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Anatomical and Functional Study of Interleukin-2 in the Brain: Possible Neuromodulatory Significance

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

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Anatomical and Functional Study of Interleukin-2 in the Brain: Possible Neuromodulatory Significance

Abstract

Interleukin-2 (IL-2), an immunomodulatory cytokine first isolated from the immune system, plays an essential role in the maturation of T lymphocytes. IL-2 was also found to have effects on selected cells of the nervous system. The behavioural and cognitive adverse effects observed following the clinical use of systemic IL-2, coupled with pathological observations that associate IL-2 to neurodegenerative events, suggest that IL-2 exerts modulatory effects on neurological functions. This thesis focusses on the neuroanatomical features of IL-2 and IL-2 receptors in the central nervous system, the neuromodulatory role of IL-2 on hippocampal acetylcholine release, and possible intracellular signalling mechanisms following IL-2 receptor activation in the brain.

Using immunoautoradiography, IL-2-like immunoreactivities were observed in a selected pattern in the central nervous system, with particularly high densities seen in the hippocampal formation and the hypothalamus of the rodent brain. The cellular localisation of the immunostaining using immunohistochemical approaches reveals that this staining was seen most evidently on cell perikarya especially in areas of high labelling density. The distribution of IL-2 receptor binding sites using both *in vitro* receptor autoradiography and immunoautoradiography (anti-TAC antibody against the IL-2 receptor α chain) shows that IL-2 receptors are selectively distributed in the rodent brain, with the highest densities observed in the hippocampal formation and the hypothalamus, in accordance with the localisation of IL-2 peptide immunostaining. The postulated neuromodulatory role of IL-2 and IL-2 receptors in the hippocampus was investigated next focusing on cholinergic parameters (acetylcholine release) on the basis of previous results from our laboratory (Araujo *et al.*, 1989).

The neuromodulatory effects of IL-2 on acetylcholine (ACh) release was investigated

using *in vitro* rat brain slices superfusion. IL-2 exerted potent effects on hippocampal ACh release, acting as a potentiating agent at low (p*M*) concentrations, while inhibiting release at higher (low n*M*) concentrations. An inhibitory effect (10 n*M* IL-2) on ACh release was also observed in the frontal cortex, but not in the parietal cortex or the striatum. This action was not shared by other interleukins such as IL-6. Both the stimulatory and inhibitory effects of IL-2 in hippocampal ACh release were blocked by an anti-IL-2 receptor antibody (TAC), suggesting the requirement of a genuine IL-2 receptor for both effects. The potentiating, in contrast to the inhibitory effect, was insensitive to tetrodotoxin, suggesting a direct action (or in close proximity) of IL-2 on cholinergic terminals to stimulate hippocampal ACh release. The inhibitory effect of IL-2 on ACh release was abolished by both bicuculline and phaclofen, suggesting the involvement of GABA acting on both $GABA_A$ and $GABA_B$ receptors present in the rat hippocampal formation.

The signalling mechanisms of the IL-2 receptor in the rat brain was studied next *in vitro* by measuring the effects of IL-2 on cytidine-diphosphate diacylglycerol (CDP-DAG) turnover in rat brain slices. IL-2 potently inhibited basal CDP-DAG turnover in the frontal cortex and hypothalamus, but not in the hippocampus or striatum. However, IL-2 inhibited carbachol-stimulated CDP-DAG turnover in the hippocampus. This decrease was in parallel to an increase in choline production, suggesting a role for phospholipase D in brain IL-2 receptor signalling.

These results collectively identify IL-2 as a new endogenous modulator of neuronal function, being particularly potent as a regulator of rat hippocampal ACh release. Our results also provide additional evidence that molecules originally thought to be selective for the immune system possess potent effects on the central nervous system, hence linking the immune and nervous systems in a tightly integrated neuro-immune complex.

Etude anatomique et fonctionelle sur l'interleukine-2 dans le cerveau

Résumé

Isolée d'abord du système immunitaire, l'interleukine-2 (IL-2) est une cytokine immunomodulatrice qui joue un rôle essentiel dans la maturation des lymphocytes T. De plus, il a été observé que l'IL-2 a des effets chez certaines populations de cellules du système nerveux. Couplés aux observations pathologiques qui associent l'IL-2 aux événements neurodégénératifs, les effets adverses comportementaux et cognitifs observés suite à l'usage clinique de l'IL-2 systémique suggèrent que l'IL-2 exerce des effets modulateurs sur les fonctions neurologiques. Cette thèse se concentre sur les aspects neuroanatomiques de l'IL-2 et des récepteurs à l'IL-2 dans le système nerveux central, du rôle neuromodulateur de l'IL-2 sur la libération de l'acétylcholine à partir de l'hippocampe, et sur les mécanismes d'action intracellulaires possibles de l'IL-2 suite à l'activation de son récepteur dans le cerveau.

En utilisant l'immunoautoradiographie, le marquage immunopositif à l'IL-2 a été observé selon un patron particulier dans le système nerveux central, avec des densités particulièrement élevées observées dans l'hippocampe et l'hypothalamus du rat et de la souris. La localisation immunocytochimique révèle que l'immunoréactivité associée à l'IL-2 est observée dans les périkaryons cellulaires. La distribution des sites de liaison à l'IL-2 par l'autoradiographie *in vitro* et par immunoautoradiographie (anticorps anti-TAC contre la chaîne α du récepteur à l'IL-2) démontre que ceux-ci sont distribués sélectivement dans le cerveau du rongeur, avec les densitiés les plus élevées observées dans la formation hippocampale et l'hypothalamus, en accord avec la localisation immunocytochimique de l'IL-2. Le rôle neuromodulateur postulé pour l'IL-2 dans l'hippocampe portant sur la libération d'acétylcholine a été étudié par la suite en se basant sur des résultats antérieurs de notre laboratoire (Araujo *et al.*, 1989).

Les effets neuromodulateurs de l'IL-2 sur la libération de l'acétylcholine (ACh) ont été étudiés en utilisant une technique de superfusion de tranches de cerveau de rat. On a observé que l'IL-2 exerce des effets puissants sur la libération d'ACh à partir de l'hippocampe, agissant comme un agent potentialisateur aux concentrations faibles (p*M*), et comme inhibiteur à des concentrations plus élevées (faible n*M*). Un effet inhibiteur (10 n*M* IL-2) sur la libération d'ACh a été aussi noté dans le cortex frontal, mais non pas dans le cortex pariétal et le corps strié. Cette action n'est pas observée pour d'autres interleukines, tel que l'IL-6. Les effets potentialisateur et inhibiteur de l'IL-2 sont bloqués par un anticorps anti-récepteur de l'IL-2 (TAC), suggérant l'implication d'un récepteur typique de l'IL-2. L'effet potentialisateur, par contraste à l'effet inhibiteur, est sensible à la tétrodotoxine, suggérant une action directe (ou proximité) de l'IL-2 sur les terminaisons cholinergiques pour stimuler la libération de l'ACh à partir de l'hippocampe. L'effet inhibiteur de l'IL-2 sur la libération de l'ACh est aboli à la fois par la bicuculline et le phaclofène, suggérant l'intervention du GABA agissant aussi bien sur des récepteurs $GABA_A$ et $GABA_B$ localisés dans l'hippocampe du rat.

Les mécanismes de signalisation du récepteur à l'IL-2 ont été étudiés *in vitro* en mesurant les effets de l'IL-2 sur le renouvellement du cytidine-diphosphate diacylglycérol (CDP-DAG) dans des tranches de cerveau du rat. L'IL-2 inhibe de façon puissante la production basale du CDP-DAG dans le cortex frontal et l'hypothalamus, mais non pas dans l'hippocampe et dans le corps strié. De plus, l'IL-2 inhibe la production du CDP-DAG stimulé par le carbachol dans l'hippocampe. Cette diminution est en parallèle à une augmentation de production de la choline, suggérant un rôle pour la phospholipase D dans le mécanisme de signalisation intracellulaire de l'IL-2 dans le cerveau.

En bref, nos résultats identifient l'IL-2 comme un nouveau modulateur endogène de la fonction neuronale, étant particulièrement puissant comme modulateur de la libération

de l'ACh dans l'hippocampe du rat. Nos résultats fournissent aussi une preuve additionnelle que des molécules présumées d'origine sélective pour le système immunitaire possèdent des effets puissants sur le système nerveux central, reliant ainsi les systèmes immunitaire et nerveux dans un complex neuro-immunitaire bien intégré.

CHAPTER 1:

GENERAL INTRODUCTION

PREFACE TO CHAPTER 1

The principal aim of this General Introduction is to provide a framework of understanding on the different aspects of the cytokine interleukin-2 that are to be elaborated in the subsequent Chapters. The General Introduction starts with sections describing interleukin-2 and its receptor. Then there is a detailed synthesis of the knowledge of interleukin-2 and its receptors in the nervous system. Finally, this ends with a section that establishes the general objectives of this thesis.

1. Introduction

A wealth of studies have collectively provided evidence that cytokines and their receptors are endogenous to both the nervous and immune systems. The first documented observations were reported by Metal'nikov and Chorine (1926) who showed that immune responses, like other physiological reactions, can be conditioned. These findings were later supported and extended by Selye (1936) whose descriptions of the involution of the thymus, an important immune organ, during stress, provided an anatomical basis for these observations. However, progress in this field was impeded by the lack of adequate approaches. Hazum *et al.* (1979) reported that opioid binding sites were observed on lymphoblastoid cells. In addition, O'Dorisio *et al.* (1980) reported the presence of vasoactive intestinal polypeptide in granulocytes. Smith and Blalock (1981) detected corticotropin and endorphins in lymphocytes. The opening of a bidirectional pathway between these two systems came with the observation that interleukin-1 (IL-1) functions as an endogenous pyrogen, acting on hypothalamic sites, and is synthesized in the brain (Fontana *et al.*, 1984).

1.1 Cytokines and Growth Factors

Cytokines and growth factors can be described as polypeptide hormones that modulate homeostatic processes usually in the tissue of origin, but can also recruit external mechanisms *via* the bloodstream to facilitate restoration of homeostasis. Expression and activity of these hormones are increased under conditions of increased cellular metabolism, such as rapid growth and injury. These hormones include the interleukin series from 1 to 15 and a wide variety of growth factors.

The interleukins are a class of signalling hormones within the cellular network of the immune system. These molecules function as autocrine, paracrine, endocrine factors

involved in the control of a variety of physiological and pathological states such as normal and malignant growth, recognition and elimination of pathogens by immune cells and inflammation. Among the interleukins, interleukin-2 was one of the first few employed in the treatment of a disease state in human subjects. Furthermore, only IL-1, -2, -3 and -6 have been documented to be associated with the nervous system. These factors have made it crucial to gain a deeper understanding of interleukin-2 as it relates to the brain.

1.2 Interleukin-2

First described by Morgan *et al.* (1976), interleukin-2 (IL-2) is a small protein of 15,42 kD encoded by a single gene spanning 3,5 kb located in the q26-28 region of human chromosome 4 (Sykora *et al.*, 1984; Shows *et al.*, 1984; Seigel *et al.*, 1984). The IL-2 gene, similar to other cytokines, is composed of 4 exons and 3 introns that encodes a message of 153 amino acids. The 20-residue N-terminal signalling sequence is removed to yield the mature 133-amino acid product (Fujita *et al.*, 1983; Taniguchi *et al.*, 1983; Holbrook *et al.*, 1984). The polypeptide is glycosylated at threonine 3 and has cysteines at positions 58, 105 and 125, in which the disulfide bond between cysteines 58 and 105 is essential for bioactivity. The cysteine at position 125 causes aggregation and disulfide exchange which leads to the formation of less active IL-2 molecules *in vitro* (Bailon and Weber, 1988).

IL-2 is an α-helical protein (Brandhuber *et al.*, 1987). It consists of 4 anti-parallel helices, without any segments of ß secondary structure, with an overall helical content of about 65%. It has been proposed that granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4) and interleukin-15 (IL-15), as well as granulocyte colony-stimulating factor (G-CSF), 4 members of the cytokine family, share protein folding patterns similar to that of IL-2 (Bazan, 1992; Hill *et al.*, 1993; Grabstein *et al.*, 1994). Studies exploring the structure-activity relationships of IL-2 analogues have

revealed that replacement of threonine 51 by proline indicate that although this analogue's receptor binding affinity is weaker than that of the original IL-2, this mutant is able to evoke an equivalent biological effect with a lowered receptor occupancy (Chang *et al.*, 1995). The authors conclude that these observations point to conformation-dependent changes upon IL-2 receptor binding necessary for signalling.

1.3 Receptors for interleukin-2

Advances in the knowledge of the IL-2R was made possible through the use of a sensitive IL-2 assay, the receptor binding assay (Robb *et al.*, 1981) using radiolabelled IL-2 isolated from constitutively IL-2-producing cell lines (Gillis and Watson, 1980) or expression of the cloned IL-2 cDNA (Taniguchi *et al.*, 1983). Another equally important advance was the generation of anti-IL-2R monoclonal antibodies (Uchiyama *et al.*, 1981), termed anti-TAC (T cell activation; also CD25) because the antibody reacted with activated normal T cells but not with resting normal T cells. This antibody recognized the IL-2 binding site of the IL-2R by blocking IL-2 binding to high-affinity receptors, and conversely, IL-2 blocked binding of anti-TAC. This antibody precipitated a 55-kD glycoprotein (Leonard *et al.*, 1982) which permitted the eventual isolation of the full-length IL-2Rα cDNA clones (Leonard *et al.*, 1984; Nikaido *et al.*, 1984; Cosman *et al.*, 1984).

IL-2Rα is encoded by a gene spanning 35 kb on chromosome 10p14-15 (Leonard *et al.*, 1985; Ishida *et al.*, 1985) and is organized into 8 exons and 7 introns. Full-length TAC cDNA clones have an opening reading frame that encodes a primary product of 272 amino acids. Removal of the 21-amino acid signalling fragment results in the mature 251-amino acid TAC polypeptide of 33 kD. Extensive post-translational processing involving glycosylation, sulphation and serine/threonine phosphorylation results in the 55-kD IL-2Rα subunit (p55). There are 11 cysteine residues, some of which are involved in disulphide bonding (Rusk *et al.*, 1988). Mutational analyses

revealed residues 1-6 and 35-43 to be important for IL-2 binding (Rusk *et al.*, 1988). Its structure does not exhibit any sequence similarity to members of the Ig superfamily. Near the C-terminal portion is a stretch of 19 hydrophobic amino acids which consists of the single transmembrane domain. The C-terminal intracytoplasmic region of 13 amino acids, too short for the tyrosine kinase activity that is a typical feature of cytokine receptors, but contains six positively charged residues which presumably serve to anchor the receptor within the membrane.

The structure of the IL-2Rα strongly suggests the existence of other components that participate in IL-2R binding and activation, since it was obvious that IL-2Rα did not suffice to account for the activity of IL-2. It was observed that in the presence of anti-TAC, certain cells can respond to high concentrations of IL-2 (Ortaldo *et al.*, 1984). Subsequent studies revealed the existence of a new IL-2 binding protein of about 70-75 kD on cells expressing high-affinity receptors (Tsudo *et al.*, 1986; Dukovich *et al.*, 1987; Siegel *et al.*, 1987). Antibodies raised against this protein, such as Mikß1 (Tsudo *et al.*, 1989) enabled the successful isolation of the p70 cDNA clones (Hatakeyama *et al.*, 1989).

IL-2Rß is encoded by a gene spanning 24 kb on chromosome 22q11.2-12 and is organized into 10 exons and 9 introns (Gnarra *et al.*, 1990; Shibuya *et al.*, 1990). Full-length IL-2Rß cDNA contains an opening reading frame yielding a primary translational product of 551 amino acids. Following removal of the 26-amino acid signalling fragment, the mature protein has 525 amino acids, consisting of 214 in the extracellular, 25 in the transmembrane and 286 in the intracytoplasmic domains. Like TAC, IL-2Rß is not a member of the Ig superfamily. However, the recently cloned receptors for erythropoietin (D'Andrea *et al.*, 1989), IL-4 (Mosley *et al.*, 1989) and interleukin-6 (IL-6) (Yamasaki *et al.*, 1988) and p70 appear to comprise a new group of cytokine receptors (D'Andrea *et al.*, 1989). They all share significant sequence similarity and certain structural features such as conserved cysteine residues and a conserved tryptophan-serine-X-tryptophan-serine (W-S-X-W-S; X being the

unconserved amino acid) motif in the extracellular domain and an abundance of proline and serine residues in the intracellular domain.

Several observations suggest that additional receptor components are required for full formation of intermediate- and high-affinity IL-2R. These results arose from transfections of full-length IL-2Rα and/or IL-2Rß cDNA into non-lymphoid cells. Transfection of IL-2Rß cDNA into lymphoid cells results in the expression of IL-2Rß as determined by reactivity with antibody Mikß1, and intermediate-affinity binding sites are formed (Hatakeyama *et al.*, 1989). Co-transfection of these cells with IL-2Rα and IL-2Rß cDNA results in the appearance of high-affinity receptors. However, when IL-2Rß cDNA is transfected into non-lymphoid cells, IL-2Rß is expressed but appears non-functional, as it fails to bind IL-2 (Hatakeyama *et al.*, 1989). Co-transfection of IL-2Rα and ß results in the expression of the α/ß heterodimer but the resultant affinity for IL-2 is less than that of the high-affinity receptor, and appears unable to mediate IL-2 internalization (Hatakeyama *et al.*, 1989). An additional IL-2R subunit of 95 to 110 kD, was then proposed and termed γ (Szollios *et al.*, 1987; Saragovi and Malek, 1987; Zurawski *et al.*, 1990).

The IL-2Rγ gene is encoded by a gene spanning 4,2 kb on chromosome 23q13 (X) organized into 8 exons and 7 introns (Noguchi *et al.*, 1993). Its full-length cDNA has an opening reading frame encoding a primary transcript for 369 amino acids (Takeshita *et al.*, 1992), and removal of the N-terminal signalling peptide results in a mature sequence of 347 amino acids with a calculated 39,9 kD. Analysis of hydrophobicity revealed a single transmembrane domain from residues 255 to 283. The predicted extracellular domain includes the W-S-X-W-S motif like IL-2Rß and the intracytoplasmic domain consists of 86 amino acids.

There are three forms of the IL-2 receptor (IL-2R): high, intermediate and low. The high-affinity form (kD = 10^{-12} *M*) contains 3 distinct subunits (IL-2Rα, IL-2Rß and IL-2Rγ). The intermediate-affinity form (kD = 10^{-9} *M*) contains IL-2Rß and IL-2Rγ. In

contrast, the IL-2Rα alone binds IL-2 with low affinity (kD = 10^{-8} *M*). The ß subunit by itself binds IL-2 at kD of 10^{-7} *M*, while the γ subunit alone does not bind IL-2 (Takeshita *et al.*, 1992; Minami *et al.*, 1993). Expression of IL-2Rα is undetectable in resting T cells, but is efficiently induced upon T cell activation. IL-2Rß and γ are expressed constitutively and ß subunit levels can be further increased following T cell activation (Takeshita *et al.*, 1992).

Following the identification of the ß and γ subunits, several studies brought about a convergence of other interleukin receptors to the IL-2R complex. IL-4 and interleukin-13 (IL-13), 2 α-helical proteins, were observed to share a common receptor component for signal transduction (Zurawski *et al.*, 1993). In addition, Pitton *et al.* (1993) reported that IL-4- and interleukin-9 (IL-9)-dependent cells transfected with IL-2Rα and/or IL-2Rß cDNA were able to proliferate in the sole presence of IL-2, indicating that IL-4 and -9 receptor components were able to render the IL-2Rα,ß complex to a functional state by providing additional subunits common to IL-2, -4 and -9. Russell *et al.* (1993), using their antibody against the IL-2Rγ, R878, and Kondo *et al.* (1993), using their own anti-IL-2Rγ, TUGm2, independently reported that IL-2Rγ was a component of the IL-4R. Another piece of evidence was provided when antibodies against IL-2Rγ were shown to inhibit binding of IL-2 and -4 (Kondo *et al.*, 1993). Using immunoprecipitation, it was also observed that IL-2Rγ was part of the interleukin-7 (IL-7) complex (Noguchi *et al.*, 1993; Kondo *et al.*, 1994).

Early evidence provided by Martinez *et al.* (1990) and Torigoe *et al.* (1992) hinted at IL-4 inhibition of IL-2 activity. Many mechanisms have been proposed but in light of the observation that both IL-2 and -4 share the IL-2Rγ subunit, Kondo *et al.* (1993) proposed that perhaps receptor down-regulation seen with IL-2 and -4 together may likely be attributed to competition for a limited supply of IL-2Rγ.

IL-2-deficient mice have normal immune responses (Schorle *et al.*, 1991), suggesting that IL-2R components are used by other cytokines to compensate for the lack of IL-2.

Accordingly, several groups have also raised the point that IL-2Rγ mutations, not IL-2 deficiencies, are more consistent with the profile of X-linked severe combined immunodeficiency (XSCID) since IL-2Rγ has been mapped onto the locus involved in this disease (Noguchi *et al.*, 1993).

In addition to the wide use of IL-2Rγ, it has also been reported that interleukin-15 (IL-15) (Carson *et al.*, 1994; Grabstein *et al.*, 1994) and interleukin-T (IL-T) (Bamford *et al.*, 1994) share the IL-2Rß subunit. Furthermore, there is evidence to suggest that IL-15 also uses IL-2Rγ (Giri *et al.*, 1994). Other components associated with IL-2R have been proposed on the basis of co-immunoprecipitation of molecules cross-linked with IL-2; their biochemical structure and function remain undefined (Minami *et al.*, 1993).

1.4 Interleukin-2 and the nervous system

1.4.1 Interleukin-2 in the brain

Two key observations provided the initial clues that IL-2 was able to exert effects on the nervous system. The first clue was the observation that IL-2 was a growth factor for oligodendroglial proliferation and maturation *in vitro* (Benveniste and Merrill, 1986). The second was the report of severe neurological deficits of cognitive function in the course of IL-2 pharmacotherapy (Denicoff *et al.*, 1987).

As fast as these observations were being assimilated, controversy erupted over several litigious aspects. Are the observed central effects of IL-2 caused directly by way of penetration by IL-2 of the blood-brain barrier or indirect in which IL-2 acts through another system that then penetrates the brain? If the nervous system responds to IL-2, is there IL-2 endogenous to the nervous system?

Early reports indicated that rhIL-2 (100 000U/kg) administered intravenously by a

single bolus injection in anaesthesized adult cats (2,4 to 4 kg) penetrated the brain by permeabilising the blood-brain barrier (Ellison *et al.*, 1987). All the cats infused with IL-2 or its vehicle showed increased cerebrovascular permeability as visualized using horseradish peroxidase or IgG histochemical approaches with light and electron microscopy. This altered blood-brain barrier permeability was observed in multiple loci throughout the brain, being most prominent within the white matter. The histochemical markers were also observed within perivascular basal laminae and the interstitial brain parenchyma. Numerous endothelial lesions were also detected. In addition, the authors found numerous disrupted neuronal and glial processes as well as expanded intercellular spaces. The authors conclude that some of the effects observed may be due to the presence of sodium dodecylsulphate, a detergent, and mannitol, in the excipient of the IL-2 preparation.

Using selected sources of IL-2, Kobiler *et al.* (1989) experimented with a novel and convenient method to determine the effect of IL-2 on the blood-brain barrier. The assay is based on a variant of the West Nile virus, WN-25, which had lost its neuroinvasiveness, but not its neurovirulence. WN-25, when injected intravenously in mice, can cause death only if there is a breach in the blood-brain barrier. They observed that the sodium dodecylsulphate and mannitol in the IL-2 preparation were present sufficient quantities to impair the murine blood-brain barrier. However, in preparations of IL-2 without these excipients, IL-2 did not permeabilize the murine blood-brain barrier.

Using a different source of IL-2, Saris *et al.* (1988) observed the effects of IL-2 in patients treated for metastatic cancer. They examined the concentrations of IL-2 and human albumin in both the serum and the lumbar cerebrospinal fluid at regular intervals following intravenous IL-2 infusion (10 000 U/kg three times a day). The appearance of IL-2 in the cerebrospinal fluid following IL-2 infusion indicates that IL-2 is able to cross the blood-brain barrier, and the presence of human albumin suggest that IL-2 is likely able to induce a breach in the blood-brain barrier.

In a follow-up study, Ellison *et al.* (1990) reported that some of the changes can be correlated to the number of intravenous injections, with the greatest effects observed among rats having received a single dose (600 000 U/kg) in contrast to those having received multiple injections (600 000 U/kg three times a day for 5 days). Ultrastructural examination of the cerebral vasculature and brain parenchyma of the rats 6 and 24 hours after a single dose revealed moderately increased permeability to IgG that was not seen in those rats sacrificed 6 hours after 5 days of IL-2 treatment. Alterations of the cerebrovascular morphology were detected as early as 6 hours after a single dose and were accompanied by sparse axonal degeneration and demyelination. However, such structural changes persisted, becoming more extended after 5 days of treatment, and were associated with neuronal as well as glial alterations. The authors also added that perhaps behavioural manipulations associated to the administration of a single dose of IL-2 in contrast to the habituation that develops following repeated manipulations may be a factor in changes of the blood-brain barrier, since it has been reported that fear has been demonstrated to induce similar changes (Hayes *et al.*, 1985). Additionally, they observed that myelin damage following bolus IL-2 intravenous infusions (60 000 U/kg) in the rat have been correlated with increased serum levels of TNFα, and elevated TNFα *in vitro* produce abnormalities in nervous tissue ultrastructurally indistinguishable from that of IL-2 (Selmaj and Raine, 1988; Ellison and Merchant, 1991). However, whether TNFα is causally related to IL-2-associated axonal lesions remains to be determined.

Using yet another source of rhIL-2, Banks and Kastin (1992) reported that central or intravenous IL-2 independently of the dose did not disrupt the murine blood-brain barrier in either direction. In their study IL-2 and radioiodinated bovine serum albumin was concurrently or alternately injected in the brain or periphery. Their measurements of the distribution of radioiodinated bovine serum albumin in both compartments following both modes of IL-2 administration indicate that IL-2 does not acutely disrupt the blood-brain barrier of the mouse.

Unlike IL-1α and ß, TNFα and IL-6 (Banks *et al.*, 1989, 1991, 1993; Gutierrez *et al.*, 1993), which penetrate the brain *via* a saturable bidirectional mechanism, Waguespack *et al.* (1994) reported that rhIL-2 in the mouse does not penetrate the brain by way of a saturable transport system. In this study, it was observed that labelled IL-2 penetrated the brain about 10 times faster than labelled albumin, and analysis of the labelled IL-2 recovered from the brain revealed that it was the intact molecule. Moreover, this entry of IL-2 was not impeded by excess unlabelled IL-2, suggesting that the transport process was not saturable.

However, Saija *et al.* (1995), by measuring the distribution of [^{14}C]α-aminoisobutyric acid following the intracarotid injection of rhIL-2 1000 U/rat, reported that IL-2 did indeed disrupt the blood-brain barrier in the rat. This was indicated by a greater than normal presence of the tracer in the brain. Moreover, they observed regional differences in the alterations of permeability, with the cerebral cortex, hippocampus and hypothalamus exhibiting higher levels of tracer than other areas such as the striatum. To resolve this controversy, Saija *et al.* (1995) did point out probable species differences, noting that in previous reports, IL-2 affected the blood-brain barrier in cats (Ellison *et al.*, 1987) and rats (Ellison *et al.*, 1990), but not in mice (Kobiler *et al.*, 1989; Banks and Kastin, 1992).

Thus the available evidence indicates unequivocally that systemically administered exogenous IL-2 does indeed gain access to the brain. This suggests that it is likely that IL-2 released by immune cells in the periphery are also able to enter the brain by way of the central vasculature. Much of the controversy, as Saija *et al.* (1995) attempted to reconcile, may reside in the species used. In addition, this controversy appears to relate not to the ability of IL-2 to penetrate the brain, but rather to the mechanism by which it crosses the blood-brain barrier. However these issues leave open another question: is there IL-2 that is endogenous to the brain?

IL-2-like materials have been detected in the normal mammalian brain. Using the IL-2 bioassay established by Gillis *et al.* (1978), Nieto-Sampedro and Chandy (1987) observed very low amounts of IL-2-like bioactivities on IL-2-dependent cytotoxic lymphocytes in extracts of the entorhinal/occipital area of adult rat brains. IL-2-like bioactivities were also observed in neonatal (3-day old) and aged (24-month old) rat brain extracts, with increasing amounts as a function of age. Interestingly, there were uncertainties regarding the purported molecular size of the IL-2-like bioactivity following chromatographic separation.

Using radioimmunoassay antisera against IL-2, Araujo *et al.* (1989) reported that IL-2 immunoreactivities were present in rat brain homogenates of the hippocampus, frontal cortex and striatum. According to their study, the hippocampal (0,7 ng/mg = 45,4 fmol/mg) and striatal (0,65 ng/mg = 42,1 fmol/mg) homogenates contained comparably low amounts of IL-2 immunoreactivities, but the levels detected in the frontal cortical (0,1 ng/mg = 6,4 fmol/mg) homogenates were even lower. There was also an attempt to determine whether the IL-2 immunoreactive materials detected in these brain areas can be released under both basal and evoked conditions. Results reported by Araujo *et al.* (1989) indicated that there were low levels of IL-2 immunoreactive materials detected in the supernatants of hippocampal and striatal slices incubated in normal conditions. Levels in the supernatant of frontal cortical slices, however, were undetectable. When the tissue slices were incubated in high K^+ Krebs buffer, IL-2 immunoreactivities were undetectable. Araujo *et al.* (1989) concluded that IL-2 is not released from neuronal terminals. At this point, it was not apparent whether the source of IL-2 immunoreactivities was neuronal or glial.

IL-2 immunoreactivities have also been detected in normal human brains *post mortem* (Araujo and Lapchak, 1994). Using IL-2 radioimmunoassay antisera, IL-2 immunoreactivities were observed in normal human (average age, 68 years old) hippocampal homogenates (5,41 fmol/mg). It is unclear whether the differences in the

reported values of IL-2 immunoreactivities between adult rat (45,4 fmol/mg) and aged human (5,4 fmol/mg) hippocampal homogenates are comparable since they they were performed under different and independent conditions.

Using immunoautoradiographic approaches, Lapchak *et al.* (1991) was able to observe the distribution of IL-2 immunoreactivities in rat brain slices. These observations indicated, in addition to the results of the IL-2 immunoreactive content reported by Araujo *et al.* (1989), that IL-2 immunoreactive distribution is present in discrete and selected brain areas. Furthermore, within IL-2-enriched areas such as the hippocampus and the cerebral cortex, there is a differential pattern of distribution. In the hippocampus, IL-2 immunostaining is present in the pyramidal layer and the granular layer of the dentate gyrus. In the frontal cortex, IL-2 immunoreactivities were observed to be more prominent in layer IV than in the other laminae.

Immunohistochemical approaches were used to gain a better insight on the distribution and localization of IL-2 in the brain (Villemain *et al.*, 1990). In the normal mouse brain, high levels of IL-2 immunoreactive staining were observed in the arcuate nucleus-median eminence complex. However, vascular staining was virtually absent, suggesting that peripheral IL-2 is not a likely source of the IL-2 immunostaining.

The evidence presented thus far indicates that IL-2 immunoreactive materials are present in the brain and are distributed in selected areas. Moreover, it appears that IL-2 immunoreactivities reside in brain cells, thus suggesting that uptake of peripheral IL-2 is not the key source of IL-2 immunoreactivity in the central nervous system. These descriptions, however, remain tentative, and more extensive investigations are necessary to establish the central origin for IL-2 expression.

