY (A), [Leu^{31}Pro^{34}]NPY (B), NPY$_{13-36}$ (C) or PYY (D). Each value represents the median and interquartile range of the number of vocalizations and flinching reactions for a given treatment group. *p<0.05 and **p<0.01 vs control (saline & saline) group. ↔p<0.05 vs saline & MK-801 -treated group. #p<0.05 and ##p<0.01 vs the first training session.

Table 2. Effects of CGRP-related peptides (pmol, i.c.v.) and MK-801 (0.2 mg/kg, i.p.) on sensitivity to electric footshocks.

Treatment		n	First Training median/range	Second Training median/range
A) CGRP				
saline	saline	26	9.5 [7.0-12.0]	6.0 [4.0-9.0] ##
750	saline	15	9.0 [6.0-11.0]	8.5 [6.0-10.0]
1000	saline	23	7.0 [3.25-9.75]	6.0 [4.5-10.0]
1500	saline	28	8.0 [4.0-12.0]	8.0 [6.25-12.5]
saline	MK	27	11.0 [8.0-14.75]	8.0 [7.0-12.5]
750	MK	18	7.5 [5.0-11.0]	14.5 [10.0-18.0] **##
1000	MK	25	11.0 [7.0-14.0]	10.0 [6.5-15.25]
1500	MK	34	9.5 [7.0-12.0]	12.0 [9.0-15.0] **#
ANOVA			F= -1468 (p<0.001)	F= -1199 (p<0.001)
B) [Cys(ACM)^{2,7}]hCGRPα				
saline	saline	16	10.0 [7.0-12.0]	10.0 [6.75-14.75]
750	saline	13	11.0 [9.0-13.5]	12.0 [6.0-13.0]
1000	saline	15	15.0 [7.25-18.25]	9.0 [6.75-16.75]
1500	saline	15	9.0 [7.0-13.5]	9.0 [6.0-13.75]
saline	MK	19	14.0 [13.25-17.75] *	15.0 [10.0-20.5]
750	MK	15	9.0 [8.25-25.75]	10.0 [8.0-15.75]
1000	MK	26	16.5 [10.0-21.0]	21.0 [9.0-33.5] #
1500	MK	26	11.0 [7.0-23.0]	15.0 [9.0-30.0] #
ANOVA			F= 18.6 (p<0.01)	F= 14.4 (p<0.05)
C) CGRP$_{8-37}$				
saline	saline	18	10.0 [7.0-12.0]	8.0 [6.0-10.0]
750	saline	11	9.0 [8.25-10.0]	6.5 [6.0-9.0]
1000	saline	10	8.0 [6.0-10.0]	7.0 [6.0-9.0]
1500	saline	16	9.0 [8.0-19.5]	8.0 [7.0-10.0]
saline	MK	17	14.0 [10.75-15.25]	13.0 [8.5-17.5]
750	MK	12	11.0 [9.5-13.0]	12.5 [8.5-13.0]
1000	MK	18	8.5 [7.0-17.0]	10.5 [7.0-18.0]
1500	MK	31	11.0 [10.0-18.75]	15.0 [10.5-29.5] **#
ANOVA			F= 21.0 (p<0.01)	F= 24.8 (p<0.001)

Number of vocalizations and flinching reactions of mice that received either hCGRPα (A), [Cys(ACM)^{2,7}]hCGRPα (B) or CGRP$_{8-37}$ (C). Each value represents the median and interquartile range of the number of vocalizations and flinching reactions for a given treatment group. *p<0.05 and **p<0.01 vs control (saline & saline) group. $^{+}$p<0.05 and $^{++}$p<0.01 vs saline & MK-801 -treated group. #p<0.05 and ##p<0.01 vs the first training session.

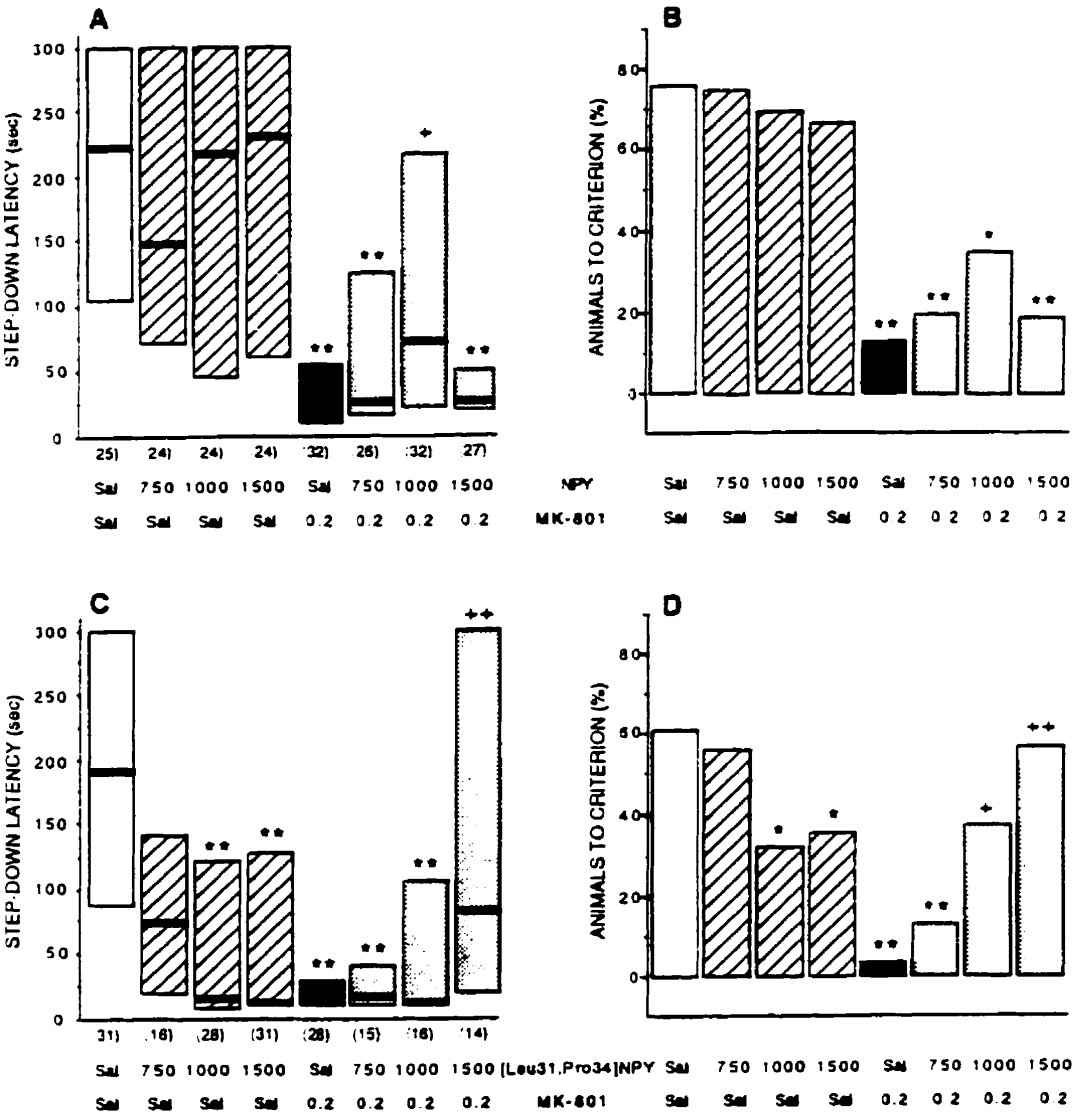
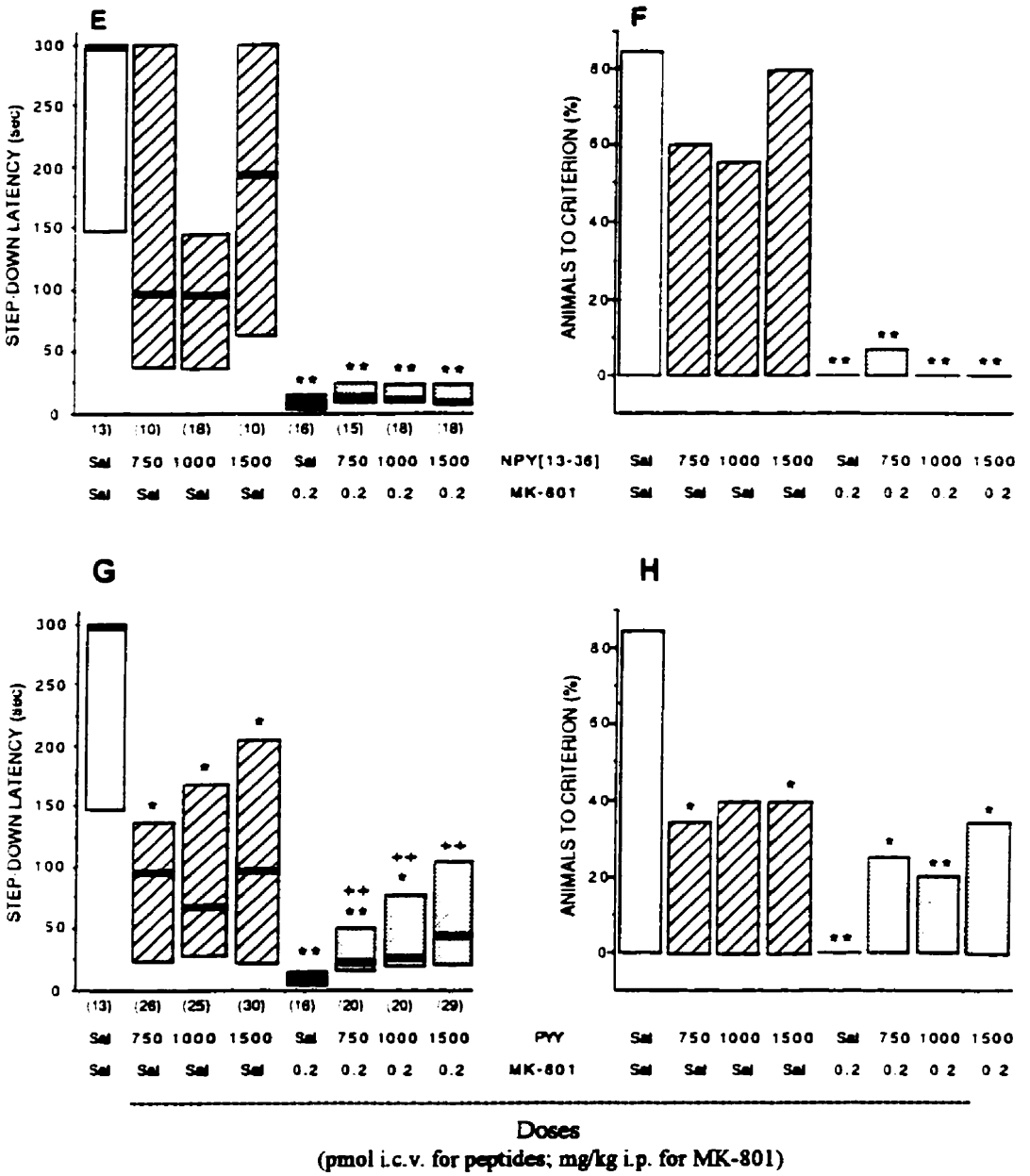


Figure 1. Dose-response effects of NPY (A and B), [Leu^{31}Pro^{34}]NPY (C and D), NPY$_{13-36}$ (E and F) and PYY (G and H) on MK-801 -induced learning impairments in the passive avoidance task. Results are expressed as median and interquartile range of the number of mice. While NPY (1000 pmol), [Leu^{31}Pro^{34}]NPY (1500 pmol) and PYY (750-1500 pmol) significantly prevented learning impairments induced by MK-801, NPY$_{13-36}$ had no effect. *p<0.05 and **p<0.01 vs control animals; $^{+}$p<0.05 vs MK-801 -treated animals.



