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ALPHA-BUNGAROTOXIN SENSITIVE NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS

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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 Master of Science

This thesis is dedicated to my parents, Maro Sahakian and Eduardo J. Komourian. All that I am and all that I ever hope to be, I owe to them...

Press on. Nothing can take the place of persistence. Talent alone will not; the world is full of unsuccessful people with talent. Genius will not; unrewarded genius is almost a proverb. Education alone will not; the world is full of educated derelicts. Persistence and determination alone are omnipotent.

-Calvin Coolidge

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Last, but certainly not least, I would like to thank all my family, especially my mom and dad, for all their love, support and encouragement. "Perfection is expected, but excellence will be tolerated"...

PREFACE

A note on the format of this thesis:

In accordance with the regulations of the Faculty of Graduate Studies and Research, the candidate has the option of including as part of the thesis the text of original papers already published by learned journals. The five paragraphs relating to this option are as follows:

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The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

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The thesis is divided into three experimental chapters that will be referred to as:

Chapter 2:	Characterization of nicotinic receptors in immortalized hippocampal neurons (*Brain Research* **718**, 37-45 [1996]).
Chapter 3:	Stable transfection of nicotinic receptor subunit $\alpha 7$ in rat pituitary GH_4C_1 cells (represents part of *Journal of Neurochemistry* **67**, 145-154 [1996]).
Chapter 4:	c-*fos* mRNA levels in PC12 cells following nerve growth factor and/or nicotine exposure.

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LIST OF ABBREVIATIONS

5-HT	Serotonin
α-BGT	Alpha-bungarotoxin
BAC	Bromoacetylcholine
BDNF	Brain derived neurotrophic factor
B_{max}	Maximum number of binding sites
bp	Base pair(s)
BSA	Bovine serum albumin
CNS	Central nervous system
DMEM	Dulbecco's modified Eagles medium
DMPP	1,1-Dimethyl-4-phenylpiperazinium
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	1,2 Di-2-aminoethoxyethane-N,N,N',N'-tetraacetic acid
FBS	Fetal bovine serum
Fura-2AM	Fura-2 acetoxymethyl ester
$GABA_A$	Gamma-aminobutyric acid
h	Hour(s)
HEK293	Human embryonic kidney 293
K_d	Affinity constant
mAb	Monoclonal antibody
min	Minute(s)
nAChR	Nicotinic acetylcholine receptor
NGF	Nerve growth factor
NMJ	Neuromuscular junction
PC12	Pheochromocytoma 12
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
SCLC	Small cell lung carcinoma
SDS	Sodium dodecyl sulfate
S.E.M.	Standard error of the mean

ABSTRACT

Current evidence indicates there exists two populations of neuronal nicotinic acetylcholine receptors, the α-bungarotoxin (α-BGT) sensitive and α-BGT insensitive receptors. While the latter are thought to be involved in neurotransmission, the physiological significance of the former are not fully understood. However, evidence suggests that the toxin sensitive receptors may mediate trophic functions. The present studies were carried out to develop and validate novel and unique model systems for studying the structure, function and regulation of the nicotinic α-BGT receptor. To this end, two such systems are presented here. First, the immortalized rat hippocampal H19-7 cell line was investigated. This cell line was shown to express a similar set of mRNA encoding for nicotinic receptor subunits as adult rat hippocampus. Further, binding studies using [^{125}I]-α-BGT and other nicotinic ligands demonstrated a similar pharmacological profile between α-BGT sites expressed in H19-7 cells and those described in rat brain and primary cultures. The second model system developed involved the stable transfection of the rat α7 nicotinic receptor subunit into a rat clonal cell line (GH_4C_1) which does not endogenously express any nicotinic receptor subunits. RNase protection assays demonstrated that untransfected cells expressed no nicotinic receptor subunit mRNAs, while their transfected counterparts (α7/GH_4C_1 cells) expressed the α7 subunit message exclusively. Northern analysis confirmed that the size of the α7 message expressed by the transfected cells was similar to the predicted length based on the cDNA used for transfection. The two novel model systems developed and validated in the present studies may serve as an impetus for future studies to delineate the role of the α-BGT sensitive neuronal nicotinic acetylcholine receptor.

RÉSUMÉ

L'évidence actuelle indique qu'il existe deux populations de récepteurs nicotiniques à l'acétylcholine, soit les récepteurs sensibles à l'α-bungarotoxine (α-BGT) et ceux non-sensibles à l'α-BGT. Bien que ces derniers semblent impliqué dans la transmission nerveuse, la signification physiologique des premiers n'est pas complètement comprise. Néanmoins, la littérature suggère que les recepteurs sensible à la toxine sont impliqués dans un rôle trophique. Les études présentes furent effectués afin de développer et valider des systèmes modèles pour étudier la structure, la fonction et la réglementation des recepteurs sensibles a l'α-BGT. Dans ce but, deux modèles sont presentés dans cette oeuvre scientifique. Premièrement, la lignée cellulaire immortalisée de l'hippocampe de rat H19-7 était recherché. C'était demontré que cette lignée cellulaire exprime un ensemble des ARNm qui encodent les élements des recepteurs nicotiniques similaire au rat adulte. Par la suite, les études de liasion utilisant la [^{125}I]-α-BGT et des autres ligands nicotiniques ont démontré un profil pharmacologique semblable entre les sites α-BGT exprimés dans les cellules H19-7 et ceux trouvés dans le cerveau de rat et dans les cultures primaires. Le deuxième modèle a impliqué la transfection stable du sous-unité α7 du récepteur nicotinque dans une lignée cellulaire clonale de rat (GH_4C_1) qui habituellement n'exprime aucune sous-unité du récepteur nicotinique. Les essais *RNase protection* ont demontrés que les cellules non-transfectées n'exprimaient aucune ARNm pour des sous-unités nicotiniques, tandis que les cellules transfectées (cellules α7/GH_4C_1) exprimaient uniquement le messager génétique pour l'α7. Les analyses *Northern* ont confirmé que la grandeur de l'ARMm α7 exprimé par les cellules transfectées correspondaient à la longeur prédit par l'ADNc utilisé pour la transfection. Les deux

systèmes modèles developés et validés dans les études présentes peuvent servir comme une impulsion pour les études futures afin d'essquiser le rôle du récepteur nicotinique à l'acétylcholine sensible à l'α-BGT.

SUMMARY OF CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

In this thesis, two model systems were developed and validated for use in the examination of the structure, function and regulation of the α-BGT sensitive nicotinic receptor. The first such model was the immortalized rat hippocampal cell line H19-7, while the second system involved the stable transfection of the rat α7 nicotinic receptor subunit into a rat clonal cell line (GH_4C_1) which does not endogenously express any nicotinic receptor subunits. The novel findings of this thesis are summarized below.

1. a) RNase protection assays demonstrated that similar to adult rat cortex, differentiated H19-7 cells express mRNA for the α4, α7 and ß2 nicotinic receptor subunits, while their undifferentiated counterparts do not express transcripts for any nicotinic receptor subunit.

   b) Binding studies using [^{125}I]-α-BGT showed that differentiated H19-7 cells express saturable, high-affinity, cell surface α-BGT receptors, while undifferentiated cells express do not. Binding studies also confirmed a similar pharmacological profile between α-BGT sites in H19-7 cells and those described in rat brain and primary cultures.

2. a) RNase protection assays demonstrated that α7 transfected GH_4C_1 cells

express mRNA exclusively for the $\alpha 7$ nicotinic receptor subunit, while their untransfected counterparts do not express transcripts for any nicotinic receptor subunit.

b) Northern analysis confirmed that the size of the $\alpha 7$ transcript in $\alpha 7$ transfected GH_4C_1 cells matched the predicted length based on the cDNA used for transfection.