IL-2 expression can also be modulated under certain conditions. Nieto-Sampedro and Chandy (1987) observed that IL-2-like bioactivities were increased in the entorhinal/occipital area of the neonatal and adult rat brain following a lesion of that

area, with the IL-2-like bioactivities maximally expressed at 10 days post-lesion. In addition, these rat brain extracts were able to promote proliferation of astrocytic cultures, providing an additional index of IL-2-like bioactivity. Most importantly, they were able to demonstrate that IL-2-like bioactivities from lesioned brain extracts on both astrocytes and IL-2-dependent cytotoxic lymphocytes were blocked by a monoclonal anti-IL-2 receptor antibody. This observation thus provides additional support that brain-derived IL-2-like bioactivities are likely due to IL-2.

When the rat striatum was lesioned by the local application of 1-methyl-4-phenylpyridinium (MPP^+), increased IL-2-like molecules as detected by polyclonal anti-IL-2 antisera were observed in the vicinity of the lesioned site 2 weeks post-lesion (Liang *et al.*, 1989). Although it is clear that the increased IL-2-like expression is associated to the lesion, since it is not detected on the contralateral control side, it is not apparent from these results whether the increased IL-2-like expression is specific to the brain or due to a recruitment of immune cells as a consequence of the inflammation in response to neurotoxicity.

Increased IL-2 immunoreactive materials detected by radioimmunoassay were also observed in hippocampal homogenates of brains obtained from patients dying of Alzheimer's disease (average age, 74 years)(Araujo and Lapchak, 1994). The IL-2 immunoreactive levels were reported to be 12,4 fmol/mg, which are significantly higher than normal or Parkinson's-diseased brains. Using immunocytochemistry, Hofman *et al.* (1986) identified the presence of IL-2 immunoreactivity in brains from patients with multiple sclerosis. The IL-2 immunostaining was localized to the plaque centre and its edge. On the other hand, Hofman *et al.* (1986) did not observe any IL-2 immunostaining on normal or Alzheimer's-diseased brains. Similar observations in support of IL-2 immunostaining in brains with multiple sclerosis, but none in normal brains were reported by Woodroofe *et al.* (1986).

It is unclear whether detection of normal or increased expression of IL-2 in the brain

can be pinpointed to all cells of the brain or to defined subsets such as glia or neurons, or immune cells that have penetrated the blood-brain barrier. While detailed immunocytochemical studies can identify the cell(s) bearing the IL-2 immunoreactivity, at this point it is not possible to exclude that the IL-2 be taken up from the circulation, i.e., the periphery. To address this issue, studies targeting the IL-2 mRNA may provide an indication that if the gene is expressed in the brain, it would suggest that IL-2 can be genuinely synthesized within the brain.

The presence of IL-2 mRNA in nervous tissues was recently described by Eizenberg *et al.* (1995). Transcripts for IL-2 were observed in mouse, rat and human brains as analyzed by Northern blot. These brain-derived IL-2 transcripts are of high molecular weight (~ 5 kB) compared to that from lymphocytes (~ 1 kB) and appear to be unique to the brain. These high-molecular weight IL-2 transcripts in human brain tissues were observed in all parts of the adult brain (cerebellum, grey and white matter), and were even more prominent in the human foetal brain (20-week old). Using a polymerase chain reaction (PCR) method, Eizenberg *et al.* (1995) determined that the sequences of human brain-derived and lymphocytic IL-2 mRNA were indeed for IL-2 as they all hybridized with a specific IL-2 probe.

Similar manipulations were also performed with whole-brain preparations from newborn rats and mice and adult rats and mice (Eizenberg *et al.*, 1995). As in human brains, rodent brains expressed a high-molecular weight transcript in comparison to the low-molecular weight transcript seen in lymphocytes. In addition, young rat brains appear to express more IL-2 transcripts than the adult rat brain.

Additional hybridizations were performed on mRNA extracts from rat cortical astrocytic and neuronal cultures. Eizenberg *et al.* (1995) observed the presence of the high-molecular weight IL-2 transcript in the astrocytic, but not neuronal mRNA preparation. These observations identify astrocytes as a possible source of IL-2 although it is unclear whether conditions of culture may have modified the expression of IL-2 in

neurons. Furthermore, these results collectively identify in the brain a rather unique IL-2 transcript not observed in lymphocytes. Eizenberg *et al.* (1995) advanced many possibilities from gene transcription to translational events to explain the differences in size observed between brain-derived and lymphocytic IL-2 transcripts.

Observations by Eizenberg *et al.* (1995) supporting astrocytic expression as the primary source of central IL-2 appear to be at variance with the discrete distribution of IL-2 immunoreactivities in the brain. It is obvious that other approaches are needed to resolve this issue. IL-2 mRNA as revealed by *in situ* hybridization can be considered as one method to determine the type of brain cells that expresses endogenous IL-2.

Villemain *et al.* (1991) described the distribution and localization of murine IL-2 mRNA in the normal brain. They detected IL-2 mRNA in a subset of arcuate neurons, confirming that IL-2 detected immunohistochemically in this area was synthesized by those cells. Moreover, IL-2 mRNA was also observed within the hippocampal formation and the cerebral cortex in accordance with earlier immunohistochemical findings. These results provide support for the expression of IL-2 by discrete brain areas consistent with its immunoreactivity and point to neurons as a likely source of endogenous IL-2.

1.4.2 Interleukin-2 receptors in the brain

Receptors for IL-2 have also been observed in the normal mammalian brain. Although results from functional observations in brain-derived cells using IL-2 suggest that IL-2 receptors exist in the brain to mediate unique effects (see below), these approaches do not allow for a detailed description of the IL-2 receptor. One of the first reports of IL-2 receptors in the brain came from Saneto *et al.* (1986, 1987). They observed that IL-2 was able to inhibit the proliferation of oligodendrocyte progenitor cells in culture. After 5 days of continuous exposure to IL-2, the effect disappeared, and the disappearance of TAC staining on these cells suggests that the expression of the IL-

2Rα was necessary for the effect. When the cells were stimulated with IL-1 to induce IL-2Rα expression, the cells were once again responsive to IL-2. Similarly, Smith *et al.* (1989) was able to purify the IL-2Rα subunit from membrane preparations of the murine anterior pituitary tumour cell line AtT-20 and primary female rat pituitary cultures by using IL-2 and anti-IL-2 receptor affinity chromatography. This receptor protein co-purified with a control splenocytic preparation at the expected size for the IL-2Rα. This result strongly suggests the expression of the IL-2Rα on pituitary cells. However neither Saneto *et al.* (1986, 1987) nor Smith *et al.* (1989) used the anti-IL-2Rα to block the effects of IL-2, which would have functionally demonstrated the necessary participation of the IL-2Rα in these effects, a procedure well established for T cells (Leonard *et al.*, 1982).

The anti-IL-2Rα antisera can also be used to reveal the IL-2 receptor on thin brain slices *in vitro*. In this manner, Lapchak *et al.* (1991) reported that high levels of IL-2Rα immunoreactive labelling detected by autoradiography were distributed discretely in particular areas of the normal rat brain. These include the pyramidal layer of the hippocampus, granular layer of the dentate gyrus, layer IV of the cerebral cortex and the arcuate nucleus-median eminence complex of the hypothalamus. In the cerebellar cortex, high densities of labelling were noted in the molecular layer while lower levels were observed in the granular layer. Other areas of the brain displaying moderate to low levels of IL-2Rα immunoreactive labelling include the striatum, other layers of the cerebral cortex, the hypothalamus and the locus coeruleus.

Using the anti-IL-2Rα, Luber-Narod and Rogers (1988) revealed the presence of the IL-2 receptor in normal and Alzheimer's-diseased human brains *post mortem* and localized the IL-2Rα immunostaining to microglia. However, they did not comment on nor show any comparison between the intensity of the IL-2Rα immunostaining between normal and Alzheimer's-diseased human brains. On the other hand, Hofman *et al.* (1986) did not observe any IL-2Rα immunoreactivity in normal human brains. In parallel, using a monoclonal anti-mouse IL-2Rß, Sawada *et al.* (1995) did not

observe any IL-2Rß immunoreactivities in normal murine microglial cultures.

Receptor binding studies with [^{125}I]IL-2 to brain homogenates provided new information about the density and distribution of IL-2 receptors in the brain. Using rat hippocampal homogenates, Araujo *et al.* (1989) reported that IL-2 binds specifically with limited capacity (B_{max} = 0,48 ± 0,08 fmol/mg) and with high affinity (K_d = 25 ± 6 p*M*) to a single class of sites (n_H = 0,95 ± 0,02). However no specific binding using [^{125}I]IL-2 could be observed in rat striatal or frontal cortical homogenates. Using hippocampal homogenates from normal human brains (average age, 68 years), the IL-2 binding capacity B_{max} was observed to be 0,63 ± 0,06 fmol/mg (Araujo and Lapchak, 1994).

Receptor autoradiography using [^{125}I]IL-2 revealed the widespread but discrete pattern of distribution in the normal rat brain (Araujo *et al.*, 1989). The highest IL-2 binding levels were observed in the hippocampus. Lower IL-2 binding levels were observed in the septum and the cerebellum, and much lower and diffuse levels were seen in the striatum. However, unlike the high levels of IL-2Rα immunoreactivity observed by Lapchak *et al.* (1991), only very low and diffuse [^{125}I]IL-2 receptors in the cerebral cortex and the hypothalamus were detected by Araujo *et al.* (1989).

The immunoautoradiographic distribution of IL-2Rα matches very closely the autoradiographic distribution of [^{125}I]IL-2 binding sites in the hippocampal formation, where high levels of both markers are found to be predominantly associated with the pyramidal layer of the hippocampus and the granular layer of the dentate gyrus. However, mismatches between the 2 markers are clearly evident in many other areas. For example, high levels of anti-IL-2Rα immunoreactive labelling were observed in the hypothalamus and layer IV of the cerebral cortex, yet [^{125}I]IL-2 binding densities are low in these areas. On the other hand, closer matches can be observed between IL-2Rα and IL-2 immunoreactivities in many of the areas that express both markers, such as the hippocampus, hypothalamus and cerebral cortex and also in other areas

expressing moderate to low amounts of IL-2Rα and IL-2 immunoreactivities, as in the septum and the corpus callosum. The overlap between these 2 immunoreactive markers strongly implies that perhaps IL-2 is synthesized or stored in the brain, in proximity to its receptors. Discrepancies between IL-2Rα immunoreactivities and [^{125}I]IL-2 binding sites remain evident. Several hypotheses were advanced to interpret this mismatch. For example, Lapchak *et al.* (1991) suggested that anti-IL-2 antisera may be recognizing the IL-2 already bound onto the IL-2 receptors in the brain, thus giving an IL-2 receptor-like pattern of distribution. Immunodetection with anti-IL-2Rα may also be more sensitive than [^{125}I]IL-2 binding to determine IL-2 receptor distribution (Lapchak *et al.*, 1991). Alternatively, central IL-2 receptors may be occupied by endogenous IL-2 and thus inaccessible to the radioiodinated ligand, while some epitopes still remain available to the IL-2Rα antisera. Lapchak *et al.* (1991) has suggested that IL-2 rapidly dissociates from one subunit while remaining bound onto the other one. According to this hypothesis, this would allow the anti-IL-2Rα access to its antigen, but at the same time, remain bound to the IL-2Rß. IL-2Rα and ß bind to IL-2 with different on-off rates that were determined in defined receptor subunit preparations from cell lines. It remains to be confirmed whether there is a differential kinetic binding activity of IL-2 to its receptor subunits once binding has occurred.

Although the precise nature of the IL-2 receptor remains to be defined, brain-derived IL-2 expression can be modulated under certain conditions. Ohno *et al.* (1993) reported that IL-2R immunoreactivities were not seen in the nervous system of normal mice. However, in the demyelinating lesions of the twitcher mice strain, a model for human globoid cell leukodystrophy caused by a deficiency of galactosylceramidase, IL-2R immunoreactivities were found in the striatum and the deep layer of the cerebral cortex of 30-day old mice. The immunoreactivities seem to be localized on microglial cells. However, despite an intact blood-brain barrier in this model in contrast to experimental allergic encephalomyelitis (Kondo *et al.*, 1987), immunoreactivities were also observed on lymphocyte-like cells in the brain parenchyma. In parallel,

using Western blot Sawada *et al.* (1995) observed that contrary to normal murine microglial cultures, lipopolysaccaride-stimulated cultures increased their immunoreactive expression of IL-2Rß, and more interestingly, the anti-IL-2Rß antibody identified the antigen that is similar in size to that expressed on T cells.

Using a model of lesioned brains, Araujo *et al.* (1989) investigated the specific binding of [^{125}I]IL-2 in rat hippocampus. Rats were unilaterally lesioned at the dorsal hippocampus by a local application of kainic acid. After 6 days, analysis of ChAT activity revealed no significant changes between lesioned and contralateral control hippocampi. However, [^{125}I]IL-2 binding sites were significantly increased in the lesioned compared to the contralateral intact side. Araujo *et al.* (1989) concluded that this increase may be attributed to an increased microglial expression of IL-2 receptors. Alternatively, a breach of the blood-brain barrier during the surgical procedure which in course of an inflammatory response leads to a recruitment of immune cells bearing IL-2 receptors.

Hofman *et al.* (1986) reported an increased IL-2 receptor immunoreactivity in brains with multiple sclerosis in comparison to controls and other neurological disorders, including Alzheimer's disease. They observed profuse staining in the plaques of brains with multiple sclerosis. This staining was most intense in the centre and outer edge of the plaque, with a pattern of staining similar to that observed with anti-IL-2. In contrast, Araujo and Lapchak (1994) were able to detect IL-2 receptors in normal human hippocampal homogenates and observed an increase in IL-2 receptor binding sites in human hippocampal homogenates derived from Alzheimer's (1,06 $\pm$ 0,09 fmol/mg)- and Parkinson's (1,12 $\pm$ 0,09 fmol/mg)-diseased brains. The significance of this increased central IL-2 receptor expression in Alzheimer's disease in view of the increased expression of IL-2 remains enigmatic. It is undeniable, however, that IL-2 and IL-2 receptors are associated with these pathological processes. These observations lead an emerging consensus that there is a major neuronal component to IL-2 receptor expression.

The cellular localization of the IL-2 receptor was greatly facilitated by the use of hybridization for IL-2Rα mRNA. Using a human IL-2Rα probe, Shimojo *et al.* (1993) observed using Northern blot a greater expression of IL-2Rα mRNA in rat cortical neuronal cultures than in rat cortical astroglial or microglial cultures. They also reported that the normal adult rat brain contains IL-2Rα mRNA.

In addition, the blot observed for both neurons and adult spleen cells by Shimojo *et al.* (1993) was located at the 1,4-kB position. Petitto and Huang (1995) pointed out that rat immune cells express IL-2Rα mRNA that is 3,5 kB (Page and Dallman, 1991). Incidentally, Merrill (1990) observed that a human T cell line that constitutively expresses IL-2 receptors, MO, contains both the 1,4-kB and 3,5-kB species. However, under low stringency, Merrill (1990) detected 2 novel fragments of 2,8 kB and 2,2 kB using a preparation from a human oligodendroglioma cell line 5D7. So it is unclear whether these differences in size observed between brain-derived and lymphocytic IL-2Rα mRNA represent differences in coding sequence or in untranslated segments. Moreover, there appear to be differences in IL-2Rα levels in normal mammalian brains among various reports using either the antibody (Hofman *et al.*, 1986; Luber-Narod and Rogers, 1988; Ohno *et al.*, 1993) or a probe for IL-2Rα mRNA (Shimojo *et al.*, 1993). To resolve these discordant observations regarding IL-2Rα expression, Petitto and Huang (1995) proceeded to clone the IL-2Rα cDNA from the murine brain. They were able to clone the IL-2Rα cDNA from the normal murine forebrain. They observed that translated brain-derived and lymphocytic IL-2Rα sequences are identical and differences are likely due to untranslated regions since it has been reported that murine T cells express 5 distinct mRNA transcripts which differ primarily in their 3' untranslated sequences (Miller *et al.*, 1985). These results suggest that IL-2Rα mRNA is likely the same in the brain as in T cells. Moreover, they found that unlike the immune system, the brain constitutively expresses the IL-2Rα mRNA.

However, in the immune system, the IL-2Rα subunit alone is inactive and it is likely

that its sole presence in the brain may not account for effects of IL-2. It is believed that the expression of other IL-2 receptor subunits is necessary. To that end, Petitto and Huang (1994) were able to clone the IL-2Rß cDNA from the normal murine forebrain. In their study, they provided evidence that the normal brain expresses IL-2Rß mRNA which is sequentially identical to the T cell transcript.

Sawada *et al.* (1995) observed IL-2Rα expression from murine microglial preparations. Using reverse transcriptase PCR, they were able to identify a product of the predicted size of 700 bp. Moreover, microglial IL-2Rα mRNA expression can only be detected after stimulation with lipopolysaccaride (LPS). They also observed that astrocytic cultures did not express IL-2Rα mRNA under either normal or LPS-stimulated conditions. It is obvious that the observations of Sawada *et al.* (1995) regarding the absence of IL-2Rα mRNA expression in cultured neurons is in contrast to the constitutive expression detected by Petitto and Huang (1995), and may in part be attributable to conditions of culture that may modify the expression of certain genes.

Using *in situ* hybridization, Petitto and Huang (1994) described the distribution and localization of IL-2Rß mRNA in the normal murine brain. They observed IL-2Rß expression in CA1 of the hippocampus and the dentate gyrus. It is believed that the IL-2Rß mRNA be localized in neurons since the dentate gyrus is relatively devoid of glial cells (Rickmann *et al.*, 1987). They were also able to isolate the message for IL-2Rß from the murine frontal cortex and striatum using PCR. In addition, they extended their results by detecting IL-2Rß gene expression by PCR in 2 unrelated neuroblastoma cell lines, Neuro 2A and NB41A3.

In a recent report, Otero and Merrill (1995) were able to detect the 4,0-kb IL-2Rß mRNA fragment by Northern blot under highly stringent conditions in several areas (amygdala, caudate nucleus, corpus callosum, hippocampus, hypothalamus, substantia nigra, subthalamic nucleus and thalamus) of the adult human brain.

However they were unable to observe any IL-2Rα or γ mRNA in their preparations. On the other hand, in the human foetal spinal cord, they were able to detect the 1,5-kb IL-2Rα mRNA by Northern blot. IL-2Rß and γ mRNA were not observed in the human foetal spinal cord.

In addition, Otero and Merrill (1995) were able to clone by reverse transcriptase PCR the cDNA for the IL-2Rα, ß and γ from an oligodendroglioma cell line TC620.6A2. They observed multiple fragments of IL-2Rα mRNA as reported by others. The predicted products of IL-2Rß and γ gene sequences were found to be identical to those observed in immune cells. This suggests that the full complement of IL-2 receptor subunits are expressed concurrently in brain-derived cells to allow for the effect of IL-2 to be manifested.

1.4.3 Functional effects of interleukin-2

1.4.3.1 Cellular approaches

The presence of IL-2 receptors in cells of the nervous system was first suggested on the basis of growth-promoting effects of IL-2, allowing the speculation that IL-2 receptors must exist on these cells to mediate these effects. Benveniste and Merrill (1986) first reported that IL-2 at a concentration range of 10-100 U/mL was able to stimulate rat cortical oligodendroglial proliferation and maturation *in vitro*. No effects were observed on astrocytic cultures at the concentrations of IL-2 used. Initially no such effects were observed using IL-2 from supernatants of the T cell line HSB2 or with purified IL-2 at the concentrations used (Merrill *et al.*, 1984) However Saneto *et al.* (1986, 1987) reported that IL-2 inhibited the proliferation of oligodendrocyte progenitor cells *in vitro*. This IL-2 effect occurred at a concentration range of 0,3 to 100 U/mL. Saneto *et al.* (1986, 1987) found that prolonged exposure of these cells to IL-2 resulted in the disappearance of the IL-2 effect and a parallel disappearance of TAC-positive expression. More importantly, Saneto *et al.* (1986, 1987) were able to

restore sensitivity of these cells to IL-2 and induce a parallel TAC expression by the addition of IL-1 at 3 U/mL. The authors concluded that the TAC immunoreactivity detected on these cells is not identical to those on lymphocytes despite the many similar features observed. They based their conclusions mainly on the differences in kinetics of TAC immunoreactivities between these brain cells and lymphocytes following exposure to IL-2.

Another group observed that a soluble factor derived from injured fish neurons was oligodendrocytotoxic, and when applied to injured adult rabbit optic nerves, permits regenerative axonal growth (Cohen *et al.*, 1990). This factor was neutralized by anti-IL-2 antisera and in Western blot analysis, migrates to a position that is twice the size for IL-2, leading them to suggest that this permissive factor could be an IL-2 dimer (Eitan *et al.*, 1992). Furthermore it was reported that regenerating optic nerves release a Ca^{+2}-dependent transglutaminase that dimerizes IL-2 (Eitan and Schwartz, 1993) This enzyme was purified by raising a polyclonal antibody against sequences known to be conserved among various transglutaminases and using the antibody to precipitate the enzyme from the supernatants. They were able to test this enzyme in the presence of various concentrations of IL-2 and at 100 U/mL, only about 25% of the IL-2 was dimerized. The dimerized IL-2, unlike monomeric IL-2, is cytotoxic to rat oligodendrocytes in culture. They conclude that the estimate of 25 U/mL of dimeric IL-2 is within the concentration range (1 to 100 U/mL) of the physiological activity of IL-2. When this nerve-derived transglutaminase was applied to injured rat optic nerves, it promoted the recovery of the visual response within 6 weeks after injury (Eitan *et al.*, 1994). Morphological analyses of the treated rats 2 weeks post-injury revealed that an abundance of unmyelinated nerve fibres resembling newly growing axons embedded in astrocytes at the proximal edge of the injured nerve, while in controls, very few unmyelinated axons were observed and none at the site of injury. By the sixth to eighth week postinjury, nerve fibres resembling newly growing axons were found throughout the entire length of the nerve in the treated rats, while in the controls, no viable fibres can be seen in any of the sections. These observations suggest that

oligodendrocytes secrete factors inhibiting neuronal regeneration (Schwab and Thoenen, 1985; Schwartz *et al.*, 1985; Schwab and Caroni, 1988; Sivron *et al.*, 1994) and that dimeric IL-2, not IL-2 itself, procures the toxicity to oligodendrocytes that is permissive for neuronal regeneration.

Another series of studies used cultures from sympathetic and sensory neurons from embryonic chick and neonatal rats to investigate the growth effects of IL-2 (Haugen and Letourneau, 1990). They observed that in cultures of chick sympathetic chain and rat superior cervical ganglia, IL-2 enhances the number of neurons with neurites and neurite outgrowth *in vitro* in chick and rat sympathetic neurons (Haugen and Letourneau, 1990). However, IL-2 did not affect cultures of sensory neurons from chick and rat dorsal root ganglia. They also determined that the optimal response of the sympathetic neurons occurred at concentrations of IL-2 ranging from 0,2 to 2 U/mL. In addition, they were able to observe their effects in cultures containing or depleted of Schwann cells, suggesting the IL-2 did not act through the major supporting cells of the peripheral nervous system. Moreover, they were able to visualize by immunofluorescence the expression of the TAC antigen on sympathetic, but not on sensory neurons, suggesting that IL-2 receptors may be localized on sympathetic neurons to account for the IL-2 effect and that IL-2 is able to act directly on neurons.

Furthermore, three groups independently demonstrated that IL-2 enhanced survival and neurite extension in primary cultures of hippocampal, septal and cortical neurons (Shimojo *et al.*, 1993; Awatsuji *et al.*, 1993; Sarder *et al.*, 1993). Using cultures of primary neocortical neurons from the embryonic rat brain, Shimojo *et al.* (1993) observed that IL-2 was able to enhance the viability of these neurons in a concentration-dependent manner, with an optimal response between 10 to 100 U/mL for rat and murine IL-2 and 100 U/mL for human IL-2. As mentioned previously, they found that neuronal cultures responded better to IL-2 than astroglial or microglial cultures. Furthermore they were able to demonstrate by Northern blot the greater presence of IL-2Rα mRNA in neuronal than in astroglial or microglial cultures, which is

in accordance with their observations of preferential IL-2 growth effects with regard to cell type. Awatsuji *et al.* (1993) studied the effects of IL-2 in supporting the survival of cultured neurons from selected foetal rat brain areas. They observed that IL-2 at concentrations between 2 to 200 U/mL supported the survival and enhanced neurite extension of cultured hippocampal neurons. In addition they found that IL-2 supported the survival of cultured cortical, striatal and septal neurons. They also observed that IL-2 did not affect levels of striatal choline acetyltransferase activity, suggesting that IL-2 did not affect differentiation of striatal cholinergic neurons. Sarder *et al.* (1993) investigated the effects of IL-2 on the survival and morphology of primary cultured neurons from selected foetal rat brain areas. They found that IL-2 from 0,1 to 100 ng/mL was able to enhance the survival of neuronal cultures from the hippocampus, cerebral cortex, septum and cerebellum. In parallel, they found that the effects of IL-2 was observed only in high-density (100 000/cm^2 at initial plating), but not low-density (5000/cm^2 at initial plating) cultures. Morphological studies concentrating on hippocampal neurons revealed that IL-2 promoted neurite extension in low-density cultures. Sarder *et al.* (1993) believed that the IL-2 effects on high-density cultures suggest that it not be a direct action on neurons. However, Sarder *et al.* (1993) also added that the IL-2 effects on hippocampal neurite extension in low-density cultures indicated a direct neuronal effect of IL-2.

IL-2 under subchronic/chronic conditions in cultured cells has provided evidence of growth-promoting effects of IL-2. However, because of potential artefacts on growth and differentiation arising from cultures *in vitro*, this approach may not be entirely adequate to serve as a marker of central IL-2 activity. In addition, a review of these reports have indicated that IL-2 may act on both neurons and glia of brain cells. Accordingly, studies that pinpoint selected neuronal systems are necessary to establish IL-2 activities in the brain as a marker for IL-2 receptors.

1.4.3.2 Interleukin-2 and the hypothalamic-pituitary axis

The presence of IL-2 receptors in the normal brain has also been described using functional markers. One of the first observations revealed the hypothalamic pituitary complex as a focal point for IL-2 action. Bindoni *et al.* (1988) and Bartholomew and Hoffman (1993) reported evidence of IL-2 being able to influence bioelectric activity of the supraoptic, paraventricular and ventromedial nuclei and the anterior hypothalamus. Application of IL-2 15-30 U into the third ventricle of the rat brain under anaesthesia resulted in a marked and significant decrease in neuronal discharge frequency in the ventromedial nucleus of the hypothalamus and a marked increase in the supraoptic and paraventricular nuclei (Bindoni *et al.*, 1988). On the other hand, IL-2 at the dose range used was ineffective in modulating bioelectric activity in the dorsomedial, arcuate, and medial mamillary nuclei and in the dorsal and lateral areas of the hypothalamus. Interestingly, they observed that their hypothalamic IL-2 effects were antagonized by a monoclonal antibody shown to inhibit the IL-2-dependent effects by acting with MHC class I antigens (Osawa *et al.*, 1985), suggesting similarities in IL-2 pathways between the immune and central nervous systems. Bartholomew and Hoffman (1993) administered IL-2 60 U by peripheral injection to mice and observed a decreased electrical activity in the lateral margin of the anterior hypothalamus as measured in freely moving subjects. They also found that IL-2 effects appear to be confined to this area of the anterior hypothalamus.

IL-2 at a concentration of 25-100 U/mL was reported to potentiate the basal release of corticotropin-releasing factor (CRF) from superfused hypothalamic slices (Cambronero *et al.*, 1992). These effects were dexamethasone-sensitive. However, the CRF response to IL-2 was similar between 8-day adrenalectomized and sham-operated rats, indicating that the presence of glucocorticoids *in vivo* does not seem to be necessary for the response to IL-2. Antagonism of lipo-oxygenase or cyclo-oxygenase did not alter the IL-2 effect, suggesting that metabolites of arachidonic acid do not seem to be involved. Similarly, addition of naloxone did not result in any differences in the effects to IL-2, indicating that opioid peptide receptor activation does not seem to

not seem to play a role in this phenomenon. However, using static incubation of hypothalamic slices, effect of IL-2 on CRF release was suppressed by Nᵍ-methylmono-L-arginine acetate (L-NMMA), an inhibitor of nitric oxide synthase (Karanth *et al.*, 1993). This finding indicates that IL-2 can activate NO synthase leading to increased NO release which activates CRF release. Haemoglobin, a scavenger for NO, has also been observed to antagonize hypothalamic IL-2-evoked CRF release (Raber *et al.*, 1995). Addition of $CoCl_2$ also antagonized IL-2-induced CRF release from the incubated hypothalamic slices, suggesting that the effect be Ca^{+2}-dependent (Raber *et al.*, 1995). Similarly, IL-2 was observed to induce basal CRF release from the amygdala *in vitro*, occurring at concentrations of 1-100 U/mL (Raber *et al.*, 1995). A comparison of the IL-2-induced hypothalamic and amygdalar release of CRF to equal concentrations of IL-2 suggests that the hypothalamic slices release more CRF expressed as a ratio of basal levels. There was also an attenuation over time of both the hypothalamic and amygdalar response to higher concentrations of IL-2, suggesting time-dependent effects of IL-2 (Raber *et al.*, 1995). This may be due to IL-2 receptor desensitization or downregulation (Raber *et al.*, 1995), or perhaps an exhaustion of the stores of readily releasable CRF. The basal amygdalar CRF response to IL-2 was also observed to be attenuated in the presence of L-NMMA, haemoglobin and $CoCl_2$, suggesting that the IL-2 can also activate NO synthase to release NO to stimulate CRF release, and that IL-2 effects in the amygdala occurs by Ca^{+2}-dependent mechanisms (Raber *et al.*, 1995). In parallel, IL-2 was also observed to stimulate the basal release of arginine vasopressin (AVP) from both rat hypothalamic and amygdalar slices *in vitro* (Raber and Bloom, 1994). While the effects in both brain areas were concentration-dependent, the hypothalamus responded with IL-2 as low as 1 U/mL, while for the amygdala, stimulation of AVP release was noted only from 50 U/mL. The IL-2-stimulated release of AVP in both areas were abolished by *N*ᵍ-methyl-L-arginine, an inhibitor of nitric oxide synthase, suggesting that IL-2 can activate local NO synthesis and release to induce CRF release in both areas. The IL-2 effects in both areas were also sensitive to $CoCl_2$, suggesting that the observed effects on AVP release represent Ca^{+2}-dependent

events. A comparison of the hypothalamic release of CRF and AVP in response to IL-2 reveals that AVP is released sooner and at lower concentrations of IL-2. This finding suggests that under physiological conditions, AVP may be more important than CRF in mediating IL-2 effects on plasma corticotrophin and cortisol (Raber *et al.*, 1995). These conclusions seem to be in line with those of Hillhouse (1994) who reported that IL-2 induces the release of AVP from the intact rat hypothalamus *in vitro* and cultured hypothalamic cells, but not of CRF from both preparations. The threshold response to IL-2 occurred at 10^{-11} *M* for the intact hypothalamus and at 10^{-8} *M* for the cultured cells.

IL-2 has also been reported to target other functional markers in the hypothalamus as measured by its effects on other transmitter systems. Lapchak and Araujo (1993) observed that IL-2 at concentrations of 10^{-9} to 10^{-8} *M* was able to modulate the K^+-evoked overflow of [^{3}H]noradrenaline from incubated hypothalamic slices, but was not effective in altering that of [^{3}H]dopamine or [^{14}C]5-HT, nor that of [^{14}C]glutamate or [^{14}C]GABA. The spontaneous overflow of these monoamines was also unaltered in the presence of IL-2. Furthermore, they observed that IL-2 was able to potentiate the K^+-evoked, but not basal, release of methionine-enkephalin and to a lesser extent, ß-endorphin, from hypothalamic slices. However, the release of leucine-enkephalin was unaltered under both conditions.