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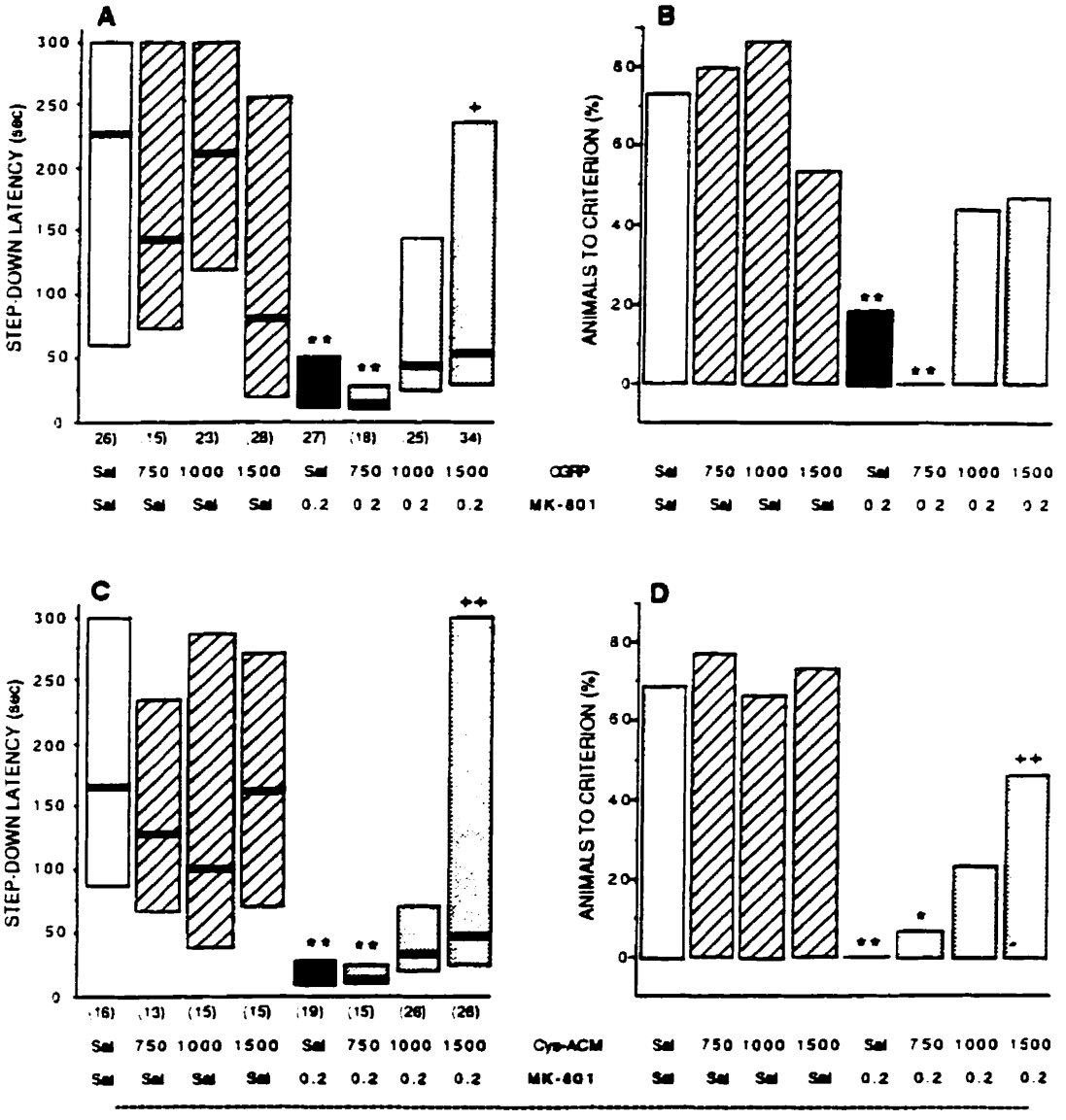

Figure 2. Dose-response effects of hCGRPα (A and B), [Cys(ACM)^{2,7}]CGRP (C and D) and CGRP$_{8-37}$ (E and F) on the MK-801 -induced learning impairments in the passive avoidance task. hCGRPα (1500 pmol) and [Cys(ACM)^{2,7}]CGRP (1500 pmol) significantly reduced the learning impairment induced by MK-801 whereas CGRP$_{8-37}$ had no effect. *$p<0.05$ and **$p<0.01$ vs control animals; $^{+}p<0.05$ vs MK-801 -treated animals.

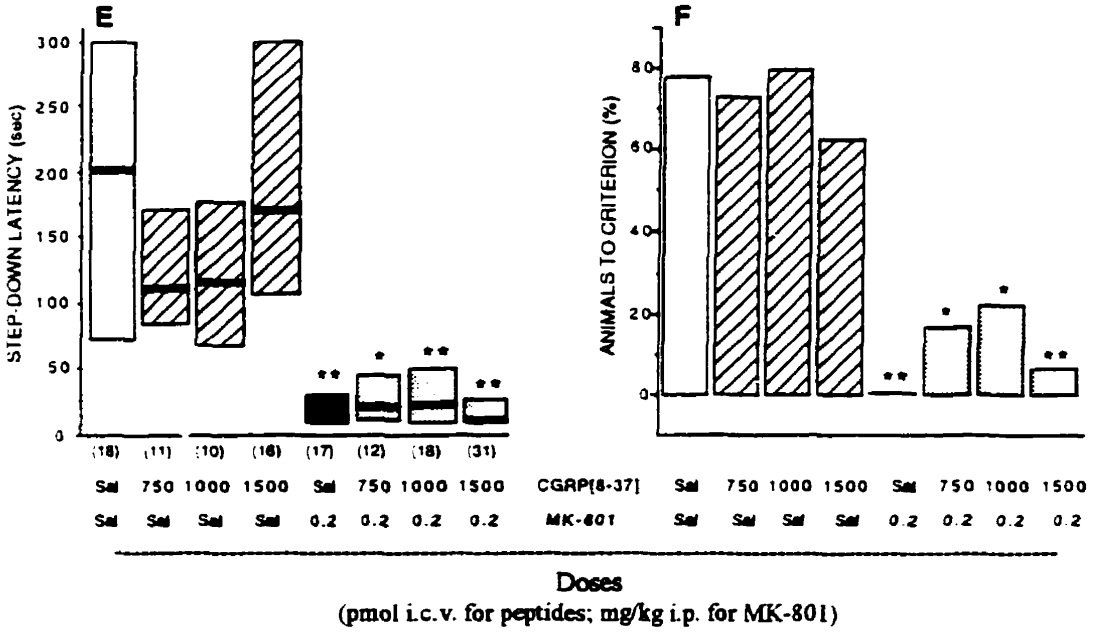

Figure 2. Dose-response effects of hCGRPα (A and B), [Cys(ACM)^{2,7}]CGRP (C and D) and $CGRP_{8-37}$ (E and F) on the MK-801 -induced learning impairments in the passive avoidance task. hCGRPα (1500 pmol) and [Cys(ACM)^{2,7}]CGRP (1500 pmol) significantly reduced the learning impairment induced by MK-801 whereas $CGRP_{8-37}$ had no effect. *$p<0.05$ and **$p<0.01$ vs control animals; $^{+}p<0.05$ vs MK-801 -treated animals.

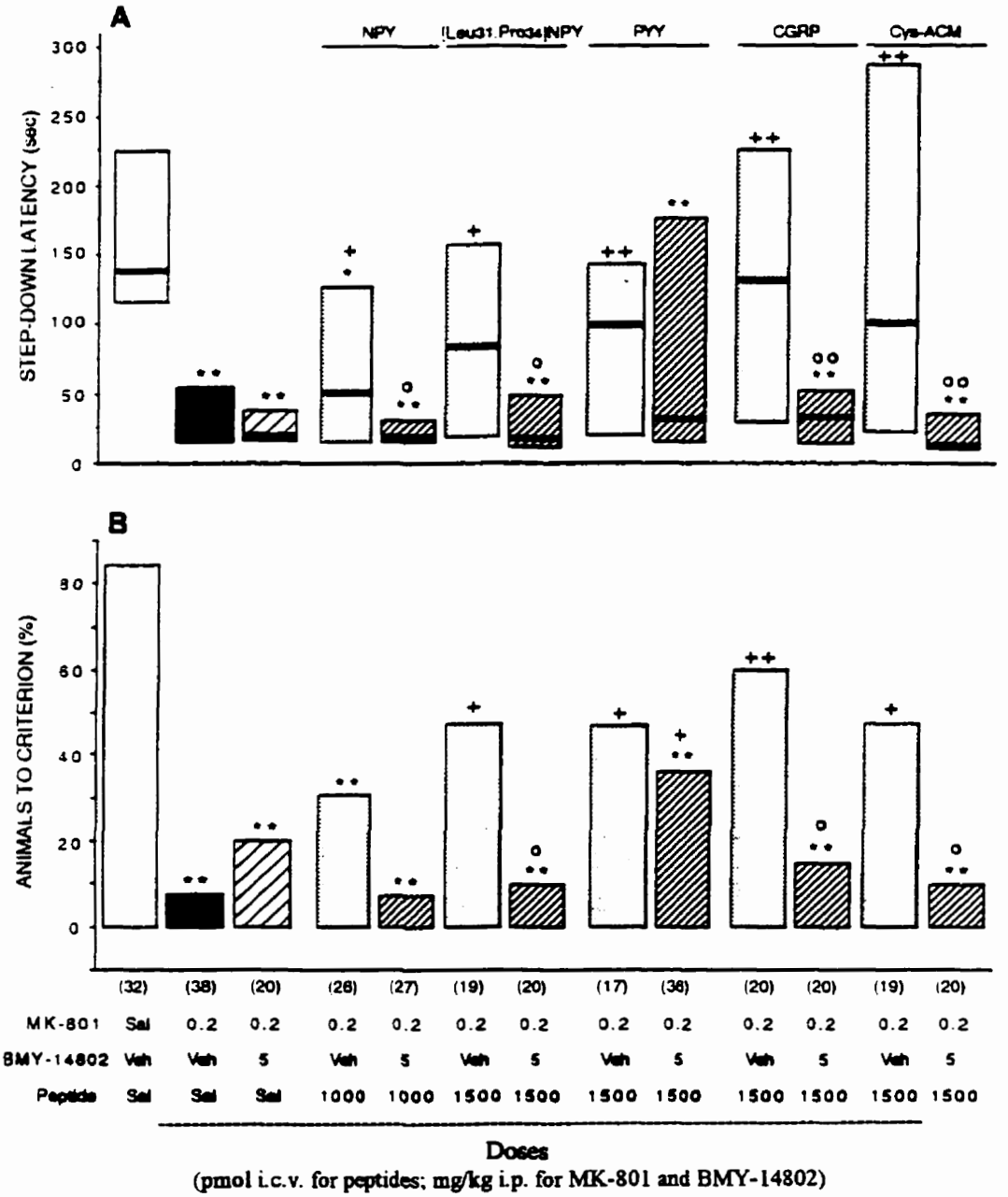

Figure 3. Antagonism, by BMY-14802, of the effects induced by NPY- and CGRP- related peptides on MK-801 -induced learning impairments in the passive avoidance task. When injected simultaneously with MK-801 (0.2 mg/kg, i.p.), BMY-14802 (5 mg/kg, i.p.) did not alter, by itself, learning impairments induced by MK-801 (third column). However, BMY-14802 significantly blocked the effects of NPY, [Leu^{31}Pro^{34}]NPY, hCGRPα, and [Cys(ACM)$^{2-7}$]CGRP on MK-801 -induced learning impairments. In comparison, BMY-14802 did not antagonize the effect of PYY. * $p<0.05$ and ** $p<0.01$ vs control animals; + <0.05 and ++ $p<0.01$ vs MK-801 -treated animals; °$p<0.05$ and °° $p<0.01$ vs peptide plus MK-801 -treated animals.

Chapter VI

DISCUSSION and CONCLUSION

DISCUSSION

The results obtained from the experiments leading to this thesis confirmed the existence of interactions between σ receptors and NPY-related peptides, *in vivo*. Furthermore, it was demonstrated for the first time, that certain CGRP-related peptides can interact with σ receptors *in vivo*, in a manner similar to NPY.