1.0. INTRODUCTION

The nicotinic acetylcholine receptor (nAChR) is a member of the ligand-gated ion channel superfamily of receptors. The binding of two molecules of the neurotransmitter acetylcholine results in the opening of the gate situated at the pore of the ion channel which subsequently allows for the passage of cations through the channel. Included in this family of receptors are the serotonin ($5\text{-}HT_3$), glutamate, glycine and the gamma-aminobutyric acid ($GABA_A$) receptors. These receptors all share a common defining characteristic: the conversion of a chemical signal (the binding of the ligand to the receptor) to an electrical signal (ionic flow) in the cell, which is referred to as neurotransmission. Nicotinic, glutamate and serotonin receptors are found at excitatory synapses; activation of these receptors results in cation (Na^+ and K^+) flux into the cell which causes cellular depolarization. Upon stimulation of the glycine or gamma-aminobutyric acid receptors, located at inhibitory synapses, anions (Cl^-) are fluxed through the ion channel, thus hyperpolarizing the cell. The nicotinic acetylcholine receptor located at the neuromuscular junction is the focus of many experiments examining the biophysical, biochemical and pharmacological properties of neurotransmitter-gated ion channels. The abundance of this particular receptor in the electric organ of *Torpedo californica* has allowed for the isolation and extensive characterization of this protein and has been the basis for much of our knowledge concerning ligand-gated ion channels.

Nicotinic acetylcholine receptors may be divided into two groups based on their

anatomical localization and distribution. In skeletal muscle, there are neuromuscular junction (NMJ) nicotinic receptors, while neuronal nicotinic receptors are situated on autonomic neurons and adrenal chromaffin cells in the peripheral nervous system as well as on many neurons in the central nervous system (Sargent 1993). The architecture of these two classes of nicotinic acetylcholine receptors are very similar: both NMJ and neuronal receptors are oligomers of homologous subunits arranged in a ring which spans the lipid bilayer, with the central axis forming the channel for ion flow (Unwin 1993). Most of the protein mass is on the synaptic side of the bilayer, which contains the site for neurotransmitter binding (Unwin 1993). The differences between the two groups of receptors lie essentially in the subunit composition and stoichiometry of the different receptors. To date, 16 nicotinic receptor subunit genes have been identified and cloned: alpha1 to 9, beta1 to 4, gamma, delta and epsilon (Luetje et al. 1990; Deneris et al. 1991; Galzi et al. 1991; Role 1992; Papke 1993; Sargent 1993; Elgoyhen et al. 1994). NMJ receptors are pentamers composed of two alpha1 subunits (which both bind the neurotransmitter acetylcholine), a beta1 subunit, a delta subunit and either a gamma subunit (in the case of fetal type receptor, prior to muscle innervation) or an epsilon subunit (seen in the adult type receptor, post innervation). The case is not as well-defined for neuronal nicotinic receptors. Although neuronal nicotinic receptors appear to be composed of two and not four different types of subunits, they exhibit a greater diversity of subunits which include $\alpha 2$ to $\alpha 9$, which may represent ligand binding units, and ß2 to ß4, which are thought to be structural subunits (Luetje et al. 1990; Deneris et al. 1991; Galzi et al. 1991; Role 1992; Papke 1993; Sargent 1993). Although

there is evidence to suggest that neuronal nicotinic receptors may be pentamers (Anand et al. 1991; Cooper et al. 1991), the stoichiometry and composition of these receptors have not been fully elucidated. In addition to the architectural similarities between NMJ receptors and neuronal receptors, there are also similarities between the individual NMJ receptor subunits and their neuronal nicotinic receptor counterparts. Hydropathy plots suggest that all the nicotinic receptor subunits have four significant hydrophobic stretches which may correspond to four putative membrane spanning domains, referred to as M1 to M4 (Deneris et al. 1991; Galzi et al. 1991; Sargent 1993; Unwin 1993). In addition, there is significant homology between the subunits (up to 60%, Sargent 1993) of both receptor subtypes. Furthermore, all subunits have an N-terminal signal sequence, suggesting that the N-terminus is located on the extracellular side of the membrane. Assuming the presence of 4 membrane spanning domains leads to the conclusion that the C-terminus of nicotinic receptor subunits is also located extracellularly (Unwin 1993).

1.1. Neuronal Nicotinic Receptors

Monoclonal antibodies (mAbs) raised against the purified *Torpedo* nicotinic receptor were also found to interact with the skeletal muscle receptor due to the high level of homology, which allowed for the immunopurification, isolation and characterization of the muscle nAChR. In addition, Patrick and Stallcup (1977a; 1977b) reported that nicotinic responses elicited from rat pheochromocytoma cell line PC12 cells were blocked by the antiserum raised against electric organ receptors, thus allowing for the immunopurification of neuronal nAChRs by also using the antibodies raised against

electric organ receptors (Whiting and Lindstrom 1986; Whiting and Lindstrom 1987; Whiting et al. 1987). From the protein sequence information, cDNA sequences encoding neuronal nAChR subunits were determined (Schoepfer et al. 1988). This homology between *Torpedo*, NMJ and neuronal nicotinic receptors suggested to investigators an alternate approach for identifying new neuronal nAChRs: a radiolabelled cDNA probe for the muscle receptor α gene was used under conditions of low stringency to screen PC12 and rat brain cDNA libraries (Boulter et al. 1986; Boulter et al. 1987; Wada et al. 1988). This novel approach led to the identification of the first member of the neuronal nAChR subunit encoding gene family (Boulter et al. 1986). Other cDNAs encoding neuronal subunits were identified by homology cloning as well; in addition, newly discovered cDNAs were being used as probes to further screen the libraries (Sargent 1993).

These newly discovered neuronal genes were classified as either an α or a ß subunit. If the gene product had adjacent cysteine residues at positions 192 and 193 (numbered according to the muscle α1 sequence) and was affinity alkylated by the acetylcholine agonist bromoacetylcholine (BAC) then it was called an α subunit (Clarke 1992; Sargent 1993). The muscle α gene is designated α1 and the eight neuronal α subunits are designated α2-α9 (Boulter et al. 1986 (α3); Goldman et al. 1987 (α4); Wada et al. 1988 (α2); Boulter et al. 1990 (α5); Schoepfer et al. 1990 (chick α7 and α8); Séguéla et al. 1993 (α7); and Elgoyhen et al. 1994 (rat α9)). The neuronal nicotinic subunits lacking the vicinal cysteine residues are referred to as ß (or non-α) subunits. To date, three such subunits have been described, ß2 (Deneris et al. 1988), ß3 (Deneris et al. 1989) and ß4

(Duvoisin et al. 1989).

In addition to the division of nicotinic acetylcholine receptor subunits based on anatomical distribution, these receptors may also be divided into two classes based on their affinity for the snake toxin α-bungarotoxin (α-BGT). α-BGT sensitive nicotinic receptors have nanomolar affinity for the ligand and display a lesser affinity for nicotine, while the toxin-insensitive receptors have a higher affinity for nicotine and do not demonstrate nanomolar affinity for α-BGT (Luetje et al. 1990; Deneris et al. 1991; Galzi et al. 1991; Role 1992; Papke 1993; Sargent 1993). Alpha1, which is found at the NMJ, is α-BGT sensitive, as are the neuronal subunits α7, α8 (avian only) and α9 (described in rats only).

Experiments involving the injection of different neuronal nicotinic receptor subunit cDNAs or cRNAs, either alone or in combination, into *Xenopus* oocytes, suggest that α2-α4 subunits, possibly in some combination with the ß2-ß4 subunits, may form pentameric channels which may mediate the effects of acetylcholine activation and may be involved in synaptic transmission (Luetje et al. 1990; Deneris et al. 1991; Galzi et al. 1991; Role 1992; Papke 1993; Sargent 1993). The α5 subunit does not form functional channels in combination with ß subunits alone; nevertheless, it may participate in the formation of functional channels in conjunction with the α4 and ß2 subunits, forming receptors that are distinct from the α4/ß2 type (Ramirez-Latorre and Role 1995). The α6 subunit remains an enigma in that it neither forms functional channels alone nor in combination with other α and ß subunits, perhaps it combines with as yet unidentified subunit(s) to form functional channels.