On the basis of the presence of the IL-2Rα subunit in the pituitary, Smith *et al.* (1989) investigated the possible effects of rmIL-2 on hormone release. They observed that IL-2 at a range of 100-250 U/mL was approximately equipotent to 0,5 µg/mL of CRF in the stimulation of corticotropin release from AtT-20 cells. Karanth and McCann (1991) observed that femtomolar concentrations of IL-2 increased basal prolactin, corticotropin and thyroid-stimulating hormone release from incubated male rat anterior pituitary slices *in vitro*, while inhibiting follicule-stimulating hormone, luteinizing hormone and growth hormone release under the same conditions. Subsequent exposure to high K^+ alone resulted in the decrease of prolactin, follicle-stimulating

hormone and luteinizing hormone release, and no effect on the release of corticotropin, thyroid-stimulating hormone and growth hormone. Furthermore, IL-2 effects on prolactin, follicle-stimulating hormone and luteinizing hormone appeared to be dopamine-sensitive (Karanth *et al.*, 1992). Various concentrations of dopamine with IL-2 exerted a complex profile of effects on the release and content of prolactin, follicle-stimulating hormone and luteinizing hormone under basal and high K^+ conditions. These results indicate that IL-2 exhibits a profile of complex and multiple effects in the anterior pituitary that are particular to each hormone.

In addition to modulatory effects on hormone release, IL-2 *in vivo* was observed to influence gene expression in the central nervous system. Using *in situ* hybridization, a peripheral IL-2 injection (100-2500 U) was observed to enhance pro-opiomelanocortin (POMC, the precursor for specific pituitary peptides, such as corticotropin, endorphins, and melanocyte-stimulating hormone) mRNA expression in the rat in a dose-dependent manner (Harbuz *et al.*, 1992). These effects were significant for both 500 and 2500 U/injection, and were noted as soon as 4 hours after the injection and persisted for as long as 24 hours. Interestingly, they also observed in parallel that IL-4 decreased the POMC signal under identical conditions. Furthermore, they found that there was no effect on CRF mRNA expression (Harbuz *et al.*, 1992), suggesting that the increase in POMC mRNA in the anterior pituitary was not driven by an increase in CRF. Using the murine anterior pituitary tumour cell line AtT-20 and primary female rat pituitary cultures, Brown *et al.* (1987) reported that IL-2 at 25-50 U/mL was able to enhance POMC gene transcription in both models. In another study, a single peripheral injection of rmIL-2 at 40 000 U/kg increased both vasopressin and oxytocin gene expression in the hypothalamus of the Swiss nude mouse, a model of impaired immunocompetence (Pardy *et al.*, 1993). The effect on vasopressin mRNA expression was apparent within 12 hours after injection and was present even at the fifth day after injection. A similar profile was also observed for oxytocin mRNA expression. These effects appear to be specific to the Swiss nude mouse, since there were no significant changes in vasopressin mRNA levels in the normal, tumour-

bearing or cyclosporin A- or radiation-immunosuppressed AKR/J mouse. In addition, injection of rmIL-2 at 40 000 U/kg in the normal rat failed to induce any changes in vasopressin or oxytocin gene expression in the supraoptic nucleus, an area known to be a major site of vasopressin and oxytocin synthesis (Pardy *et al.*, 1993). However, this may be due to the use of murine, rather than rat or human IL-2. These experimental observations are in line with clinical reports of the neuroendocrine effects of IL-2 treatment. Denicoff *et al.* (1989) reported that IL-2 infusion increased the blood levels of stress-related hormones, including CRF, corticotropin, ß-endorphin and cortisol.

Studies on IL-2 administered by continuous central infusion in the rat revealed that IL-2 increases plasma levels of corticotropin in the dark phase and total corticosterone in the light phase (Hanisch *et al.*, 1994). In parallel, there were decreases in corticosterone-binding globulin levels in the dark phase, which contribute to increased free corticosterone levels. Histological observations indicated that this regimen of IL-2 administration resulted in periventricular tissue alterations and gliosis. These results add further evidence to the neuroendocrine effects of IL-2.

1.4.3.3 Interleukin-2 and dopamine

Application of IL-2 to striatal slices *in vitro* produced significant increases in both spontaneous and K^+-evoked overflow of [^{3}H]dopamine (Lapchak, 1992). The evoked tritium overflow was observed to be both Ca^{+2}-dependent and tetrodotoxin-sensitive. Both the spontaneous and evoked overflow exhibited a biphasic profile to increasing concentrations of IL-2, with stimulation of basal overflow from 10^{-11} to 10^{-8} *M* and no effect at 10^{-7} *M*, and potentiation of evoked overflow from 10^{-11} to 10^{-10} *M* and no effect at higher concentrations of IL-2.

1.4.3.4 Interleukin-2 and effects related to behaviour

Using dissociated guinea pig hippocampal CA1 neurons, Plata-Salamán and ffrench-Mullen (1993) reported that IL-2 was able to depress inward voltage-dependent Ca^{+2} currents. By means of whole-cell patch clamp recording, they observed that these effects were rapid, reversible and concentration-dependent ($6,8 \times 10^{-12}$ to $6,8 \times 10^{-9}$ *M*). Tancredi *et al.* (1990) examined the effects of IL-2 on the potentiation of synaptic transmission of rat hippocampal slices *in vitro* by recording extracellular field potentials in the Schaffer collateral/commissural-CA1 pathway. The application of IL-2 inhibited the induction of both short-term and long-term potentiation in a reversible and dose-dependent manner (200-3000 U/mL). IL-2 was also able to reduce both the post-tetanic potentiation and the maintenance phases. However, when IL-2 was applied before the conditioning stimulus, there was no change in basal synaptic transmission, but the post-tetanic potentiation was reduced and the induction of both short-and long-term potentiation was inhibited.

IL-2 has also been applied centrally *in vivo* in other models of investigation. Park *et al.* (1995) studied the effect of topical application of IL-2 (0,1-5 U) on the cortical surface of the anaesthesized rat and analyzed the response on afferent sensory transmission in the primary somatosensory cortex following cutaneous stimulation. They observed that IL-2 was able to suppress in a dose-dependent and reversible manner the afferent sensory transmission. It is not apparent, however, whether other effects may have escaped detection due to the narrow dose range. In addition, observations under conditions of urethane anaesthesia did not permit investigation of the IL-2 response in the freely moving rat under both normal and stimulated conditions.

Application of IL-2 (5-50 U) into the third ventricle of the brain in the freely moving rat resulted within 5-10 minutes in typical behavioural sedation associated with electrocorticogram (ECoG) synchronization and a dose-dependent increase in its total voltage power (De Sarro *et al.*, 1990). During sedation, alterations in activity were

observed in the cortex. When IL-2 (1-10 U) was injected into the locus coeruleus, De Sarro *et al.* (1990) observed a behavioural and sedation profile similar to that seen after intracerebroventricular infusion, with increased ECoG activity in the cortex and hippocampus. Pretreatment with naloxone into the locus coeruleus 15 minutes prior to the experiment attenuated the effects of IL-2 applied locally into the locus coeruleus.

IL-2 (5-50 U) applied into other brain areas such as the dorsal hippocampus, caudate nucleus, substantia nigra or ventromedial hypothalamus was ineffective in inducing behavioural and ECoG slow-wave sleep. However, IL-2 injection into the caudate nucleus and substantia nigra pars compacta induced dose-dependent asymmetric body posture with ipsilateral rotating behaviour and periodic ipsilateral rotations. These manifestations were apparently longer-lasting when the IL-2 was applied in the substantia nigra than when injected into the caudate nucleus. In addition, IL-2 injection into the dorsal hippocampus or ventromedial hypothalamus resulted in a dose-dependent increase in locomotor and exploratory activity.

Bianchi and Panerai (1993) measured the effects of IL-2 using behavioural parameters. Prior to acquisition, mice were treated with scopolamine 1 mg/kg to induce amnesia. By measuring the passive-avoidance response in mice injected peripherally with or without IL-2 (625-10 000 U), they observed that IL-2-treated mice had a greater memory deficit. IL-2 administered alone had no effects on the passive-avoidance response and did not affect nociceptive thresholds. In addition, mice that received 2500 U of IL-2 subchronically for 10 days did not exhibit any effect on passive avoidance. However subchronic IL-2 significantly enhanced the amnesic effect of scopolamine. Locomotor responses to single-dose IL-2 were also observed in mice. IL-2 alone did not modify spontaneous locomotor activity. Scopolamine alone increased locomotor activity relative to controls, and when IL-2 was also administered, mice exhibited a potentiated response to both treatments.

In an other set of studies, Nemni *et al.* (1992) investigated the effects of intermittent

peripheral IL-2 injection 18 000 000 U/m^2 a day every other week for 3 weeks in each of adult and 20-month old mice. Using a model of passive avoidance, they observed no differences among the untreated and treated young and untreated aged mice. However, they found that IL-2-treated old mice exhibited a decrease in response latency with respect to aged controls. Furthermore, neurohistological examination of the brain tissues did not reveal any evident changes in the young between the 2 groups. On the other hand, there were signs of damage in CA1-2, CA3 and CA4 hippocampal subfields of the IL-2-treated old mice. They also observed slight perivascular oedema in all areas explored in both IL-2-treated groups. More importantly, they did not detect any presence of inflammatory cells or immunoglobulins in the tissues of IL-2-treated mice. Additionally, they did not observed any neuronal loss or degenerating neurons in the dentate gyrus, cerebellum or the frontal cortex of IL-2-treated old mice. The authors conclude that aging must be a crucial factor to bring about the IL-2 neurotoxic and amnesic effect *in vivo*.

1.4.3.5 Clinical use of interleukin-2 and its adverse effects

The use of IL-2 in human immunotherapy against selected types of cancer has also provided insightful information regarding the central effects of IL-2. Denicoff *et al.* (1987) first reported that patients receiving 30 000 to 100 000 U/kg/day of IL-2 by peripheral injection exhibited marked alterations in behaviour, cognition and mood. Caraceni *et al.* (1993) collaborated this report by presenting detailed studies that suggest that cognitive failure resulting from IL-2 treatment affects attention and spatial memory. Furthermore, there were 2 case reports which pointed to IL-2 as the culprit in acute fatal leukoencephalopathy following systemic IL-2 treatment (Vecht *et al.*, 1990) and a case of delayed neurotoxicity of central administration of IL-2 resulting in death (Meyers and Yung, 1993). In addition, another case report suggests that mood alterations by IL-2 may be linked to depression and suicide in patients undergoing IL-2 immunotherapy (Baron *et al.*, 1993). These few clinical observations among the several case reports published strongly indicate that IL-2 is indeed able to exert

powerful effects on the brain.

1.4.3.6 Interleukin-2 and acetylcholine release

The release of central acetylcholine (ACh) has also been revealed as a marker of IL-2 activity in the brain. Araujo *et al.* (1989) investigated the possible function of IL-2 in the rat brain using a model of static incubation, targeting areas associated with IL-2 activities in relation to the cholinergic system. Araujo *et al.* (1989) reported that IL-2 reduced at rather low concentrations the K^+-evoked release of ACh from hippocampal slices in a concentration-dependent manner (10^{-9} to 10^{-8} *M*) without affecting hippocampal basal release. A similar examination of IL-2 activity in the frontal cortex revealed a feeble response, but was devoid of effect on the basal release from this brain area. However, the release of striatal ACh was unaffected by IL-2 at the concentration range studied.

Moreover, Araujo *et al.* (1989) studied the response of IL-2 in lesioned hippocampus. Rats were subjected to a unilateral local application of kainic acid in the dorsal hippocampus. An analysis of ChAT activity 6 days later revealed that the lesion did not affect this cholinergic marker. On the other hand, examination of the K^+-evoked ACh release revealed that the lesioned hippocampi released less ACh than the contralateral control in the presence of IL-2 at 12 n*M*, indicating that the lesions enhanced the sensitivity of the hippocampal cholinergic system to IL-2.

1.5 Objectives of the present studies

In summary, these reports have collectively presented a strong description of IL-2 activity in the brain. It is evident that brain areas which display IL-2 receptors are also very sensitive to IL-2. In accordance with the IL-2 receptor distribution, IL-2 exerts selected effects on the brain. This neuromodulatory action of IL-2 suggests that IL-2 may be a putative modulator of synaptic function under physiological conditions that

occurs by way of local release of the endogenous peptide. The cellular source of central IL-2 has not been clearly identified, and many cell types have been reported to express the gene for the subunits of the IL-2 receptor. However it is not clear whether the neuromodulatory effects of IL-2 are exclusively associated to neurons or if they also involve other cell types such as glia. Furthermore, it is not at this point feasible to draw conclusions with regards to any correlation between the IL-2 receptor density in any given brain area and the magnitude of its sensitivity to IL-2. Other unexplored factors need to be accounted, such as receptor coupling in IL-2 action.

Results obtained from diverse sources indicate that IL-2 activity in the brain appears to be coincident with the cholinergic system. Even though cholinergic markers were not analyzed directly, many of the brain areas exhibiting responses to IL-2 both *in vivo* and *in vitro*, be it induction of rotational behaviour when IL-2 is applied to the caudate nucleus (De Sarro *et al.*, 1990), increased locomotor activity upon local IL-2 application in the hippocampus (De Sarro *et al.*, 1990), attenuation of afferent somatosensory transmission in the cortex (Park *et al.*, 1995) all occur in brain areas of known cholinergic innervation. Whether the observed increase in locomotor activity can be related to specific cholinergic brain areas is unclear (Bianchi and Panerai, 1993), but it is tempting to draw parallels to the observations of De Sarro *et al.* (1990). It is clear, on the other hand, that the cholinergic system is targeted in the IL-2 enhancement of scopolamine-induced amnesia. In addition, studies at the cellular level in the hippocampus in which long-term potentiation or voltage-dependent Ca^{+2} currents are used as end points indicate that hippocampal cells are endowed with the ability to respond to IL-2.

In brief, because of the overlap between IL-2 receptors and IL-2 activity with the cholinergic system in many of the brain areas studied, ACh release as an end point emerges as a potentially sensitive marker of IL-2 action in various brain areas. Preliminary results reported by Araujo *et al.* (1989) suggest that this is a reliable marker for IL-2 activities. Moreover, this approach allows IL-2 to gain access to the

system targeted and provides an immediate indication of IL-2 activity under acute conditions.

The aim of the present studies is to: 1) provide evidence supporting the presence of endogenous IL-2 in the brain, and determine the cell types containing IL-2; 2) provide information regarding the IL-2 receptor in the brain; 3) correlate the presence of IL-2 in the brain to a functional marker, ACh release, in the areas of enriched IL-2, IL-2 receptors and ACh; and 4) explore possible intracellular signalling mechanisms through which IL-2 is believed to exert its effects following IL-2 receptor activation in the brain.

CHAPTER 2

PREFACE TO CHAPTER 2

At the time, the presence of interleukin-2 in the brain was a subject of intense controversy. Emerging research knowledge had associated interleukin-2 and its receptor to various elements of the nervous system using isolated cellular approaches. Accordingly, very little information was available regarding the origin of interleukin-2 in the brain. In this first study, the aim was to provide evidence of interleukin-2-related elements in brain tissues. The determination of the possible central origin of interleukin-2 is of capital importance since it provides a point of reference to understand the relevance of how the brain uses interleukin-2.

Anatomical and Functional Approaches to Study of Interleukin 2 and its Receptors in Brain

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Introduction

Interleukin-2 (IL-2), a 133-amino acid 15,42-kDa protein, is a cytokine secreted by T lymphocytes, known to act on receptors present on a subset of these cells (1). Receptors for IL-2 (IL-2R) are membrane-associated proteins that exist either as single units or as heterodimers. The low-affinity unit (p55, IL-2Rα, TAC) binds IL-2 with an affinity in the range of 10-20 n*M*. The combination of these two units in a heterodimer demonstrates high affinity for IL-2 [Kd = 10-50 p*M* (2)].

Biochemical and immunohistochemical evidence has revealed the presence of IL-2-like immunoreactivity in mammalian brain (3-6). Moreover, IL-2 was found to induce various biological effects in the CNS following either direct injection into the brain or peripheral administration. For example, intracerebroventricular administration of IL-2 produces sedation, sleep, and synchronization of electrocorticogram spectra (7). Interleukin-2 is also able to suppress the induction and expression of long-term potentiation (LTP) in the rat hippocampus, and to modulate K^+-evoked acetylcholine release in rat brain slices (3, 8, 9, 10). These effects appear to be exerted through specific membrane receptors of the type found on the surface of T cells (3, 5).

Here we briefly describe some of the methods and approaches that have been used thus far to investigate the presence and distribution of IL-2-like immunoreactivity and IL-2 receptors in the central nervous system (CNS), as well as the possible modulatory role of this cytokine on neurotransmitter release.

Immunohistochemical detection of brain IL-2-like materials

The topographic distribution of IL-2-like immunoreactivity has been reported for the rat and mouse brain (4, 5). The major advantage of mouse studies rests with the availability of homologous antibodies against IL-2 and IL-2 receptors of this species.

For the rat, studies had to rely on heterologous antibodies against recombinant human IL-2 in the absence of commercially available homologous probes. However, the anti-human serum demonstrated good cross-reactivity toward rat IL-2, as expected from the structural homologies between human and rat IL-2 and the high potency of human IL-2-like molecules in the rat brain (3, 6, 7-10).

Immunoautoradiography

For regional localization of IL-2 immunoreactivity, adult male Sprague-Dawley rats (200 - 250 g) or adult CD-1 mice (4 -5 weeks old) are deeply anaesthetized with chloral hydrate [3,5 mg/kg, intraperitoneal (ip)] and perfused transaortically with a mixture of 4% paraformaldehyde and 0.2% saturated picric acid in 0.1 *M* phosphate buffer (*p*H 7,4). Brains are then removed fom the skull and postfixed in the same solution for 1 hour at 20 - 21 C, and immersed overnight in a 30% sucrose phosphate-buffered solution. Next, tissues are snap frozen by immersion in 2-methylbutane at - 40 C, after which they are kept frozen at - 80 C until use. Coronal sections (30 μm thick) were cut on an American Optical (Buffalo, NY) freezing microtome and collected in 0,1 *M* sodium-potassium phosphate buffer. Sections are then preincubated for 30 minutes at room temperature in TRIS-HCl (100 m*M*; *p*H 7,4) containing 0.2% bovine serum albumin (Sigma Chemicals, St-Louis, MO) and 1.8% lysine (Sigma Chemicals) and sequentially incubated in normal donkey serum (rats; Sigma; dilution 1:30) or normal rabbit serum (mice; ICN Immunobiologicals, Costa Mesa, CA; dilution 1:30) for 30 minutes at room temperature. Sections from rat brain are then incubated with a rabbit antiserum directed against recombinant IL-2 (Araujo *et al.*, 1989) (dilution 1:8000; Amersham, Arlington Heights, IL) for 18-20 hours at room temperature. This antiserum is documented to cross-react only marginally with other interleukins, such as human IL-1α (< 0,39%), IL-1ß (< 0,19%), IL-3 (< 0,30%) IL-4 (< 0,04%) TNF and IFNγ (< 0,01%). Control sections are incubated under the same conditions, either by omitting the primary antiserum or by pre-absorbing it with rhIL-2 (Sigma Chemicals or

UBI (Lake Placid, NY) 0,1 μM in TRIS-NaCl buffer overnight at 4 C). Sections from mouse brain are incubated in the same fashion, using a rat anti-mouse IL-2 monoclonal antibody (5 μg/mL) derived from the S4B6 hybridoma line (11). Rat brain sections are then incubated with donkey anti-rabbit and mouse brain sections with rabbit anti-rat ^{125}I-labelled immunoglobulins (IgG) (0,2 mg/mL; Amersham) for 30 minutes at room temperature. All sections are next rinsed thoroughly in TRIS-HCl, followed by a rinse in distilled water. Sections are mounted onto gelatinized glass slides, dehydrated in graded ethanols, cleared in xylene, and rehydrated. They are finally air-dried in a dust-free atmosphere and juxtaposed against tritium-sensitive film (Hyperfilm; Amersham).

Prototypical examples of results obtained in the rat by using this protocol are illustrated in Fig. 1. Comparable selective, albeit widespread, immunostaining patterns are evident in sections from mouse brain (F. Villemain and A. Beaudet, unpublished). Low to moderate labelling densities are apparent in cerebral cortex, neostriatum, lateral spetum, thalamus, and the cerebellar cortex. High immunostaining densities are confined in both species to the pyramidal cell layer of the hippocampus, the granule cell layer of the dentate gyrus, and a number of hypo- and epithalamic nuclei including the arcuate nucleus-median eminence complex, the zona incerta, and the habenula (4, 5).

Peroxidase-anti-peroxidase immunohistochemistry

For cellular localization of IL-2 immunoreactivity, sections from selected mouse brain regions are prepared and immunolabelled as above, using the S4B6 anti-IL-2 monoclonal antibody. After overnight incubation in S4B6, sections are washed three times (10 minutes each) in TRIS-saline containing 0.2% bovine serum albumin (BSA) and 1% normal rabbit serum (NRS) and sequentially incubated at room temperature with: (1) a 1/50 dilution of rabbit anti-rat IgGs (Jackson Immunoresearch Laboratories, Bar Harbour, ME) for one hour and (2) a 1/100 dilution of polyclonal rat peroxidase-

anti-peroxidase (PAP) complex (Sternberger-Meyer Immunocytochemicals, Baltimore, MD) for one hour. These two steps are then each repeated once for 30 minutes. After two rinses in TRIS-NaCl, the sections are reacted for 6 minutes with 0,5% 3,3'-diaminobenzidine (DAB) in 0,1 *M* TRIS-HCl buffer containing 0,01% H_2O_2. Sections are briefly rinsed in distilled water, mounted onto gelatin-coated slides, dehydrated in graded ethanols, defatted in xylene, coverslipped and examined with a Leitz Aristoplan (Wetzlar, Germany) microscope.

At low magnification, the regional distribution of IL-2-like immunoreactivity conforms to that observed by immunoautoradiography. At high magnification, reaction product is seen to be accumulated over both neuronal perikarya and intervening neuropil. Perikaryal labelling was most evident in areas of high labelling densitiy, such as the arcuate nucleus (Fig. 2a) and the hippocampus (Fig. 2b). Electron microscope examination of the arcuate nucleus confirmed the association of IL-2 immunolabelling with neuronal perikarya and indicated that dendritic processes accounted for the bulk of neuropil labelling (5).

Immunohistochemistry of brain IL-2 receptors (TAC antigen-like immunoreactivity)

One possible means to study the presence and distribution of putative IL-2R in the CNS is to use immunochemical approaches to reveal the presence of the TAC (p55) receptor subunit in this tissue (4).

For this purpose, rat brains are fixed and sectioned as described precedingly for IL-2 immunostaining. Following a pre-incubation in TRIS-NaCl buffer containing BSA and lysine, sections are incubated for 30 minutes in normal sheep serum followed by a monoclonal antibody directed against the TAC antigen of the human IL-2 receptor (dilution 1:500) for 18-20 hours at room temperature. The anti-human TAC

monoclonal antibody has been shown to detect IL-2 receptors in primary cultures of rat cortex (12, 13) and on rat sympathetic neurons (14) neurons. Pre-absorption of the antibody with rhIL-2 (0,1 μM in TRIS-NaCl buffer overnight at 4 C) did not reduce the intensity of TAC immunostaining. However, immunolabelling was reduced somewhat if IL-2 (0,1 μM) was added to the antibody during the overnight incubation period, suggesting that the latter recognizes an epitope onto or close to the IL-2 binding site. Control sections are incubated under the same conditions, either in the presence of mouse anti-human monoclonal antibody (Sigma Chemicals) or in the absence of anti-TAC monoclonal IgG antibody. All sections are then incubated with sheep anti-mouse ^{125}I-labelled immunoglobulin (0,2 mg/mL; Amersham) for 30 minutes at room temperature. Following this last incubation, sections are autoradiographically processed, using trutium-sensitive film as described above.

As shown in Fig. 1b, human TAC immunoreactivity is present and selectively distributed in the rat brain. As for IL-2 immunoreactivity (compare Fig. 1a and b), the highest densities of labelling are detected in the hippocampal formation (stratum pyramidale of Ammon's horn and granule cell layer of the dentate gyrus), the arcuate nucleus of the hypothalamus, the median eminence, and the molecular layer of the cerebellar cortex. Preliminary results in the mouse obtained with a monospecific anti-mouse IL-2Rα chain antibody reveal a distribution comparable to that seen in the rat (F. Villemain and A. Beaudet, unpublished), thereby strengthening the validity of the results observed in the rat.

Quantitative IL-2 receptor autoradiography

In vitro receptor autoradiography has also be used to study the discrete distribution of IL-2 receptor binding sites in the rat brain (4). However, because of the low abundance of receptors in the normal rat brain (poor signal-to-noise ratio), only highly sensitive probes and optimal assay conditions allow the detection of specific labelling

by this approach. Slides must be cleaned and gelatinized, and sections prepared as described (15). Additionally, radioiodinated IL-2 must be used as fresh as possible and even repurified before its use in the binding assay. Otherwise, the level of specific labelling is too low for meaningful analysis.

Rats are sacrificed by decapitation and their brains rapidly removed from the skull, snap-frozen by immersion in 2-methylbutane at - 40 C, and stored at - 80 C. Sections (20-μm thick) are cut with a cryostat at -17 C, mounted on pre-cleaned gelatin-coated slides, air-dried and stored at -80 C until use. For labelling of ^{125}I-labelled IL-2 binding sites, sections were pre-incubated for 30 minutes at 22 C in a buffer of the following composition: 20 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 120 m*M* NaCl, 5 m*M* KCl, 2 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$ (*p*H 7,4), also containing bovine serum albumin (1 mg/mL). The sections are then incubated (2 hours, 22 C) in the same buffer containing 50 p*M* of ^{125}I-labelled human IL-2 (700-1100 Ci/mmol; Amersham). Specific binding is determined in the presence of an excess (0,1 μ*M*) of unlabelled IL-2 (Sigma). At the end of the incubation, slides are rinsed in five washes (2 minutes each) of cold TRIS-HCl (50 m*M*; *p*H 7,4; 4 C) buffer. Slides are then dipped in cold distilled water to remove salts and air-dried before exposing against Hyperfilms with ^{125}I-labelled microscale standards (Amersham) for up to 3 months, depending on the brain regions studied. Films are then developed as described earlier (4, 15) and autoradiograms are quantitated by computerized image analysis.

Under these assay conditions, ^{125}I-labelled IL-2 binding sites are distributed in a manner similar, but not identical, to that described above for TAC immunoreactivity (Fig. 1c). For example, both signals are highly enriched in the hippocampal formation, whereas the arcuate hypothalamic nucleus is better stained with the TAC antiserum than with ^{125}I-labelled IL-2 (compare Fig. 1b and c). This may relate to the existence, in certain regions, of only one subunit of the IL-2 receptor (i.e., TAC/p55) with too low an affinity for IL-2 for autoradiographic detection using picomolar concentrations of

radioligand.

Modulatory role of IL-2 on acetylcholine release

The presence of IL-2-like immunoreactivity and receptor binding sites in the CNS strongly argues for biological roles for this cytokine in the brain. As a means to address its possible role as neuromodulator, we investigated the action of IL-2 on the release of acetylcholine (ACh) in slices of rat hippocampus, in view of the concomitant enrichment in ACh terminals, IL-2 immunoreactivity, and IL-2 receptor binding sites in this structure. A superfusion method was preferred here to static incubations (3) as it allows the tissue slices to be continuously exposed to fresh, oxygenated incubation medium, and permits a better evaluation of time onset and duration of drug effects.

The protocol described here was established for adult (300 - 350 g) rat brain but is applicable to other species as well as to other tissues, the critical variable being the use of a sufficient amount of tissue to ensure reliable measurements of ACh levels.

Various brain regions (hippocampus, striatum, cortex) are removed on ice and sliced with a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, England) at 0,4 mm. An entire sliced hemispheric region is transferred to one superfusing chamber (Brandel Instruments, Gaithersburg, MD) and perfused with Krebs buffer (composition: 120 m*M* NaCl, 4,6 m*M* KCl, 2,4 m*M* $CaCl_2$, 1,2 m*M* KH_2PO_4, 1,2 m*M* $MgCl_2$, 9,9 m*M* dextrose, 25 mM $NaHCO_3$) at *p*H 7.4, 37 C, using a flow rate of 0,5 mL/minute for 30 - 45 minutes to equilibrate the tissue and establish a stable basal efflux of ACh. The buffer also contains physostigmine (30 μM; Sigma Chemicals), an esterase blocker, and choline choride (10 μM; Sigma Chemicals) to ensure the stability of the released ACh and to support a constant supply of its precursor. Collection of the superfusate is initiated at various intervals depending on the time resolution required in a given experiment. The tissue is then stimulated with

either a high K^+ (26,2 m*M*) Krebs buffer (with concomitant reduction in NaCl to conserve isotonicity) or electrical stimulation, in the presence or absence of various concentrations of human IL-2 (Sigma Chemicals or UBI). After variable periods of stimulation and exposure to IL-2, tissues are returned to superfusion with normal Krebs buffer.

The collected superfusates are next spun (13 000 rpm; 4 min; 25 C; Biofuge, Baxter Co., Montréal, Québec, Canada) to remove any extraneous protein and an aliquot is either frozen at -70 C or subjected immediately to ACh analysis. The superfused tissues are removed at the end of the experiment and kept for protein determination according to Lowry *et al.* (16).

Samples for ACh analysis are subjected to extraction according to Fonnum (17) as modified by Goldberg and McCaman (18) and to a radioenzymatic reaction for its quantifiaction. The protocol used is as follows.

1. ACh is extracted from a volume (400 μL) of superfusate by the addition of an equal volume of tetraphenylboron in butyronitrile (Aldrich Chemicals, Milwaukee, WI)(10 g/L) mixed and spun for 4 minutes each.

2. Each volume (300 μL) of the organic phase is removed and placed in clean plastic tubes and a half volume of $AgNO_3$ (20 g/L; 150 μL) is added, mixed and spun (13 000 rpm, 25 C) for 4 minutes each to recover the ACh from the organic phase.

3. Each volume (110 μL) of the aqueous phase is removed and placed in another set of clean plastic tubes and excess silver is precipitated by adding 10 μL of $MgCl_2$ (1 *M*) per 110 μL of sample, mixed and spun (13 000 rpm, 25 C) for 4 minutes each.

4. A final volume (100 μL) is then removed for evaporation under vacuum, and either stored at -20 C or sujected immediately to the radioenzymatic reaction.

5. For this reaction, each sample is redissolved in 32 μL of a mixture containing ATP (0,8 m*M*; Boehringer-Mannheim, Indianopolis, IN), dithiothreitol (5 m*M*; Boehringer-Mannheim, Indianopolis, IN), $MgCl_2$ (12,5 m*M*), glycylglycine at *p*H 8,0 (25 m*M*;Sigma), and choline kinase (0,005 U; Sigma) and incubated at 30 C for 25 minutes to phosphorylate the choline but not the ACh contained in the samples.

6. Ten microlitres of a solution containing acetylcholinesterase (2 U) and [γ-^{32}P]ATP (*ca.* 0,45 μCi) are added to each sample, which are then incubated at 30 C for 20 min. During this second incubation period, sample ACh is hydrolysed and the choline formed phosphorylated to [^{32}P]phosphorylcholine.

7. The reaction is stopped by the addition of 100 μL of NaOH (50 m*M*), and radioactive phosphorylcholine is separated from the radiolabelled ATP by ionic-exchange chromatography on a 5 X 20 mm column of Amberlite CG-400 (converted to formate form; Sigma) that had previously been equilibrated with 50 m*M* NaOH (Fisher, Pittsburgh, PA) before use. Phosphorylcholine is eluted by adding 3 mL of NaOH (50 m*M*).

8. Radioactivity is determined by liquid scintillation counting using Eco(+)lite (ICN, Costa Mesa, CA) as the solvent system.

For each set of extractions, internal ACh standards dissolved in Krebs buffer used for experimental samples are extracted and analysed along with these samples to determine recovery and generate standard curves.

A prototypical example of the effects of IL-2 on hippocampal ACh release is shown in Fig. 3. Interleukin-2 is an extremely potent modulator of ACh release, acting as a stimulatory agent at low concentrations (femto- to picomolar range) but inhibiting at higher concentrations (nanomolar). These results reveal that IL-2 immunoreactivity

and IL-2 binding sites present in the rat hippocampal formation are biologically relevant and involved in the regulation of neurotransmitter release. It remains to be established if the inhibitory and stimulatory effects of IL-2 on ACh release are mediated by similar or different transduction mechanisms.