1. In vivo *interactions between NPY-related peptides and σ sites*

Our first study demonstrated that selected NPY-related peptides can modulate σ receptor binding in the mouse hippocampal formation, *in vivo* (Bouchard et al., 1992; 1993). NPY and its homologue PYY, as well as the non-selective probe NPY_{2-36} and the Y_1 agonist [$Leu^{31}Pro^{34}$]NPY significantly inhibited, in a dose-related manner, the *in vivo* binding of the prototypical σ ligand [^{3}H](+)SKF 10047 in the mouse hippocampal formation. In contrast, the Y_2 agonist NPY_{13-36} and the inactive analog, NPY-OH, did not compete for *in vivo* [^{3}H](+)SKF 10047/σ binding. The lack of effect of other neuroactive peptides such as VIP and neurotensin as well as the vasoactive substance adrenaline, further demonstrated the specificity of the observed effects. The involvement of σ receptors was confirmed with the potent inhibition (90%) of [^{3}H](+)SKF 10047/σ binding by the high-affinity σ ligand, haloperidol.

The *in vivo* interactions between NPY and σ systems seen in our study are in keeping with data obtained by other groups (Monnet et al., 1990b; 1992d,e; Roman et al., 1993). Similarly to various σ ligands (Monnet et al., 1990a; 1992b), microiontophoretic applications of selected NPY-related peptides in the CA_3 sub-field of the rat hippocampus were shown to selectively potentiate NMDA-induced firing of

pyramidal neurons, *in vivo* (Monnet et al., 1990b; 1992d,e; 1994). Intravenous administration of low doses of the σ antagonists haloperidol or BMY-14802, but not of the dopamine antagonist spiperone, suppressed the potentiating effect of NPY-related peptides demonstrating the involvement of σ sites. Thus, the interactions between NPY and σ systems seen in our *in vivo* binding experiments (Bouchard et al., 1992; 1993) corroborated these electrophysiological data (Monnet et al., 1990b; 1992d,e; 1994), albeit with some discrepancies. In our study, the Y_2 agonist NPY_{13-36}, did not compete for *in vivo* [^{3}H](+)SKF 10047/σ binding in the mouse hippocampus, while it potentiated NMDA-induced firing of CA_3 pyramidal neurons of the rat hippocampus. In addition NPY-OH, which has been found inactive in most NPY assays (Allen et al., 1987; Chang et al., 1985; Wahlestedt et al., 1986; for review see Dumont et al., 1992) as well as in our binding study (Bouchard et al., 1992; 1993) dose-dependently inhibited, in a haloperidol- and BMY-14802 -sensitive manner, the NMDA-induced response (Monnet et al., 1992e). An adequate explanation for these apparent discrepancies is presently lacking but likely relates to methodological differences. Furthermore NPY, in a manner similar to certain σ ligands, modulated NMDA-evoked [^{3}H]noradrenaline release from preloaded rat hippocampal slices, this potentiation being blocked by haloperidol (Roman et al., 1991a,b) providing further support for a role of NPY in the modulation of σ receptor function, *in vivo*.

NPY receptor subtype(s) involved

The existence of four subtypes of NPY receptors (denoted Y_1, Y_2, Y_3 and the recently defined Y_4/PP_1) have been reported so far. The cloned Y_1 receptor subtype (Eva et al., 1990; Herzog et al., 1992; Larhamar et al., 1992) is characterized by the high affinity of NPY and its homologue PYY, and of the Y_1 agonists [$Leu^{31}Pro^{34}$]NPY

and [Leu^{31}Pro^{34}]PYY (Dumont et al., 1992; 1993; 1995; 1996; Fuhlendorff et al., 1990; Krstenansky et al., 1990; Schwartz et al., 1989), and its very low (μM) affinity for C-terminal fragments such as NPY_{13-36} (Dumont et al., 1992; Gehlert, 1994; Grundemar et al., 1993; Wahlestedt and Reis, 1993). The recently purified (Wimalawansa, 1995) and cloned (Gehlert et al., 1996; Gerald et al., 1995; Rose et al., 1995) Y_2 receptor subtype is characterized by its high affinity for NPY, PYY and C-terminal fragments such as NPY_{13-36} and a lower affinity for [Leu^{31}Pro^{34}]NPY (Dumont et al., 1992; 1994; 1996; Grundemar et al., 1993; Wahlestedt and Reis, 1993). In contrast, the Y_3 subtype displays low affinity for PYY and high affinity for NPY-related peptides including the Y_1 agonist [Leu^{31}Pro^{34}]NPY and the Y_2 agonist NPY_{13-36} (Dumont et al., 1994; Grundemar et al., 1993; Wahlestedt and Reis, 1992; Wahlestedt et al., 1992). Recently, a fourth subtype of NPY receptors has been cloned. The Y_4/PP_1 receptor subtype is characterized by its high affintiy for PP-related peptides (Bard et al., 1995; Lundell et al., 1995; 1996; Yan et al., 1996) as well as for [Leu^{31}Pro^{34}]PYY (Bard et al., 1995; Gackenheimer et al., 1995).

Considering this receptor classification, the ligand selectivity profile observed in our studies (Bouchard et al., 1992; 1993; 1996a) suggests that the Y_1 receptor subtype likely mediates the interaction between NPY-related peptides and σ sites. Given the high affinity of the Y_2 probe NPY_{13-36} for the Y_2 and Y_3 receptors, it would seem unlikely that these NPY receptor subtypes could be involved in the effects observed since the C-terminal fragment NPY_{13-36} was inactive in our assays. Additionally, the Y_3 receptor subtype has low affinity for PYY which was found to be as potent as NPY in our experimental paradigm. However, an involvement of the newly characterized Y_4/PP receptor, which has rather high affinity for PP-related peptides as well as for PYY and

[Leu^{31}Pro^{34}]PYY, cannot be excluded at this time since PP-like peptides were not investigated in our study.

2. In vivo *interactions between CGRP-related peptides and σ sites*

The second study demonstrated, for the first time, that selected peptides of the CGRP family can modulate, *in vivo*, σ receptor binding in the mouse hippocampal formation and NMDA-induced activation of the CA_3 pyramidal cells of the rat hippocampus (Bouchard et al., 1995). We have shown that CGRP (hCGRPα and rCGRPβ), the $CGRP_2$ receptor subtype agonist [Cys(ACM)^{2,7}]hCGRPα, and amylin, a peptide that shares 50% homology with CGRP, significantly inhibited *in vivo* [^{3}H](+)SKF 10047/σ binding in the mouse hippocampal formation. In comparison, the purported $CGRP_1$ receptor subtype antagonist, $CGRP_{8-37}$, as well as other vasoactive peptides including atrial natriuretic peptide (ANF), substance P and substance P_{1-7} did not compete for [^{3}H](+)SKF 10047/σ binding, demonstrating the specificity of the observed effects. The pharmacological profile obtained in the binding experiments correlates with that found in the electrophysiological paradigm. In the CA_3 subfield of the rat dorsal hippocampus, hCGRPα decreased while [Cys(ACM)^{2,7}]hCGRPα enhanced NMDA-induced activation of CA_3 pyramidal cells in a current-dependent manner, in agreement with a receptor-mediated effect. As observed for *in vivo* binding experiments, the antagonistic fragment $CGRP_{8-37}$ did not modulate NMDA-induced firing of the CA_3 pyramidal cells. The involvement of σ receptors was confirmed by the potent inhibition (90%) of *in vivo* [^{3}H](+)SKF 10047/σ binding by haloperidol as well as by the blocking effect of haloperidol on hCGRPα and [Cys(ACM)^{2,7}]hCGRPα - induced actions.

CGRP receptor subtype(s) involved

Although *in vitro* binding experiments were not very successful in discriminating CGRP receptor subtypes, two classes of CGRP receptors have been identified on the basis of functional bioassays in various tissues, in addition to an atypical site characterized using binding assays (for review see van Rossum et al., 1996). The putative $CGRP_1$ subtype is defined by its high sensitivity to the potent antagonistic properties of various C-terminal fragments such as $CGRP_{8-37}$ (Chiba et al., 1989; Dennis et al., 1990; Quirion et al., 1992b). On the other hand, the putative $CGRP_2$ receptor subtype is defined by its sensitivity to the agonistic properties of the linear analogue [Cys(ACM)^{2,7}]hCGRPα, while being less sensitive to the antagonistic properties of $CGRP_{8-37}$ (Dennis et al., 1989; Quirion et al., 1992b). The existence of a third class of CGRP receptors is supported by binding data. This "atypical" receptor subtype is characterized by its high binding affinity for salmon calcitonin and rat amylin in selected areas of the rat brain including the nucleus accumbens and fundus striati. In comparison, $hCGRP_{8-37}$ and [Cys(ACM)^{2,7}]hCGRP have a reduced affinity in these brain regions (Dennis et al., 1991; Quirion et al., 1992b; Sexton et al., 1988; van Rossum et al., 1994; 1996).

In keeping with the pharmacological profile observed in our *in vivo* binding and electrophysiological experiments (Bouchard et al., 1995; 1996a), it seems rather unlikely that the effects observed were mediated through either the $CGRP_1$ or $CGRP_2$ receptor subtypes. The involvement of $CGRP_1$ sites is unlikely since $CGRP_{8-37}$ did not compete for σ/[^{3}H](+)SKF 10047 binding *in vivo*, nor did it antagonize the potentiating effect of hCGRPα on NMDA-induced activation of CA_3 pyramidal neurons. Given the potent modulatory effect of hCGRPα and of the $CGRP_2$ agonist

[Cys(ACM)^{2,7}]hCGRPα, it could be that the *in vivo* interactions seen between CGRP-related peptides and σ sites are mediated via $CGRP_2$ receptors. However, the high potency of amylin, which is relatively weak in most CGRP bioassays (for review see van Rossum et al., 1996), in competing for *in vivo* [^{3}H](+)SKF 10047/σ binding is puzzling. However, an involvement of the putative atypical CGRP receptor subtype, which has high affinity for CGRP, salmon calcitonin and rat amylin, cannot be discarded on the basis of our results. It is thus conceivable that the *in vivo* interactions between CGRP-related peptides and σ receptors observed in this study were mediated through the activation of an "atypical" CGRP receptor, or via a yet-to-be fully characterized receptor subtype. The recent cloning of the $CGRP_1$ receptor (Kapas and Clark, 1995) and of a novel receptor protein, apparently not G-protein coupled, that interacts with CGRP (Luebke et al., 1996) may provide interesting clues in that regard.

3. In vivo *interactions between σ receptors and NPY & CGRP: Neuroanatomical profile*

The *in vivo* inhibition of [^{3}H](+)SKF 10047/σ binding by NPY- and CGRP-related peptides seen in the mouse hippocampal formation was confirmed and extended to various other brain areas using an *in vivo/ex vivo* autoradiographic approach. The NPY homologue PYY, the Y_1 agonist [Leu^{31}Pro^{34}]NPY and the CGRP homologue rCGRPβ, but not the Y_2 agonist NPY_{13-36}, were found to significantly inhibit specific [^{3}H](+)SKF 10047/σ binding in most brain areas enriched with [^{3}H](+)SKF 10047/σ labeling including the pyramidal cell layer of the hippocampus, several cortical areas, the thalamus and hypothalamus, various mesencephalic structures as well as most cranial nerve nuclei (Bouchard et al., 1996a). This study adds further support to the

hypothesis that certain NPY- and CGRP-related peptides could act as global neuromodulators of σ receptor systems *in vivo* (Bouchard et al., 1993; 1995; Monnet et al., 1990b; 1992d,e).

4. *σ receptor subtype involved*

[^{3}H](+)SKF 10047 binds primarily to σ_1 sites *in vitro* (for review see Quirion et al., 1992a). It is then most likely that the *in vivo* interactions observed in our studies (Bouchard et al., 1993, 1995, 1996a) were mediated, at least in part, via activation of the σ_1 receptor subtype. Nevertheless, our data do not exclude the possible involvement of σ_2 sites. As pointed out earlier (Bouchard et al., 1993), the use of another σ ligand would have been more appropriate. For example [^{3}H]DTG, which acts on σ_1 and σ_2 sites with almost equal potency and [^{3}H](+)pentazocine, which selectively binds σ_1 sites, could have been used to distinguish between a σ_1- and a σ_2-mediated effect. However, previous attempts to label σ sites in the mouse brain, *in vivo*, were unsuccessful as the amount of binding recovered in the CNS using these two radiolabeled probes was too low to allow for adequate characterization (Bouchard, unpublished results).