Alpha 7, 8 and 9 subunits, which have substantial homology to each other, and less with the other α subunits, are unique in that they are capable of forming channels on their own, without combining with ß subunits (Couturier et al. 1990; Gerzanich et al. 1990; Schoepfer et al. 1990; Gotti et al. 1991; Bertrand et al. 1992; Séguéla et al. 1993; Gotti et al. 1994). As indicated previously, these three subunits encode α-BGT binding subunits. Receptors containing these subunits are quite distinct from receptors formed with α2 to α4 subunits (which may mediate synaptic transmission) in terms of subunit composition, pharmacological properties and distribution in the nervous system. The α-BGT sensitive neuronal nicotinic receptors differ functionally from their insensitive counterparts in that α-BGT generally does not block nicotinic receptor activation (Luetje et al. 1990; Deneris et al. 1991; Galzi et al. 1991; Role 1992; Papke 1993; Sargent 1993). Nevertheless, α-BGT sensitive recordings have been detected recently by different groups in neurons (Alkondon et al. 1992; Vijayaraghavan et al. 1992; Zorumski et al. 1992; Alkondon et al. 1993) and the recent development of rapid recording techniques have led to the findings that α-BGT nicotinic receptors flux calcium and that these responses desensitize very rapidly (Couturier et al. 1990; Séguéla et al. 1993), perhaps explaining why they were difficult to initially detect.

Radioligand binding experiments using α-BGT demonstrated that α-BGT binds with high affinity to neuronal membranes and that this binding could be displaced by nicotinic but not muscarinic ligands (McQuarrie et al. 1976; Morley et al. 1977; Morley et al. 1979). These nicotinic α-BGT receptors have been shown to be distinct from other neuronal nicotinic receptors. Studies using [^{3}H]nicotine, [^{3}H]acetylcholine and

[^{125}I]-α-bungarotoxin have demonstrated that while the binding distribution of the former two radioligands are similar and may represent the same class of nicotine receptors, the binding pattern of α-BGT was distinct from the other nicotinic ligands and represented a different population of nicotinic receptors (Clarke et al. 1985). In addition, *Xenopus* oocyte expression studies demonstrated that chick or rat α7 expression in oocytes result in homo-oligomeric channels that are activated by application of nicotine and blocked by nanomolar concentrations of the snake toxin (Couturier et al. 1990; Gotti et al. 1991; Anand et al. 1993; Séguéla et al. 1993; Gotti et al. 1994). Also, *in situ* hybridization studies, which, using [^{35}S] labelled antisense mRNA probes, have demonstrated that the pattern of radiolabelled α-BGT binding in rodent brain closely overlaps with the α7 gene transcript distribution (Clarke et al. 1985; Séguéla et al. 1993). This suggests that the α7 subunit comprises most, if not all, of the high affinity [^{125}I]-α-BGT sensitive nicotinic receptors. In addition to linking the α7 subunit with α-BGT sensitive nicotinic receptors, the *in situ* work demonstrated that the most abundant neuronal nicotinic receptor subunit gene transcripts in brain are α4 and ß2 and that their overlapping distributions are similar to the binding pattern observed using radiolabelled nicotine and acetylcholine (Clarke et al. 1985; Wada et al. 1989), suggesting that these subunits constitute most of the α-BGT insensitive neuronal nicotinic binding sites observed in brain.

1.2. Role of the α-BGT Sensitive Neuronal Nicotinic Receptor

The fact that α-BGT generally does not block nicotinic receptor activation, combined with the finding that α-BGT binding sites are detected in brain regions devoid of

cholinergic innervation (Hunt and Schmidt 1979), suggests that unlike their toxin insensitive counterparts, α-BGT sensitive neuronal nAChRs do not appear to mediate synaptic transmission. Rapid recording techniques have led to reports describing α-BGT nicotinic receptor responses that flux calcium (Alkondon et al. 1992; Vijayaraghavan et al. 1992; Zorumski et al. 1992; Alkondon et al. 1993; Zhang et al. 1994). The ability of α-BGT receptors to raise intracellular calcium levels by fluxing calcium directly (Séguéla et al. 1993) and by activating voltage-gated calcium channels (Vijayaraghavan et al. 1992; Rathouz and Berg 1994; Zhang et al. 1994) may point to roles for the α-BGT receptor not traditionally associated with nicotinic receptors. Indeed, evidence exists implicating α-BGT receptors in cellular growth, differentiation and development, as well as in the modulation of synaptic transmission in the CNS. In addition, reports have linked α-BGT receptor regulation to various neurological disease states.

Evidence pointing towards a role for the α-BGT receptor in cellular growth, differentiation and development includes the observation that a major increase in choline acetyltransferase (an enzyme which serves as a neuronal marker; it catalyses the conversion of choline to the neurotransmitter acetylcholine) occurs after the appearance of α-BGT receptors (Greene 1976; Fiedler et al. 1987). This suggests a role for the α-BGT receptor in development, namely in the guidance of incoming cholinergic nerve fibres. Furthermore, in rat developing rat sensory cortex, there is a regulated temporal pattern of α-BGT binding sites which parallels synapse formation (Fuchs 1989). In line with this interpretation of a trophic/developmental role for the α-BGT, it has been reported that α-BGT binding sites mediate a role in neurite extension (Chan and Quik

1993; Pugh and Berg 1994). Further evidence establishing a trophic/developmental role of α-BGT binding sites comes from studies which show that α-BGT may block the nicotine receptor mediated increase in cell numbers in neuroendocrine small cell lung carcinoma (SCLC) cells (Codignola et al. 1994; Quik et al. 1994) and that α-BGT receptors regulate apoptosis in chick embryogenesis (Renshaw 1994).

The very ancient lineage of the neuronal α-BGT receptors and their conservation throughout the course of evolution may suggest that they play a significant role in neuronal function (Ballivet et al. 1996). Two recent reports demonstrate that the α-BGT sensitive α7 subunit may be involved in the modulation of synaptic transmission in the CNS (McGehee et al. 1995; Gray et al. 1996). Specifically, Gray et al. (1996) have shown that α-BGT sensitive nAChRs containing the α7 subunit modulate the enhancement of synaptic transmission that occurs in rat hippocampal neurons (where α-BGT sensitive receptors are present in relatively high concentrations) when nicotine is applied.

In addition to the evidence suggesting the α-BGT receptor modulates synaptic transmission and/or serves a trophic role, there are many findings which suggest other possible roles for the α-BGT receptor. The observation that α-bungarotoxin can regulate the mRNA levels of neurotrophic factors such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the α-BGT receptor rich rat hippocampus (Freedman et al. 1993), when taken with the fact that the neurotrophins play a neuroprotective role, may provide evidence for a possible neuroprotective role for the snake toxin binding sites. α-BGT receptors have also been shown to be associated with

various neurological disease states. A possible involvement of the receptor in epileptic seizures may be indicated by the higher levels of [^{125}I]-α-BGT binding sites in a mouse strain possessing a heightened sensitivity to nicotine induced seizures (Miner et al. 1986). In addition, α-BGT receptors have been linked to an auditory sensory gating deficit (Adler et al. 1992; Luntz-Leybman et al. 1992; Adler et al. 1993; Freedman et al. 1995), a hallmark psychophysiological deficit observed in schizophrenia. This may explain why changes in the expression levels of the α7 receptors have been associated with schizophrenia (Adler et al. 1993; Freedman et al. 1995). In conclusion, the α-BGT sensitive neuronal nicotinic subtype may be involved in numerous diverse neurological roles.