Conclusions

It is now clear that IL-2, by acting on specific receptor sites, can induce a variety of biological effects in the CNS, including neuromodulation of transmitter release. However, major questions are still pending, among which are those concerned with the nature of the IL-2-like immunoreactivity present in the CNS. For example, is the primary sequence of brain IL-2 identical to that produced by T lymphocytes? Already, some evidence suggests that in the mouse, T lymphocyte IL-2 mRNA is present in the brain (19) but it remains to be established if it is fully translated and if the posttranscriptional maturation is identical in the CNS and immune cells. Attempts have been made to extract and purify IL-2-like materials directly from brain tissues (6, 11), but it has until now proved difficult to exclude possible contamination from blood-borne IL-2.

The present results clearly identify neuronal cells as the primary source of IL-2-like materials in regions such as the arcuate nucleus and the hippocampus. However, these findings do not exclude the possibility that in other brain regions some of the immunoreactive IL-2 might be produced by glial cells, particularly by resident microglial cells, which bear resemblance to immune cells. Further studies will also be needed to determine the cellular localization of brain IL-2 receptors, of which virtually nothing is currently known (4, 13, 14). Moreover, it remains to be established if the functional organization of brain IL-2 receptor is identical to that of its peripheral counterparts. For example, are the respective roles of the two IL-2 receptor subunits (p55 and p70) the same in the CNS as in the immune system? Are these subunits

identical molecular moieties in brain and periphery? Is their association necessary to ensure high affinities for IL-2 in the CNS? Is an identical structural organization required to ensure functional activities in all brain regions? Are brain IL-2 receptors coupled to transduction mechanisms similar to those of T lymphocyte receptors? It is also of interest that the p55/TAC antigen is widely expressed under normal resting conditions in the brain. This is in contrast to the immune cells, in which expression is seen only on challenge. It may suggest unexpected functions for this protein in the CNS as well as differential mechanisms of expression for the TAC antigen in brain *vs* immune cells. Information regarding most of these questions should be available soon, as research activities in this field have markedly expanded. It should then be easier to establish the genuine functional relevance of IL-2 in normal brain organization and function.

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Figure 1

Comparative distribution of IL-2-like immunoreactivity (a), TAC antigen-like immunoreactivity (b), and ^{125}I-labeled IL-2 binding sites (c) in rat brain sections. Film autoradiograms prepared after tagging anti-IL-2 (a) and anti-TAC (b) primary antibodies with iodinated IgGs, or after incubation of fresh frozen sections with ^{125}I-labeled human recombinant IL-2 (c). Note the similarity between the distributions of IL-2 and TAC antigen immunoreactivity. By contrast, ^{125}I-labeled IL-2 binding distribution shows both points of similarity (e.g., in the hippocampus) and divergence (e.g., in the hypothalamus) with that of TAC immunolabeling. (Modified from Lapchak *et al.* (1991) with permission.)

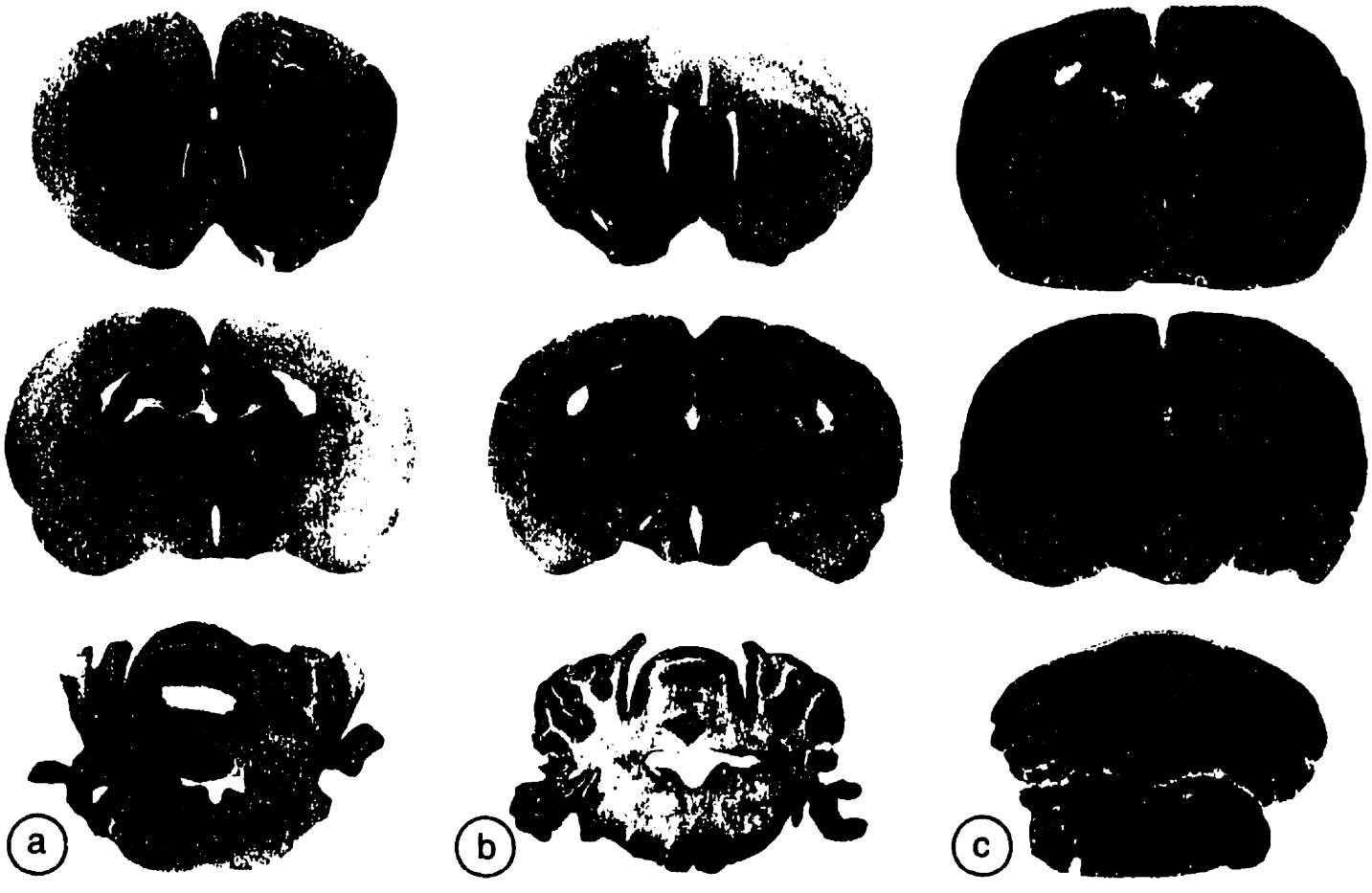
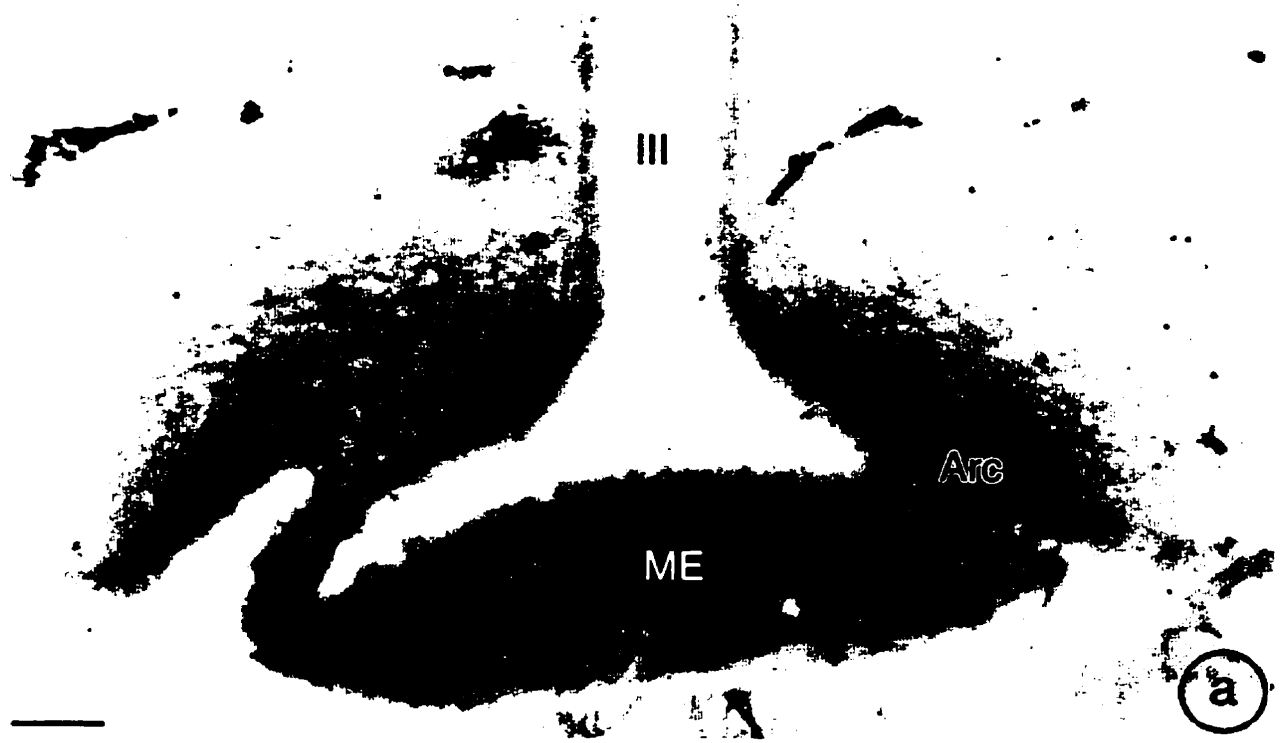

a
b
c

Figure 2

Light microscopic localization of IL-2-like immunoreactivity as revealed by the PAP technique in the arcuate nucleus-median eminence complex of the hypothalmus (a) and in the hippocampus (b). (a) In the arcuate nucleus (Arc), IL-2-like immunoreactivity is intense and distributed over both perikarya and neuropil. (b) In the hippocampus, the immunoreactivity is mainly confined to the perikarya of pyramidal cells (SP, stratum pyramidale). Note that the reaction product pervades the cytoplasm of the cells but spares the nucleus. III, Third ventricule; ME, median eminence; SO, stratum oriens; SR, stratum radiatum. Scale bars: 50 μm. (Personal data.)


III
Arc
ME
a

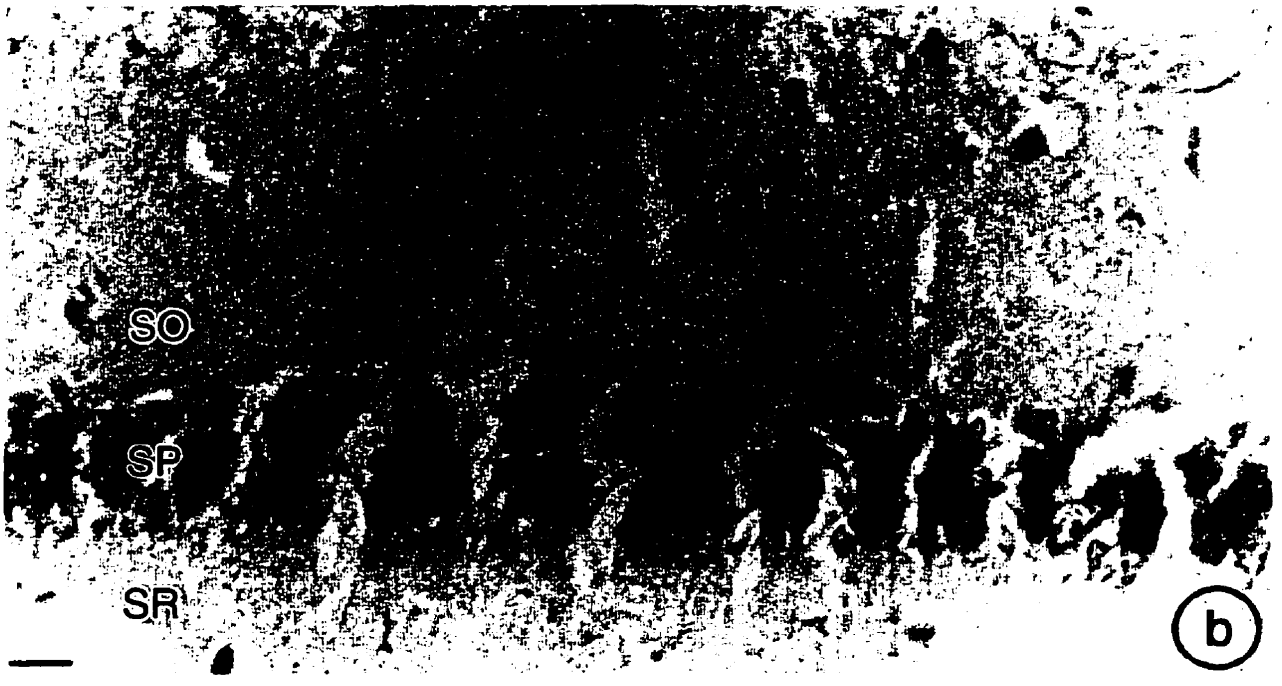
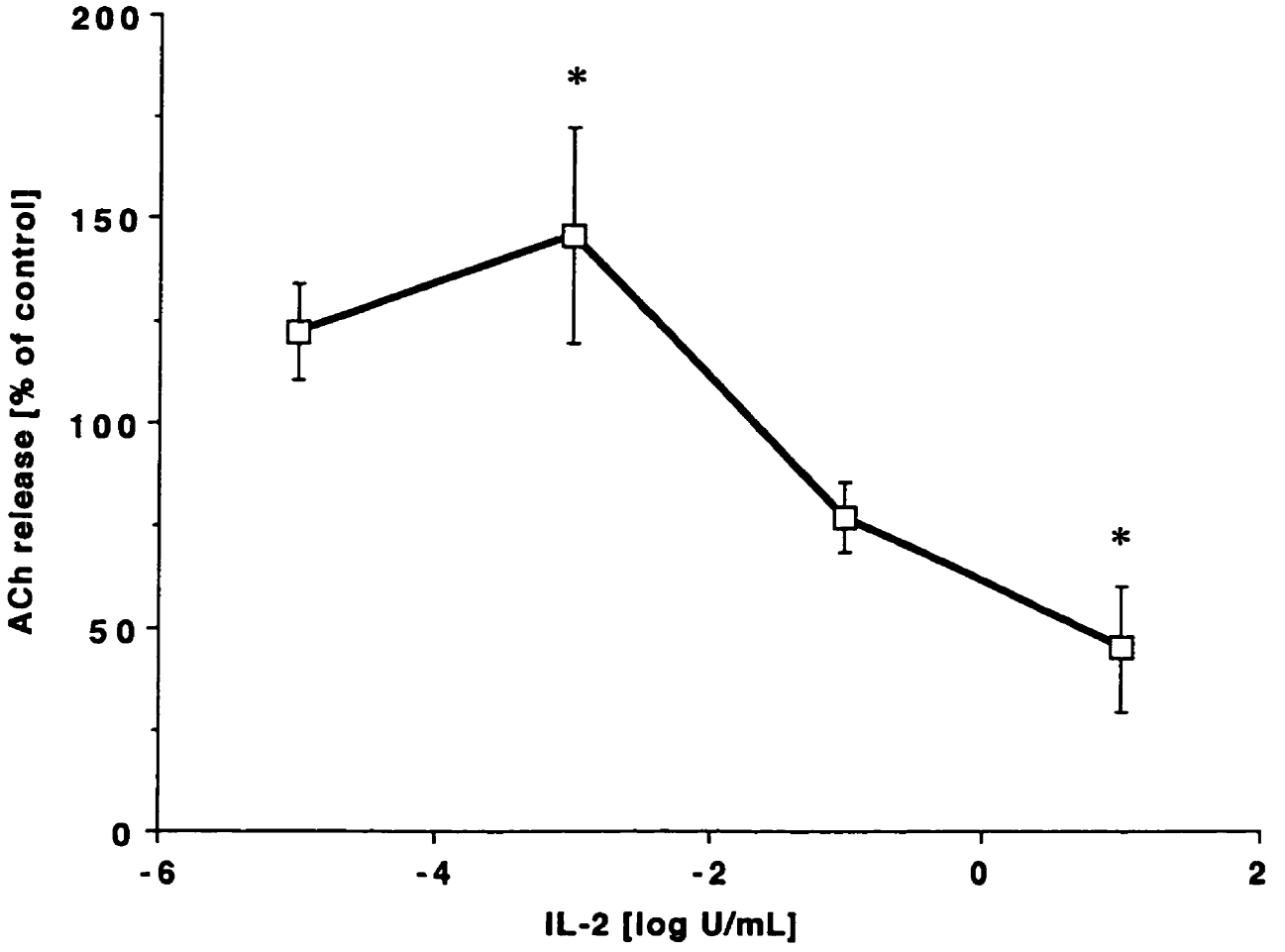

SO
SP
SR
b

Figure 3

Modulatory effects of human IL-2 on acetylcholine release in superfused rat hippocampal slices *in vitro*. Low concentrations of IL-2 potentiate K^+-stimulated acetylcholine release whereas higher concentrations significantly inhibit the release of the transmitter. Means ± SEM of at least six to eight determinations. Statistical significance was determined using student *t* test (parallel or paired two-tailed) following *F* test, (*) $p < 0.05$ being considered significant. * $p < 0.05$. (Personal data.)


200
150
100
50
0
ACh release [% of control]
*
*
-6
-4
-2
0
2
IL-2 [log U/mL]

CHAPTER 3

PREFACE TO CHAPTER 3

The preceding Chapter provided evidence that interleukin-2 elements exist in the brain. This Chapter proposes to correlate the selective presence of these elements in the brain to a particular function associated to these areas, namely, the release of acetylcholine.

Modulation of Hippocampal Acetylcholine Release: a Potent Central Action of Interleukin-2

Uwe- Karsten Hanisch, David Seto, and Rémi Quirion

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The first two authors contributed equally to this work.

The potential of the T cell growth factor interleukin-2 (IL-2) to modulate the release of ACh from rat hippocampus was studied *in vitro*, as a means to investigate the possible functional significance of this cytokine in the CNS. Hippocampal slices were superfused with Krebs buffer medium, and endogenous ACh released into the superfusate was measured using a radioenzymatic assay. Recombinant human IL-2 present during a stimulation with 25 m*M* KCl altered, in a concentration-dependent manner, the evoked transmitter release. At a concentration of 15 U/mL (≤ 1 n*M*), IL-2 inhibited ACh release by more than 50% of the control level (evoked ACh release from the untreated contralateral hemispheres). Inhibition was observed within 20 minutes of tissue exposure to IL-2 and lasted for up to 1 hour. The inhibitory effect of IL-2 was reversible since transient tissue exposure to IL-2 did not affect subsequent evoked ACh release. IL-2 at this concentration also significantly decreased evoked ACh release in frontal cortical slices, but was ineffective in the parietal cortex and striatum, revealing that IL-2 selectively modulates the release of ACh from certain, but not all, cholinergic nerve terminals in the CNS. At very low concentrations (1,5 mU/mL, ≤ 0,1 p*M*), IL-2 transiently increased hippocampal evoked ACh release, resulting in a biphasic dose-response profile with no significant effect observed at 0,015 mU/mL (≤ 1 f*M*). Other cytokines (IL-1α, IL-3, IL-5, IL-6, interferon α), tested in hippocampal slice incubations, failed to modulate ACh release. These results suggest that the immunoregulator IL-2 is a potent and selective modulator of ACh release in selected regions of the rat brain, an effect apparently not shared with other cytokines. The potent action of IL-2 on ACh release most likely relates to the discrete distribution of IL-2(-like) antigen and ^{125}I-IL-2 binding sites in rat brain tissues (Lapchak *et al.*, 1991).

Key words: interleukin-2, cytokines, neuromodulation, transmitter release, ACh, hippocampus

Cytokines are signalling peptides of the immune system. Interleukin-2 (IL-2) was one of the first cytokines to be characterized in detail (for a review, see Hatakeyama and Taniguchi, 1991). IL-2 is predominantly synthesized by antigen- or mitogen-activated T cells and regulates, as an autocrine/paracrine growth hormone, their clonal expansion. IL-2, in conjunction with antigen, mitogen, or anti-immunoglobulin antibodies, also controls B cell proliferation and differentiation into antibody-secreting cells. Furthermore, by acting on cytolytic T lymphocytes, and natural killer (NK) and lymphokine-activated killer cells, IL-2 participates in the activation of cytotoxic immune responses. These diverse immunoregulatory functions are mediated by specific interactions with cell membrane IL-2 receptors (IL-2R), which are composed of two moieties, IL-2Rα/p55/TAC and IL-2Rß/p70, respectively (Kuziel and Greene, 1990; Waldmann, 1991). IL-2Rα and IL-2Rß bind IL-2 with low (kD = 10^{-8} *M*) and moderate (kD = 10^{-9} *M*) affinity; upon activation-induced association to the non-covalent αß heterodimer they form the high-affinity IL-2 receptor complex. (kD = 10^{-11} *M*). Other putative components of the receptor/effector complex are predicted that may control ligand binding and receptor-mediated IL-2 endocytosis, in addition to enzymatic activities involved in IL-2-triggered signal tranduction(s). Because of its potent immunoregulatory properties, IL-2 has been introduced as an anti-tumour drug (for review, see Rosenberg, 1990) and is now clinically used in various immunotherapeutic strategies.

IL-2, among other cytokines, may also act as a neuromodulator in the CNS. Regulatory functions in the nervous tissue have been proposed, for example, for the growth and differentiation of glial and neuronal cells as well as for the control of neuronal activity in the hippocampus, locus coeruleus, and certain hypothalamic nuclei (for a review, see Merrill, 1990, 1991; Nisticò and De Sarro, 1991; Plata-Salamán, 1991). As a modulator of the neuroendocrine system, IL-2 can directly alter the release of pituitary hormones (Karanth and McCann, 1991). The presence of biological activities as well as IL-2(-like) immunoreactive material (IRM), IL-2R-associated IRM, IL-2 mRNA, and ^{125}I-IL-2 binding sites has been demonstrated in the

rodent and, partly, in the human brain, under normal and pathophysiological conditions (Nieto-Sampedro and Chandy, 1987; Luber-Narod and Rogers, 1988; Araujo *et al.*, 1989; Merrill, 1990; Lapchak *et al.*, 1991; Villemain *et al.*, 1991). Moreover, IL-2-induced disruption and penetration of the blood-brain barrier has been subject to experimental studies and clinical observation (Ellison *et al.*, 1987, 1990; Saris *et al.*, 1988; Alexander *et al.*, 1989; Watts *et al.*, 1989; Banks *et al.*, 1991; Ellison and Merchant, 1991). It thus appears that effects of IL-2 in the brain could be due to the genuine existence of IL-2(-like) peptides intrinsic to the CNS, or to elevated blood-borne IL-2.

Elevated amounts of peripheral IL-2 in patients undergoing high-dose IL-2 immunotherapy are reflected by IL-2 concentrations in the cerebrospinal fluid reaching up to 9 U/mL (Saris *et al.*, 1988). At these concentrations, IL-2 immunotherapy is accompanied by a variety of side effects, including adverse neurologic symptoms such as memory impairments (Denicoff *et al.*, 1987; Plata-Salamán, 1991). The hippocampus, a brain structure enriched with IL-2/IL-2R IRM, ^{125}I-IL-2 binding sites (Lapchak *et al.*, 1991) , and IL-2 mRNA (Villemain *et al.*, 1991), is most probably involved in certain aspects of memory and therefore one of the likely targets of CNS-directed IL-2 activites. Of the various neurotransmitters present in the hippocampal formation, ACh is the candidate that has most often been releated to learning and memory.

The objective of the present study was thus to investigate the potential of IL-2 to modulate ACh release in the rat hippocampal formation using *in vitro* brain slice superfusion. The results indicate that IL-2 is able to modify in a concentration-dependent manner ACh release in the hippocampus. Its minimal effective dose being in the (sub)picomolar range, it demonstrates that IL-2 is one of the most potent modulators of ACh release characterized so far. This effect seems to be specific, since various other cytokines tested failed to alter ACh release.

Parts of the results have recently been presented in abstract form (Seto *et al.*, 1991).

Materials and Methods

Male albino Sprague-Dawley rats (300 - 325 g) were obtained from Charles River (breeding colony St-Constant, Québec, Canada). Recombinant human IL-2 (rhIL-2), frequently shown to be effective in the rat CNS (see Karanth and McCann, 1991), was purchased from Sigma Chemical Co. (catalogue number T-3267; St-Louis, MO). Interferon α (rhIFNα) was from Jannsen/Cedarlane Laboratories (Hornby, Ontario, Canada). All other cytokines, in the human recombinant form (IL-1α, IL-3, IL-5, and IL-6), were from Upstate Biotechnology (Lake Placid, NY). Adenosine triphosphate (ATP), dithiothreitol, and bovine serum albumin (BSA) were from Boehringer-Mannheim (Laval, Québec, Canada); choline kinase (ATP: choline phosphotransferase, EC 2.7.1.32), acetylcholinesterase type V-S (acetylcholine hydrolase, EC 3.1.1.7), acetylcholine chloride, physostigmine sulphate, and Amberlite CG-400 (chloride form) were from Sigma Chemical Co. (St-Louis, MO); tetraphenylboron and butyronitrile were from Aldrich Chemical Co. (Milwaukee, WI); [γ-^{32}P]ATP (2 - 10 mCi/mmol) was from New England Nuclear (Mississauga, Ontario, Canada); Ecolite was from ICN (Montréal, Québec, Canada). All other chemicals were of analytical grade and purchased from Fisher Scientific (Montréal, Québec, Canada).

Tissue preparation and release studies. Rats were decapitated and the brains were removed into chilled Krebs buffer medium (KBM) composed of (in m*M*) NaCl 120 m*M*, KCl 4,6 m*M*, $CaCl_2$ 2,4 m*M*, KH_2PO_4 1,2 m*M*, $MgCl_2$ 1,2 m*M*, D-glucose 9,9 m*M*, $NaHCO_3$ 25 m*M*; saturated with O_2/CO_2 (95%/5%), and adjusted to *p*H 7,4. Tissue regions (hippocampus, striatum, frontal, or parietal cortex) were removed rapidly on ice and sliced at 400 μm intervals with a McIlwain tissue chopper. Slices of one hemisphere were immediately placed in a superfusion chamber, built according to Richter (1976), and superfused at a rate of 0,5 mL/minute with KBM containing

choline chloride (10 μM), to support synthesis of ACh, and physostigmine (30 μM for the hemisulphate) to prevent enzymatic degradation of ACh. The buffer reservoir was continuously bubbled with O_2/CO_2 (95%/5%) mixture. The temperature of the chambers and the medium was kept at 37 C. After 45 minutes, superfusate fractions (10 or 20 minutes each) were collected. Aliquots were spun in a microcentrifuge (Biofuge B13, Heareus, 13 000 rpm, 3 min), and 1,3 mL of the supernatant was stored at - 70 C until further processing. Tissue slices were stimulated with KBM containing 25 mM KCl (K^+ KBM), with equimolar reduction in NaCl, in the presence of IL-2. Controls were stimulated in parallel with K^+ KBM omitting the interleukin. At the end of the superfusion, tissue slices were removed from the chambers and protein content was measured in triplicate according to Lowry *et al.* (1951), using BSA dissolved in KBM as a standard.

Evoked release of ACh from hippocampal slices was also determined in tissue incubations carried out according to Araujo *et al.* (1989). Briefly, hippocampal slices (300 μm) were incubated at 37 C in KBM (1 mL) for 70 min, with changes of the medium after 30 and 60 min. Subsequently, slices of one hemisphere were incubated in 1,35 mL of KBM containing BSA (1 mg/mL) and one of the following cytokines: IL-1α, IL-3, IL-5, IL-6, or IFNα. After 20 min, the slices were incubated for another 20 min with the cytokine dissoved in K^+ KBM containing BSA (1 mg/mL). As controls, slices from the contralateral hemisphere were treated in parallel, but in the absence of the cytokine. Supernatants and tissues were separated by centrifugation and treated as described for the superfusions.

Determination of ACh. ACh was determined using a radioenzymatic assay (Goldberg and McCaman, 1973). Briefly, ACh was extracted from samples by mixing an aliquot of 400 μL with an equal volume of tetraphenylboron in butyronitrile (10 g/L). Separation of the phases was completed by centrifugation (Biofuge B 13, Heareus, 13 000 rpm, 4 min); 300 μL of the organic phase was removed and shaken (4 min) with a half volume of $AgNO_3$ solution (20 g/L). The mixture was spun (Biofuge B 13,

Heareus, 13 000 rpm, 4 min) and 110 μL of the aqueous phase was placed in a new tube containing 10 μL of $MgCl_2$ solution (1 *M*). After shaking and spinning, 100 μL of the supernatant was transferred into a glass tube and evaporated. The pellet was dissolved in 32 μL of a reaction mixture composed of choline kinase (0,005 U), ATP (0,8 m*M*), $MgCl_2$ (12,5 m*M*), and dithiothreitol (5 m*M*) in glycylglycine buffer (25 m*M*; *p*H 8,0). Following 30 minutes of incubation at 30 C, 10 μL of [γ-^{32}P]ATP solution (*ca.* 0,45 μCi) containing acetylcholinesterase (2 U) was added to the sample. The mixture was incubated for another 20 min at 30 C. The reactions were terminated by addition of 100 μL NaOH (50 m*M*). As a result, only the choline derived from ACh was ^{32}P-phosphorylated. The samples were placed on glass pipette columns filled with Amberlite CG-400 (approximately 800 μL bed volume) that was converted to formate form and equilibrated with NaOH (50 m*M*). The columns were rinsed with 3 mL of NaOH (50 m*M*) to elute any phosphorylcholine, while excessive [γ-^{32}P]ATP (accounting for more than 99% of the activity added) was retained on the column. The eluate, collected in scintillation vials, was mixed with scintillation cocktail, and radioactivity was measured by liquid scintillation spectrometry. Standards of ACh chloride (0 - 400 pmol in 400 μL of KBM) were processed in parallel. All determinations were carried out in triplicate.

Calculations and statisitics. Concentrations are given for IL-2 on the basis of biological activity to BRMP units. Approximative molar concentrations were calculated from the specific activity. Evoked transmitter release during superfusions was expressed as pmol ACh/min/mg protein and regarded as the net transmitter release over basal efflux. The basal efflux was determined from the superfusate samples collected prior, between, as well as following periods of K^+ stimulations, respectively, depending on the experimental paradigm. To calculate the evoked release during depolarization, the baseline was estimated for this period, and the values obtained for the basal efflux were subtracted from the total amount of ACh found in the superfusate. Statistical evaluations were carried out using the program NCSS (parallel or paired two-tailed) *t* test, respectively, following *F* test, $p < 0,05$ being considered significant.

Results

Stimulation of ACh release from brain slices was carried out with 25 m*M* KCl, increasing the total concentration of K^+ in the medium from 5,8 to 26,2 m*M*. At this concentration, evoked release is known to be at submaximal level (Lipton, 1985; Pearce *et al.*, 1991). Submaximal stimulation was regarded appropriate to reveal both drug-dependent attenuation as well as augmentation of the evoked neurotransmitter release. Figure 1 shows the hippocampal release profile for endogenous ACh as determined for a stimulation period of 60 minutes. The average basal release was 0,98 ± 0,29 pmol/min/mg protein (mean ± SEM, n = 98). The average decline of the baseline value was determined to be 1,1% (n = 18), demonstrating the stability and viability of the tissue preparations under the present incubating conditions.