5. *Possible mechanisms of action*

The mechanism(s) underlying the *in vivo* interactions between σ receptors and NPY- and CGRP-related peptides observed in our studies remains to be established. However, a direct action of the peptides on σ receptors seems rather unlikely since no interaction was observed *in vitro*. Using *in vitro* binding assays, NPY- or CGRP-related peptides failed to compete for either [^{3}H]DTG or [^{3}H](+)pentazocine/σ binding sites in rat brain membrane homogenates. Conversely, σ ligands did not compete for

either [^{125}I]NPY or [^{125}I]hCGRPα binding, *in vitro* (Bouchard et al., 1995; Quirion et al., 1991; Tam et al., 1991). Although the possibility of a direct interaction cannot be completely exluded (Roman et al., 1989), an indirect mode of action between NPY, CGRP and σ systems appears more likely to explain our *in vivo* data.

The endogenous σ ligand(s) is(are) yet to be characterized, but the existence of endogenous substance(s) with affinity for σ sites has been reported by several groups (Connor and Chavkin, 1991; 1992a,b; Contreras et al., 1987a; Su et al., 1987; Zhang et al., 1988). NPY- and CGRP-related peptides may induce the release of such endogenous substance(s) that would then interfere with *in vivo* [^{3}H](+)SKF 10047/σ binding. However, NPY and CGRP belong to distinct peptide families and their structure, their modulatory action on various neurotransmitter systems, their physiological effects as well as the distributional profile of NPY and CGRP receptors are clearly different (for reviews see Dumont et al., 1992; Poyner, 1992; van Rossum et al., 1996; Wettstein et al., 1995). This suggests that if the *in vivo* interactions between those peptides and σ sites are related to the release of one or more endogenous substance(s), it may not be the same one(s) for both NPY and CGRP-related peptides.

However, this hypothesis seems rather unlikely given the similar nature of the modulatory effects induced by NPY- and CGRP-related peptides on *in vivo* [^{3}H](+)SKF 10047/σ binding and NMDA-induced activation of CA_3 pyramidal cells in the hippocampus (Bouchard et al., 1992; 1993; 1995; 1996a; Monnet et al., 1990b; 1992d,e; 1994). Furthermore, our *in vivo* autoradiographic study (Bouchard et al., 1996a) failed to reveal significant difference between the potency of PYY, [Leu^{31}Pro^{34}]NPY and rCGRPβ in their respective inhibitory effect on [^{3}H](+)SKF 10047/σ binding in any of the brain areas examined suggesting that both peptides act via a similar mechanism. Furthermore, the combined administration of maximally

effective doses of rCGRPβ with either NPY, PYY or [Leu^{31}Pro^{34}]NPY provided no evidence of additive or antagonistic activity (Bouchard et al., 1995) suggesting that both classes of peptides exert their effects via a common signaling pathway.

Accumulated evidence suggests that σ (most likely σ_1 sites; Bowen, 1994; Itzhak, 1989; Itzhak and Khouri, 1988; Itzhak and Stein, 1991b; Connick et al., 1992b; Monnet et al., 1992a; 1994), NPY (Bard et al., 1995; Dumont et al., 1992; Gerald et al., 1995; Herzog et al., 1992; Larhammar et al., 1992; Michel, 1991; Wahlestedt et al., 1992) and CGRP (van Rossum et al., 1993; 1996; Poyner; 1992) receptors are members of the G-protein -coupled receptors superfamily. Therefore, NPY- and CGRP-related peptides, by acting on similar G-protein subtypes, may activate a cascade of events that, in turn, could alter the integrity of the σ receptor complex leading to inhibition of *in vivo* [^{3}H](+)SKF 10047/σ binding. One of the events involved could include G-protein -mediated changes in ion channel dynamics since an increasing body of evidence suggests that NPY, CGRP and σ receptors can modulate intracellular Ca^{2+} fluxes either through G-proteins, or directly via receptor-operated Ca^{2+} or K^+ channels (for reviews see Bouchard et al., 1996; Dumont et al., 1992; van Rossum et al., 1996). Unfortunately, the study of second messenger systems associated with σ receptors is still in its infancy and only allow for speculations.

6. *Behavioral relevance*

As stated above, NPY and CGRP are unlikely to act as direct endogenous σ ligands. Nevertheless, we have shown that their modulatory effects on σ receptor binding are behaviorally relevant (Bouchard et al., 1996c). Using a step-down passive avoidance paradigm, we demonstrated that selected NPY- and CGRP-related peptides, in a manner similar to σ ligands (Maurice et al., 1994a,c), were able to significantly

antagonize MK-801 -induced learning impairments in the mouse (Bouchard et al., 1996c). NPY, PYY and the Y_1 agonist [$Leu^{31}Pro^{34}$]NPY (but not the Y_2 agonist NPY_{13-36}), as well as hCGRPα and the $CGRP_2$ agonist [Cys $(ACM)^{2,7}$]hCGRPα (but not the $CGRP_1$ antagonist $CGRP_{8-37}$) significantly attenuated learning impairments induced by the systemic administration of the non-competitive NMDA receptor antagonist, MK-801. Except for PYY, the effects of these peptides were blocked by the purported σ receptor antagonist BMY-14802, confirming the involvement of σ receptors. The similarity between the peptide-selectivity profile observed in the behavioral study and our previous *in vivo* binding and autoradiographic studies (Bouchard et al., 1992; 1993; 1995; 1996a) is striking and suggests that a common mechanism(s) of action may underlie the effects of NPY- and CGRP-related peptides on *in vivo* [^{3}H](+)SKF 10047/σ binding and on MK-801 -induced learning impairment in the mouse.

The involvement of σ receptors in learning and memory has recently been suggested (Earley et al., 1991; Matsuno et al., 1993a,b; 1994; 1995; Maurice et al., 1994a-c). For example, Maurice et al. (1994a,c) have shown that σ ligands including DTG, (+)SKF 10047, (+)pentazocine and PRE-84 attenuated MK-801 -induced learning impairments in the mouse. The attenuation of learning impairments by these σ ligands was blocked by the administration of σ antagonists such as BMY-14802 and NE-100 supporting the involvement of σ sites (Maurice et al., 1994a,c). This contention was strengthen further by the fact that down-regulation of σ receptors following a subchronic pre-treatment with haloperidol prevented the attenuating effect induced by σ ligands on MK-801 -induced learning impairments (Maurice et al., 1994c). MK-801 being a non-competitive NMDA receptor antagonist (Loo et al., 1987; Wong et al., 1986), the effects induced by σ ligands in this behavioral paradigm

are likely representative of an interaction between NMDA and σ systems as suggested by electrophysiological data obtained earlier by Monnet et al. (1990a; 1992b). Since similar modulatory effects were observed with NPY- and CGRP-related peptides in the electrophysiological (Bouchard et al., 1995; Monnet et al., 1990b, 1992d,e) and behavioral (Bouchard et al., 1996c) paradigms, it appears that these peptides can modulate NMDA-dependent learning processes via σ receptor activation. A direct effect of NPY- and CGRP-related peptides on the NMDA receptor complex can be ruled out since BMY-14802, which blocked NPY and CGRP effects, has very low affinity for the NMDA receptor (Largent et al., 1988). Moreover, NMDA itself did not reverse MK-801 -induced amnesia (Maurice et al., 1994a; Parada-Turska and Turski, 1990). It thus appears that selected NPY- and CGRP-related peptides, by acting as neuromodulators of σ systems *in vivo*, can indirectly modulate certain NMDA-dependent learning processes.

7. *NPY, CGRP and endogenous σ ligands*

The putative existence of endogenous ligand(s) for the σ receptor is now rather well accepted. However, its or their identification remains to be accomplished. NPY and related peptides were proposed as putative σ ligands (see section 10.4). However, the results reported in the present thesis strongly suggest that this is not the case since *in vitro* interaction between NPY-related peptides and σ sites failed to be observed by our group as well as others. On the other hand, a growing body of evidence has accumulated demonstrating that σ sites can interact with peptides of the NPY family *in vivo*, and the present thesis provides a strong case for the existence of such interactions. Moreover, the observation, well documented in this thesis, that certain peptides of the CGRP family could, in a manner similar to NPY, interact with σ sites *in*

vivo, supports further the contention that NPY and related peptides do not behave as endogenous σ ligands. However, our results strongly support the hypothesis that NPY- and CGRP-related peptides can act as neuromodulators of σ receptor function *in vivo*. Given the potent effects of selected NPY- and CGRP-related peptides in *in vivo* paradigms including electrophysiological recordings and binding assays, the reported lack of direct *in vitro* receptor binding interaction between these systems is intriguing. One could argue that the effects mediated by both peptide families are, in fact, non-specific and relate to global cell membrane perturbations. It is true that peptide interactions with σ receptors are not exclusive to NPY as it was believed at first. Nevertheless, we demonstrated, using binding, electrophysiological and behavioral approaches, that the effects observed, if not exclusive to NPY, were selective to certain peptide families and restricted to particular analogues and fragments, providing arguments against the likelyhood of non-specific effects. Our results further suggest that specific receptor subtypes for NPY and CGRP likely mediate their effects on σ systems.

Several mechanisms could be suggested to attempt to explain the mechanisms underlying NPY and CGRP interactions with σ sites, *in vivo* (see Chapter 4. *Discussion*) However, the rather poor knowledge of the σ receptor *per se*, does not allow for the identification of such a mechanism. Additional data on the intracellular consequences of σ receptor activation will be needed in order to shed light on the mechanisms behind the *in vivo* interactions reported in the present thesis. In that regard, the cloning of a putative σ_1 receptor has recently been achieved (Glossman et al., *Proc Natl Acad Sci*; in press) and should be most useful to elucidate the intracellular events following σ receptor activation. This receptor, cloned from human and mouse cDNA libraries, may be an enzyme involved in cholesterol synthesis and its

mRNA, identified by Northern blotting, was found in the liver, adrenal gland, ovary and foetal tissue. Further studies of mRNA localization using *in situ* hybridization should provide important clues regarding the functions of the σ_1 site in the nervous system.

CONCLUSION

Although the exact mechanisms of action of σ receptors are yet to be fully understood, the demonstrated existence of interactions between σ receptors and various major neurotransmitter systems such as glutamatergic, cholinergic, noradrenergic and dopaminergic systems indicates that σ sites may primarily act as neuromodulators. This neuromodulatory role would provide σ receptors with a wide range of effects in the nervous system. Elucidating the mechanisms regulating σ receptors activities would be of great value toward the understanding of several other neurotransmitter systems and could provide the opportunity to develop new means to indirectly modulate them. This strategy, in turn, may assist in the development of new drugs for pathologies related to these neurotransmitter systems.

The term "σ receptor" was used throughout this thesis. However, the fact that an endogenous σ ligand(s) is yet to be identified and that the cloning of σ receptor subtypes is yet to be reported may not allow to qualify σ sites as receptors. Nevertheless, they fulfill many requirements to be considered as such: 1) They were shown to be widely but discretely distributed in different brain areas and various peripheral tissues; 2) their binding parameters were shown to be modulated upon drug treatments; 3) their activation is followed by the modulation of various

neurotransmitter systems and second messenger pathways and finally, 4) certain σ ligands were shown to have significant behavioral effects including the modulation of posture and movement, and of cognitive functions such as learning and memory. However, the elucidation of the role(s) of σ receptors has been hampered by their significant affinity for a wide range of structurally dissimilar compounds and the lack of very selective ligands allowing for a good discrimination between σ receptor subtypes. Among the pressing needs to clarify the functional relevance of σ receptors are 1) the development of highly selective σ ligands for each identified subtype, 2) the identification of endogenous σ ligand(s), 3) their purification to homogeneity 4) as well as the cloning of each σ receptor subtypes. Achievement of these goals should be most helpful toward the resolution of the "σ enigma" (Chavkin, 1990).

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INTRODUCTION and DISCUSSION

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Appendix I

[^{3}H]DTG AND [^{3}H](+)PENTAZOCINE BINDING SITES IN THE RAT BRAIN: AUTORADIOGRAPHIC VISUALIZATION OF THE PUTATIVE SIGMA-1 AND SIGMA-2 RECEPTOR SUBTYPES

Preface.