1.3. Genetic Manipulation and Neuronal nAChRs

Most of the model systems used for studying the structure, function and regulation of the α-bungarotoxin sensitive neuronal nicotinic receptor may be classified as either involving primary cell cultures (Barrantes et al. 1995; Dávila-García et al. 1995; Didier et al. 1995) or else malignant (neuroblastoma) cell lines (Lukas 1993; Lukas et al. 1993; Puchacz et al. 1994) which endogenously express nAChRs. Both of these approaches have advantages, however, there are also potential drawbacks. Primary cells in culture may be difficult to maintain and do not readily yield sufficient material for biochemical analyses, while tumor cells line usage is limited by its malignant nature. The present studies introduce a novel CNS model system for examining α-BGT nicotinic receptors, the H19-7 immortalized rat hippocampal cell line (Eves et al. 1992). These cells were

shown to express α-BGT receptors having properties similar to the native rat α-BGT receptor (discussed further in Chapter 2). These findings, when taken together with the fact that immortalized cell lines may provide a limitless supply of cells as opposed to primary cells, but yet are not malignant in origin, suggest that the H19-7 cell line may represent a useful CNS model system for examining the structure, function and regulation of α-BGT receptors.

Recently, the advent of molecular techniques has led to two other major approaches for studying neuronal nAChRs, which may be broadly divided as involving either the introduction of genes responsible for nAChR expression into systems that do not endogenously express nAChRs or else involving the removal or silencing of the genetic information that encodes nAChRs in systems that normally express nAChRs. The former approach seeks out new properties that are conferred by expressing nAChRs while the latter aims to determine what changes occur in a system as a result of losing the innate ability to express nAChRs.

The introduction of nAChRs to cells that do not otherwise express them may either be transient (short term) or stable (long term). As mentioned previously, the *Xenopus* oocyte system is a model system used quite extensively to investigate neuronal nAChRs (Couturier et al. 1990; Bertrand et al. 1991; Bertrand et al. 1992; Role 1992; Sargent 1993; Séguéla et al. 1993). This is an example of transient expression, that is, the desired gene products are present only for a limited window of time and the novel genetic information which is introduced to the system does not integrate into the host genome. Protein expression ceases shortly after the introduced genetic material is

degraded by the host's nucleases. Although this system is useful for answering questions regarding receptor structure and composition, as well as for providing information regarding agonist and antagonist sensitivities, the procedure is quite labour intensive; oocytes must be individually injection with genetic material (cRNA or cDNA) and the period of protein expression is short lived (only a few days) (Bertrand et al. 1991). Transient transfection of cells in culture is an alternate approach for examining receptor characteristics. Examples of this approach include the transient expression of both the rat neuronal α3/ß4 nAChR (Wong et al. 1995) and of the human neuronal α4/ß2 nAChR (Monteggia et al. 1995) in human embryonic kidney 293 (HEK293) cells. In addition to examining neuronal nicotinic receptor subtypes, transient transfection is the method of choice for investigating nAChR subunit gene promoters as well as tissue specific transcription factors which regulate nAChR expression. Plasmids are constructed with a reporter gene (luciferase or chloramphenicol acetyltransferase) upstream of a putative promoter or promoter fragment and then the plasmids are used to transiently transfect cells in culture. This approach has helped us to understand the limited spacial and temporal patterns of nAChR expression by identifying critical positive and negative regulatory promoter elements upstream of the coding sequence (Matter-Sadzinski et al. 1992; Bessis et al. 1993; Boyd 1994; Hu et al. 1994; Bessis et al. 1995; Hernandez et al. 1995; Hu et al. 1995; Milton et al. 1995; Yang et al. 1995; Boyd 1996). Furthermore, this technique has led to the discovery of putative tissue specific transcription factors responsible for regulating neuronal nicotine receptor subunit expression (Hu et al. 1995).

An alternate mode of introducing novel genetic material to a cell model system is referred to as stable transfection, that is, the newly introduced genetic information is incorporated into the host genome under selective pressure. Examples of this include the stable expression of $\alpha 4$ and ß2 subunits in M10 fibroblast cells (Whiting et al. 1991), of rat $\alpha 2/ß2$, $\alpha 2/ß4$, $\alpha 3/ß4$ and $\alpha 4/ß2$ receptors in HEK293 cells (Xiao et al. 1996), of human $\alpha 3/ß2$ and $\alpha 4/ß2$ receptors in HEK293 cells (Chavez-Noriega et al. 1996), of the human $\alpha 7$ subunit in human kidney cells (Gopalakrishnan et al. 1995) and the rat $\alpha 7$ subunit in rat pituitary cells (Quik et al. 1996, discussed further in Chapter 3). As the host cells do not normally express the nAChR subunits, studies with stably transfected cells may more clearly define the results of nAChR expression.

Diametrically opposed to the introduction of novel genetic information to a system is the approach of impeding, reducing or even eliminating altogether the expression of preexisting genetic messages; this approach is also a very useful for investigating the role and regulation of neuronal nicotinic acetylcholine receptors. Reduction or elimination of a particular message may be achieved at a cellular level by using antisense oligonucleotides. These short (15-20 base pair) oligonucleotide sequences are complementary to the cellular mRNA, they bind the transcript and the duplex does not allow protein translation to occur, causing a great reduction or elimination of target gene expression (Albert and Morris 1994; Wagner 1994; Wahlestedt 1994). Listerud et al. (1991), Yu et al. (1993) and McGehee et al. (1995) have demonstrated differences in measured currents in cells after antisense treatment.

In scaling up from the cellular level to a whole animal level, gene knockouts have

been used to eliminate target gene expression form the embryo stage throughout adulthood. Embryonic stem cells are used; the targeted gene is allowed to homologously recombine with a deleted version of the gene in order to disrupt the target gene (Picciotto et al. 1995). The first report of a neuronal nicotinic receptor subunit gene knockout was from Picciotto et al. (1995). It was demonstrated that mice containing a ß2 knockout displayed altered behavioural patterns; however, the lack of a particular gene throughout the entire course of development may cause novel compensatory mechanisms to come into play and thus make interpretations of such results problematic at best. More recently, the development of an $\alpha 7$ knockout mouse was reported (Orr-Urtreger et al. 1996). Preliminary results indicate that the brains of these mice appear normal on routine histological examination, show no differences when examined for acetylcholinesterase activity and show normal barrel fields in the somatosensory cortex at age eight weeks. This apparent lack of effect observed upon silencing of the $\alpha 7$ subunit expression may reflect compensatory developmental mechanisms; alternatively, further, closer examination may show cognitive and/or behavioural differences between $\alpha 7$ knock out mice and their wild type counterparts.

1.4. Neuronal Nicotinic Receptors and Immediate Early Genes

c-*fos* is an inducible proto-oncogene (Morgan and Curran 1991) and a member of the immediate early genes (IEG) family (Sheng and Greenberg 1990). Oncogenes are transforming genes; cellular transformation (cancerous growth) occurs when these genes are expressed in an inappropriate fashion, either as a result of mutation or deregulated

expression (Reddy et al. 1988). The normal cellular genes from which oncogenes are derived are more properly referred to as proto-oncogenes, as they represent the progenitors of oncogenes (Bishop 1985). Over the last 10 years, it has been discovered that proto-oncogenes are involved in several aspects of signal transduction and that these same signalling molecules which mediate cell growth are also involved in cellular differentiation and neuronal physiology (Morgan and Curran 1991).