Effects of IL-2 on hippocampal ACh release

The effect of IL-2 on the evoked hippocampal ACh release is displayed in Table 1 for various times of tissue exposure to the interleukin. Hippocampal slices were first superfused with normal KBM for 60 min to establish the basal release. Subsequently, the tissue was stimulated for up to 60 minutes with K^+ KBM containing various concentrations of IL-2. The ACh release in the presence of IL-2 was compared to controls that were stimulated in the absence of IL-2.

Figure 2 represents an overlay of the concentration-response relations obtained from the various intervals of tissue stimulation, revealing the time dependency of the IL-2-mediated effects. The augmentation of ACh release by IL-2 at 1,5 mU/mL was obvious during the early period of stimulation and attenuated rapidly with time. On the contrary, the inhibition of ACh release by 15 U/mL IL-2 was a consistent finding even over a prolonged period of stimulation. Only tendencies toward decreased or

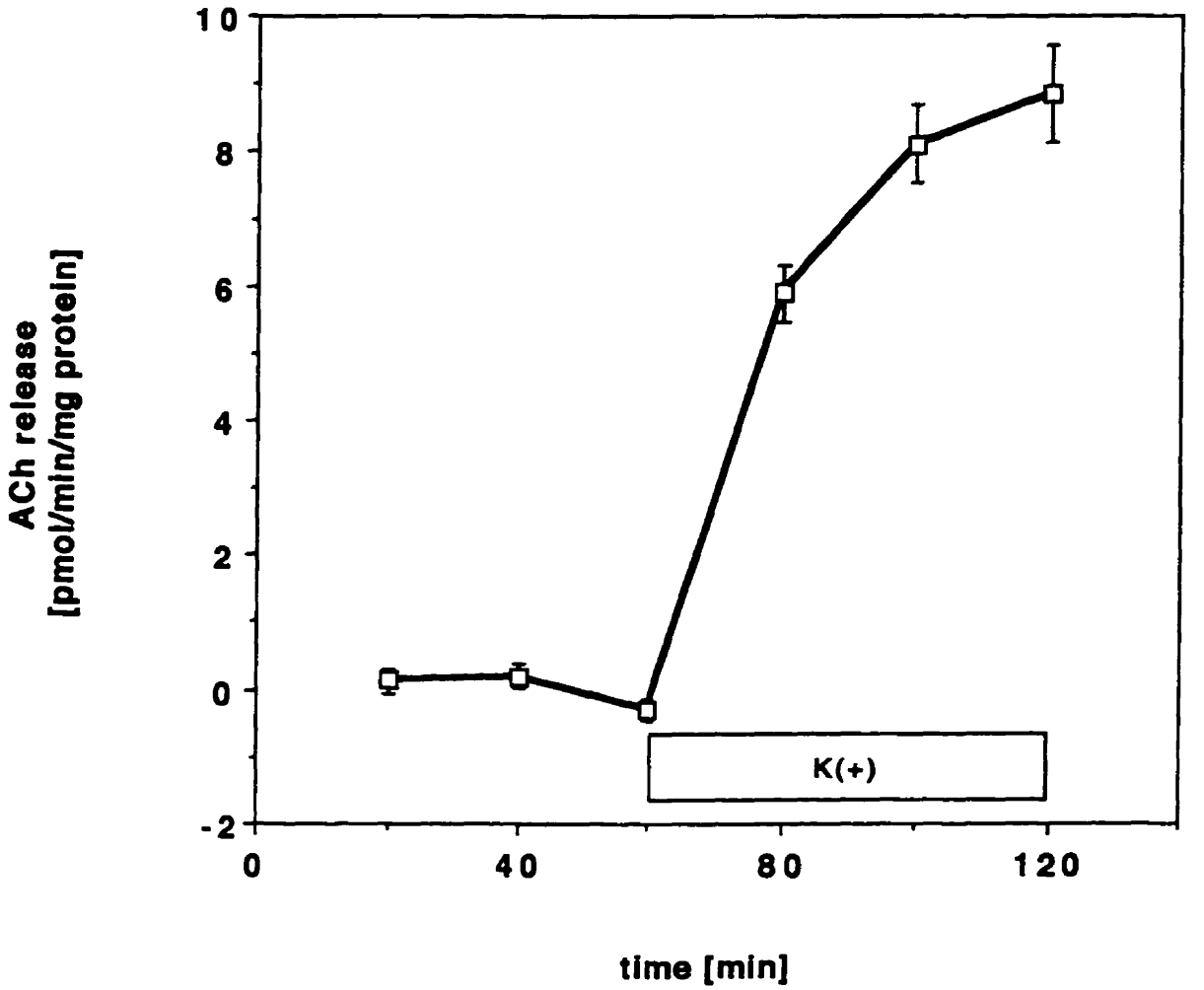


Figure 1. K^+-evoked hippocampal ACh release using in vitro slice superfusion. Slices were superfused with normal KBM and then stimulated for 60 min with KBM containing 25 mM KCl (K^+). Data represent the mean ± SEM of 36 superfusions.

Table 1. Effect of IL-2 on the evoked release of endogenous ACh from hippocampal slices during superfusion

Time (min)	IL-2 (mU/mL)			
	0.015	1.5	150	15 000
0-20	122.2 ± 11.5 (n = 10)	145.9 ± 26.4 (n = 7)*	77.1 ± 8.3 (n = 9)	44.8 ± 15.6 (n = 10)*
20-40	115.1 ± 15.4 (n = 10)	123.2 ± 7.0 (n = 8)	87.8 ± 9.1 (n = 9)	38.6 ± 10.8 (n = 10)*
40-60	108.8 ± 10.1 (n = 10)	96.5 ± 16.2 (n = 8)	75.9 ± 8.7 (n = 9)	62.0 ± 12.5 (n = 8)*

Data are evoked hippocampal ACh release, expressed as percentage of control, determined for various periods of tissue exposure to IL-2 in the presence of 25 mM KCl. Release over basal efflux was determined as pmol ACh/min/mg protein. Data for slices treated with IL-2 were converted to percentage of the average release evoked from control slices. The data for a given concentration represent the mean ± SEM (n) of two experiments.

*, $p < 0.05$.

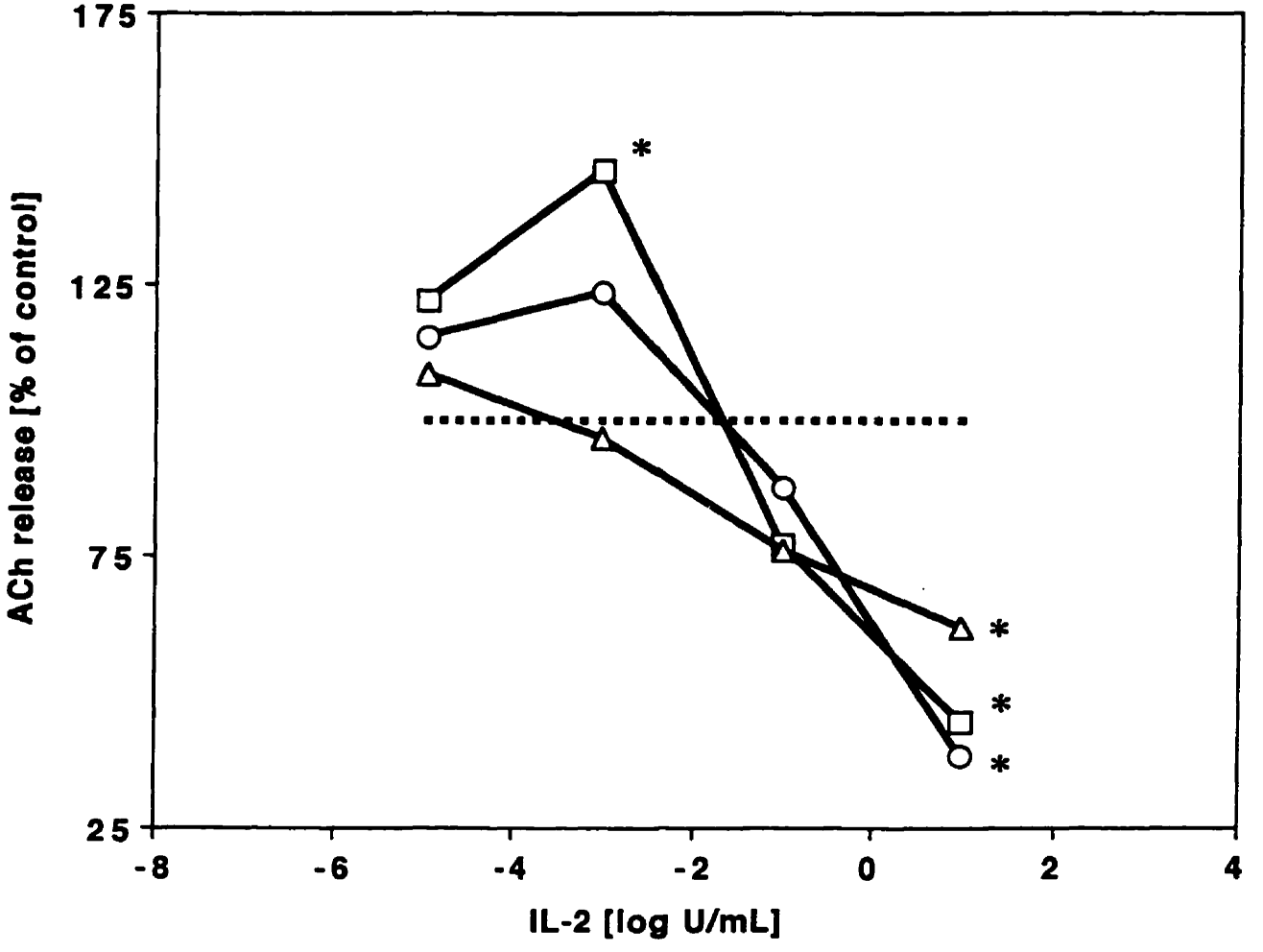


Figure 2. Effects of IL-2 on the evoked release of ACh from hippocampal slices. The dose-response curves were obtained from three intervals during tissue stimulation with 25 mM KCl (—□— , 0-20 min; —○— 20-40 min; —△— 40-60 min). Data are expressed as percentage of control measurements carried out in the absence of IL-2. For clarity, error bars were omitted, but values are given in Table 1. *, $p < 0.05$.

values were found for 150 and 0,015 mU/mL of IL-2.

During the first 20 minutes of K^+ stimulation, evoked ACh release was modulated by IL-2 in a concentration-dependent manner. At 15 U/mL, IL-2 inhibited ACh release by more than 50 % of the control value. At a 100-fold lower concentration (150 mU/mL), ACh release was only nonsignificantly attenuated (Fig. 2). However, when the concentration of IL-2 was further lowered to 1,5 mU/mL, an increase in the amount of ACh released by a 20-minute K^+ KBM exposure was detected (Fig. 2). No significant change was observed for the tissue samples with 0,015 mU/mL IL-2.

During the subsequent period of stimulation, that is, the interval between 20 and 40 minutes, there was still a marked decrease in the evoked ACh release from the slices exposed to 15 U/mL IL-2 (Fig. 2). With lowering the concentration of IL-2, ACh release returned to control levels. There was no longer a significant increase of evoked transmitter release for IL-2 at 1,5 mU/mL.

An inhibitory effect of IL-2 was also detected during the interval between 40 and 60 minutes of continuous superfusion with K^+ KBM (Fig. 2). Almost 40% reduction in ACh release was found for chambers superfused with 15 U/mL IL-2. However, the IL-2 effect was significantly weaker, when compared to the period before, that is, the interval between 20 and 40 minutes ($p < 0,05$ in a paired *t* test) (Fig. 2). Calculated for the total stimulation period of 60 minutes, the overall evoked release was (percentage of control) 115.4, 121.9, 80.3, and 48.5 for the experimental groups treated with 0, 015 mU/mL, 1,5 mU/mL, 150 mU/mL and 15 U/mL IL-2, respectively.

IL-2 also inhibited ACh release when added to an ongoing stimulation. As shown in Fig. 3, IL-2 at 15 U/mL transiently decreased the evoked release when it was added to stimulated hippocampal slices. The profile returned to control levels after 40 minutes of IL-2 presence, equivalent to 70 minutes of continuous stimulation.

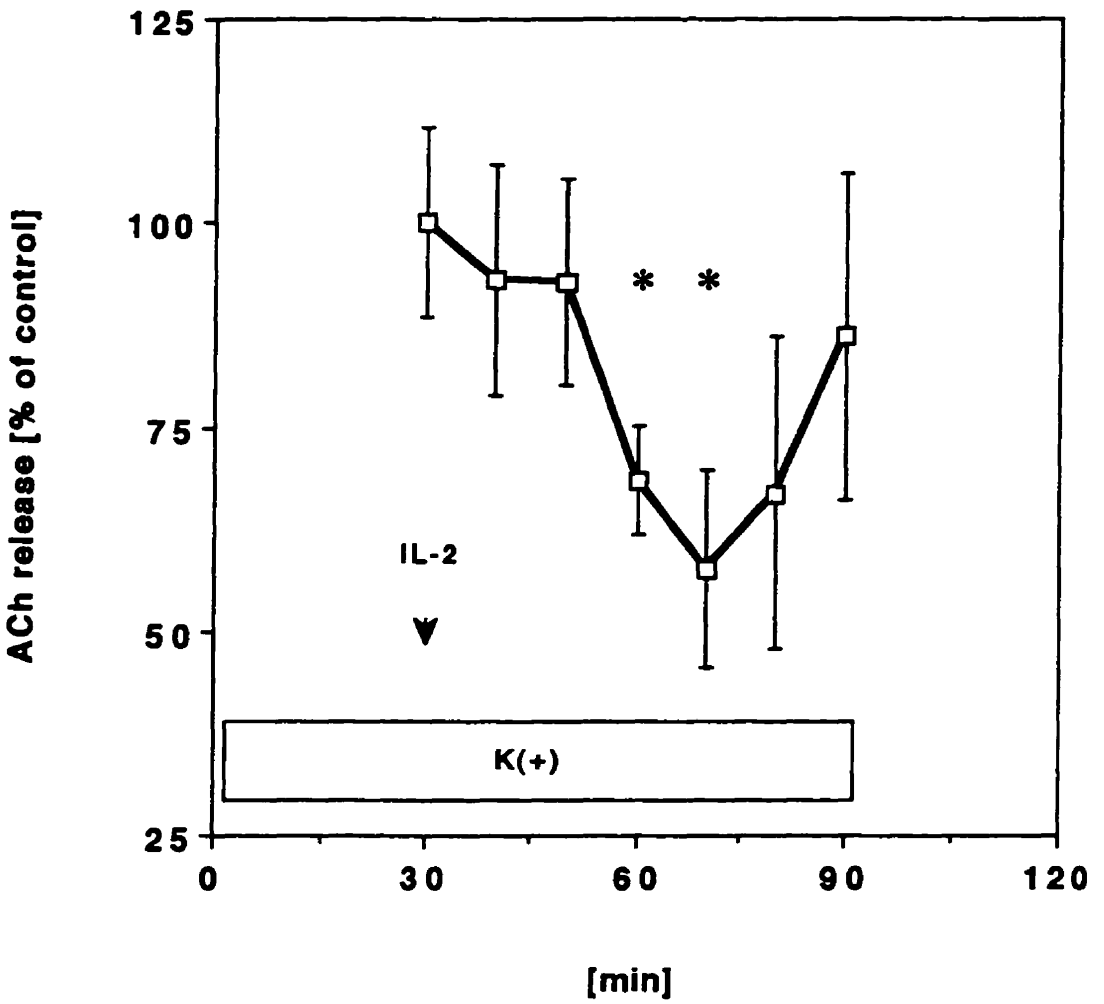


Figure 3. Effect of IL-2 on an ongoing stimulation of evoked ACh release from hippocampal slices. The tissue was stimulated with 25 mM KCl for a total period of 90 min (K+); 30 min after the onset of the stimulation IL-2 (15 U/mL) was added to the superfusion medium (arrowhead). Data are given as percentage (mean ± SEM, n = 5) of untreated controls. *, $p < 0.05$.

Reversal of the IL-2-mediated inhibition of ACh release

When hippocampal slices were stimulated with K^+ KBM for a first period of 30 minutes (S1) and, following a 30-minute rinsing with normal KBM, were stimulated a second time (S2, 30 minutes), the amounts of ACh release by S1 and S2 were identical (1,04 for the ratio S2:S1). To test whether the presence of IL-2 during S1 would alter subsequent ACh release during S2 in the absence of IL-2, hippocampal slices were exposed to 150 U/mL IL-2 and rinsed for 30 minutes, before being restimulated. The profiles in Figure 4 show that transient presence of IL-2 did not alter the efficacy of later stimulations. Consequently, toxic or permanent effects of IL-2 on the tissue could be excluded for the duration of the experiments.

Regional differences in the effect of IL-2

Besides the hippocampal formation, IL-2 and IL-2R-associated IRM has been localized in a variety of rat brain regions, including the neostriatum and the cerebral cortex (Araujo *et al.*, 1989; Lapchak *et al.*, 1991). To test whether IL-2 could also modulate evoked ACh release in those regions, slices of striatum as well as of frontal and parietal cortices were superfused and K^+- stimulated over 60 minutes in the absence (controls) or presence of IL-2. The results are summarized in Figure 5. The average basal release was (in pmol/min/mg protein) 5,94 $\pm$ 0,84 (n = 32), 2,56 $\pm$ 0,48 (n = 27), and 3,27 $\pm$ 0,64 (n = 21) for the striatum, frontal, and parietal cortices, respectively.

IL-2 at 15 U/mL did not affect evoked ACh release in striatal tissue (Fig. 5A). The relase in the presence of IL-2 was in the range between 82,1 $\pm$ 12,2% and 126,0 $\pm$ 20,2% when compared to the corresponding control values. Neither was there any effect of IL-2 on slices from the parietal cortex; evoked ACh release was between 90,2 $\pm$ 16,6% and 98,5 $\pm$ 13,3% of the corresponding control level (Fig. 5C). However, 15 U/mL IL-2 reduced the evoked ACh release from frontal cortex during the first

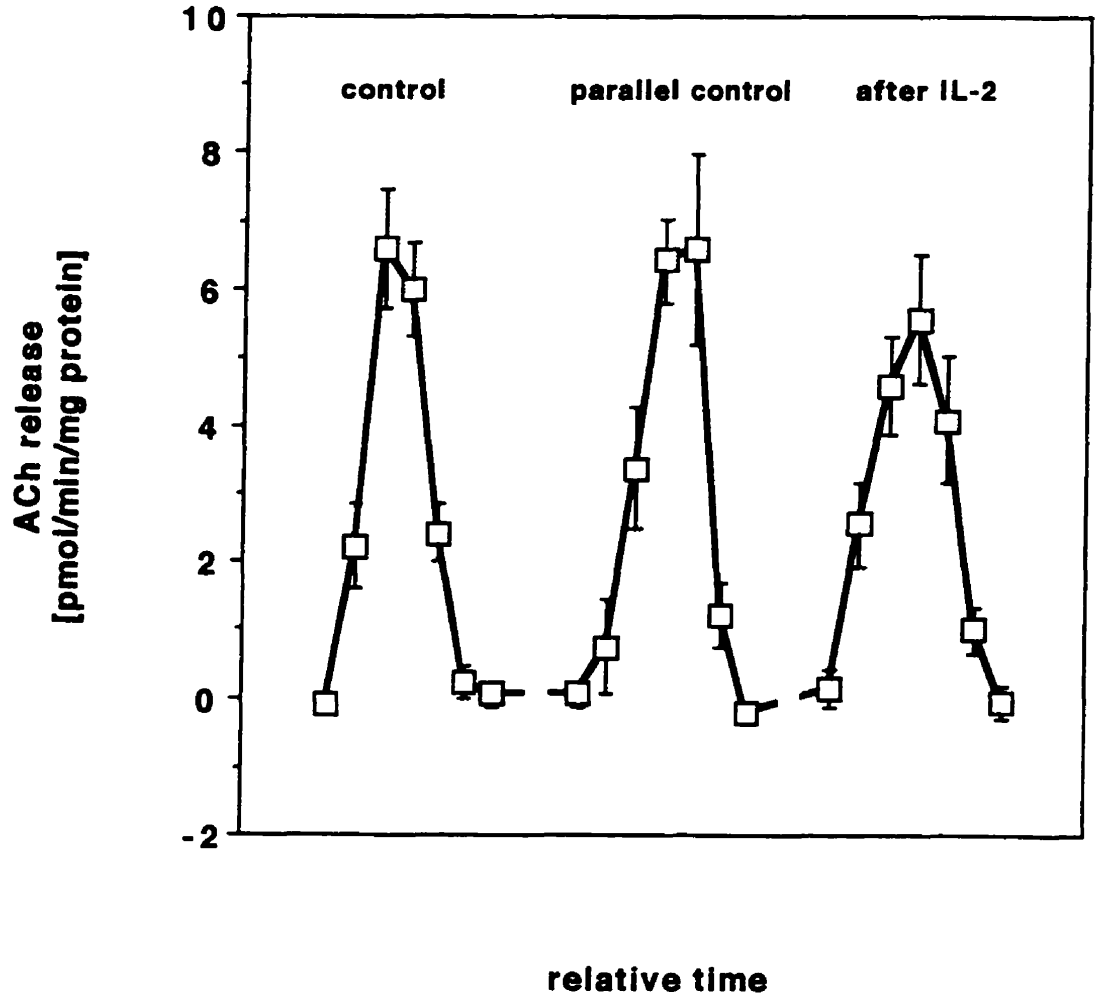
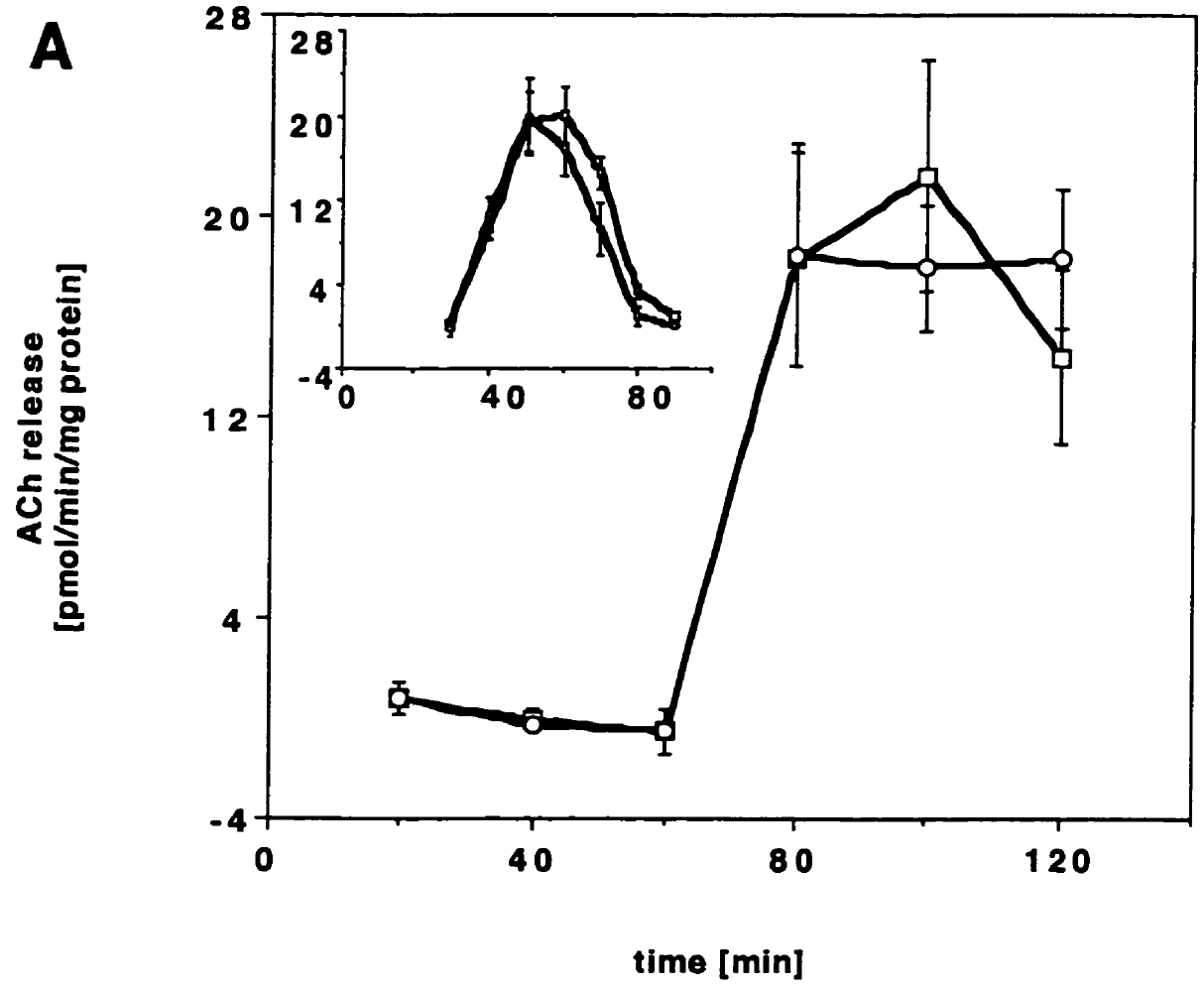


Figure 4. Evoked hippocampal ACh release following a transient exposure of the tissue to IL-2. Slices were stimulated for a first period of 30 min (S1, n = 11) with 25 mM KCl, rinsed for 30 min, and stimulated a second time for 30 min (S2). Controls showed identical amounts of released ACh for S1 and S2 (left). Another group of tissue samples (n = 6) was exposed to IL-2 (150 U/mL) during S1. Following rinsing, the tissue was stimulated in the absence of IL-2 (S2 shown on the right). From the left to the right, the peak areas (corresponding to the amounts of released transmitter) were calculated to be 100.0%, 104.4%, and 102.8%. Data are given as mean ± SEM.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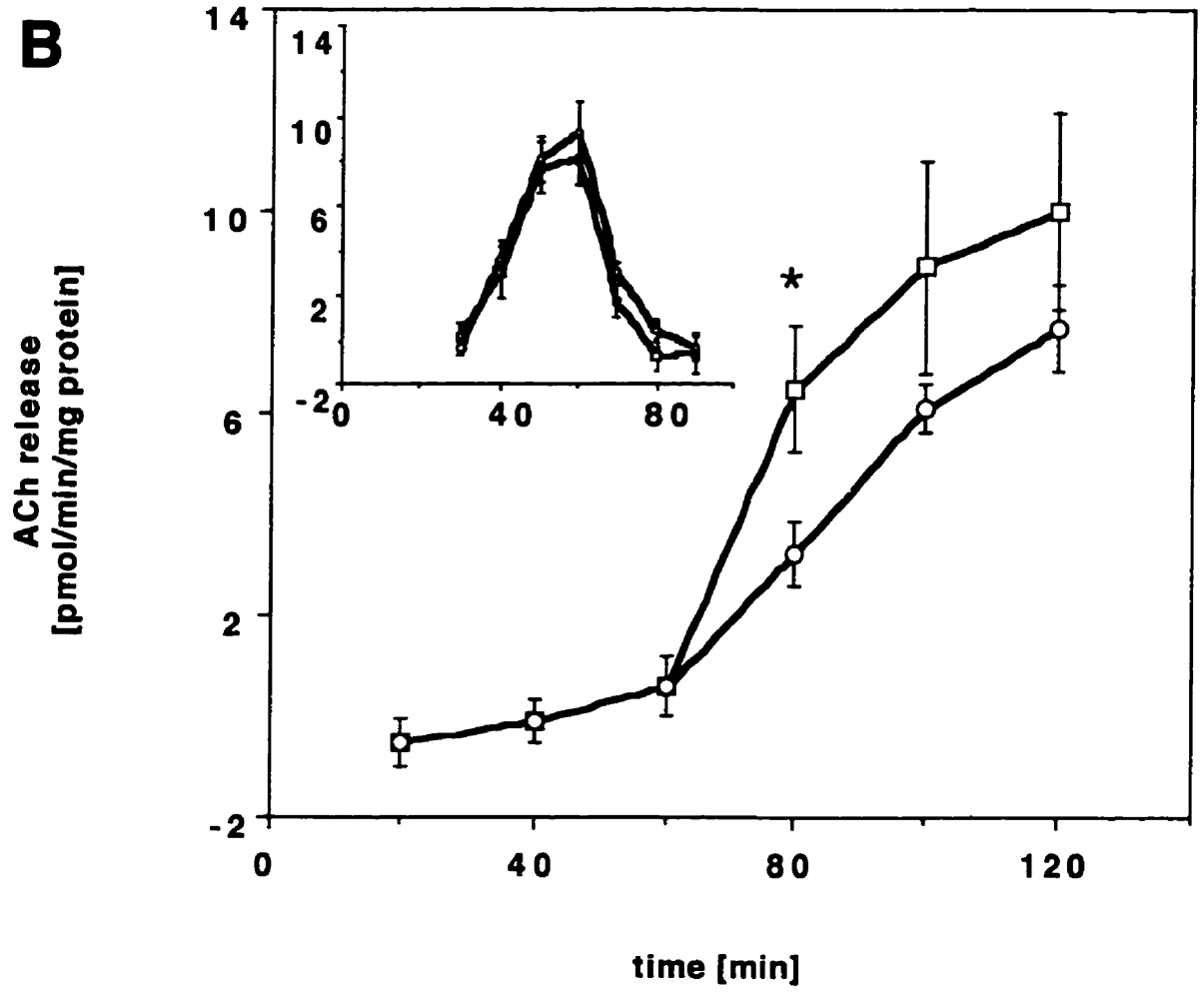
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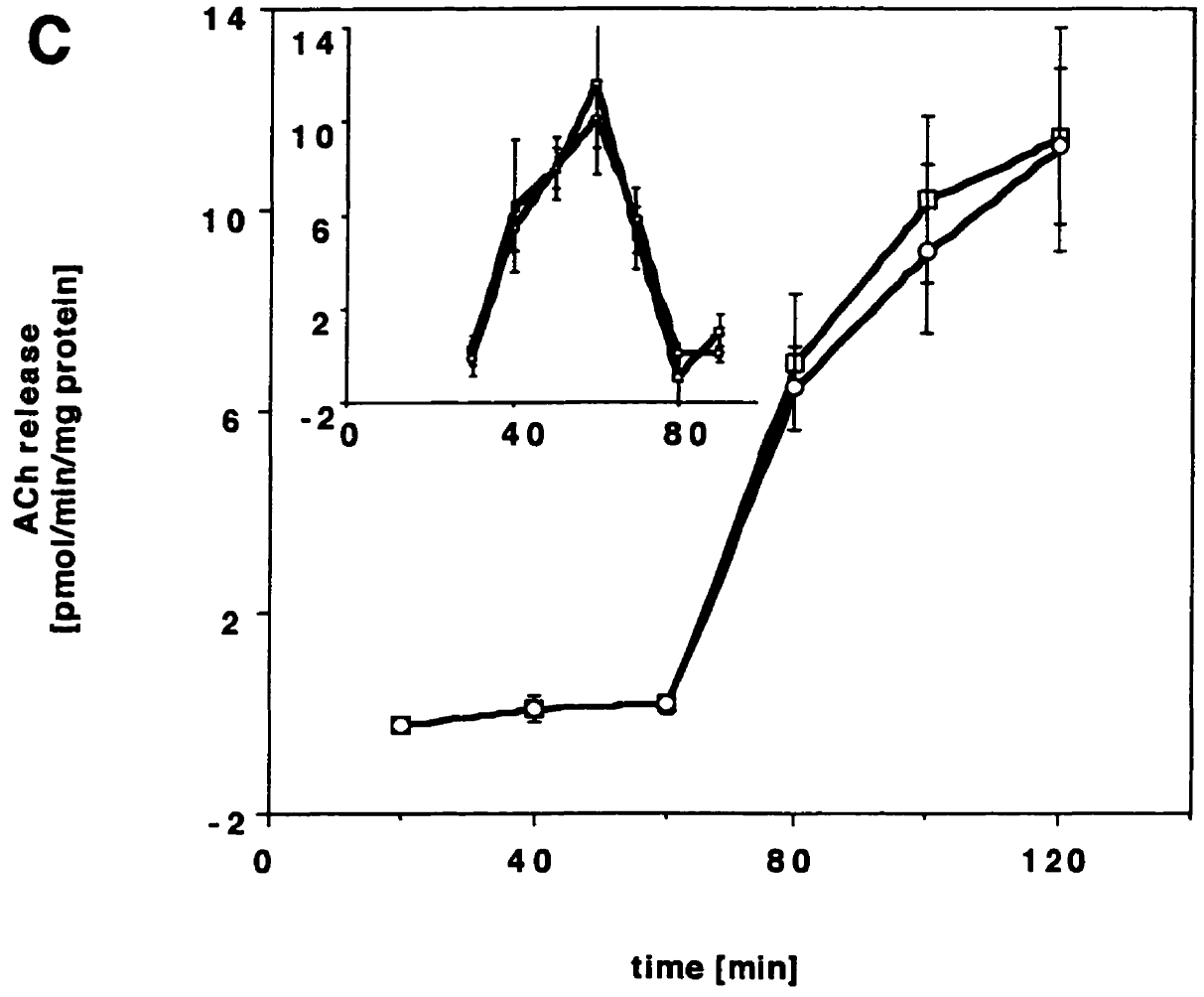

Figure 5. Effects of IL-2 on the evoked ACh release from slices of the striatum (A, n = 5), frontal cortex (B, n = 5), and parietal cortex (C, n = 6). Tissue slices were stimulated with KBM containing 25 mM KCl in the presence (o) or absence (□) of 15 U/mL IL-2. The insets in A-C represent the release profiles obtained with 30 min of KCl stimulation from controls (□) and from slices following to an exposure to IL-2 (150 U/mL, o). Axis dimensions in the insets are the same as in the full-size graphs. For further explanation see Results. All data are given as mean ± SEM. *, $p < 0.05$.