The aim of this manuscript was to describe, using an *in vitro* autoradiographic approach, the discrete distributional profile of σ_1 and σ_2 receptor subtypes in the rat brain. Using [^{3}H]DTG and [^{3}H](+)pentazocine as σ ligands, we show that σ_1 and σ_2 receptor sites are widely but discretely distributed in the rat brain. Furthermore, we demonstrate that the distributional profiles for both σ receptor subtypes are similar, but that the ratio of σ_1 to σ_2 receptor densities is different among brain regions.

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[^{3}H]DTG AND [^{3}H](+)PENTAZOCINE BINDING SITES IN THE RAT BRAIN: AUTORADIOGRAPHIC VISUALIZATION OF THE PUTATIVE SIGMA$_1$ AND SIGMA$_2$ RECEPTOR SUBTYPES.

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ABSTRACT

Sigma (σ) receptors have generated a great deal of interest on the basis of their possible role in psychosis and on locomotor behaviors. The effects of σ drugs on these various functions are apparently mediated by different σ receptor subtypes (σ_1 and σ_2). However, little information is currently available on the discrete anatomical distribution of these putative σ receptor subtypes in the rat brain. The aim of the present study was to investigate, by quantitative autoradiography, the respective distribution of purported σ_1 and σ_2 receptor subtypes in the rat brain using [^{3}H]DTG, a universal σ ligand[86], and [^{3}H](+)pentazocine, a selective σ_1 ligand[7]. Putative σ_2 receptor sites were visualized using [^{3}H]DTG in presence of a saturating concentration of (+)pentazocine. Specific [^{3}H]DTG and [^{3}H](+)pentazocine binding sites were found to be widely but discretely distributed in the rat brain. The highest densities of specific labeling were seen in various cranial nerve nuclei, followed by certain hippocampal sub-fields and laminae, the red nucleus, the interpeduncular nucleus and mid-layers of primary and secondary motor cortices. Lower amounts of specific binding were present in various other structures including most thalamic and hypothalamic nuclei, and the cerebellum. Interestingly, [^{3}H]DTG binding in the motor cortex was found to be particularly resistant to a saturating concentration of (+)pentazocine suggesting an enrichment in the putative σ_2 receptor subtype. This also apply for few other structures such as the nucleus accumbens, substantia nigra pars reticulata, central gray matter, oculomotor nucleus and cerebellum. On the other hand, the σ_1 subtype is more abundant in most other regions with the highest densities seen in the dentate gyrus of the hippocampal

formation, facial nucleus, and various thalamic and hypothalamic nuclei. The comparative localization of the σ_1 and σ_2 receptor binding sites likely relates to the differential effects of σ_1 and σ_2 drugs in the rat brain.

INTRODUCTION

The proposal for the existence of a new class of receptors termed sigma (σ) on the basis of its prototypical ligand SKF 10,047 (N-allylnormetazocine[24,44]) generated substantial interest, likely because of its possible involvement in psychosis such as schizophrenia[9,18,20,22,33,36,60,74,77,84]. The role of σ receptors in psychotic behaviors originally stems from observations suggesting that certain drugs with high affinity for σ sites such as SKF 10,047 induced hallucinations in man[40] and delirium in the chronic spinal dog[44]. High densities of σ binding sites found in various limbic structures[16,26,56] and the high affinity of classical (haloperidol and chlorpromazine) and atypical (remoxipride, rimcazole, umespirone, Dup 734) neuroleptics for σ sites[22,42,74,75,76,80] argue in favor of their implication in psychosis. Further, post-mortem studies have reported a slight reduction in the levels of σ receptors in the brain of schizophrenic patients[72,88]. Finally, chronic treatments with the high-affinity σ ligand, haloperidol, were reported to down-regulate σ binding sites in animals and humans[31,48,67]. In spite of all these informations, a genuine involvement of σ sites in the induction of psychotic behaviors remains to be fully established[60,65,84].

More recently, σ receptors have been associated with various other functions such as the modulation of posture and movement[84,85], neuroprotection[15,17,63], anxiety and depression[4,18], nociception[10] and drug abuse[57,71]. σ drugs may also be implicated in the regulation of certain endocrine and immune functions[76,78] as well as in cognitive behaviors[21,51,52,53,54].

Receptor binding homogenates and autoradiography using various radioligands ([^{3}H]haloperidol, [^{3}H]DTG, [^{3}H](+)SKF 10,047, [^{3}H](+)pentazocine, [^{3}H](+)3-PPP, [^{3}H]dextromethorphan and [^{3}H]ifenprodil) have demonstrated the widespread distribution of putative σ sites throughout the brain of various species including mouse[3,6,23,45,73,87], rat[26,29,73], guinea-pig[1,26,56,75], cat[25], monkey[46] and human[72,81,88,89]. These studies showed that σ binding sites are different from opioid, dopaminergic and phencyclidine receptors[64,65], and provided evidence for the existence of at least two classes (σ_1 and σ_2) of σ sites[27,33,34,35,64,82]. These two putative subtypes demonstrate similar affinity for "classical" σ ligands such as [^{3}H]haloperidol and [^{3}H]DTG, while the dextrorotatory benzomorphans (eg. (+)SKF 10,047 and (+)pentazocine) preferentially recognize the σ_1 subtype[27,64,82]. The levorotatory isomers of these benzomorphans only have low to moderate affinity for σ sites and do not distinguish between the σ_1 and σ_2 subtypes[27,64]. Accordingly, radiolabeled (+)benzomorphans such as [^{3}H](+)pentazocine have been used as σ_1 ligand[7]. Other σ ligands such as dextromethorphan[61,62] and carbetapentane[68] apparently also behave as selective σ_1 ligands. Additionally, phenytoin can allosterically modulate σ_1 sites without affecting the σ_2 subtype[61], and σ_1 and σ_2 sites are differentially regulated by G-protein modifying agents[1,30,58]. Finally, the partial purification and isolation of σ binding sites further support the existence of multiple σ receptor subtypes. The molecular size of σ_1 site determined using photoaffinity labeling and/or gel electrophoresis, was found to be between 25-29 kDa, while that of the σ_2 subtype is lower (18-21 kDa)[27,28,37,38,39,69].

Little information is currently available on the discrete, comparative distribution of the σ_1 and σ_2 receptor subtypes in the CNS. This is particularly significant for the rat

brain for which homogenate binding assays and functional data clearly support the existence of σ receptor subtypes[33,34,35,43,64]. The aim of the present study was to investigate, using *in vitro* quantitative receptor autoradiography, the comparative distribution of the σ_1 and σ_2 receptor subtypes in the rat brain. Our data show the differential but overlapping distribution of σ_1 and σ_2 sites in the rat brain.

MATERIALS & METHODS

Materials:

[^{3}H]DTG (37 Ci/mmol) and [^{3}H](+)pentazocine (42 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Tritium-sensitive films and tritium standards were from Amersham Canada (Oakville, Ontario, Canada). NaCl, Tris buffer and sucrose were purchased from Sigma Chemicals (St-Louis, MI, USA) and bovine serum albumin (BSA) was purchased from Boehringer Mannheim (Laval, Québec, Canada). Gelatin, chromium alum and other chemicals were of the highest analytical grade and were obtained from Fisher Scientific Co. (Montréal, Québec, Canada).

Animals:

Male Sprague-Dawley rats (250-300 g.) were purchased from Charles River (St-Constant, Québec, Canada). Animals were housed in a temperature and humidity controlled room on a 12 hour light/dark cycle, and were fed *ad libitum* with standard laboratory chow and tap water. They were kept under these conditions for at least 24 hours prior to experiments. Animal care was according to protocols and guidelines approved by McGill University and the Canadian Council for Animal Care.

Tissue preparation:

Following decapitation, brains were quickly removed and immersed for 20 sec in 2-methylbutane at -40°C and then kept frozen at -80°C until sectioning. Slicing was performed using a refregerated cryostat; 20 µm-thick coronal sections being mounted on

gelatin-coated slides, desiccated overnight at 4°C and stored at -80°C for no more than two weeks until use.

In vitro *quantitative autoradiography:*

7nM [^{3}H]DTG was used to label both the σ_1 and the σ_2 receptor subtypes while 5nM [^{3}H](+)pentazocine was employed to visualize the σ_1 subtype. 7nM [^{3}H]DTG in the presence of a saturating concentration of (+)pentazocine (5μM) was used in order to occlude the σ_1 sites and hence reveal the distribution of the putative σ_2 subtype. Non-specific binding was determined using 10μM haloperidol.

After warming up to 4°C, brain sections were preincubated for 15 min in Tris-HCl buffer (50mM, 1 mg/ml BSA or 25mM sucrose, pH 7.4, 22°C), followed by a 45 min incubation at room temperature in a Tris-HCl buffer either containing 7nM [^{3}H]DTG (σ_1 and σ_2), 5nM [^{3}H](+)pentazocine (σ_1) or 7nM [^{3}H]DTG in presence of 5μM (+)pentazocine (σ_2). At the end of the incubation period, sections were washed twice for four min in ice-cold buffer (Tris-HCl, 10mM, 2 mg/ml BSA, pH 7.4) before dipping into distilled water to remove ions. Slides were then dried under a cold stream of air, and apposed to tritium-sensitive films for six weeks and then developed as described elsewhere[66].

Data analysis:

At least three different animals were used for each experiment. Optical densities (expressed as a mean ± SEM in fmol/mg tissue, wet weight) were quantified by computerized densitometry using an MCID image analysis system (Imaging Research Inc., St-Catharines, Ontario, Canada) and low activity tritium standards as described

elsewhere[66]. The apparent ratio of σ_1 to σ_2 sites was determined using readings from sections incubated with 7nM [^{3}H]DTG plus 5μM (+)pentazocine for the σ_2 subtype, while σ_1 values were derived by substracting the σ_2 readings from total [^{3}H]DTG binding.

RESULTS

Total σ/ [^{3}H]DTG labeling:

Specific [^{3}H]DTG binding is widely but discretely distributed throughout the rat brain (Fig. 1A). Highest densities of specific [^{3}H]DTG labeling are found in various cranial nerve nuclei (Fig. 2E), and in the dentate gyrus and pyramidal layer of the hippocampal formation (Fig. 2B). Various areas associated with motor functions such as the substantia nigra, the red nucleus and the cerebellum were also enriched with [^{3}H]DTG binding (Fig. 2D). The superficial gray layer of the superior colliculus and the central gray matter contained lower, but still significant levels of [^{3}H]DTG labeling (Fig. 2D).

Various cortical areas were also enriched with σ labeling (Fig. 1A) with the highest amounts of binding seen in the retrosplenial cortex (Fig. 2A). Most other brain regions such as the non-pyramidal layer of the hippocampus, the caudate-putamen, the nucleus accumbens as well as various thalamic and hypothalamic nuclei were not as enriched in σ sites (Fig. 1A). White matter areas such as the corpus callosum (Fig. 2C) contained only very low levels of specific [^{3}H]DTG binding.

σ_1/ [^{3}H](+)pentazocine binding:

As for specific [^{3}H]DTG binding, specific [^{3}H](+)pentazocine labeling, most likely representing σ_1 sites, are discretely distributed in the rat brain (Fig. 1B). Quantification of labeling revealed that brain stem nuclei are particularly enriched with

specific [^{3}H](+)pentazocine binding (Fig. 2E). For example, the oculomotor, trigeminal and facial cranial nerve nuclei are most enriched with [^{3}H](+)pentazocine binding (Fig. 2E), in addition to the red nucleus and the substantia nigra (Fig. 2D). The pyramidal layer of the hippocampal formation contained very high levels of specific [^{3}H](+)pentazocine labeling (Fig. 2B) while most cortical areas contained lower but still significant amounts of specific labeling (Fig. 2A). Sub-cortical forebrain structures such as the striatum, thalamus and hypothalamus were not as enriched with specific [^{3}H](+)pentazocine labeling (Fig. 2C).

For comparison, the apparent density of σ_1 sites in the rat brain was also determined by substracting from total [^{3}H]DTG binding, putative σ_2 labeling as determined using [^{3}H]DTG in the presence of a saturationg concentration of (+)pentazocine. While absolute values are not necessarily identical to those evaluated for specific [^{3}H](+)pentazocine labeling, the observed distributional profile is very similar with highest levels of specific binding found in cranial nerve nuclei (Fig. 2E) and in the pyramidal cell layer of the hippocampus (Fig. 2B).