IEGs were first identified in nonneuronal cells as the genes that control the reentry of quiescent cells into the cell cycle and c-*fos* was among the first ones identified (Greenberg and Ziff 1984; Sheng and Greenberg 1990). IEGs share the following characteristics: their expression is low or undetectable in quiescent cells, but is induced within minutes of extracellular stimulation (Sheng and Greenberg 1990). This induction is rapid and transient in nature and is independent of new protein synthesis (Greenberg et al. 1986b). The subsequent return to basal levels (transcription shut-off) however, does depend on new protein synthesis. The mRNAs transcribed form these genes have a very short half-life (only 12 min in the case of c-*fos*) (Greenberg and Ziff 1984; Greenberg et al. 1985; Greenberg et al. 1986a; Sheng and Greenberg 1990). This tightly controlled regulation of IEG expression suggests that IEGs have a critical regulatory role. Additional evidence demonstrating the key regulatory role of IEG expression is provided by the finding that growth factors activate the transcription of IEGs such as c-*fos*, whose mutation or deregulated expression may lead to cell transformation. Indeed, more direct evidence stems from the finding that many IEGs encode for transcription factors (nuclear proteins) that bind target DNA sequences found in the promoter regions of genes (Sheng

and Greenberg 1990).

Many types of stimuli, some relating to the process of differentiation and others linked to neuronal excitation, lead to a transient induction of c-*fos* mRNA and protein (Greenberg and Ziff 1984; Morgan and Curran 1986). mRNA levels peak at 10-15 min post-stimulation and then decline back to basal levels, with the transcripts having a relatively short half-life of about 10 to 15 min (Greenberg and Ziff 1984; Greenberg et al. 1985; Greenberg et al. 1986a). Synthesis of c-*fos* protein follows mRNA expression, and the protein is turned over with a half life of about 2 hours (Koistinaho 1991; Ren and Sagar 1992; Koistinaho et al. 1993; Matta et al. 1993). In PC12 cells, examples of stimuli that induce c-*fos* expression include growth factors such as nerve growth factor (NGF) and epidermal growth factor (EGF) (Greenberg et al. 1985). In addition, depolarizing events such as calcium influx through voltage-gated channels brought about by stimuli such as high extracellular K^+ (Greenberg et al. 1986a; Morgan and Curran 1986) or nicotine (Greenberg et al. 1986a), which acts on the nicotinic receptor and subsequently results in an opening of voltage gated Ca^{+2} channels, also induce c-*fos* expression in PC12 cells. In addition to the exposure of rat cell cultures to nicotine, direct administration of nicotine to rats also results in an increase in c-*fos* protein levels in brain 1-3 h post-injection as detected by immunohistochemistry (Koistinaho 1991; Ren and Sagar 1992; Koistinaho et al. 1993; Matta et al. 1993; Valentine et al. 1996).

It has been demonstrated that α-bungarotoxin (α-BGT) sensitive nicotinic receptors are capable of raising intracellular calcium levels by fluxing calcium directly (Séguéla et al. 1993) and by activating voltage-gated calcium channels (Vijayaraghavan et al.

1992; Rathouz and Berg 1994; Zhang et al. 1994). This calcium influx may be related to a growth/developmental role for the α-BGT receptor as previously stated, such as neurite extension (Chan and Quik 1993; Pugh and Berg 1994). Calcium influx mediated by nicotinic receptors is known to induce c-*fos* expression, which also has a growth/development related role. Although NGF is known to induce IEG expression and evidence exists implicating α-BGT receptors in the regulation of NGF levels (Freedman et al. 1993), it has not been fully elucidated whether the nicotinic receptors involved in c-*fos* activation and regulation are exclusively the α-BGT sensitive nicotinic receptors or whether another population of neuronal nicotinic receptors are also implicated.

1.5. Statement of the Problem

As discussed in the Introduction, many lines of studies have distinguished the α-BGT sensitive neuronal nicotinic acetylcholine receptor from its toxin insensitive counterparts. Evidence for this distinction includes localization studies which demonstrate different distribution patterns for the different populations of nicotinic receptors. In addition, purification studies have shown a different subunit composition for the α-BGT sensitive receptor as opposed to the α-BGT insensitive neuronal nicotinic acetylcholine receptor. Also, genes which encode the α-BGT binding proteins have been cloned and shown to have different properties than genes encoding the α-BGT insensitive receptor subunits.

Various model systems have been developed to study the α-BGT sensitive nicotinic receptor with the aim of elucidating the structure, function and regulation of the toxin-sensitive receptor. While these model systems have provided tremendous insights about the α-BGT receptor, the models used may have potential drawbacks, as discussed in the Introduction. Therefore, one of the objectives of my studies was to develop novel and unique model systems for studying the α-BGT receptor in order to gain further insight into the structure, function and regulation of this receptor subtype. To this end, an immortalized rat hippocampal cell line model system was developed; α-BGT receptors expressed by these cells were shown to have similar properties to the extensively characterized native rat CNS α-BGT receptor. Additionally, the first stable transfection of the rat α7 nicotinic subunit in a rat clonal cell line which does not endogenously express any nicotinic receptor subunits was established. This model system may be used to assess what novel properties of the cells are observed upon α-BGT receptor

expression.

Through the examination of various models, the α-BGT receptor has been linked to a trophic/developmental role, as discussed previously. Nicotinic stimulation of PC12 cells has been shown to transiently increase the level of the proto-oncogene c-*fos*, an immediate early gene which is involved in cellular differentiation and neuronal physiology. As it has not been demonstrated whether α-BGT sensitive or insensitive nicotinic receptors are involved in upregulating c-*fos* levels in PC12 cells, the possibility exists that the α-BGT receptor exerts its trophic effects via proto-oncogene activation. In line with previous evidence linking the α-BGT receptor in a trophic/developmental role and in the scope of investigating the function of the α-BGT receptor, another goal of the present studies was to delineate the role of α-BGT receptors in proto-oncogene induction and regulation.

2.0. CHARACTERIZATION OF NICOTINIC RECEPTORS IN IMMORTALIZED HIPPOCAMPAL NEURONS

A form of this manuscript by J. Komourian and M. Quik is in print in *Brain Research* **718**, 37-45 (1996). Contributions by the authors are as follows: J. Komourian performed all experiments and M. Quik provided supervisory support.

2.1. SUMMARY

Nicotinic acetylcholine receptors, particularly nicotinic α-bungarotoxin (α-BGT) receptors, are present in relatively high concentrations in rat hippocampus. Because of the difficulties encountered in studying receptors using primary cells in culture, especially for biochemical work, we investigated the possibility of using an immortalized cell line from embryonic rat hippocampus (H19-7). RNase protection assays show that α4, α7 and ß2 neuronal nicotinic receptor subunit mRNAs are present in differentiated but not undifferentiated H19-7 cells, while α2, α3, α5 and ß3 subunit mRNAs were not detectable under either condition. In line with these results, the present data demonstrate that the H19-7 cells express cell surface nicotinic α-BGT binding sites, which were maximal after seven days of differentiation in culture. The receptors were saturable, of high affinity (K_d = 1.30 nM and B_{max} = 11.70 fmol/10^5 cells) and had a pharmacological profile similar to that observed for CNS α-BGT receptors. On the other hand, although α4 and ß2 neuronal nicotinic subunit mRNAs were present in differentiated H19-7 cells, no [^{3}H]cytisine binding was observed. Because immortalized cell lines have the advantage that they provide a limitless supply of cells as compared to primary cell cultures, but yet are not malignant in origin, the present results may suggest that the H19-7 immortalized hippocampal cell line represent a useful CNS model system for examining α-BGT nicotinic receptors.