Table 2. K^+-evoked ACh release from hippocampal slices incubated in the presence of various cytokines.

	Hippocampal ACh release (% of control)
IFNα (5 nM)	99.8 ± 23.7 (n = 7)
IL-1α (1 nM)	93.3 ± 8.2 (n = 8)
IL-3 (5 nM)	101.9 ± 5.3 (n = 10)
IL-5 (1.3 nM)	93.5 ± 4.0 (n = 10)
IL-6 (5 nM)	95.1 ± 6.5 (n = 10)

Total ACh release during a stimulation of 20 min with 25 mM KCl was determined and expressed as percentage of transmitter release from the corresponding controls (untreated contralateral hemispheres). The overall control value was 5.95 ± 0.27 pmol/min/mg protein (n = 44). Data are given as mean ± SEM.

20 minutes of K^+ stimulation (Fig. 5B). The evoked release during this period was determined to be 49,8 ± 9,9% (n = 5) of the control value.

As was shown for hippocampus, preexposure to IL-2 did not modify the subsequent release pattern in any of these other tissues (insets in Fig. 5A-C). There was thus no evidence for an altered transmitter release as a consequence of a previous incubation of the tissues with IL-2. The striatal evoked ACh release during S2 (Fig. 5A) was 85,7% of the control amount, and 108,5% in a second experiment. When compared to the corresponding controls, ACh releases over baseline were 114,0% and 93,7% for the S2 periods in the frontal and parietal cortices, respectively.

Effects of other cytokines

The specificity of the IL-2 effect on hippocampal ACh release was determined in slice incubations as described in Araujo *et al.* (1989), in the presence or absence (control) of several other cytokines. As shown in Table 2, IFNα, IL-1α, IL-3, IL-5, and IL-6 did not significantly alter ACh release in this tissue. In preliminary experiments, we also observed that rat IL-2, the peptide homologous to human IL-2, modulated ACh release at 50 U/mL.

Discussion

The results point to a neuromodulatory action of IL-2 on certain populations of cholinergic neurons. At a higher concentration (15 U/mL, ≤ 1 n*M*), IL-2 showed a pronounced inhibition of evoked ACh release in the hippocampus and frontal cortex, while being ineffective in the striatum and parietal cortex. Interestingly, a lower concentration of IL-2 (1,5 mU/mL, ≤ 0,1 p*M*) stimulated ACh release in the hippocampal formation. It is thus evident that the effect of this cytokine on ACh release is (1) concentration-dependent and (2) region-specific, with terminals of selected

cholinergic projections (but not striatal intrinsic neurons) being sensitive to its modulatory activity.

Modulation of ACh release has been reported for various agents, including muscarinic agonists and antagonists (Raiteri *et al.*, 1984; Lapchak *et al.*, 1989), excitatory amino acids (Scatton and Lehmann, 1982), neurotensin (Lapchak *et al.*, 1990), and somatostatin (Araujo *et al.*, 1990). However, micromolar concentrations of these agents are required to produce significant effects while IL-2 is effective at markedly lower concentrations ($\leq$ 0,1 p*M*) that most likely have physiological relevance. Similarly, sympathetic neurite outgrowth is optimally enhanced by 0,2-2 U/mL IL-2 (Haugen and Letourneau, 1990) while neuroendocrine effects of IL-2 were detected using picomolar to femtomolar concentrations (Karanth and McCann, 1991). IL-2, in picomolar quantities, is known to produce soporific effects and to modulate EEG spectrum (Nisticò and De Sarro, 1991). Higher concentrations (200 to $\geq$ 3000 U/mL) were required to block long-term potentiation (LTP) in the rat hippocampus (Tancredi *et al.*, 1989).

For the duration of our experiments potential neurotoxic effects of IL-2 were not observed, but have been documented in neuronal cell culture following a 24-hr exposure to IL-2 (Araujo and Cotman, 1991). In the present series of experiments, subsequent ACh release during K^+ stimulation was not altered by previous exposure to IL-2. Furthermore, over prolonged periods of stimulation in the presence of IL-2, inhibition of hippocampal evoked ACh release attenuated, being significantly less pronounced or absent after 40 minutes. In the IL-2Rα/IL-2Rβ receptor complex, the α subunit determines the fast association with IL-2 (t 1/2 = 37 s), whereas the β subunit contributes to the slow dissociation of the ligand (t 1/2 = 285 minutes) and to signal transduction (Kuziel and Greene, 1990; Waldmann, 1991). Similar kinetics likely apply in the CNS, the putative kD value being very low, rendering rapid dissociation ("washout") most unlikely. Taken all together, this suggests the termination of the IL-2 signalling by endocytosis of the ligand/receptor complex (Legrue *et al.*, 1991). As

shown in YT+ cells, ^{125}I-IL-2 is almost completely internalized after 30-40 minute incubation period (Fung *et al.*, 1988).

While IL-2 was shown to be a very potent modulator of hippocampal ACh release, several other cytokines with established CNS activities (Plata-Salamán, 1991), that is IFNα, IL-1α, IL-3, IL-5, IL-6 (in the present study), and IL-1ß, as well as IL-4 (in Araujo *et al.*, 1989), were ineffective, indicating that ACh release in the hippocampus is sensitive to a specific effect of IL-2 that is not shared by other well known cytokines.

IL-2(-like) and IL-2R-associated (TAC antigen-like) IRM are present in the rat hippocampal formation, especially in the stratum pyramidale of the hippocampus proper as well as in the granule cell layer of the dentate gyrus (Lapchak *et al.*, 1991). It is well known that cholinergic septal projections terminate throughout the hippocampal region, but most heavily in the strata oriens and radiatum of subfields CA2 and CA3, as well as in the infra- and supragranular zones of the dentate gyrus (Semba and Fibiger, 1989). The proximity of IL-2-related IRM and cholinergic terminals is very striking and may thus explain our functional observation, that is, the modulation of ACh release by the cytokine in the hippocampal formation. On the other hand, IL-2/IL-2R-like IRM in the frontoparietal cortex originate from cells in the vicinity of the substantia innominata and the ventromedial globus pallidus (Semba and Fibiger, 1989). Although some intrinsic ACh neurons exist in the cortex (layers II/III), the laminar pattern of cholinergic terminals predominantly arises from these projections, with high densities seen in layer V, and to a lesser extent in layer IV. It thus appears that most prominent effects of IL-2 on ACh release were observed in cortical and hippocampal areas that contain cholinergic projection terminals in proximity to IL-2/IL-2R IRM.

In contrast, IL-2 failed to modulate ACh release in the striatum, an area enriched with ACh interneurons (1-2% of the cells; Semba and Fibiger, 1989) and detectable quantities of IL-2/IL-2R IRM (Lapchak *et al.*, 1991). This illustrates that IL-2 is not a

universal modulator of ACh release, but exerts its effects in regions where IL-2-related IRM was localized in vicinity to the terminals of far-projecting ACh neurons.

The modulatory effect of IL-2 on ACh release, especially in the hippocampus, is of potential interest in regard to the impairments of cholinergic functions in Alzheimer's disease (AD) (Whitehouse *et al.*, 1982; Quirion *et al.*, 1990). In that context, it is intriguing that cholinergic neurons of the striatum, an area usually spared in AD, are insensitive to IL-2. It is also known that various markers of the immune system, such as major immunohistocompatibility complex glycoproteins, immunoglobulin, complement, and cytokine receptors, infiltrating T cells, cytokines, and complement factors, associated with neurodegenerative plaques and neurofibrillary tangles, are present in AD brains, implicating immune-mediated autodestruction as an important pathological event in this disorder (McGeer and Rogers, 1992). IL-2, though not being the sole candidate, could play a crucial role in the central cascading of immune-related markers, as it does in the periphery.

Regarding the biphasic profile of action observed in the hippocampus, IL-2, normally expressed in low amounts, could usually stimulate ACh release. However, under pathological conditions, levels of IL-2 are likely increased (McGeer and Rogers, 1992) and could conceivably contribute to decreased levels of ACh released by terminals especially in the regions primarily affected in AD, such as the hippocampus and the frontal cortex. High concentrations of IL-2 are known to interfere with LTP in the hippocampus (Tancredi *et al.*, 1989), an observation that could also relate to neurological side effects associated with high-dose IL-2 immunotherapies (Denicoff *et al.*, 1987; Plata-Salamán, 1991).

Bidirectional communication between the immune system and the CNS is likely mediated by both direct cellular contacts, for example, via innervation of immunocompetent organs, and soluble factors triggering effects in remote tissues or providing humoral feedbacks (Bateman *et al.*, 1989; De Micco, 1989; Dunn, 1989;

Jankovic, 1989; Rabin *et al.*, 1989; Sternberg, 1989; Merrill, 1990; Plata-Salamán, 1991). Some of these mediators can be produced by cells of both systems, obliterating the "classical" demarcation between immuno- and neuroregulators. Immunoregulators likely play pivotal roles in normal CNS development and function, in the induction and coordination of post-traumatic events (Plata-Salamán, 1991; Hall and Rao, 1992), and probably in pathogenetic processes associated with neurological disorders (Blume and Vitek, 1989; Matsuyama *et al.*, 1991; Vandenabeele and Fiers, 1991; McGeer and Rogers, 1992; Royston *et al.*, 1992). Upon challenge of an immune or inflammatory response or during cytokine-based immunotherapy, cytokines may become more active in the CNS, provided that they have access to functionally coupled receptors in the brain. In the case of IL-2, evidence has been reported that fulfills these predictions. However, it remains to be shown whether other factors known to be induced by IL-2, such as tumour necrosis factor, are partly responsible for the effects ascribed to IL-2 (Ellison and Merchant, 1991). It is thus of great interest to determine whether the modulatory action of this cytokine on cholinergic nerve terminal is due to a direct effect or whether it involves other mediators.

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CHAPTER 4

PREFACE TO CHAPTER 4

The previous Chapter provided a detailed description of interleukin-2 action on the release of acetylcholine from selected areas that contain interleukin-2 elements and are known to release acetylcholine. As the results suggest, interleukin-2 exerts effects on neurotransmitter release that correlate well to the presence of interleukin-2 elements in those areas. The following Chapter examines the putative mechanisms by which interleukin-2 modulates the release of acetylcholine.

Evidence for direct and indirect mechanisms in the potent modulatory action of interleukin-2 on the release of acetylcholine in rat hippocampal slices

David Seto, Satyabrata Kar and Rémi Quirion

D. Seto, S. Kar & R. Quirion. Evidence for direct and indirect mechanisms in the potent modulatory action of interleukin-2 on the release of acetylcholine from rat hippocampal slices. Br J Pharmacol 1997; 120: 1151-1157.

Summary

1 The biphasic nature of the potent modulatory action of interleukin-2 (IL-2) on hippocampal acetylcholine (ACh) release was investigated using brain slice superfusion.

2 Both the potentiating (10^{-13} *M*) and inhibitory (10^{-9} *M*) effects of IL-2 on hippocampal ACh release were stimulation-dependent and were blocked by a neutralizing IL-2 receptor antibody, suggesting the activation of typical IL-2 receptors in both cases.

3 Tetrodotoxin (TTX; 10 μM) failed to block the potentiation of ACh release induced by a very low concentration of IL-2 (10^{-13} *M*) suggesting a direct effect on cholinergic nerve terminals.

4 In contrast, the inhibitory effect seen at a higher concentration (10^{-9} *M*) was TTX-sensitive, and hence indicative of an indirect action.

5 To establish the nature of this intermediate mediator, blockers of nitric oxide synthesis, and of opioid and GABA receptors were used. Only $GABA_A$ and $GABA_B$ receptor antagonists altered the inhibitory action of IL-2, suggesting the participation of GABA as mediator.

6 Taken together, these results provide further evidence on the potent role of IL-2 in the modulation of cholinergic function in the rat hippocampus.

Key words: interleukin-2, cytokine, acetylcholine, release, GABA, hippocampus

Introduction

A wealth of evidence supports the genuine existence of interleukin-2 (IL-2) in the mammalian brain (for reviews, see Merrill, 1990; Nistico & de Sarro, 1991; Plata-Salamán, 1991; Seto *et al.*, 1993). IL-2-like immunoreactivities and IL-2 mRNA were found to be widely, but discretely distributed in the rodent brain with greater densities observed in the hippocampus and hypothalamus. (Lapchak *et al.*, 1991; Villemain *et al.*, 1990; Seto *et al.*, 1993). In parallel, IL-2 receptor (IL-2R) binding sites or IL-R immunostaining as revealed using an antibody against the α subunit (TAC), and IL-2Rß mRNA are expressed in the mammalian brain with a pattern mirroring that of the endogenous protein (Araujo *et al.*, 1989; Lapchak *et al.*, 1991; Petitto & Huang, 1994). Furthermore, recent evidence provided by *in situ* hybridization studies clearly shows that both IL-2 and IL-2R mRNA are expressed by certain neural populations (Villemain *et al.*, 1991; Shimojo *et al.*, 1993; Petitto & Huang, 1994, 1995; Eizenberg *et al.*, 1995; Sawada *et al.*, 1995), demonstrating the local synthesis of IL-2 in the rodent brain.

Functionally, IL-2 exerts various biological effects in the normal mammalian brain. In addition to its effects on growth and differentiation of neurons and oligodendrocytes (Benveniste & Merrill, 1986; Haugen & Letourneau, 1990; Eitan *et al.*, 1992; Awatsuji *et al.*, 1993; Eitan & Schwartz, 1993; Sarder *et al.*, 1993; Shimojo *et al.*, 1993), IL-2 is able to modulate the release of hypothalamic (Cambronero *et al.*, 1993; Karanth *et al.*, 1993; Raber & Bloom, 1994; Hillhouse, 1994; Raber *et al.*, 1995) and pituitary (Karanth & McCann, 1991) hormones, to block long-term potentiation in the hippocampus (Tancredi *et al.*, 1990) and to inhibit electrocorticogram spectrum (de Sarro *et al.*, 1990). Moreover, IL-2 has been shown to potentiate scopolamine-induced amnesia (Bianchi & Panerai, 1993), to inhibit Ca^{++} currents in hippocampal cells (Plata-Salamán & ffrench-Mullen, 1993) and to modulate the release of catecholamines from the hypothalamus *in vitro* (Villemain *et al.*, 1992; Lapchak & Araujo, 1993) and dopamine from striatal slices (Lapchak, 1992) and cultured

mesencephalic cells (Alonso *et al.*, 1993).

In light of evidence pointing to the particularly high densities of IL-2 and IL-2R in the hippocampus, we focused on the possible modulatory role of IL-2 on acetylcholine (ACh) release from the septo-hippocampal pathway. Indeed, this is appropriate given the observed effects of IL-2 on the inhibition of long-term potentiation (Tancredi *et al.*, 1990) and its facilitation of scopolamine-induced amnesia (Bianchi & Panerai, 1993), two models for which the involvement of this projection is clearly established. Early on, we reported that n*M* concentrations of IL-2 potently inhibited K^+-evoked ACh release from rat hippocampal slices *in vitro* (Araujo *et al.*, 1989). In a subsequent study, we observed that IL-2 was among the most potent modulators of ACh release known to date, having a potentiating action at low concentrations (f*M*-p*M*) while inhibiting ACh release at n*M* concentrations (Hanisch *et al.*, 1993).

The aim of the present study was thus to explore the nature of the concentration-dependent modulatory actions of IL-2 on hippocampal ACh release. We first determined if the effect of IL-2 was dependent on the nature of the depolarizing agents used before establishing whether the IL-2R subtype mediating these effects was similar to that expressed on T cells using a highly specific anti-rat IL-2Rα antibody. Tetrodotoxin (TTX) was then employed to explore if the potentiating and inhibitory effects of IL-2 were likely due to a direct action on cholinergic terminals. Since the inhibitory effect observed at n*M* concentrations appeared to be indirect (TTX-sensitive), the possible nature of some likely mediators (nitric oxide, opioid peptides, GABA) was finally investigated.

Methods

Materials

Recombinant human IL-2, acetylcholine chloride, choline chloride, physostigmine hemisulphate, veratridine, tetrodotoxin, N^g-monomethyl-L-arginine acetate (L-NMMA), glycylglycine, choline kinase (ATP: choline phosphotransferase, EC 2.7.1.32) and acetylcholinesterase type V-S (acetylcholine hydrolase, EC 3.1.1.7) were purchased from Sigma Chemical Co. (St-Louis, MO, U.S.A.). (-)-Bicuculline methochloride and phaclofen were obtained from Trocris Cookson (Bristol, England, U.K.), while monoclonal mouse anti-rat IL-2Rα antiserum (NDS-61) was purchased from Serotec/Cedarlane (Hornby, Ontario, Canada). Dithiothreitol and adenosine triphosphate (ATP) were obtained from Boehringer-Mannheim (Laval, Québec, Canada). Sodium tetraphenylboron was obtained from Fisher Scientific (Montréal, Québec, Canada) while butyronitrile was purchased from Aldrich (Milwaukee, WI, U.S.A.). [γ-^{32}P]ATP (30 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Eco(+)lite scintillating cocktail was purchased from ICN (Montréal, Québec, Canada). The chromatographic resin Duolite was a generous gift from Gerald D. Button (Rohm & Haas Co., Philadelphia, PA, U.S.A.). Other chemical reagents were obtained from either Sigma Chemical Co. or Fisher Scientific Co.

Superfusion of Brain Slices

Adult male Sprague-Dawley rats (300 - 325 g) obtained from Charles River (St-Constant, Canada) maintained according to guidelines of the Canadian Council for Animal Care and McGill University, were used in the study. Rats were decapitated and hippocampi dissected out on ice and sliced at 0.4 mm with a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, England). Slices of one whole hemispheric region were transferred to one superfusing chamber (12 parallel chambers; Model SF-12, Brandel, Gaithersburg, MD, U.S.A.). The slices were continuously superfused with oxygenated Krebs buffer (m*M*: NaCl 120; KCl 4.6; $CaCl_2$ 2.4; KH_2PO_4 1.2; $MgCl_2$ 1.2; dextrose 9.9; $NaHCO_3$ 25; choline chloride 0.01;

physostigmine 0.03, adjusted to *p*H 7.4) at 37 °C at a flow rate of 0.5 ml/min as described in detail elsewhere (Seto *et al.*, 1993). Following a 45-min stabilization period, samples were collected every 20 min during basal efflux and depolarisation and/or drug treatment. After one hour of basal efflux, the slices were stimulated with 25 m*M* K^+ Krebs buffer (with concomitant decrease in Na^+ to maintain iso-osmolarity) or veratridine (30 μM) in absence or presence of IL-2, or in the presence of IL-2 and other drug/agent. At the end of each experiment, protein content of each hemisphere was measured according to Lowry *et al.* (1951). Superfusates collected every 20 min were spun (21800 X *g*), and frozen at -70 °C until further processing.

Radioenzymatic analysis of ACh

Samples were processed in triplicate for ACh analysis according to the procedure of Fonnum (1969) and Goldberg & McCaman (1973). Briefly, ACh was extracted by mixing with an equal volume of tetraphenylboron in butyronitrile (30 m*M*) and recovered from the organic phase by shaking with a half volume of $AgNO_3$ (120 m*M*). Excess $AgNO_3$ was precipitated by the addition of $MgCl_2$ (1 *M*) and finally 110 μl of the supernatant was removed and evaporated to dryness. Dried samples were redissolved in 32 μl of a mixture containing ATP (0.8 m*M*), dithiothreitol (5 m*M*), $MgCl_2$ (12.5 m*M*), glycylglycine (25 m*M*, *p*H 8.0), and choline kinase (0.005 U) and incubated at 30 °C for 25 min to phosphorylate the choline and not ACh contained in the samples. 10 μl of a solution containing acetylcholinesterase (2 U) and [γ-^{32}P]ATP (0.45 μCi) were added to each sample during which ACh was hydrolysed and the choline formed phosphorylated to [^{32}P]phosphorylcholine. The reaction was stopped by the addition of 100 μl of NaOH (50 m*M*), and radioactive phosphorylcholine was subsequently separated from the radioactive ATP by ionic-exchange chromatography (Duolite) followed by determination of radioactivity using liquid scintillation. For each experiment, known standard amounts of ACh (0 - 200 pmol) were processed in parallel to monitor recovery (≥ 85%; sensitivity of about 5 pmol). Evoked transmitter release was calculated by subtracting the basal efflux from the total release and is expressed as pmol ACh/min/mg protein. Replicate determinations for each condition

were derived from independent experiments and the data were analysed using one-way ANOVA followed by Fisher post-hoc test and the level of significance was set at $p < 0.05$.

Results

Effects of IL-2 on hippocampal ACh release under stimulated conditions

In keeping with our earlier findings (Hanisch *et al.*, 1993), IL-2 at 10^{-13} *M* potentiated whereas a higher concentration (10^{-9} *M*) inhibited K^+-evoked ACh release from hippocampal slices (Fig. 1A). To assess whether this biphasic response of IL-2 can be mimicked under other depolarizing agents, hippocampal slices were superfused with submaximal concentration of veratridine (30 μM) either in the presence or absence of IL-2. IL-2 at 10^{-9} *M* can significantly inhibit whereas at 10^{-13} *M* it potently stimulated veratridine-evoked ACh release from hippocampal slices (Fig. 1B). These effects exhibited time dependency, since both became significant between 20 to 40 min of stimulation. The spontaneous, unstimulated release of ACh from hippocampal slices was not significantly altered by IL-2 in the p*M*-n*M* range (throughout the experiment, in the absence or presence of IL-2 at either concentration, basal ACh release was between 0.1 to 1.4 pmol/mg/min). Slight variations in the potentiating effect of IL-2 at 10^{-13} *M* and in the evoked controls (Fig. 1, 2, 3 and Tables 1 and 2) are likely attributable to variations in basal release, assay procedures and sample size.

Role of IL-2 receptor in IL-2 effects on ACh release

It is well known that the IL-2 receptor consists of three subunits, α, β, and γ, and that the binding of IL-2 to the α subunit is important for activity (Depper *et al.*, 1983; Taniguchi & Minami, 1993). Interestingly, the subunits of the IL-2 receptor are shared by other related cytokines, i. e., IL-4, IL-7, IL-9, IL-13 and IL-15 (Taniguchi & Minami, 1993; Bamford *et al.*, 1994; Giri *et al.*, 1994; Grabstein *et al.*, 1994) such that IL-2 effects may be exerted through other interleukin receptors. To determine the specificity of IL-2 effects on ACh release, hippocampal slices were superfused with 25 m*M* K^+ Krebs buffer in presence or absence of an anti-rat IL-2Rα antibody, NDS-61 (1:500) (Wood *et al.*, 1992). Fig. 2 shows that both the potentiating as well as the inhibitory effects of IL-2 on ACh release were sensitive to NDS-61, suggesting the involvement of a typical IL-2 receptor.

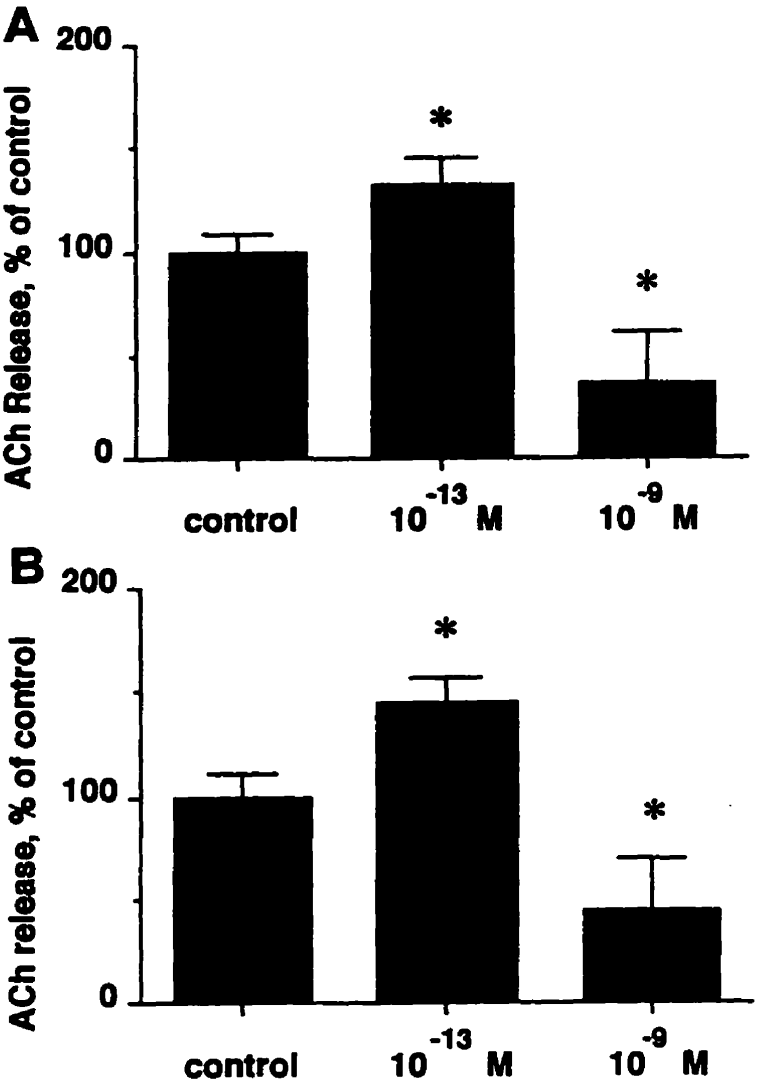

Figure 1 Effect of IL-2 concentrations on KCl (25 m*M*; A) and veratridine (30 μM; B)-evoked ACh release from hippocampal slices. The slices were stimulated with Krebs buffer containing either 25 m*M* KCl or 30 μM veratridine in the absence of IL-2 (control) or presence of IL-2 at 10^{-13} *M* or 10^{-9} *M* concentrations. Values are expressed as mean ± SEM of at least 4 determinations from independent experiments for each condition in reference to control set at 100% (for K^+ stimulation 5.7 ± 0.5 pmol/mg/min and veratridine stimulation 3.8 ± 0.5 pmol/mg/min). * $p < 0.05$ *vs* control.

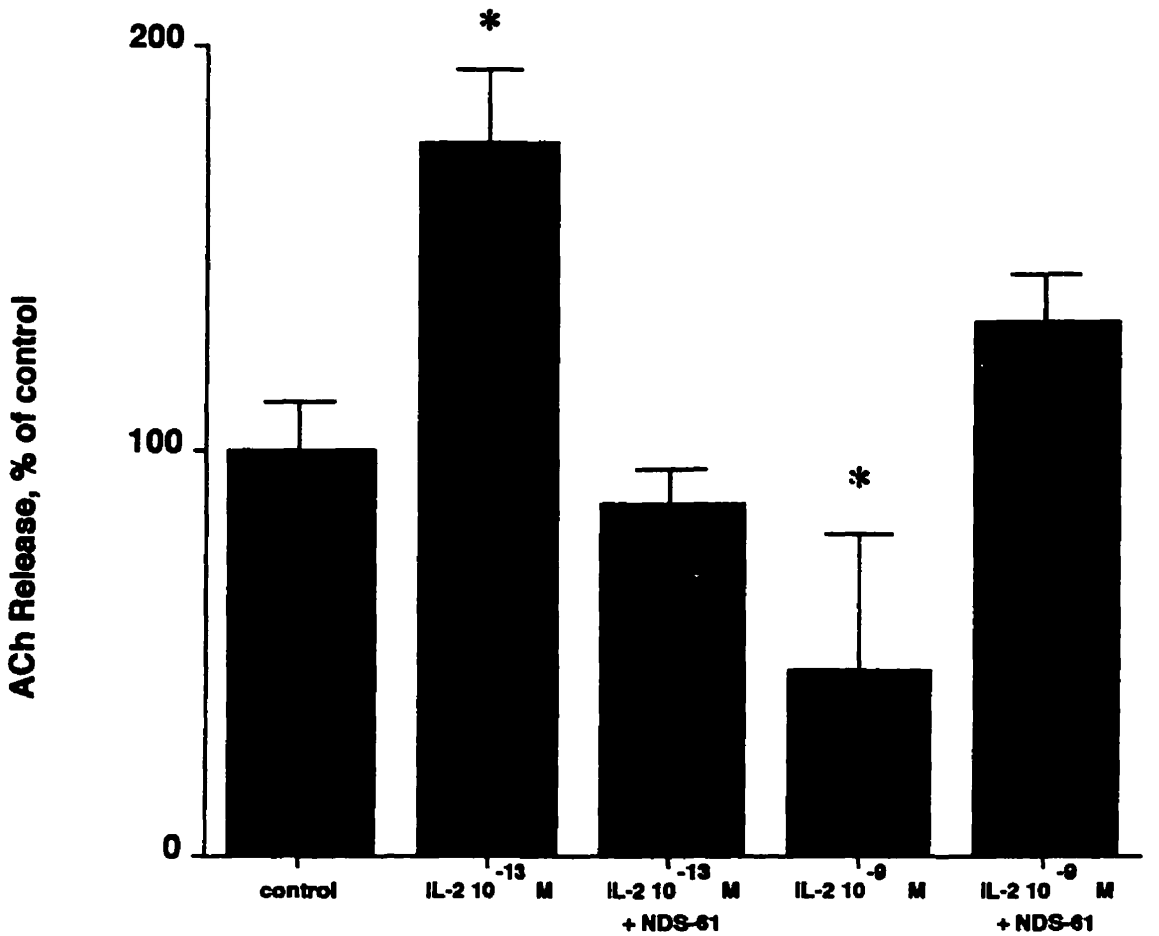

Figure 2 Effect of IL-2 receptor α antibody NDS-61 (1:500) on IL-2-mediated K^+-evoked ACh release from hippocampal slices. Control groups were exposed to NDS-61 alone. Data are expressed as percent of control (mean ± SEM) of at least 4 determinations from independent experiments for each condition. * $p < 0.05$ *vs* control (4.0 ± 0.5 pmol/mg/min).