σ_2/ [^{3}H]DTG in the presence of (+)pentazocine as blocker:

The apparent levels of putative σ_2 subtype is generally lower than that of σ_1 sites in the rat brain although also broadly distributed (Fig. 2). However, few areas such as the substantia nigra pars reticulata (Fig. 2D), central gray (Fig. 2D), oculomotor nucleus (Fig. 2E), nucleus accumbens (Fig. 2C), cerebellum (Fig. 2D) and motor cortex area (Fig. 1C) displayed higher amounts of σ_2 than σ_1 sites.

DISCUSSION

The present study shows that σ receptor sites labeled using either [^{3}H]DTG or [^{3}H](+)pentazocine are widely, but discretely, distributed in the rat brain. Highest densities of sites for both radioligands were observed in areas related to motor functions such as the motor nuclei of the cranial nerves, the red nucleus and the substantia nigra pars compacta, as well as in limbic structures including the dentate gyrus and pyramidal cell layer of the hippocampus. Overall, the distributional profile of σ receptor sites in the rat brain observed in this study is consistent with previous observations[41,56,83], except that σ receptors appear to be more abundant in the guinea pig brain, which also displays a greater proportion of σ_1 over σ_2 binding sites[83].

Although [^{3}H](+)pentazocine is fairly selective for the σ_1 receptor subtype[7,19,27,64] while [^{3}H]DTG labels both the σ_1 and σ_2 subtypes[64,84] the present study reveals that the anatomical distribution of specific [^{3}H]DTG and [^{3}H](+)pentazocine is overlapping in the rat brain, which is consistent with an earlier study performed in the guinea-pig brain[83]. Our results also demonstrate the selective enrichment in σ_1 and σ_2 receptor binding sites in most areas of the rat brain. This is in apparent contrast with the study of Leitner et al.[43] which demonstrated that, in the rat brain, σ receptors are mostly of the σ_2 subtype. This apparent discrepancy may be due to the different experimental approaches used, namely homogenate binding vs quantitative receptor autoradiography. However, a more likely explanation may relate to the use of a high concentration of (+)pentazocine (5μM) in our study in order to fully mask σ_1 receptor sites and hence unveil the unique distributional pattern of the genuine σ_2 binding

subtype. It is known that (+)pentazocine has an apparent K_D of 7nM for σ_1 sites[8] while its affinity for the σ_2 subtype is about 300 times lower[27]. It has also been shown that (+)pentazocine can bind to a second, low affinity site in the rat brain[43]; the apparent affinity and identity of this site remaining to be established, but it possibly represents the σ_2 subtype. It is likely that at a concentration of 5μM, (+)pentazocine could mask a certain (low) proportion of σ_2 sites. In any case, the present study allowed, for the first time, the visualization of the respective anatomical localization of putative σ_1 and σ_2 binding sites in the rat brain. Few brain areas like the substantia nigra pars reticulata, central gray matter, oculomotor nuclei, cerebellum and the motor cortex are particularly enriched with σ_2 sites. Such a distributional profile strengthens the hypothesis that the σ_2 receptor subtype may be particularly involved in the control of posture and movement[84,85]. However, a recent study failed to report the presence of high levels of σ_2 sites in the brainstem and cerebellum, while the highest amounts of sites were found in the hippocampus[55].

Several arguments have been recently put forward specifically associating the σ_2 subtype with posture and movement. In behavioral studies using dystonia induced by microinjections of various σ ligands in the red nucleus, Walker and co-workers[49] observed a significant correlation between the degree of torticolli induced by σ ligands and their affinity for [^{3}H]DTG binding in the rat brain. However, no such correlation was observed in the guinea-pig brain, purportedly because the σ_1 receptor subtype is preponderant in the brain of this specie[27,83]. More recently, Matsumoto et al.[47] provided additional evidence for the involvement of the σ_2 subtype in the control of posture and movement. Using two new ligands, BD1047 and BD1063, these authors observed that BD1047, which displays a 10-fold higher affinity than BD1063 for the σ_2 subtype,

showed a higher potency to antagonize the dystonic posture induced by DTG, suggesting a prominent role for the σ_2 subtype in the studied behavior. However, the high affinity of both ligands for the σ_1 receptor subtype in addition to the significant amount of σ_1 sites in the red nucleus (this study) make it most difficult to preclude a role for the σ_1 subtype in the observed motor effects. Additionally, DTG and purported σ_1 ligands such as (+)pentazocine and dextrallorphan were shown to inhibit the firing of rubral neurons[50]. If these electrophysiological data are relevant to postural changes seen following the injection of σ ligands in the red nucleus, a role for the σ_1 subtype in dystonia cannot be fully excluded. Further studies using more selective σ_2 ligands will be required to resolve this issue. Interestingly, the development of an apparently σ_2-selective ligand has recently been documented ([^{3}H]BIMU-1[5]), but no functional data are available yet.

Another interesting effect of σ ligands relates to the modulation of cognitive behaviors[21,51,52,53,54]. For example, using short- and long-term memory paradigms, Maurice et al.[52] demonstrated that the memory impairments induced by intraperitoneal injections of MK-801 in the mouse were antagonized by DTG, (+)SKF 10,047 and (+)pentazocine. Furthermore, the effects of these σ ligands were prevented by the administration of purported σ receptor antagonists such as NE-100 and/or BMY-14802[52,53,54]. The modulation of cognitive performance induced by σ ligands has been attributed to the σ_1 receptor subtype[52] since the (+) isomers of the benzomorphans SKF 10,047 and pentazocine were more potent than the (-)isomers. However, the non selective σ ligand, DTG, was the most effective drug in antagonizing the memory impairments induced by MK-801[52]. Thus, the potential involvement of the σ_2 subtype cannot be fully excluded, and the use of selective σ_2 ligands will be necessary in order

to determine if this receptor subtype is involved in cognitive processes such as learning and memory.

It has recently been reported that certain steroids such as dehydroepiandrosterone sulfate (DHEA-S) and pregnenolone sulfate (PREG-S) modulate NMDA-evoked [^{3}H]noradrenaline (NA) release in the rat hippocampus, via a σ receptor-mediated mechanism[59]. The involvement of σ receptors was suggested on the basis that two purported σ antagonists, haloperidol and BD1063, completely prevented the effects of DHEA-S and PREG-S on NMDA-evoked NA release. Moreover, the observed effects of the steroids were similar to that of the non-selective σ ligand, DTG. The σ receptor subtype involved in the modulation of NMDA-evoked NA release by DHEA-S and PREG-S was postulated to be σ_1 since pertussis toxin suppressed the effects of the steroids[59]. In keeping with this hypothesis it has been proposed that the σ_1 but not the σ_2 receptor subtype, is coupled to pertussis toxin-sensitive guanine nucleotide binding proteins[2,11,30,32,34,58,64].

These latter data are also relevant to the potential existence of endogenous σ ligands whose nature are yet to be fully determined[12,13,14,78,79,90]. Interestingly, it was reported that some steroids including progesterone, testosterone and pregnenolone sulfate inhibited the binding of [^{3}H](+)SKF 10,047 and [^{3}H]haloperidol in guinea-pig spleen and brain membranes[78]. However, controversies still remain as to the relevance of these *in vitro* binding data, given the relatively low affinity of these steroids to compete for σ binding sites[70].

In summary, the present study reveals, using *in vitro* receptor autoradiography, the comparative anatomical distribution of σ_1 and σ_2 receptor subtypes in the rat brain. Our results show the prevalence of the σ_2 subtype in regions related to motor functions and support the likely involvement of this site in the modulation of posture and movement induced by σ ligands[84,85]. Furthermore, the high levels of σ_1 labeling observed in the hippocampal formation and other limbic areas argue in favor of the significance of this σ receptor subtype in learning and memory[51,52,53,54] and in some psychiatric disorders[9,18,20,22,33,36,60,74,76,77,84].

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FIGURES LEGENDS

Figure 1A.

Total and non-specific [^{3}H]DTG binding in the rat brain. Total [^{3}H]DTG binding was determined using 5nM [^{3}H]DTG, and the residual non-specific labeling using 10μM haloperidol. *Abbreviations used:* Amb.n: ambiguous nucleus; Amy.n.: amygdala; CA_1-CA_3: CA_1 and CA_3 sub-fields of the hippocampus, pyramidal layer; CG: central gray matter; CPu: caudate-putamen; Hypothal.: hypothalamus; LC: locus coeruleus; n.3: occulomotor nucleus; n.5: trigeminal nucleus; n.7: facial nucleus; n.TS: nucleus tractus solitarius; Red.n.: red nucleus; SC: superior colliculus, superficial gray layer; SNc: substantia nigra pars compacta.

Figure 1B.

Total and non-specific [^{3}H](+)pentazocine binding in the rat brain. Total [^{3}H](+)pentazocine binding was determined using 7nM [^{3}H](+)pentazocine and the residual non-specific labeling using 10μM haloperidol. *Abbreviations used:* Amb.n: ambiguous nucleus; Amy.n.: amygdala; CA_1-CA_3: CA_1 and CA_3 regions of the hippocampus, pyramidal layer; CC: corpus callosum; CG: central gray matter; CPu: caudate-putamen; Gl: olfactory bulb, glomerular layer; IGr: olfactory bulb, internal granular layer; LC: locus ceruleus; Mi: olfactory bulb, mitral layer; n.Acc.: nucleus accumbens; n.5: trigeminal nucleus; n.7: facial nucleus; n.TS: nucleus tractus solitarius; ON: olfactory bulb, olfactory nerve layer; Pir.: piriform cortex; SC: superior colliculus, superficial gray layer; Sep.: septum; SNc: substantia nigra, pars compacta.

Figure 1C.

Putative σ_2 receptors in the rat brain. First column: Total [^{3}H]DTG binding using 5nM [^{3}H]DTG. Second column: Total [^{3}H]DTG binding in the presence of 5μM (+)pentazocine (to mask the σ_1 sites), revealing the distribution of putative σ_2 receptor subtypes. Third column: non-specific binding remaining in the presence of 10μM haloperidol. MoCx: motor cortex; Pir.: piriform cortex; SNc: substantia nigra pars compacta; CA_1: CA_1 sub-fields of the hippocampus, pyramidal layer.

Figure 2.

Quantitative analysis of σ_1 and σ_2 binding sites in the rat brain. First column: Specific [^{3}H]DTG labeling (σ_1 and σ_2); Second column: specific [^{3}H](+)pentazocine labeling (σ_1); Third column: Apparent density of σ_1 sites determined by substracting from specific [^{3}H]DTG binding, putative σ_2 labeling as determined using [^{3}H]DTG in the presence of a saturating concentration of (+)pentazocine. Fourth column: Apparent density of putative σ_2 receptor subtypes as determined using [^{3}H]DTG in the presence of a saturating concentration of (+)pentazocine. Data represent mean ± S.E.M. of three experiments.

Figure 2A; Cortical areas. FCx: frontal cortex; PCx: parietal cortex; TCx: temporal cortex; OCx: occipital cortex; CgCx: cingulate cortex; RSCx: retrosplenial cortex; PirCx: piriform cortex. *Figure 2B*; Hippocampus. CA1; CA2; CA3: CA_1, CA_2 and CA_3 sub-field of the hippocampus, pyramidal layer; DG: dentate gyrus. *Figure 2C*: Thal.: thalamus; Zi: zona incerta; Hypothal.: hypothalamus; CPu: caudate-putamen; Accum.: accumbens nucleus; CC: corpus callosum; Cereb.: cerebellum. *Figure 2D*: Midbrain areas. SC (SGL): superior colliculi, superficial gray layer; Red n.: red nucleus;

SNr: substantia nigra pars reticulata; SNc: substantia nigra pars compacta; CG: central gray matter. *Figure 2E:* Cranial nerve nuclei.

Figure 1A.