2.2. INTRODUCTION

Neuronal nicotinic receptors exhibit an extensive diversity. To date eleven neuronal nicotinic receptor subunits genes have been identified, including α2 - α9 which encode ligand binding subunits and ß2 - ß4 which may represent structural subunits (Luetje et al. 1990; Deneris et al. 1991; Role 1992; Papke 1993; Sargent 1993; Elgoyhen et al. 1994). Injection of different nicotinic receptor subunit cDNAs or cRNAs, either alone or in combination into *Xenopus* oocytes, indicate that these subunits may combine to form nicotinic receptors (Luetje et al. 1990; Deneris et al. 1991; Role 1992; Papke 1993; Sargent 1993). Current work suggests that α2 to α4, possibly in some combination with the ß subunits (ß2 to ß4), may form pentameric channels (Anand et al. 1991; Cooper et al. 1991) which are labelled with high affinity by radiolabeled agonists and which mediate the effects of acetylcholine activation; these have been designated the α-BGT insensitive nicotinic receptor population. The α5 subunit does not form functional channels in combination with ß subunits alone; however, in the presence of the α4 and ß2 subunits, α5 may participate in the formation of a functional complex (Ramirez-Latorre and Role 1995). The involvement of α6 is less clear since it does not form functional channels either alone or in combination with the ß subunits. Possibly α6 contributes to functional channels in combination with α2 to α4 and as yet unidentified receptor subunits. Alpha 7 and α8, which have substantial homology to each other, and less with the other α subunits, are unique in that they form channels on their own. These latter two α subunits encode the α-BGT binding subunits; receptors containing

these subunits may be quite distinct functionally from those formed from the α2 to α4 gene products (Couturier et al. 1990; Schoepfer et al 1990; Bertrand et al. 1992; Séguéla et al. 1993). The recent newcomer to the nicotinic receptor family α9 also interacts with α-BGT; its localization appears to be restricted to pars tuberalis, nasal epithelium, cochlear hair cells, tongue and some striated muscle (Elgoyhen et al. 1994).

As indicated above, the α-BGT insensitive nicotinic receptors, which contain α2-α4 in some combination with the ß subunits (ß2 - ß4) may mediate nicotinic transmission (Luetje 1990; Deneris et al. 1991; Galzi et al. 1991; Role 1992; Sargent 1993). Nicotinic responses vary tremendously in channel and kinetic properties, as well as in responses to agonists and blockade by antagonists. In line with this functional diversity, nicotinic receptor subunit mRNA distribution in brain is varied and widespread, with α4 mRNA being particularly prominent and ß2 being the most abundant of the ß transcripts (Deneris et al. 1988; Duvoisin et al. 1989; Wada et al. 1989). These two genes have distinct patterns of distribution, but are also present within the same brain region. Flores et al. (1991), using subunit specific antibodies, showed that the ^{3}H-cytisine binding site is an α4ß2 receptor. The above observations, together with oocyte studies which show that the α4ß2 is functional (Deneris et al. 1991; Role 1992; Papke 1993; Sargent 1993), suggest that the α4ß2 nicotinic receptor is the predominant nicotinic receptor in brain.

The α-BGT binding nicotinic receptors, which contain α7 (and/or α8 in chick) and possibly other subunits (Couturier et al. 1990; Gerzanich et al. 1990; Schoepfer et al.1990; Gotti et al. 1991; Alkondon et al. 1993; Anand et al. 1993; Séguéla et al. 1993;

Gotti et al. 1994) are quite distinct from the receptors described above in terms of subunit composition, pharmacological properties, and localization. As well, they also differ in terms of function since α-BGT generally does not block nicotinic receptor activation (Quik and Geertsen 1988; Clarke 1992; Sargent 1993). It now appears that α-BGT sensitive nicotinic responses desensitize very quickly (Couturier et al. 1990; Séguéla et al. 1993), which may explain why they had initially been difficult to detect. The development of more rapid recording techniques has led to several reports describing α-BGT nicotinic receptor responses that flux calcium (Alkondon et al. 1992; Vijayaraghavan et al. 1992; Zorumski et al. 1992; Alkondon et al. 1993; Zhang et al. 1994). Their rapidly desensitizing nature and calcium permeability may further suggest that α-BGT receptors are involved in activities distinct from those traditionally associated with nicotinic receptors. One of these, for which there has been accumulating evidence, is in growth/development (Quik and Geertsen 1988; Clarke 1992; Chan and Quik 1993; Pugh and Berg 1994).

As an approach to identify the molecular composition of CNS nicotinic receptors and determine their function and regulatory properties, a variety of systems/approaches are currently being used including primary cells in culture (Barrantes et al. 1995; Dávila-García et al. 1995; Didier et al. 1995) and neuroblastoma cell lines (Lukas 1993; Lukas et al. 1993). Both of these have advantages; however, there are also potential drawbacks. Primary cells in cultures may be difficult to maintain and do not readily yield sufficient material for biochemical analyses, while tumor cell line usage is limited by its malignant nature. In this study, we investigated the use of an immortalized rat

hippocampal cell line H19-7 as a model system for examining CNS nicotinic receptors. Cells of hippocampal origin were selected because hippocampus contains one of the highest densities of functional α-BGT sites (Alkondon et al. 1992; Zorumski et al. 1992; Alkondon et al. 1993).

2.3. MATERIALS AND METHODS

2.3.1. Materials

The immortalized rat hippocampal cell line (H19-7) was generously provided by Dr. Eva Eves, University of Chicago and Dr. Bruce H. Wainer, Albert Einstein College, NY. [^{125}I]-α-BGT (10-20 μCi/μg), [^{3}H]cytisine (30.1 Ci/mmol) and [α-^{32}P]UTP (800 Ci/mmol) were purchased from DuPont/NEN (Boston, MA). Fura-2AM was obtained from Molecular Probes (Eugene,OR), RNA polymerase SP6 and all restriction enzymes from New England Biolabs (Mississauga, ON), nicotine, α-BGT, carbachol, d-tubocurarine, 1,1-dimethyl-4-phenylpiperazinium (DMPP), atropine, muscarine and bovine serum albumin from Sigma Chemical Co. (St. Louis, MO). Supplies and chemicals for culture were purchased from Gibco/BRL (Montreal, QC).

2.3.2. H19-7 Cell Cultures

H19-7 cells were plated at a density of approximately 10^4 cells/cm^2 and grown in

collagen coated flasks. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin 50 units/ml, streptomycin 50 μg/ml and Geneticin (G418) 250 μg/ml, at 33°C in a humidified atmosphere of 95% O_2 / 5% CO_2. To promote differentiation, the cells were placed at 39°C and the culture medium was changed to DMEM containing 1% FBS, 20 nM hydrocortisone, 0.3 nM triiodothyronine, 0.1 mM putrescine, 20 nM progesterone, 1 pM estradiol, 30 nM $NaSeO_3$, transferrin at 1 μg/ml, insulin at 5 μg/ml, penicillin at 50 units/ml and streptomycin at 50 μg/ml. Cultures were allowed to differentiate for a period of 5-7 days, unless otherwise indicated, with medium being routinely changed every 2-3 days.

2.3.3. RNA Isolation

Total RNA was extracted from differentiated or undifferentiated H19-7 cells, rat cortex or rat hippocampus using a guanidinium isothiocyanate-phenol-chloroform mixture as previously described (Chomcynski and Sacchi 1987) except that a second phenol-chloroform extraction was performed. RNA concentrations were determined by OD_{260}. The integrity of the isolated RNA was assessed by ethidium bromide staining in agarose.

2.3.4. RNA Probes

Riboprobes were generated for rat nicotinic subunit α2 - α5, α7, ß2 and ß3 mRNAs by runoff transcription using RNA polymerase SP6 and appropriate DNA constructs. Probes were transcribed from the pcDNAI/Neo plasmid which contained the full length coding sequence encoding the genes. The α2 probe, generated by digestion of the cDNA

containing plasmid with *Stu*I, corresponds to the region between base pairs 1141 and 1546 (Wada et al. 1988). The α3 probe was produced by digestion with *Hinc*II and represents the region between base pairs 1188 and 1707 (Boulter et al. 1986). The α4 probe, generated by digestion with *Bsa*H1, corresponds to the interval between base pairs 1726 and 2111 (Goldman et al. 1987). The α5 probe was produced by digesting the cDNA with *Hind*III; the probe represents the span from base pair 945 to 1424 (Boulter et al. 1990). *Pvu*II was used to digest the α7 cDNA and the resulting probe corresponds to the region between base pairs 1617 and 1974 (Séguéla et al. 1993). The ß2 probe was also produced using *Pvu*II digestion and corresponds to base pairs 1206 to 2017 (Deneris et al. 1988). ß3 probe was formed by digestion with *Sph*I and it corresponds to base pairs 1377 to 2207 (Deneris et al. 1989). Antisense probes were transcribed using carrier free [α-^{32}P]UTP (800 Ci/mmol). Unprotected probe size and protected size (in base pairs) are: α2, 453 and 405; α3, 567 and 519; α4, 433 and 385; α5, 527 and 479; α7, 405 and 357; ß2, 859 and 811 and ß3, 878 and 830.