Effects of tetrodotoxin on IL-2 effects on ACh release

To determine whether the IL-2-induced alteration of evoked ACh release was affected by the sodium channel blocker tetrodotoxin (TTX), hippocampal slices were superfused under similar conditions in presence of 10 *μM* TTX. By itself TTX failed to alter evoked ACh release (Lapchak *et al.*, 1990). Interestingly however, the stimulatory effects of pM concentrations of IL-2 on ACh release were unaltered in the presence of TTX (Fig. 3). In contrast, the inhibitory effects of a nM concentration of IL-2 on ACh release resulted in enhanced release in the presence of TTX (Fig. 3). This indicates that IL-2 probably acts directly on hippocampal cholinergic terminals to potentiate ACh release while the inhibitory response is likely indirect and requires the initiation of impulse activity.

Possible mediators of TTX-sensitive inhibitory action of IL-2 on ACh release

In an attempt to determine the possible neurotransmitter/neuromodulator involved in mediating the TTX-sensitive effects of IL-2 on ACh release, we evaluated first the effects of opioid receptor blockade since it has been reported that opioid peptides inhibited hippocampal ACh release (Lapchak *et al.*, 1989) and could be involved, at least in part, in mediating the effects of IL-2 in the hippocampus (Araujo *et al.*, 1990). Accordingly, hippocampal slices were superfused with or without the opioid antagonist naloxone at a concentration of 10 or 100 *μM*. Naloxone even at the very high concentration of 100 *μM* failed to alter IL-2-mediated inhibition of ACh release under our assay conditions (Table 1). It also failed to significantly modulate the stimulatory effect of IL-2 on ACh release (not shown) as expected on the basis of the TTX data.

It has also been suggested that some of the modulatory effects of IL-2 were mediated by the synthesis and release of nitric oxide (NO) (Finkel *et al.*, 1992; Karanth *et al.*, 1993; Raber & Bloom, 1994; Raber *et al.*, 1995). Since NO synthase (NOS), the enzyme responsible for NO synthesis, is present in the hippocampus (Bredt *et al.*, 1991) and NO is believed to be involved the regulation of ACh release (Lonart *et al.*,

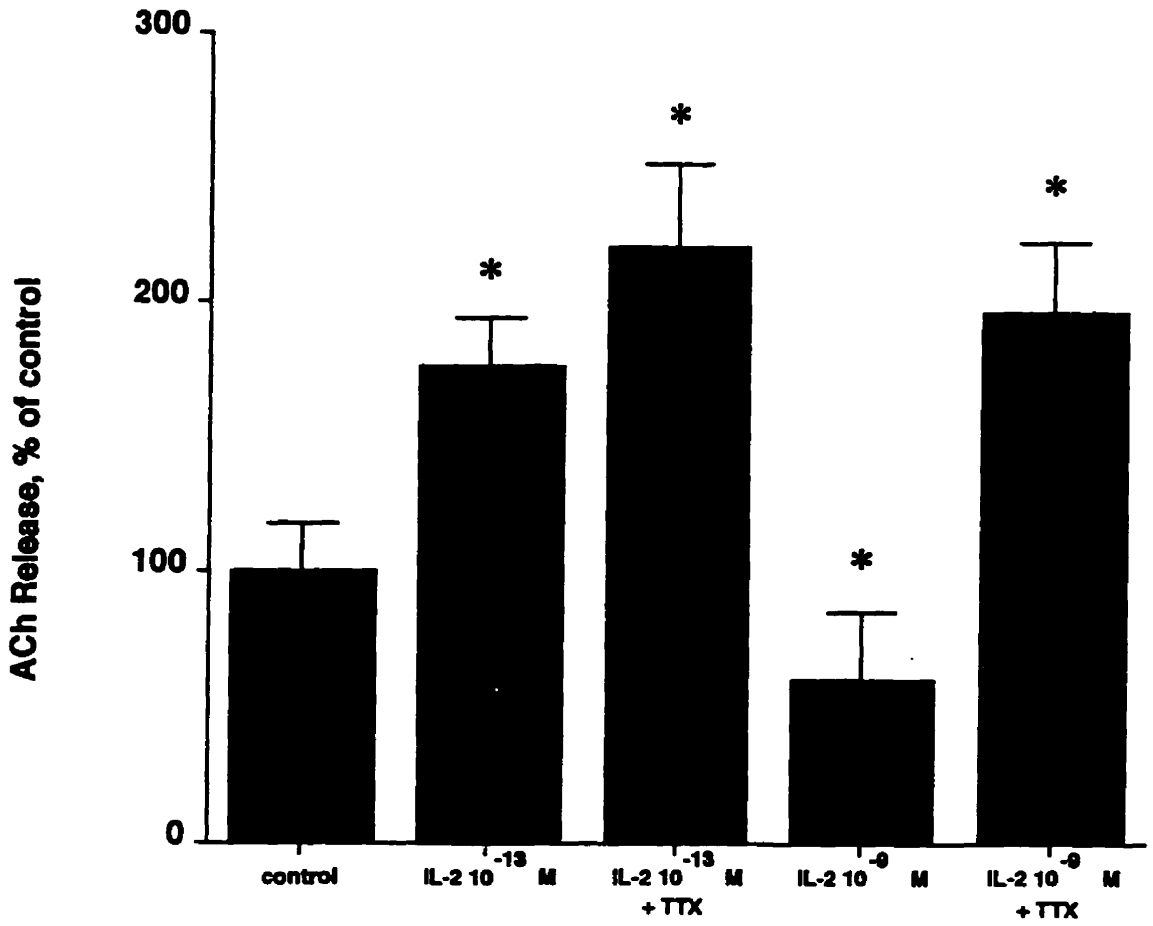

Figure 3 Effect of TTX (10 μM) on IL-2-mediated K^+-evoked ACh release from hippocampal slices. Control groups were exposed to TTX alone. While the potentiating effect of 10^{-13} *M* IL-2 was not altered by TTX, the inhibition seen at 10^{-9} *M* IL-2 was reversed in the presence of TTX. Data are expressed as percent of control (mean ± SEM) of at least 4 determinations from independent experiments for each condition. * $p < 0.05$ *vs* control (2.7 ± 0.5 pmol/mg/min).

Table 1 Effect of naloxone (10 μM and 100 μM) on IL-2 inhibition of K^+-evoked ACh release from hippocampal slices.

Treatment	% of control
naloxone 10 μM	
control	100 ± 18
IL-2 10^{-9} M	58 ± 17 *
IL-2 + naloxone	47 ± 27 *
naloxone 100 μM	
control	100 ± 20
IL-2 10^{-9} M	53 ± 3 *
IL-2 + naloxone	42 ± 21 *

The slices were stimulated with K^+ Krebs buffer containing naloxone alone (control), 10^{-9} M IL-2 alone or 10^{-9} M IL-2 plus naloxone. Data are expressed as percent of control (mean ± SEM) of at least 4 determinations from independent experiments for each condition. * $p < 0.05$ *vs* control (10.7 ± 1.9 pmol/mg/min for naloxone 10 μM and 7.0 ± 1.1 pmol/mg/min naloxone 100 μM).

Table 2 Effect of the nitric oxide synthase inhibitor L-NMMA (10 μM) on IL-2-mediated K^+-evoked ACh release from hippocampal slices.

Treatment	% of control
control	100 ± 44
IL-2 10^{-13} *M*	242 ± 18 *
IL-2 10^{-13} *M* + NMMA	244 ± 14 *
IL-2 10^{-9} *M* + NMMA	58 ± 19 *
IL-2 10^{-9} *M* + NMMA	46 ± 18 *

Control groups were exposed to L-NMMA alone. Other groups shown are 10^{-13} *M* IL-2 alone, 10^{-13} *M* IL-2 plus L-NMMA, 10^{-9} *M* IL-2 alone and 10^{-9} *M* IL-2 plus L-NMMA. L-NMMA failed to alter either the inhibiting or potentiating effects of IL-2 on ACh release. Data are expressed as percent of control (mean ± SEM) of at least 4 determinations from independent experiments for each condition. * $p < 0.05$ *vs* control (2.8 ± 0.6 pmol/mg/min).

1992), we evaluated the role of NO by blocking its production using L-NMMA (10 or 100 μM) in superfused hippocampal slices. NOS inhibition did not affect either the stimulatory or inhibitory action of IL-2 on ACh release (Table 2).

GABA is a major inhibitory neurotransmitter in the hippocampus (for a recent review, Thompson, 1994). Accordingly, its potential relevance in mediating the indirect effects of nM concentrations of IL-2 on ACh release was undertaken using prototypical $GABA_A$ and $GABA_B$ receptor antagonists. As shown in Fig. 4, both the $GABA_A$ receptor antagonist bicuculline (10 μM) and the $GABA_B$ receptor blocker phaclofen (10 μM) alone or in combination reversed the inhibitory effects of IL-2 on hippocampal

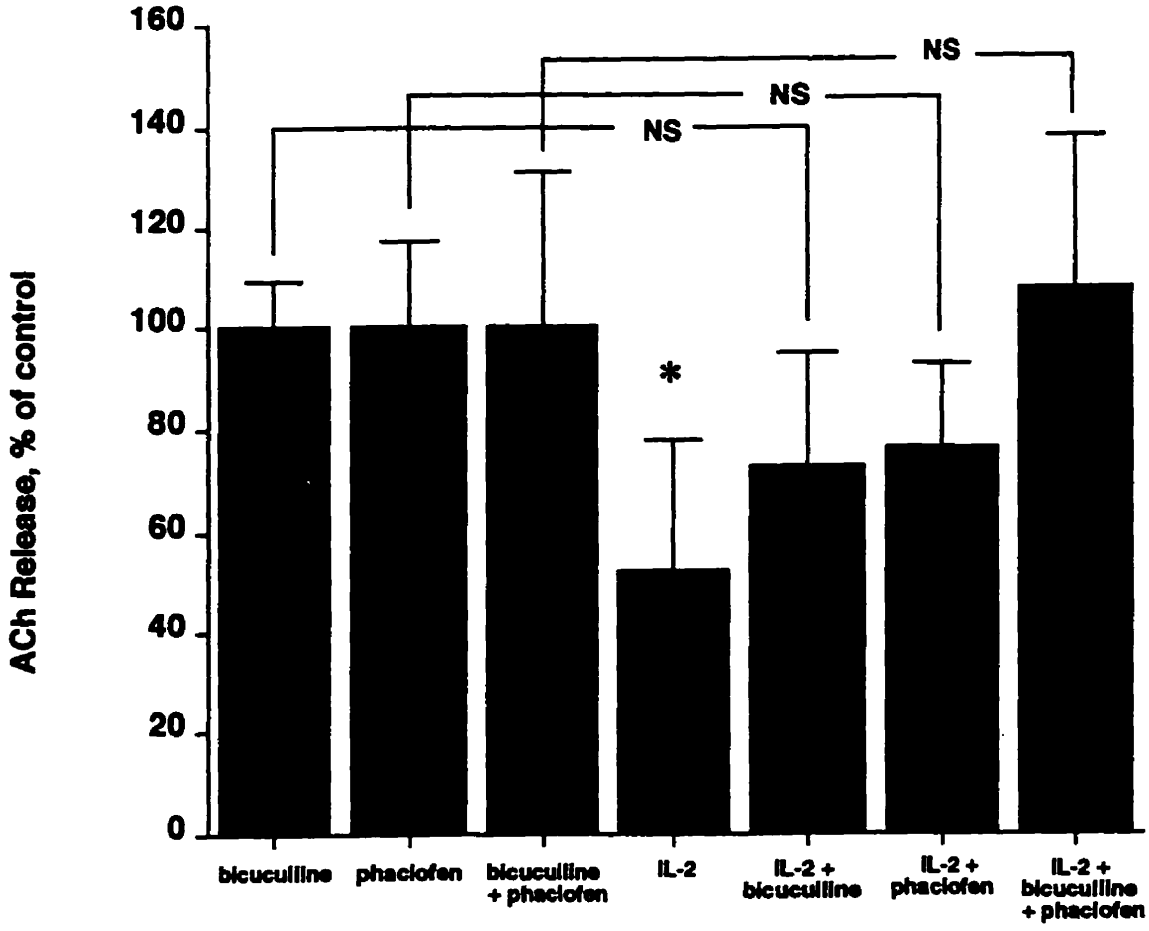

Figure 4 Effects of a $GABA_A$ antagonist (bicuculline) and a $GABA_B$ antagonist (phaclofen) alone or in combination on IL-2 inhibition of K^+-evoked ACh release from hippocampal slices. Control groups were exposed to either bicuculline or phaclofen alone or in combination at 10 μM. IL-2 10^{-9} M inhibited ACh release ($p < 0.05$), this effect being reversed by GABA antagonists alone or in combination. Data are expressed as percent of control (mean ± SEM) of at least 4 determinations from independent experiments for each condition. * $p < 0.05$ *vs* control (bicuculline 4.0 ± 0.4 pmol/mg/min; phaclofen 3.7 ± 0.6 pmol/mg/min; bicuculline and phaclofen 1.5 ± 0.5 pmol/mg/min).

ACh release, suggesting that the release of GABA and the activation of its receptors were involved in mediating the indirect inhibitory action of IL-2 on ACh release. Bicuculline or phaclofen alone or in combination at 10 μM each had no effect on the IL-2-mediated potentiation of ACh release (not shown).

Discussion

The present report provides the first detailed evidence regarding the potent modulatory action of IL-2 on hippocampal ACh release. Data obtained using non-stimulated and veratridine-evoked release in comparison to K^+ stimulation, suggest that depolarization is required for IL-2 to exert its effects on ACh release. The use of the anti-rat IL-2Rα subunit antibody revealed that both the potentiating and inhibitory effects of IL-2 on hippocampal ACh release result from an action via the same component/subunit of the prototypical IL-2 receptor. Interestingly, the potentiating and inhibitory effects of IL-2 on ACh release can be dissociated on the basis of their sensitivity to TTX which suggests that the stimulation of ACh release by a low concentration of IL-2 results from a direct action of the cytokine on cholinergic nerve terminals of the septo-hippocampal pathway. As to the nature of the TTX-sensitive inhibitory effects of a higher concentration of IL-2 on ACh release, it is unlikely to be mediated by either nitric oxide or opioid peptides but may involve GABA as it was blocked by $GABA_A$ and $GABA_B$ receptor antagonists. Hence the potent, biphasic action of IL-2 on hippocampal ACh release is complex and involves a direct effect on cholinergic nerve terminals and most likely the release of an inhibitory transmitter at higher concentrations.

Previous investigations of IL-2 effects on hippocampal ACh release were solely based on K^+ stimulation (Araujo *et al.*, 1989; Hanisch *et al.*, 1993; Seto *et al.*, 1993). Results obtained under veratridine-evoked conditions indicate that the biphasic effect of IL-2 on ACh release does not depend on the nature of the depolarizing agent, but that a depolarizing stimulus is necessary, since no effects were observed under basal unstimulated conditions. This observation is significant as effects on transmitter release under non-stimulated conditions are often not specific and associated with passive transmitter leakage, not genuine vesicular release. However, in contrast to hippocampal ACh release, corticotrophin and vasopressin releases from hypothalamic

and amygdalar slices (Cambronero *et al.*, 1992; Karanth *et al.*, 1993; Hillhouse, 1994; Raber & Bloom, 1994; Raber *et al.*, 1995) revealed that IL-2 can evoke peptide release under non-stimulated conditions. Accordingly, additional studies will be necessary to establish if the neuromodulatory action of IL-2 varies between classical transmitter and neuropeptides.

Using a highly specific antibody against the rat α subunit of the rat IL-2 receptor (Wood *et al.*, 1992), we observed that both the stimulatory and inhibitory effects of IL-2 on ACh release are mediated by a typical IL-2R and that the α subunit is required to produce an action (Depper *et al.*, 1983). It is also likely that other subunits (ß and γ) of the IL-2 receptor are involved in the observed effects as these subunits are usually essential in mediating various actions of IL-2. In any case, our results suggest that the integrity of the receptor complex with a fully available α subunit is required for the effect of IL-2 on ACh release. It could also be of interest to establish if other cytokines such as IL-4, IL-7, IL-9, IL-13 and IL-15, which all apparently share various subunits of the IL-2 receptor complex (Bamford *et al.*, 1994; Giri *et al.*, 1994; Grabstein *et al.*, 1994; Kishimoto *et al.*, 1994) can also modulate ACh release. Thus far, only IL-4 which shares the γ subunit of the IL-2 receptor, has been studied in that regard, and failed to modulate hippocampal ACh release (Araujo *et al.*,1989).

The use of TTX allowed us to distinguish between the stimulatory and inhibitory actions of IL-2 on hippocampal ACh release. The TTX-insensitive nature of the potentiating effect of IL-2 on ACh release suggests an action directly on or in very close proximity to the septo-hippocampal cholinergic nerve terminals. Accordingly, it is likely that IL-2 receptors positively modulating ACh release are directly located on cholinergic terminals. In support of this hypothesis, we have recently shown that IL-2 can stimulate choline acetyltransferase (ChAT) activity in primary septal cultures enriched in cholinergic neurons (Mennicken & Quirion, 1996). In contrast, the inhibitory effect of higher concentrations of IL-2 on hippocampal ACh release is most likely indirect as it is fully reversed by TTX. In fact, in the presence of TTX, a n*M*

concentration of IL-2 demonstrated a potent stimulatory action on ACh release (Fig. 3). Thus it would appear that IL-2 receptors are located both on hippocampal cholinergic nerve terminals as well as on other neuronal populations distal to them; the resulting effect on ACh release depending upon the concentration of IL-2 available in the intercellular space, higher concentrations leading to the release of inhibitory substance(s), counteracting the direct, stimulatory effect of IL-2 on ACh nerve terminals. To our knowledge, this is the first example of a cytokine having such a complex profile on the release of a given neurotransmitter in the brain.

The nature of the inhibitory substance possibly involved in mediating the TTX-sensitive effects of IL-2 was investigated next. Some reports have suggested the possible involvement of endogenous opioid peptides in certain CNS effects of IL-2 (Araujo *et al.*, 1990; Lapchak & Araujo, 1993; Jiang *et al.*, 1995). It has been recently proposed that IL-2 could directly act on opioid receptors to induce its effects (Jiang *et al.*, 1995). Moreover, since various opioid peptides have been shown to modulate ACh release (Lapchak *et al.*, 1989), naloxone, a potent opioid receptor antagonist was tested in our model. It failed to alter the TTX-sensitive inhibitory action of IL-2 on ACh release suggesting that opioid peptides and their receptors are unlikely to be involved at least under our assay conditions. An inhibitor of nitric oxide synthase, L-NMMA (Bredt *et al.*, 1991; Karanth *et al.*, 1993; Raber *et al.*, 1995) was investigated next since recent data have shown that some effects of IL-2 in the CNS, especially at the hypothalamus, likely involve the participation of NO or NO-related mechanisms (Karanth *et al.*, 1993; Raber & Bloom, 1994; Raber *et al.*, 1995). There is also evidence that NO may potentiate hippocampal ACh release (Lonart *et al.*, 1992). However, the inhibition of NO synthase failed to alter the inhibitory effect of a n*M* concentration of IL-2 on ACh release. Similarly, the potentiating action of a lower concentration of IL-2 failed to be modulated by the inhibition of the production of NO. Accordingly, it seems unlikely that NO play a critical role in the modulation of the biphasic effects of IL-2 in hippocampal ACh release.

GABA is a major inhibitory neurotransmitter in the hippocampus (for a recent review, Thompson, 1994) and the existence of GABA receptor sub-types in the brain is well established (Sivilotti and Nistri, 1991). Accordingly, the possible role of GABA and its receptors on the inhibitory action of IL-2 on ACh release was investigated using $GABA_A$ (bicuculline: Olpe *et al.*, 1988; Arenas *et al.*, 1990) and $GABA_B$ (phaclofen: Soltesz *et al.*, 1988; Arenas *et al.*, 1990) receptor antagonists. Interestingly and rather surprisingly, both antagonists blocked the inhibitory effects of IL-2. In addition, the combination of both antagonists was also effective in blocking the IL-2-induced inhibition of ACh release. These results suggests that the release of the inhibitory transmitter GABA induced by a n*M* concentration of IL-2 acts on both $GABA_A$ and $GABA_B$ receptor sub-types to dampen hippocampal ACh release. The simultaneous participation of both the $GABA_A$ and $GABA_B$ receptor sub-types in the inhibitory action of GABA has already been reported to occur in the human cortex on the basis of electrophysiological data (McCormick, 1989). To our knowledge, the present results are the first to suggest possible interactions between GABAergic neurotransmission and IL-2 in the CNS and studies are currently underway to establish further the nature of this association.

In summary, IL-2 is among the very most potent modulators of ACh release in the rat hippocampus. The stimulatory effect observed at a very low (10^{-13} *M*) concentration is TTX-resistant suggesting a direct action on cholinergic nerve terminals. In contrast, the inhibitory action observed at a n*M* concentration is TTX-sensitive and is likely mediated by the GABAergic system. In view of the well established role of hippocampal cholinergic innervation in learning and memory (see Björklund & Dunnett, 1995 for a recent discussion), and the marked alteration of this structure and cholinergic markers in neurodegenerative disorders such as Alzheimer's disease (AD; Bartus *et al.*, 1982; Selkoe, 1993), it is of interest that earlier studies have reported on the increased expression of IL-2 and IL-2 receptors in AD (Luber-Narod & Rogers, 1988; Araujo & Lapchak, 1994). On the basis of the present results, it is tempting to speculate that heightened IL-2 activity could exacerbate cholinergic deficits in AD by

inhibiting ACh release. Further studies in that regard are certainly warranted in view of the recent surge of interest on the possible involvement of a neuro-immune component in AD (McGeer *et al.*, 1994).

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CHAPTER 5

PREFACE TO CHAPTER 5

The preceding Chapter described interleukin-2 effects that identify the neuromodulatory function of interleukin-2. The next Chapter investigates possible intracellular signalling pathways that interleukin-2 uses to exert its powerful effects.

Brain Interleukin-2 Receptor Signalling: Possible Involvement of Cytidine Diphosphodiacylglycerol-Associated Pathway

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Abstract

Interleukin-2 (IL-2) receptor signalling in brain is mostly unknown. In immune cells, evidence suggests the involvement of phosphatidylinositol 3-kinase (PI 3-kinase) and phospholipase C hydrolysis of glycosylphosphatidylinositol (gly-PI), yielding myristyldiacylglycerol (myr-DAG). Using brain slices from regions responding to IL-2 (hippocampus, cortex, hypothalamus), effects of IL-2 on cytidine diphospho-diacylglycerol (CDP-DAG) production were studied. Under basal and carbachol-stimulated conditions, IL-2 inhibited, in a concentration-dependent manner, CDP-DAG production in the cortex and hypothalamus. In the hippocampus, inhibition was observed only under carbachol-stimulated conditions. CDP-DAG decreases may result from a reversal of the cascade by phospholipase D (PLD) activation. Accordingly, IL-2 increased choline production, an index of PLD activity, in brain slices. Taken together, these results suggest that brain IL-2 receptors are possibly coupled to CDP-DAG-associated pathways with regional differences observed in signalling efficacy.

Key Words: interleukin-2, phospholipases, diacylglycerol, choline

Abbreviations: ACh, acetylcholine; ACPD, *trans*-(1S,3R)-1-aminocyclopentyl-1,3-dicarboxylic acid; carbachol, carbamylcholine; CDP-DAG, cytidine diphospho-diacylglycerol; DAG, diacylglycerol; gly-PI, glycosylphosphatidylinositol; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; IP_1, inositol monophosphate; IP_3, inositiol 1,4,5-trisphosphate; myr-DAG, myristyldiacylglycerol; PA, phosphatidic acid; PI 3-kinase, phosphatidylinositol 3-kinase; PLC, phospholipase C; PLD, phospholipase D; rhIL-2, recombinant human interleukin-2.

Interleukin-2 (IL-2) is a 133-amino acid peptide hormone that regulates the proliferation, metabolism and differentiation of T lymphocytes [1]. In the immune system these events are mediated by IL-2 binding to a specific, high-affinity heteropolymeric receptor complex (IL-2R) composed of at least 3 sub-units: α, ß and γ (for review, see ref.2). To induce IL-2-like effects, only the ß and γ sub-units are required [3, 4]. Although many events following receptor activation in the immune system have been described, the emerging picture is that "classical" second messenger cascades are not involved [5, 6]. As a result, novel evidence has suggested the likely involvement of phosphatidylinositol 3-kinase (PI 3-kinase) [7, 8] and glycosylphosphatidylinositol (gly-PI) [9] as messengers. In the latter pathway, upon receptor activation, gly-PI is hydrolyzed, presumably through the activation of phospholipase C (PLC), to yield myristyl diacylglycerol (myr-DAG) [10].

The activation of PLC by hormones and neurotransmitters which results in the production of DAG has been extensively studied in the nervous system [11]. It has been established that cholinergic [12], noradrenergic, serotoninergic [13] or endothelin [14] receptor activation results in the production of DAG and cytidine diphospho-DAG (CDP-DAG) in nervous tissues. This phenomenon can be followed using Li^+-dependent accumulation of [^{3}H]cytidine-labelled CDP-DAG [13].

IL-2, its receptors (or mRNA) and IL-2-induced effects have been described in the nervous system [15 - 17]. In addition, there is evidence for the presence of IL-2, IL-2R binding sites and IL-2Rα sub-unit immunoreactivities in the normal rat brain [16, 18, 19], IL-2Rα mRNA in cultured brain cells [20] and IL-2 mRNA in human brain tissues [21], and on its role as a modulator of transmitter release [18, 22]. However in contrast to the immune system, very little is currently known as to transmembrane signalling events associated with IL-2R activation in the brain. This report describes a putative pathway linked to PLC and/or phospholipase D (PLD) activation in rat brain slice preparations.

Materials and Methods

Carbamylcholine (carbachol), physostigmine hemisulphate and recombinant human IL-2 (rhIL-2) were purchased from Sigma Chemical Co. (St-Louis, MO, USA). [5-^{3}H]cytidine (20-25 Ci/mmol) was obtained from New England Nuclear (Boston, MA, USA). The metabotropic glutamatergic agonist *trans*-(1S,3R)-1-aminocyclopentyl-1,3-dicarboxylic acid (ACPD) was purchased from Tocris Cookson (Bristol, England, UK). Eco(+)lite scintillation cocktail was purchased from ICN Canada (Montréal, Québec, Canada). The chromatographic resin Duolite was a generous gift of Gerald D. Button (Rohm & Haas Co., Philadelphia, PA, USA). Other chemical reagents of ACS grade or higher were obtained from usual commercial sources. Adult male Sprague-Dawley rats (300-325 g) were purchased from Charles River (St-Constant, Québec, Canada) and were housed with free access to food and water according to guidelines of the Canadian Council for Animal Care and McGill University. Animals were sacrificed by decapitation and various brain regions were dissected out on ice. Slices 0,4-mm thick were cut using a McIlwain tissue chopper.

CDP-DAG accumulation: Tissue slices were processed and CDP-DAG analyzed as described in detail elsewhere [14]. Briefly, brain slices were washed several times in oxygenated Krebs buffer (composition in m*M*: NaCl 120; KCl 4,6; $CaCl_2$ 2,4; KH_2PO_4 1,2; $MgCl_2$ 1,2; dextrose 9,9; $NaHCO_3$ 25) to dissociate them and then incubated under 5 % CO_2 atmosphere at 37 C with a change of buffer every 20 min for 1 hour. Slices were then transferred to a 24-well polystyrene culturing plate and incubated for 1 hour with 5 μCi of [5-^{3}H]cytidine/well dissolved in the same buffer to incorporate the tracer. Slices were then rinsed 3 times with the buffer containing LiCl 10 m*M*, and exposed to the experimental conditions for 60 min. All incubation buffers from this time point contain LiCl 10 m*M*. The non-stimulated condition was the normal buffer and all drugs were dissolved or diluted in normal or carbachol buffer. CDP-DAG accumulation was then quantified as described in detail elsewhere [14].

Choline production: Slices were washed several times in oxygenated Krebs buffer to dissociate them and then incubated under 5 % CO_2 atmosphere at 37 C with a change of buffer every 20 min for 1 hour. Slices were then transferred to a 24-well polystyrene culturing plate and incubated for 20 min with 30 μM physostigmine. All drugs were dissolved or diluted in normal buffer. Incubations were stopped after 30 min by transferring contents of each well into cold micro test tubes before spinning at 14900 x *g* for 4 min. Supernatants were then transferred to another set of micro test tubes and stored at -70 C until choline determination according to Fonnum [23] and protein assay according to Lowry *et al.* [24]. The data were analyzed using one-way ANOVA followed by Fisher post-hoc test and the level of significance was set at $p < 0.05$.

Results and Discussion

In the present study, IL-2, at concentrations known to modulate K^+-stimulated acetylcholine release in hippocampal slices (1 - 100 nM [18, 22]) failed to induce significant changes in basal CDP-DAG production in this area (Table 1). As the presence of IL-2 and IL-2R are documented in other brain regions such as the frontal cortex and hypothalamus [16, 18], the possible modulation by IL-2 of CDP-DAG production was also investigated in these regions. IL-2 potently inhibited basal CDP-DAG accumulation in these areas (Table 1). Accordingly, while IL-2 is apparently unable to modulate the basal production of CDP-DAG in rat hippocampal slices, it potently inhibited it in other regions such as the frontal cortex and hypothalamus which are all known to be enriched with IL-2 and IL-2R-like immunoreactivities [18, 19].

As it is well established that IL-2 is a potent modulator of evoked, but not basal acetylcholine release in the rat hippocampus [18, 22], and to explore further the apparent lack of effects of this cytokine on the production of CDP-DAG in this tissue, we investigated next the action of IL-2 in the presence of a sub-maximal (10 μM)

Table 1. CDP-DAG production as a function of increasing IL-2 concentrations under basal conditions in hippocampal, cortical and hypothalamic slices. Data are expressed as percentages of mean ± sem of 4-6 determinations per concentration, per region. Statistical significance was determined using ANOVA, (*) $p < 0.05$ being considered significant. * $p < 0,05$ *vs* basal conditions.

Region	IL-2 (*M*)	CDP-DAG production (% of basal levels ± sem)
Hippocampus	none	100 ± 8
	10^{-13}	105 ± 4
	10^{-11}	110 ± 3
	10^{-9}	112 ± 3
	10^{-8}	100 ± 8
Frontal cortex	none	100 ± 2
	10^{-15}	87 ± 6
	10^{-13}	86 ± 4
	10^{-11}	79 ± 5 *
	10^{-9}	77 ± 7 *
	10^{-8}	77 ± 3 *
Hypothalamus	none	100 ± 4
	10^{-15}	108 ± 11
	10^{-13}	73 ± 17
	10^{-11}	72 ± 6
	10^{-9}	72 ± 24
	10^{-8}	48 ± 33 *

stimulation by carbachol. At this concentration, carbachol stimulated CDP-DAG production by 2-fold, maximal effects (4,0-4,5-fold increase) being observed at 0,1 - 1,0 m*M* concentrations. This is in accordance with the well established coupling of muscarinic M1-like receptors to the inositol pathway [12]. As shown in Table 2, IL-2 reduced carbachol-stimulated CDP-DAG production in the hippocampal formation and the frontal cortex.

Taken together, these results suggest that brain IL-2 receptors are coupled to the production of CDP-DAG. To our knowledge, this is the first evidence for a putative transduction pathway associated with brain IL-2 receptors. These results are in agreement with those obtained in peripheral tissues which have shown that IL-2 receptors are coupled to a rather unique PLC-related transduction cascade associated to a PI 3-kinase and the production of myr-DAG via gly-PI [7 - 10].

The most obvious explanation for the decrease in CDP-DAG production seen in various regions following IL-2 receptors stimulation relates to decreased PLC activity (Fig. 1). Alternatively, a decrement in the availability of precursors for CDP-DAG could be evoked as well as decreases in other enzymatic activities leading to the formation of CDP-DAG (Fig. 1). For example, phosphatidic acid (PA) can revert to its precursor DAG under the influence of PA phosphohydrolase [25]. Interestingly, PA can be derived from phosphatidylcholine via the action of PLD, this representing an anti-parallel cascade opposing PLC activity. Since it was recently shown that peripheral IL-2 receptor activation can occur without phosphatidylinositol (PI) hydrolysis [26] and as gly-PI can be directly produced by PLD [27], it may be that the effect of IL-2 on CDP-DAG production is mediated, at least partly, *via* PLD [28]. To verify this hypothesis, choline production was quantified under well established conditions demonstrated to be an index of PLD activity [29]. At concentrations known to exert effects on brain slices *in vitro*, IL-2 significantly increased choline production in hippocampal slices (Table 3). This may be taken as an indication of a role for PLD in the transduction of IL-2-related effects in this tissue.