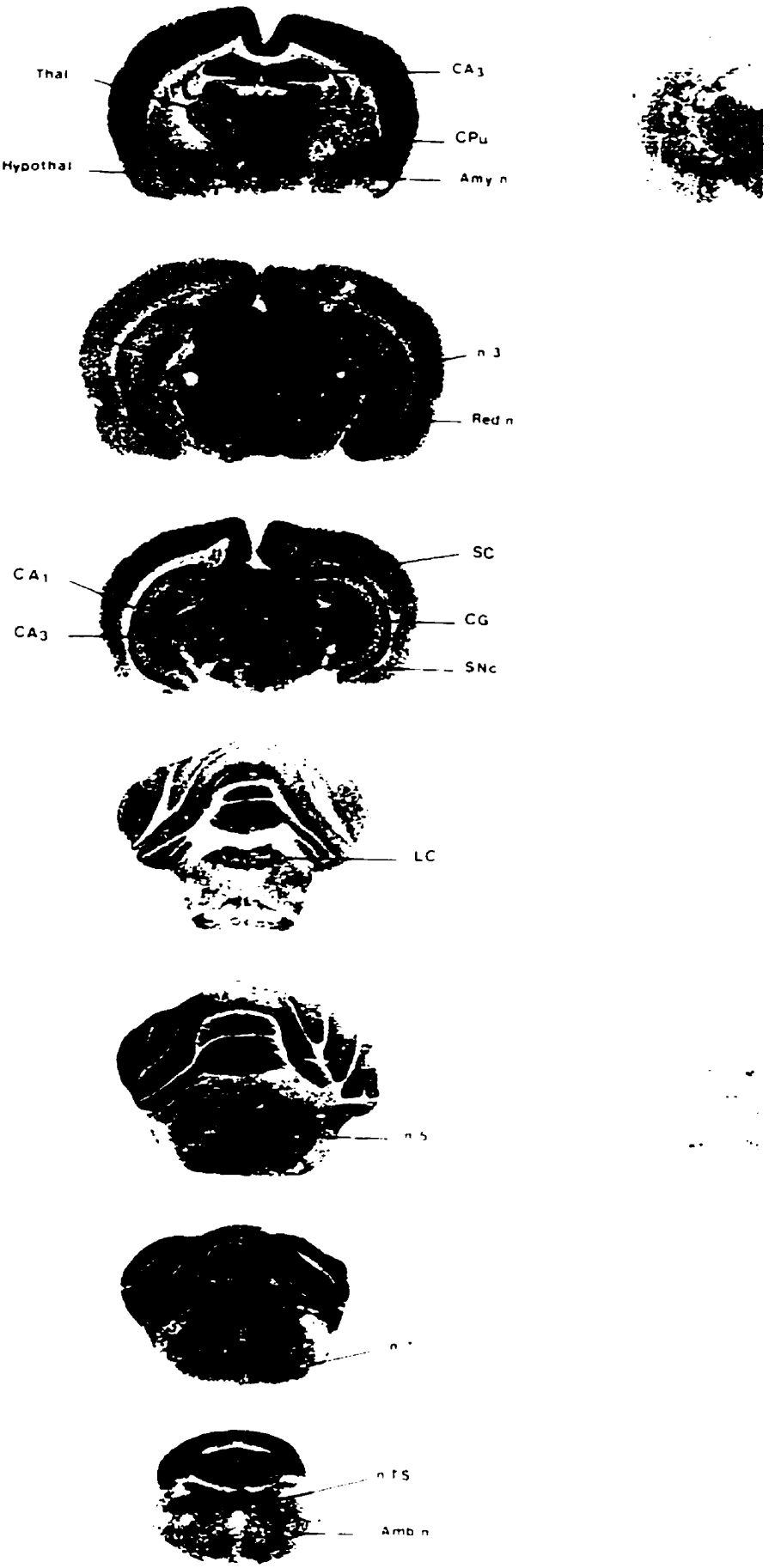
Thal
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Figure 1B.

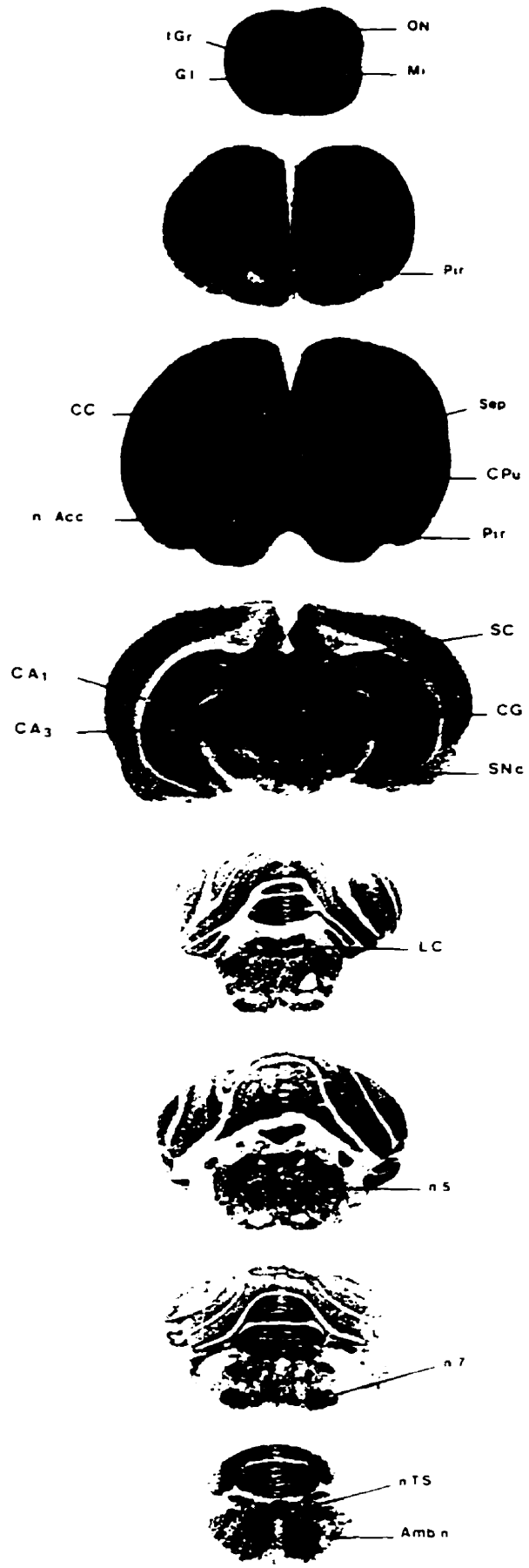
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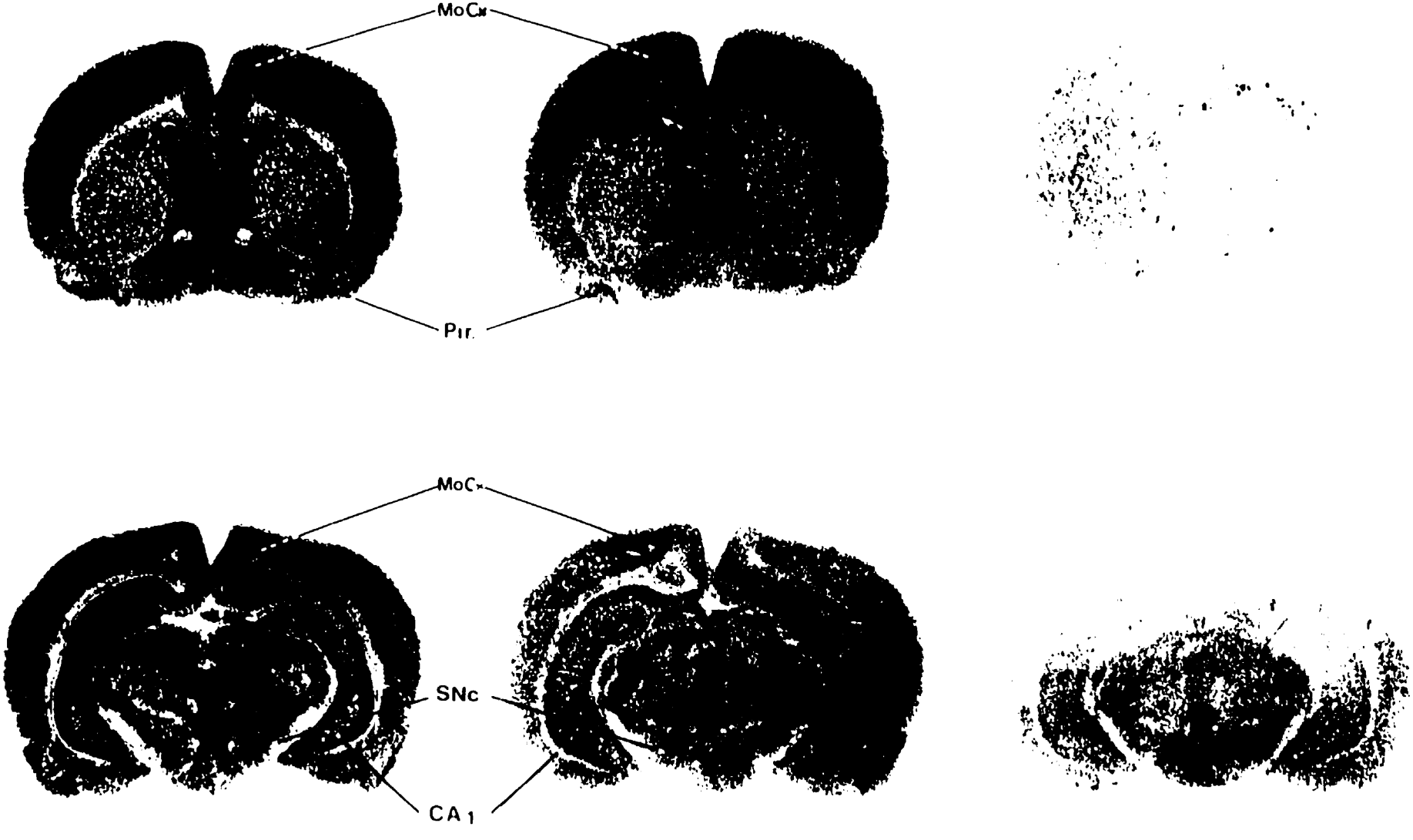
Figure 1C.



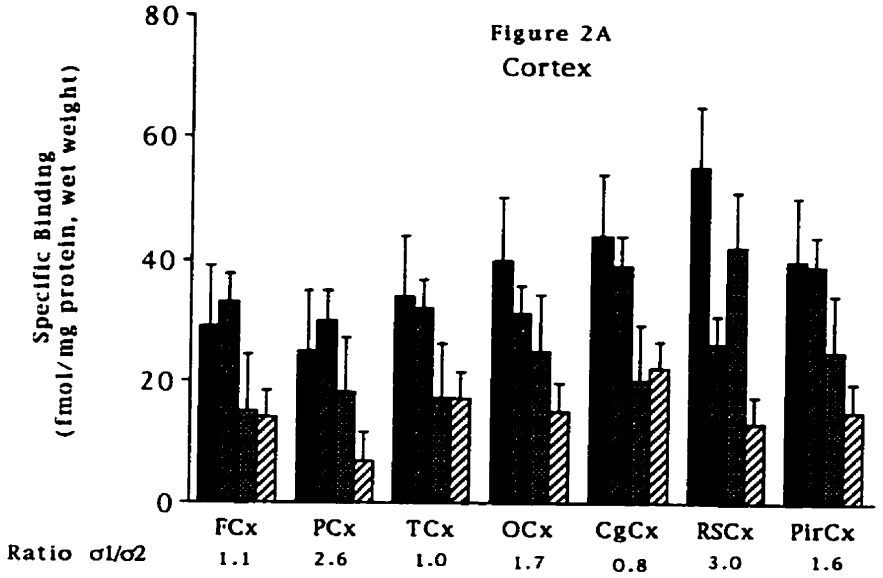


Figure 2A
Cortex
Specific Binding
(fmol/mg protein, wet weight)
80
60
40
20
0
FCx
PCx
TCx
OCx
CgCx
RSCx
PirCx
Ratio σ1/σ2
1.1
2.6
1.0
1.7
0.8
3.0
1.6