2.3.5. RNase Protection

RNase protection assays were performed as described (Krieg and Melton 1987). Briefly, 2 or 10 μg of total RNA were allowed to hybridize with a molar excess (2.50 x 10^5 cpm) of ^{32}P-labeled antisense RNA probe for 5 min at 85°C in 80% formamide, 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer pH 6.4, 0.4 M NaCl and 1 mM ethylenediaminetetra-acetic acid (EDTA). Hybridization was continued at 60°C overnight (14-18 h). Single stranded RNA was subsequently digested over a 30

min period at 37°C by adding 2 units RNase T1. The RNase was then inactivated by incubation with 10 mg/ml proteinase K and 10% SDS for 15 min at 37°C. Any protected RNA duplexes present were extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with absolute ethanol at -20°C and dissolved in 30 μl RNA loading buffer (80% formamide, 1 mM EDTA, 0.01% xylene cyanol and 0.01% bromophenol blue). Samples were then electrophoresed at 230 V for 3.5-4 h in a 5% acrylamide gel containing 8 M urea. The gel was dried on Whatman 3 MM paper and exposed to Hyperfilm ECL film for 36-72 h at -80°C, using an intensifying screen.

2.3.6. [^{125}I]-α-BGT Binding

Binding assays were performed using intact cells or cell membranes. For intact cell binding studies, 10^5 cells (per well) were washed 3 times with 10 ml of DMEM containing 2 mg/ml bovine serum albumin (BSA). Cultures were subsequently preincubated for 30 min at 37°C with varying concentrations of the indicated drugs, followed by incubation for 60 min at 37°C with the indicated concentration of labeled [^{125}I]-α-BGT. Cells were then washed 4 times over a 40 min period with DMEM containing 2 mg/ml BSA, scraped from the dish and the radioactivity counted using a gamma counter.

For cell membrane studies, cells were scraped from the dish and homogenized by polytron for 15 s in 10 mM Tris, pH 7.4 containing 10 mg/ml BSA (Tris buffer), followed by a 20 min centrifugation of the suspension at 20,000 x g at 4°C. The supernatant was discarded, the pellet resuspended in buffer and the homogenization by

polytron and the centrifugation were repeated. The pellet was resuspended in the 10 mM Tris buffer at a concentration of 10^5 cell membranes per 160 μl aliquot. The membranes were preincubated for 30 min at 37°C with 20 μl of buffer or 10^{-4} M d-tubocurarine, which served to assess non specific binding. A 20 μl aliquot of [^{125}I]-α-BGT was then added at the indicated concentration and the membranes were further incubated for 60 min at 37°C. The membranes were washed by centrifugation for 5 min at 12,000 x g, followed by aspiration of the supernatant. The pellet was resuspended in 1 ml of the Tris buffer. The wash procedure was repeated 3 more times and the radioactivity was counted using a gamma counter. Centrifugation was used in the present experiments to separate free and bound radioactivity; other studies in the laboratory indicate that similar results are obtained when a filtration assay is used.

2.3.7. [^{3}H]Cytisine Binding

Binding of [^{3}H]cytisine to rat brain cortical membranes was done as previously described at a final tissue concentration of 5 mg/sample (Pabreza et al. 1990). For binding to H19-7 cell membranes, cells were scraped off the dish in DMEM containing 2 mg/ml BSA and 20 volumes of ice cold buffer (50 mM Tris pH 7.0, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2.6H_2O$, 2.5 mM $CaCl_2.2H_2O$) added. After homogenization with a polytron for 15 s, the suspension was centrifuged for 10 min at 40,000 x g at 4°C. The supernatant was discarded, the pellet resuspended in buffer, homogenized by polytron and the centrifugation repeated. The final pellet was resuspended at a concentration of 10^5 cell membranes/160 μl buffer, and either 20 μl of buffer or 10 μM

nicotine was added, as well as various concentrations of [^{3}H]cytisine. The membranes were incubated for 75 minutes on ice. The reaction was stopped by addition of 1 ml buffer followed by filtration through GF/C filters, and 3 washes with 4 ml ice cold buffer. The filters were placed in a liquid scintillation vial and 5 ml of scintillation fluid (Formula 963, DuPont) was added. The radioactivity was counted using a beta counter.

2.3.8. Fura-2AM Assay

H19-7 cells were differentiated as described for 6-7 days. The mobilization of intracellular calcium was measured using fura-2 acetoxymethyl ester (fura-2AM, Molecular Probes) as previously described (Grynkiewicz et al. 1985). Briefly, $5x10^6$ cells were removed from the flask by treatment with 2 ml trypsin/EDTA. The cells were then centrifuged for 5 min at 800 rpm and the supernatant aspirated. The cells were then resuspended in 5 ml assay buffer (118 mM NaCl, 4.6 mM KCl, 20 mM HEPES, 10 mM d-glucose and 1 mM $CaCl_2$), followed by the addition of 4 μM of the calcium chelating dye fura-2AM. Cells were incubated for 30 min at 37°C with shaking to ensure proper mixing with the dye. Samples were then centrifuged at 800 rpm for 5 min and resuspended in 30 ml assay buffer. This wash procedure was repeated 3 times to ensure removal of any residual dye not taken up by the cells. After the final wash, the cells were resuspended in 2 ml assay buffer. Assays were carried out in a cuvette equipped with a magnetic stirrer in a Perkin Elmer Luminescence Spectrophotometer LS50 (excitation: 340/380 nm; emission: 540 nm). Drugs (nicotine or KCl) were added directly to the cuvette in aliquots of 20 or 30 μl. For calibration purposes, R_{max} (total

Ca^{+2}) and R_{min} were determined by lysing the cells using 20 μl 0.1% Triton X-100 followed by addition of 20 μl 10 mM EGTA to chelate the calcium, respectively.

2.3.9. Data Analysis

To determine IC_{50} values, a weighted, non-linear regression curve fitting program (RADLIG, Biosoft, UK) was used, with the Marquardt-Levenberg modification of the Gauss-Newton technique. The model which minimized the residual sum of squared deviations was selected as the one best representing the obtained data.

2.4. RESULTS

2.4.1. H19-7 Cell Cultures

Undifferentiated cells were flat and polygonal (Fig. 2.1A). Differentiation resulted in cell elongation, cell body thickening and the development of processes. This was first noticeable 4 days after exposure to differentiating conditions, with a readily identifiable neuronal morphology by day 7 of differentiation (Fig. 2.1B-D). Varying the fetal bovine serum (FBS) content of the differentiation medium from 0 to 10% showed that 1% FBS was optimal for cell differentiation; 0% FBS led to cell death, while 5 to 10% FBS prevented optimal differentiation. Binding studies with [^{125}I]-α-BGT confirmed that differentiation medium containing 1% FBS resulted in the highest levels of binding.