Table 2. Effect of increasing IL-2 concentrations on carbachol (10^{-5} *M*)-stimulated hippocampal and cortical production of CDP-DAG. Data are expressed as percentages of mean ± sem of 3-6 determinations per concentration. Statistical significance was determined using ANOVA, (*) $p < 0.05$ being considered significant. * $p < 0,05$ *vs* control.

Region	IL-2 (*M*)	CDP-DAG production (% of stimulated levels ± sem)
Hippocampus	control - carbachol	100 ± 6
	10^{-15}	88 ± 6
	10^{-13}	84 ± 5 *
	10^{-11}	83 ± 1 *
	10^{-9}	79 ± 1 *
	10^{-8}	67 ± 7 *
Frontal cortex	control - carbachol	100 ± 14
	10^{-15}	109 ± 14
	10^{-13}	75 ± 14
	10^{-11}	68 ± 20
	10^{-9}	62 ± 22
	10^{-8}	53 ± 13 *

Stimulated levels of CDP-DAG production were 203 ± 13 % and 181 ± 30 % of basal levels in the hippocampus and frontal cortex, respectively.

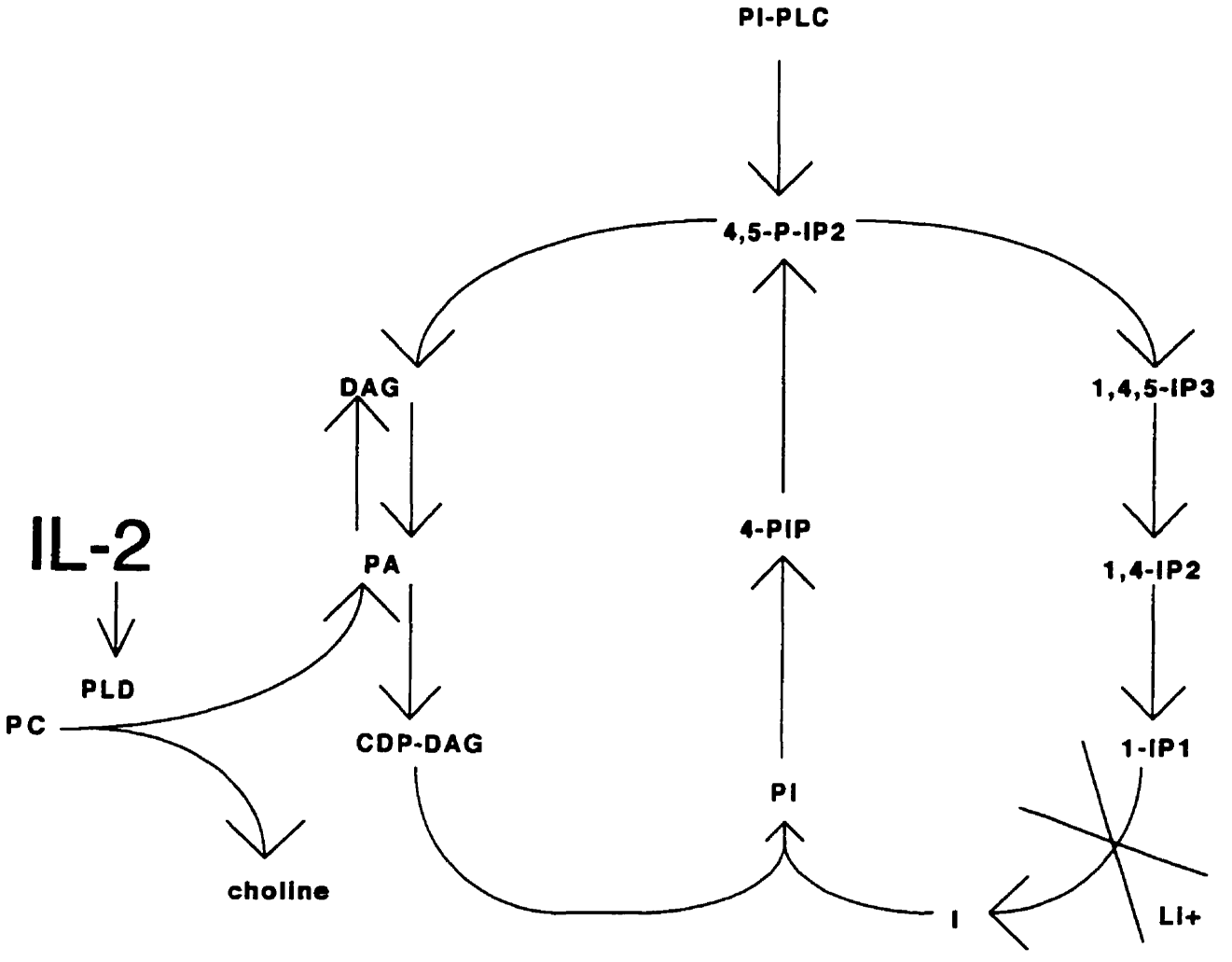

Fig. 1. Diagram of proposed IL-2 interaction and PLC and PLD pathways. PC, phosphatidylcholine; PI, phosphatidylinositol; 4-PIP, phosphatidylinositol 4-phosphate; 4,5-P-PIP_2, phosphatidylinositol 4, 5-diphosphate; 1, 4, 5-IP_3, inositol triphosphate; 1,4-IP_2, inositol diphosphate; 1-IP1, inositol monophosphate; I, inositol.

Regional differences in IL-2 receptor coupling efficacy likely exist in the brain. For example, while IL-2 was able to inhibit basal CDP-DAG production in frontal cortical and hypothalamic slices, only carbachol-stimulated CDP-DAG production was altered by IL-2 in the hippocampus. This may relate to differential IL-2 receptor coupling efficiency in these various regions known to be enriched with IL-2-like immunoreactivity and receptor sites [18, 19]. It is also of interest to note that while IL-2 is a potent modulator of K^+-evoked acetylcholine release in rat hippocampal slices [18, 22], it failed to alter the basal, unstimulated release of this transmitter [18]. Accordingly, the efficacy of IL-2 in the rat hippocampal formation may be dependent upon the state of activation of this tissue, no effect being detected under unstimulated conditions. In contrast, IL-2 was shown to be able to modulate the basal release of corticotrophin-releasing factor [30] in accordance with its direct effect on basal CDP-DAG production in hypothalamic slices.

In summary IL-2 receptor activation in the rat brain is likely coupled to an alteration in the production of CDP-DAG, possibly *via* PLD, as monitored in slice preparations. Differences in IL-2 receptor efficacy likely exist as exemplified by the differential effects of IL-2 on basal *vs* stimulated CDP-DAG production among brain regions.

Table 3. Choline production as an index of phospholipase D activity in hippocampal slices under various conditions. ACPD was used as a positive control [31], well known to stimulate PLD activity. Data are expressed as percentages of mean ± sem of 4-10 determinations per group. Statistical significance was determined using ANOVA, (*) $p < 0.05$ being considered significant. * $p < 0,05$ *vs* non-stimulated conditions.

condition	choline production (% of basal levels ± sem)
basal	100 ± 7
ACPD (1 m*M*)	114 ± 2 *
IL-2 (10^{-13} *M*)	120 ± 2 *
IL-2 (10^{-9} *M*)	118 ± 6 *

Basal level of choline production was 18,42 ± 1,3 pmol/(mg*min).

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General Summary

Interleukin-2 (IL-2) belongs to a group of substances that participate in signalling among cells of the body's defense system. Little is known, however in the way it acts on the brain, and even less clear are some of the events triggered by IL-2 inside brain cells. We used an *in vitro* brain slice model to measure products of lipid hydrolysis by phospholipases C and D. Our results indicate that IL-2 decreases CDP-DAG production under non-stimulated conditions in the frontal cortex and the hypothalamus. Decreases in the hippocampus were seen only in the presence of a carbachol stimulation. Further, IL-2-induced increases in choline may be taken as an indication of an action *via* phospholipase D. Hence, the activation of brain IL-2 receptors likely results in signalling through phospholipase C and/or D activities.

CHAPTER 6:

GENERAL DISCUSSION

6.0 General Discussion

The results presented in the previous chapters have been discussed in each respective manuscript. The objective of this general discussion is to provide a framework regarding the potential significance of the IL-2 system under normal and pathological conditions within the nervous system. Furthermore, the possible involvement of IL-2 in neuropathological processes is discussed.

6.1 Interleukin-2 in the brain

Observations of the distribution of IL-2 immunoreactivities in the normal rodent brain point to the nervous system as a potential source of IL-2. The discrete distribution of IL-2 immunoreactivities suggests a preferential presence in areas like the hippocampus and the frontal cortex (Chapter 2) that suggests particular functional roles for IL-2 in these regions. Closer examination to localize the IL-2 immunoreactivities by electron microscopy indicates that most of the immunoreactivity is associated to neuronal elements (Villemain *et al.*, 1990). Furthermore, it has also been reported that IL-2 mRNA is detected in the nervous system, strengthening the argument that the brain is a source of IL-2 (Eizenberg *et al.*, 1995). In fact, Eizenberg *et al.* (1995) reported that IL-2 mRNA was detected by Northern blot in rat cortical astrocytic cultures, as well as in human foetal and adult brains (cf. Introduction). Taken together, these results collectively identify the normal brain as capable of IL-2 synthesis. How the brain releases IL-2 under normal physiological conditions will the subject of future investigation.

The present observations regarding IL-2 immunoreactive localization in the brain open new research directions. In view of the age-related increase in IL-2-like bioactivities in the normal rat brain (Nieto-Sampedro and Chandy, 1987) and of the increased IL-2 immunoreactive materials in the hippocampus of Alzheimer's-diseased brains (Araujo

and Lapchak, 1994), it would be important to ascertain whether this increased presence of IL-2 is a result of: 1) increased permeability in the course of aging of the blood-brain barrier allowing for greater than normal penetration of blood-borne IL-2 or 2) increased synthesis of central IL-2. In this regard, Kleine and Hackler (1994) reported that there is an increased, non-specific blood-brain barrier permeability to small and large proteins in normal human elderly individuals. This increased accessibility of the brain to circulating peripheral agents would presumably allow proteins like IL-2 to gain access to the central nervous system and exert its effects in the brain. However, it seems unlikely that increased synthesis of peripheral IL-2 that has penetrated the blood-brain barrier be responsible since Doria and Frasca (1994) provided evidence that spleenocytes from aged mice produced less IL-2 than those from young animals. Additionally, Xu *et al.* (1993) have reported that T lymphocytes from healthy elderly individuals (av. age 76 years) produced less IL-2 than young adult controls (av. age 25.4 years). They demonstrated that this decrease in IL-2 secretion is a consequence of decreased IL-2 mRNA expression in T lymphocytes rather than reduced numbers of IL-2 mRNA-producing T lymphocytes.

In contrast to the observations of Araujo and Lapchak (1994), Hofman *et al.* (1986) did not detect any immunocytochemical staining of IL-2 in the normal or Alzheimer's-diseased brains, whereas they observed high levels of IL-2 in brains from patients with multiple sclerosis. Similarly, Woodroofe *et al.* (1986) observed IL-2 immunostaining in multiple sclerosis brains, but not in normal brains. Future studies will have to employ other approaches, such as looking at the IL-2 mRNA levels and expression using PCR, to resolve these apparent controversies on the genuine presence of IL-2 in the normal and pathological human brain

Studies targeting the IL-2 mRNA in the brain should help to resolve several issues. Firstly, information derived from *in situ* hybridization in the normal brain should reveal the cellular localization of the IL-2 message. Secondly, this approach will provide new information on how closely the IL-2 immunoreactive profile in the brain matches with

that of its message. Mismatches may arise in areas where there is a high degree of uptake of peripheral IL-2 or in projection neurons, the mRNA being mostly localized in perikarya, while the translated protein is mostly present at the level of the terminals. Thirdly, this approach can provide some answers regarding the level of expression of IL-2 between normal and diseased brains, and the cell types bearing the changes in levels of the message and/or protein.

6.2 Interleukin-2 receptors in the brain

The expression of IL-2 receptor subunits in the normal brain raises questions concerning the nature of brain IL-2 receptors. It is well established that in the immune system, the IL-2Rα subunit, unlike IL-2Rß and γ, is not detected under resting conditions in T cells, but its expression can be induced by stimulation (Uchiyama *et al.*, 1981; Robb *et al.*, 1981; Taniguchi and Minami, 1993). In the central nervous system, observations on the presence of IL-2Rα by Western blot in the normal pituitary (Smith *et al.*, 1989) and on the distribution of the IL-2 receptor as revealed by immunoautoradiography using anti-IL-2Rα (Lapchak *et al.*, 1991) and by receptor autoradiography using radiolabelled IL-2 (Araujo *et al.*, 1989) have been reported. Moreover, Shimojo *et al.* (1993) reported the presence of IL-2Rα mRNA in cortical neuronal cultures from the rat brain while Petitto and Huang (1995) demonstrated the presence of IL-2Rα mRNA in the normal murine brain. In addition, Otero and Merrill (1995) have recently shown that the IL-2Rα mRNA was present in the human foetal spinal cord. It is apparent that either IL-2Rα mRNA expression is regulated differently in the brain (constitutively) compared to lymphocytes (induced only), or as suggested by Petitto and Huang (1995), there may be an additional brain-specific IL-2Rα-like subunit that differs slightly in structure from the lymphocytic IL-2Rα subunit. This observation is further supported by evidence indicating that IL-2Rα gene transcripts of different sizes are expressed in the CNS and periphery (Miller *et al.*, 1985; Page and Dallman, 1991).

The presence of IL-2Rα in the brain, together with the constitutive expression of central IL-2Rß (Petitto and Huang, 1994) and possibly IL-2Rγ (Otero and Merrill, 1995) subunits, indicates that the brain expresses the necessary complement of subunits such that central IL-2 receptors are in a functional state that enables the brain to use and respond readily to IL-2. The discrete pattern of distribution of the IL-2R in selected areas of the brain such as the hippocampus and the frontal cortex (Chapter 2) endows these areas with the ability to respond to IL-2.

Thus far, IL-2 receptor binding has provided important information regarding the IL-2 receptor as a whole, and to a limited extent, its receptor subunit composition. IL-2Rα immunochemical revelation has provided evidence on the presence and levels of this subunit in the CNS (present results; Saneto *et al.*, 1987; Lapchak *et al.*, 1991). Studies of IL-2 receptor expression in the central nervous system by molecular approaches targeting the IL-2Rα, ß and γ mRNA subunits will hopefully provide additional details on their respective loci and regulation. One would expect that the expression of each subunit is selectively distributed in the central nervous system, possibly regulated according to the level of upstream elements of each subunit's gene.

There are many reasons to investigate IL-2 receptor subunit mRNA expression in the CNS. Foremost are the differences in IL-2Rα mRNA expression in both the brain and the immune system. Variations in IL-2 receptor subunit mRNA expression following selected insults or stimulations may be expected. Luber-Narod and Rogers (1988) observed the presence of IL-2R immunoreactivities in both normal and Alzheimer's-diseased brains, while Lapchak and Araujo (1994) reported an increased level of IL-2 receptor binding in human Alzheimer's-diseased hippocampal homogenates. It would now be of interest to establish whether all three known IL-2 receptor subunits are uniformily increased or differentially regulated in this neuropathological condition. In addition, it would be equally important to verify differences in cellular expression with respect to cell type and brain area. These considerations are relevant because in the

immune system, IL-2Rß and γ subunits are shared by other interleukins, such as IL-15 (Giri *et al.*, 1993; Bamford *et al.*, 1993) and that IL-2-deficient mice have normal immune responses, suggesting that other interleukins, such as IL-15, are able to compensate for this deficiency by presumably acting on IL-2-like receptors (Kündig *et al.*, 1991; Schörle *et al.*, 1993).

In contrast to observations by Luber-Narod and Rogers (1988) and Araujo and Lapchak (1994) however, Hofman *et al.* (1986) observed IL-2 receptor immunoreactivities in the cortical white matter of brains from patients with multiple sclerosis, but not in brains from patients with Alzheimer's disease. Thus it is uncertain whether these discrepancies result from differences in the studied brain area or in the level of immune activity of the neurodegenerative processes. It is likely that molecular approaches may help in resolving these discrepancies. For example, investigations of the IL-2 receptor subunit messages may provide a better understanding of the multiple events that influence IL-2 receptor levels and distribution in the nervous system.

Recently there has evidence indicating that IL-15 mRNA can be detected in cultured human brain cells (Lee *et al.*, 1995). In addition to common receptor elements between IL-2 and IL-15, the IL-15 receptor contains an additional subunit, termed IL-15Rα, whose gene is closely linked to that of IL-2Rα (Anderson *et al.*, 1995). It is of interest to note that IL-15Rα mRNA has also been detected in cultured human brain cells (Lee and Kim, 1996). It remains to be determined the extent of the overlap, if any, between IL-2 and IL-15 in terms of cytokine and receptor distribution and localization, and effects in various areas of the brain.

6.3 Neurochemical effects of interleukin-2 *in vivo*

Beyond establishing a concise profile of IL-2 and its receptor subunits in the brain under normal and altered conditions, functional approaches may reveal the central

neuronal systems that are responsive to IL-2. The results of this thesis indicate that the cholinergic system in brain areas containing relatively high levels of IL-2R are able to respond to IL-2 *in vitro*. Ultimately however, *in vivo* studies will be needed to extend these results. Following the introduction of IL-2 as a therapeutic agent for the treatment of selected cancers, surprisingly very little information has been reported regarding its central side effects. Emerging details reported by Denicoff *et al.* (1987, 1989) provided the first evidence that peripheral IL-2 can affect cognitive functions. Thus to establish the significance of the *in vitro* effects of IL-2 (for example on ACh release), detailed *in vivo* release studies will be required.

In that regard, it would be important to test whether central or systemic (or both) IL-2, following single or multiple intermittent injection or continuous infusion have any effects on the brain. For example, Ellison *et al.* (1990) showed that a single infusion of IL-2 was more toxic than a 5-day thrice daily infusion of IL-2. Thus, potential differences in response to IL-2 arising from the regimen of IL-2 administration may provide additional insights to the mechanisms involved in the expression and manifestation of a particular IL-2 response.

IL-2 has been shown to modulate the release of dopamine in striatal and hypothalamic slices (Lapchak, 1992), and hippocampal and cortical ACh in the present thesis. It would now be critical to establish if similar effects are observed *in vivo* and if they apply to other transmitters. For example, the present studies provide evidence to suggest that GABA may play a role in mediating the inhibitory action of IL-2 on the hippocampal acetylcholine release. Consequently, it will be important to establish whether IL-2 has any direct effect on the release of GABA in the hippocampus. In addition to transmitter release, there is a cascade of events preceding release that may be a target for IL-2. These include transmitter content, changes in activities of their synthetic enzymes and changes in the level of expression of these enzymes. For example, even in the absence of any direct effect on the release of a given neurotransmitter, IL-2 could modify the biosynthesis, eventually leading to altered

release under chronic conditions. Moreover, given the chronic nature of IL-2 treatment in cancer patients, it would be important to determine whether any of the effects of IL-2 on the brain are reversible or permanent and if these are dose- and time-dependent.

The present thesis also provide, to our knowledge, the first evidence for a putative transduction pathway associated with brain IL-2 receptors. Our results are in agreement with those obtained in peripheral tissues which have shown that IL-2 receptors are coupled to a rather unique PLC-related transduction cascade associated to a PI 3-kinase and the production of myr-DAG via gly-PI (Merida *et al.*, 1990, 1991; Rémillard *et al.*, 1991; Eardley and Koshland, 1991). Moreover, the present observations regarding phosphatidylcholine hydrolysis are in line with similar effects reported for other cytokines such as IL-1 (Rosoff *et al.*, 1988) and IL-3 (Durunio *et al.*, 1989; Whetton *et al.*, 1988) for which atypical signalling events, like PKC activation without inositol lipid turnover and DAG production *via* phosphatidic acid synthesis, have been observed. It remains to be established whether recent observations regarding novel peripheral IL-2 signalling mechanisms involving Jak (Janus kinase) and STAT (signal transducers and activators of transcription) (for a recent review: Ihle *et al.*, 1995) are also observed in the brain.

6.4 Some behavioural effects of interleukin-2

In parallel to neurochemical analyses of effects of IL-2 *in vivo*, there is also a large unexplored area regarding the behavioural and cognitive effects of IL-2. Evidence has suggested the key role of the hippocampus and acetylcholine in learning and cognition (Bartus *et al.*, 1983; Dunnett *et al.*, 1990; Fibiger, 1991; Jarrard, 1993). Hippocampal cholinergic dysfunctions caused by various pharmacological manipulations have been associated to cognitive deficits (Aigner and Mishkin, 1986; Jarrard, 1993). Since IL-2 *in vitro* has been shown to potently modulate hippocampal ACh release, it would be important to establish whether IL-2 can affect learning

behaviours. Recent data have also shown that IL-2 is able to block the induction and maintenance of hippocampal long-term potentiation, a cellular model of learning and memory (Tancredi *et al.*, 1990). In that regard, Nemni *et al.* (1992) and Bianchi and Panerai (1993) reported that systemic IL-2 induced various behavioural effects. Both studies revealed the effects of IL-2 in modifying responses in passive avoidance, a cognitive task partly associated with hippocampal function (Lamour *et al.*, 1989; Wilson *et al.*, 1994). It would now be important to establish if similar effects can be observed in other tasks and in humans receiving IL-2 therapy (Denicoff *et al.*, 1987; Rosenberg *et al.*, 1989).

6.5 Possible role of interleukin-2 in neuropathology

It is unclear whether acute effects of IL-2 on neurological activities share the same mechanisms as events leading to neurodegenerative processes. On the basis of evidence obtained by subchronic exposure to IL-2, Ellison and Merchant (1991) propose that TNFα may be responsible in part for the neurodegenerative effects induced by an IL-2 treatment. Gutierrez *et al.* (1993) and Saija *et al.* (1995) observed that peripheral TNF is able to gain access to the brain by an active transport process. There is also evidence to suggest that IL-2 can induce TNF production in immune cells (Larsson *et al.*, 1993). Both TNFα and its receptors are known to be expressed in the brain (Breder *et al.*, 1993; Kinouchi *et al.*, 1991). Whether TNF and TNF receptor expression can be increased in brain areas containing IL-2 receptors is currently unknown, but would certainly be worthy of investigation.

There are other factors to consider as well. For example, present results suggested that acute IL-2 exerts its neuromodulatory effects on acetylcholine release by at least 2 pathways. Low amounts of released IL-2 stimulate ACh release. However, when IL-2 release is increased possibly as a consequence of an insult, ACh release is dampened, likely leading to cognitive deficits. If local conditions favouring high

concentrations of IL-2 are maintained, it is conceivable that IL-2 will initiate other events such the production of TNFα and TNF receptor expression permitting the establishment of conditions that may trigger a neurodegenerative cascade. In that regard, observations by Hanisch *et al.* (1993) appear to indicate that infusion of IL-2 to the central nervous system under subchronic conditions exhibits neurotoxic effects. Further studies should aim at characterising the cascade of events observed following acute and chronic exposure of the central nervous system to IL-2.

6.6 Conclusion

The present study revealed the presence of IL-2 activities in the brain through the use of anatomical and functional approaches. Our results also indicate that IL-2 acts as a neuromodulator of ACh release in the CNS. These findings are consistent with both basic and clinical observations that identify IL-2 as being able to exert potent effects on selected aspects of neurological functions and provide clues to understand the basis of central adverse effects of IL-2. These may be of significance especially in neurodegenerative diseases in which altered cross-talk between the central nervous system and the immune system is becoming more evident.

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(GENERAL INTRODUCTION AND GENERAL DISCUSSION)

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Contributions to Original Knowledge

1. We have provided evidence supporting a central origin for IL-2. IL-2-like immunoreactive materials are selectively localized in the brain, particularly in areas like the hippocampus and the hypothalamus.

2. Cellular localization of the immunoreactivity indicates that it is preferentially associated to perikaryal elements.

3. In addition, brain areas containing IL-2 immunoreactive materials also contain receptors for IL-2.

4. Brain areas that are known to release ACh and containing IL-2 receptors, such as the hippocampus and the frontal cortex, are sensitive to IL-2 action on ACh release.

5. IL-2 inhibits K^+-evoked release in the hippocampus at nM range, and potentiates it at fM levels.

6. IL-2 also is able to inhibit frontal cortical ACh release at nM range, but is without effect in the parietal cortex and the striatum.

7. IL-2 is sufficiently potent to inhibit an on-going depolarisation of hippocampal ACh release.

8. The IL-2 effect on hippocampal ACh release appears to be selective since other cytokines such as IL-6 are ineffective under identical conditions.

9. Acute exposure to nM range IL-2 does not result in any toxicity as measured by residual effects on ACh release in the hippocampus, frontal cortex, parietal cortex and striatum.

10. Effects of IL-2 on hippocampal ACh release appear to be stimulation-dependent as IL-2 has no effect at both fM and nM ranges under non-stimulated conditions.

11. Effects of IL-2 on hippocampal ACh release also emerge under conditions of sub-maximal veratridine depolarisation.

12. IL-2 potentiation and inhibition of hippocampal ACh release are blocked by an antibody raised against the IL-2 receptor α subunit, indicating that this component of the IL-2 receptor is necessary to mediate IL-2 effects in the brain.

13. IL-2 potentiation of hippocampal ACh release is not sensitive to tetrodotoxin, indicating that this IL-2 action is likely effected at sites directly on the cholinergic terminal.

14. Nitric oxide synthase inhibition does not affect IL-2 potentiation of hippocampal ACh release, indicating that intracellular nitric oxide production is not a component of IL-2 action in this effect.

15. On the other hand, IL-2 inhibiton of hippocampal ACh release is sensitive to tetrodotoxin, indicating that this IL-2 action is mediated at sites indirect to the cholinergic terminal, likely through other mediators.

16. Nitric oxide and opioid peptides, neuromodulators known to affect hippocampal ACh release, do not appear to mediate IL-2 inhibition of ACh release since nitric oxide synthase inhibition and opioid peptide receptor blockade with naloxone are ineffective.

17. Both bicuculline and phaclofen, $GABA_A$ and $GABA_B$ receptors antagonists, block the IL-2 inhibition of hippocampal ACh release, indicating that GABA release and

GABA-mediated inhibition is in part responsible for IL-2 inhibition.

17. Opioid receptor antagonism by naloxone and GABA receptor antagonism by bicuculline and phaclofen are ineffective in affecting IL-2 potentiation, as expected on the basis of the tetrodotoxin data.

18. IL-2 inhibits the basal production of cytidine diphosphodiacylglycerol (CDP-DAG) in the frontal cortex (from 10^{-11} *M*) and hypothalamus (from 10^{-8} *M*), but not in the hippocampus (up to 10^{-8} *M*).

19. However, IL-2 inhibits the carbachol-stimulated production of CDP-DAG in the hippocampus (from 10^{-13} *M*), and in addition, in the frontal cortex, indicating regional differences in IL-2 receptor coupling efficacy in the brain.

20. Decreases in CDP-DAG production by IL-2 are in part due to phospholipase D activation since IL-2 at both 10^{-13} *M* and 10^{-9} *M* results in increases in choline production.

IMAGE EVALUATION
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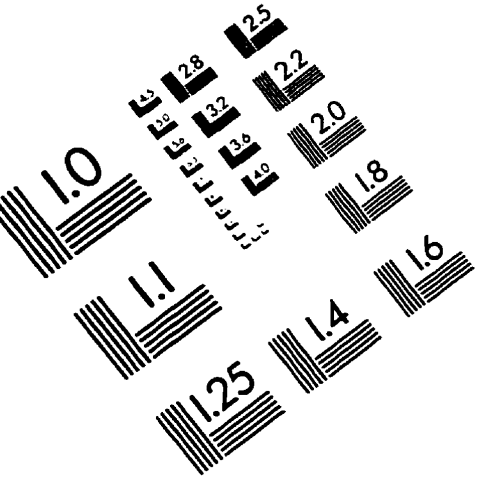

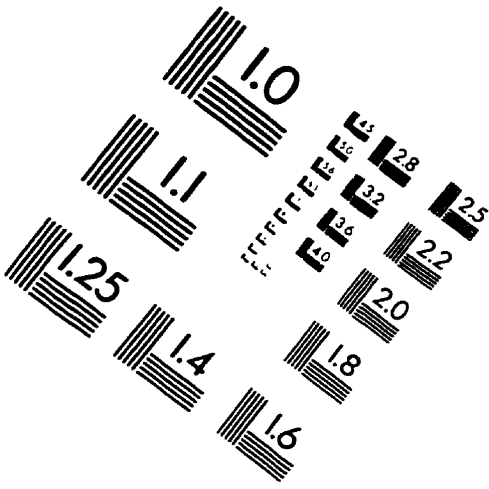

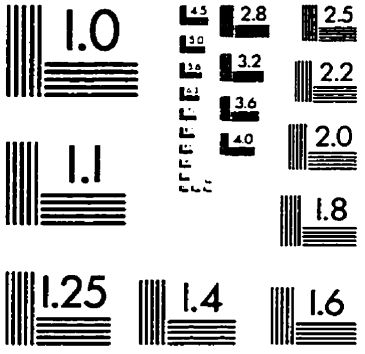

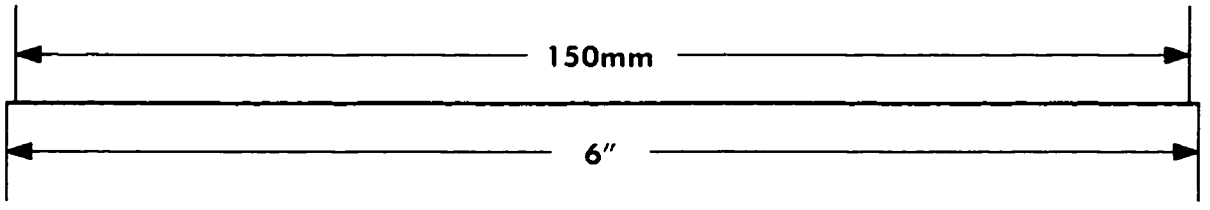

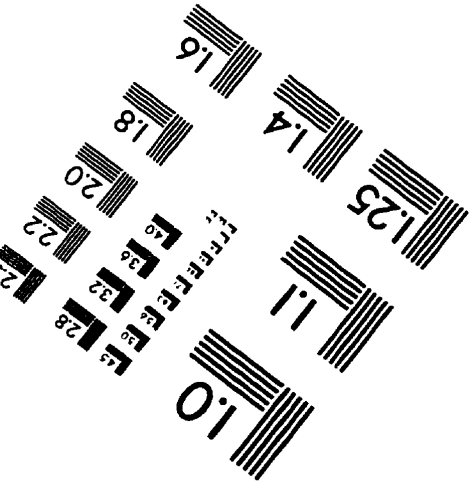

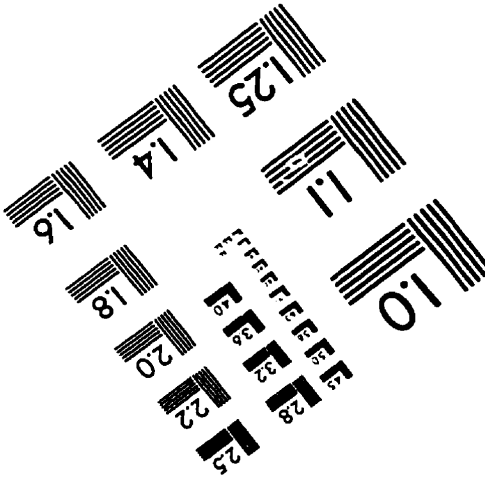

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