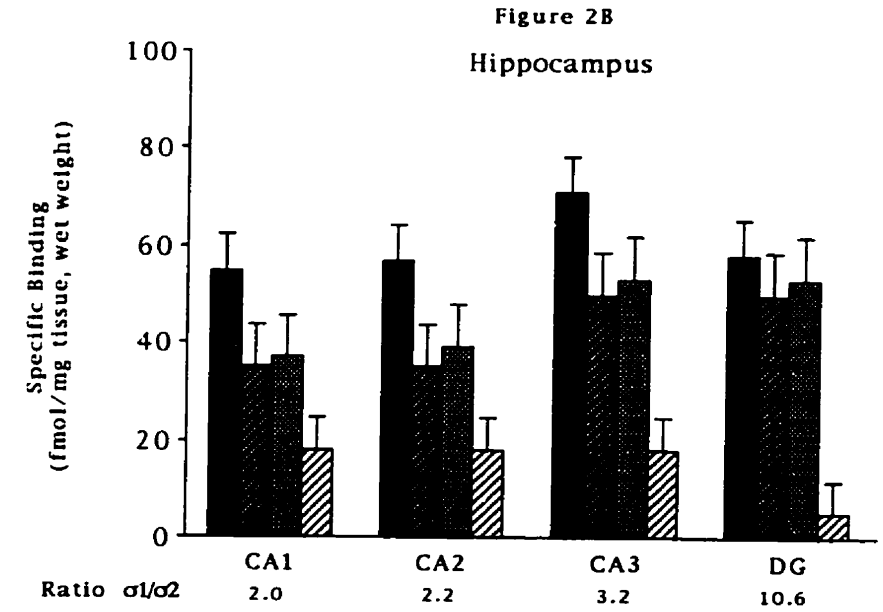


Figure 2B
Hippocampus
Specific Binding
(fmol/mg tissue, wet weight)
100
80
60
40
20
0
CA1
CA2
CA3
DG
Ratio σ1/σ2
2.0
2.2
3.2
10.6

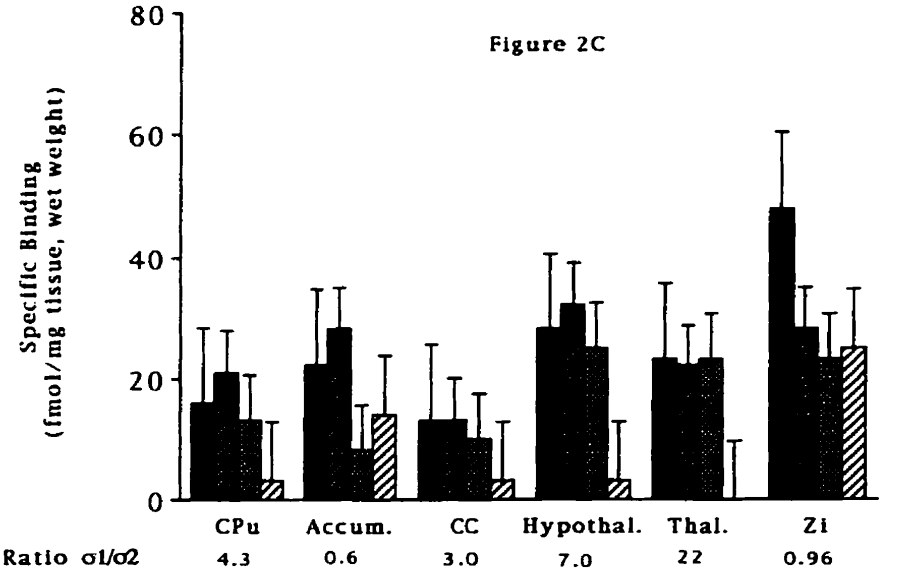


Figure 2C
Specific Binding
(fmol/mg tissue, wet weight)
80
60
40
20
0
CPu
Accum.
CC
Hypothal.
Thal.
Zi
Ratio σ1/σ2
4.3
0.6
3.0
7.0
22
0.96

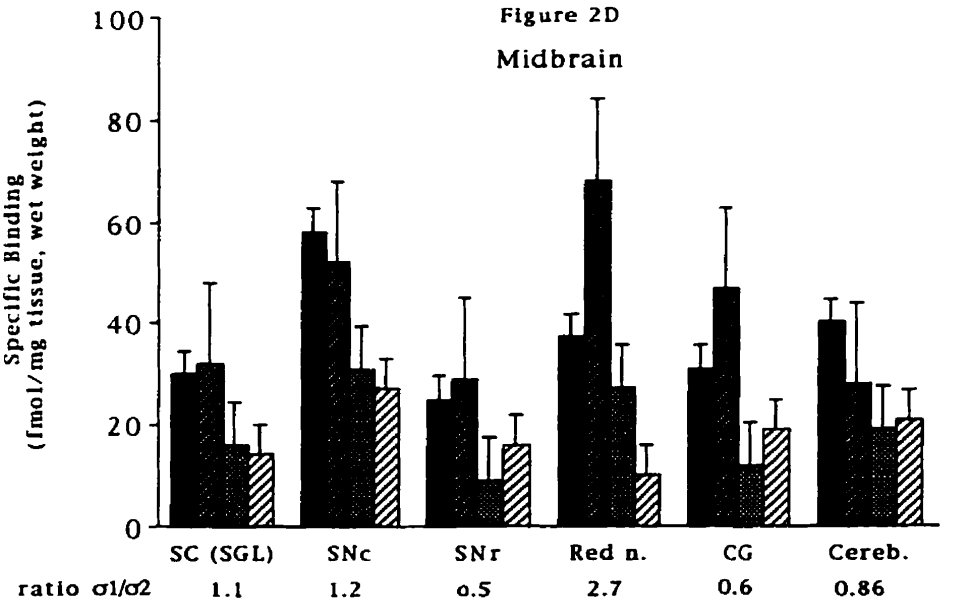


Figure 2D
Midbrain
Specific Binding
(fmol/mg tissue, wet weight)
100
80
60
40
20
0
SC (SGL)
SNc
SNr
Red n.
CG
Cereb.
ratio σ1/σ2
1.1
1.2
o.5
2.7
0.6
0.86

Figure 2E

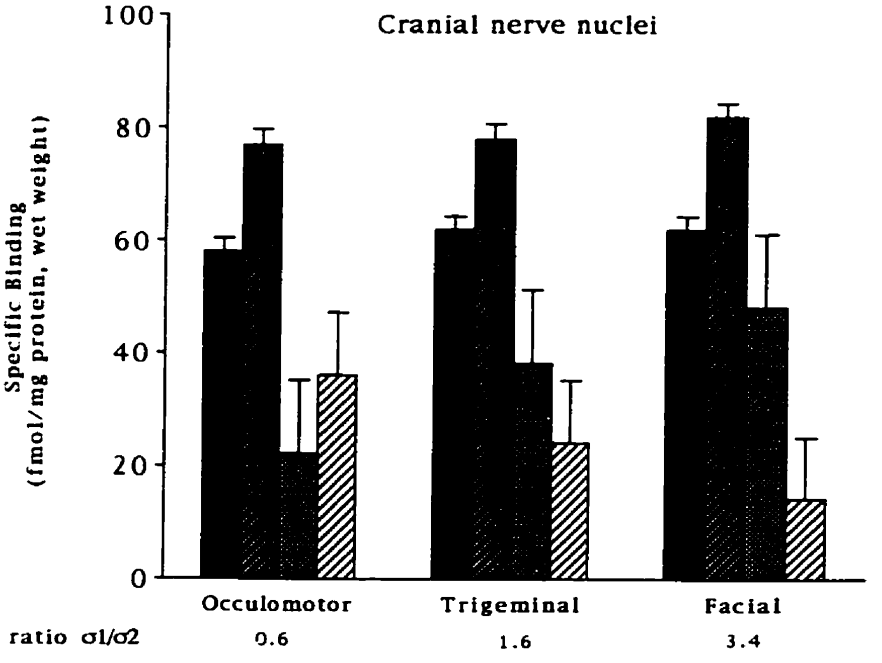
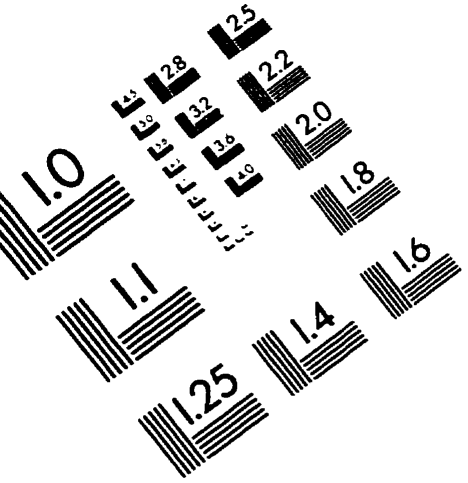
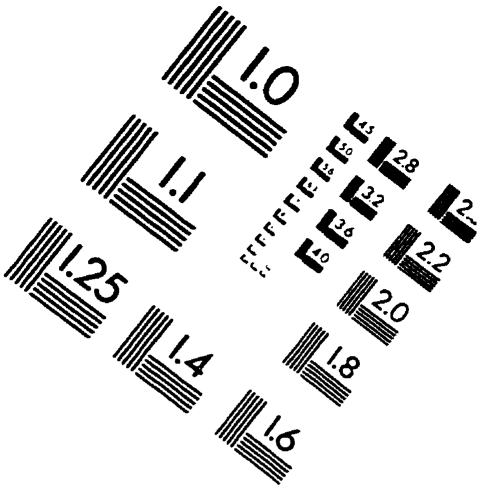
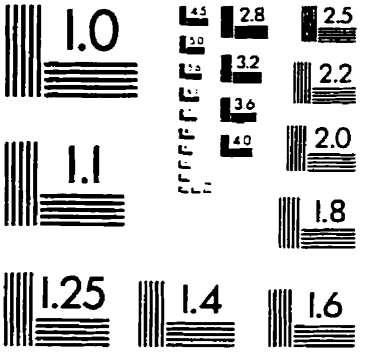
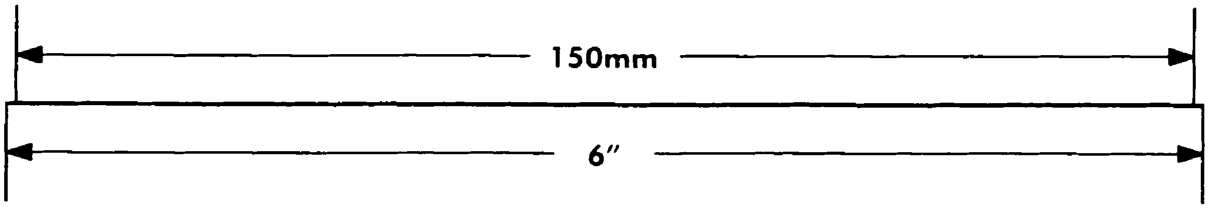

Cranial nerve nuclei
Specific Binding
(fmol/mg protein, wet weight)
100
80
60
40
20
0
Occulomotor
Trigeminal
Facial
ratio σ1/σ2
0.6
1.6
3.4

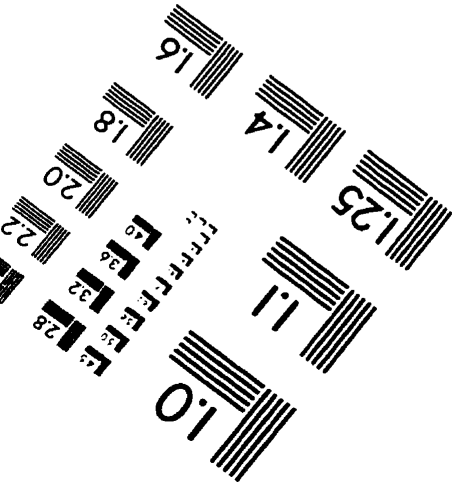
IMAGE EVALUATION
TEST TARGET (QA-3)

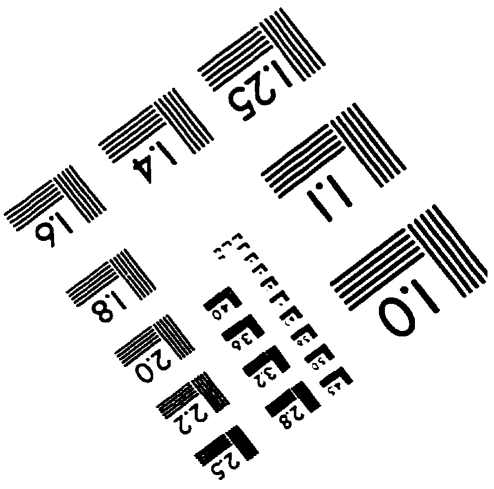












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