2.4.2. RNase Protection Assay

To determine which nicotinic receptor subunit RNAs are expressed in the H19-7 cell line, RNase protection was used (Fig. 2.2). Differentiated H19-7 cells expressed mRNA for the α4, α7 and ß2 subunits, as was observed for rat hippocampus (data not shown) and rat cortex (Fig. 2.2). Both the cells and the brain regions displayed the same size protected probes for α4, α7 and ß2. Rat cortex RNA, which was used as a positive control, protected all of the probes used, while rat liver RNA (data not shown) and yeast tRNA, which were used as negative controls, failed to protect any of the probes, thus eliminating the possibility of non-specific probe self-protection. The probe length for α4 was 433 bp, with a protected band size of 385, for α7 405 bp, with a protected band size of 357 bp and for ß2, 859 bp with a protected band size of 811. The difference between probe size and protected band length was the distance from the SP6 initiation site to the 3' end of the subunit cDNA which does not hybridize with the target RNA and was therefore not protected from subsequent RNase digestion.

Thus, cortex contains transcripts for all of the mRNA examined (α2, α3, α4, α5, α7, ß2 and ß3). Differentiated H19-7 cells displayed signals for α4, α7 and ß2 subunits but not α2, α3, α5 and ß3, while undifferentiated H19-7 cells did not show a positive signal for any subunits tested.

2.4.3. α-BGT Binding Assay

α-BGT binding studies showed very little binding (< 0.3 fmol/10^5 cells) of the radioligand to undifferentiated H19-7 cells. On the other hand, differentiation of the

cells over a several day time course (Fig. 2.3) led to the expression of α-BGT binding sites, with maximal binding on day 7; day 5 to 7 cells were therefore used in all further assays.

Saturation analysis of [^{125}I]-α-BGT binding to H19-7 cells showed that the receptors were saturable and of high affinity (Fig. 2.4) with a K_d of 1.30 ± 0.05 nM and a B_{max} of 11.70 ± 1.73 fmoles/10^5 cells (n=3). To determine whether the α-BGT receptors exhibited a nicotinic profile, the effects of various nicotinic and muscarinic agonists (Fig. 2.5A) and antagonists (Fig. 2.5B) were examined on specific [^{125}I]-α-BGT binding to H19-7 cells. Nicotinic but not muscarinic ligands inhibited the binding of ^{125}I-α-BGT to the H19-7 cells. For the antagonists α-BGT and d-tubocurarine, a one site model best fit the data, with IC_{50} values as follows: α-BGT 48 ± 3 nM (n=4) and d-tubocurarine 6.4 ± 0.4 μM (n=3). For the agonists, the data was better fit to a two site model. The IC_{50} values were as follows: carbachol 5.4 ± 0.4 μM and 180 ± 15 μM (n=3), DMPP 0.30 ± 0.04 μM and 83 ± 7 μM (n=3) and nicotine 0.32 ± 0.03 μM and 38 ± 4 μM (n=3).

The studies described above used cell membranes. To determine whether the α-BGT receptors in the H19-7 cells were located on the cell surface, intact cells were exposed to varying concentrations of cold α-BGT for 1.5 h to block any surface receptors and render them inaccessible to the radiolabeled α-BGT. The binding assay was then carried out as described, using either intact cells or lysed cells. Inhibition curves for total binding sites and external (surface) binding sites were similar (Fig. 2.6A). The results show that the majority (92.6 ± 2.4%, n=3) of receptors are located extracellularly (Fig.

2.6B).

2.4.4. Cytisine Binding Assay

H19-7 cell membranes did not display specific [^{3}H]cytisine binding using 0.5 to 10 nM of the radiolabeled ligand. On the other hand, specific binding of the radiolabel was observed to rat brain membranes with a K_d of 0.70 nM and a B_{max} of 2.9 fmol/mg brain tissue as has previously been reported (Pabreza et al. 1990).

2.4.5. Fura-2AM Assay

The fura-2AM assay was used to determine whether nicotinic receptor stimulation of H19-7 cells resulted in the mobilization of intracellular calcium. Stimulation with 30, 100 or 300 μM nicotine did not result in a measurable increase in intracellular Ca^{+2} levels above basal. On the other hand, exposure of the cells to 50 mM KCl resulted in a rapid and robust elevation of intracellular Ca^{+2} concentration to 31 $\pm$ 5 nM (n=4) above basal, confirming that the cells were viable.

2.5. DISCUSSION

RNase protection assays revealed the presence of α4, α7 and ß2 transcripts in differentiated H19-7 cells. Previous studies (Deneris et al. 1989; Duvoisin et al. 1989; Wada et al. 1989; Boulter et al. 1990; Séguéla et al. 1993) using *in situ* hybridization

had shown that adult rat hippocampus contained mRNA for α2, α3, α4, α5, α7 and ß2 and we also identified most of these subunit mRNAs (ß2 subunit not tested for in hippocampus) using RNase protection. The absence of some of these nicotinic receptor subunit mRNAs in the immortalized neurons may relate to the fact that H19-7 cells are only capable of a limited maturation (Eves et al. 1992). Alternatively, the mRNA may be present but below the level of detection of the present assay; some of these transcripts may not be well maintained in culture. Indeed, 10 μg of H19-7 cell total RNA were needed to observe the same band intensity as observed with only 2 μg of rat hippocampal or rat cortex total RNA. Previous studies by other have shown that cells in culture maintain a lower level of transcript expression than intact tissue (Corriveau and Berg 1994).

In line with our detection of α7 subunit transcripts in H19-7 cells, these studies demonstrate the presence of saturable, high affinity cell surface α-BGT sensitive receptors in the H19-7 cell line. The characteristics of the α-BGT receptors present are similar to those previously described for the rat brain receptor (Morley et al. 1977; Speth et al. 1977; Marks and Collins 1982). The K_d of 1.30 nM obtained here is similar to that reported by Barrantes et al. (1995) for the α-BGT receptor in primary rat hippocampal cultures (0.6 nM) and Didier et al. (1995) in primary cultures of cerebellar granule cells (1.5 nM). The present B_{max} value of 11.70 fmol/10^5 cells or 7.0 x 10^4 binding sites/cell is also similar to the value of 4.2 ± 0.3 x 10^4 binding sites/cell obtained for hippocampal neurons in culture (Barrantes et al. 1995). The inhibition curves for nicotinic antagonists in the present study indicated a single population of

receptor sites and IC_{50} values obtained were similar to those previously reported for the rat brain receptor (Morley et al. 1977; Speth et al. 1977; Marks and Collins 1982). Inhibition curves for the nicotinic agonists carbachol, DMPP and nicotine were biphasic, indicating the presence of two classes of receptors of different affinities. These findings may reflect the presence of an immature form of the receptor; Broide et al. (1994) have shown that embryonic α-BGT receptors exhibit a lower affinity for agonists. Meeker et al. (1986) have also shown two different affinities for agonists in competition studies.

Two site models for α-BGT binding have been reported (McQuarrie et al. 1976; Salvaterra and Mahler 1976; Lukas 1984; Meeker et al. 1986); however, Scatchard analysis seems to preclude the possibility of two different binding sites in H19-7 cells. Alternatively, there may be two different α-BGT sites in the immortalized cell line having the same affinity for α-BGT but with different affinities towards the nicotinic agonists. Other reports indicate a one site system in brain (Morley et al. 1977; Speth et al. 1977; Marks and Collins 1982; Didier et al. 1995) and primary rat hippocampal cultures in particular (Barrantes et al. 1995) which is in agreement with the saturation experiments done with the H19-7 cells.

Although [^{125}I]-α-BGT binding sites were readily detected in immortalized neurons in culture, [^{3}H]cytisine binding sites were not measurable despite the presence of mRNA for α4 and ß2. [^{3}H]Cytisine binding was observed to M10 cells transfected with α4 and ß2 (Whiting et al. 1991), confirming the ability of this subunit combination to bind the radioligand. Explanations for this apparent discrepancy may include an inability of the cells to transcribe the mRNA or to transport receptor protein to the cell surface.

Alternatively, the [^{3}H]cytisine binding may simply not allow for the detection of possibly a limited number of cell surface receptors; [^{3}H]cytisine binding sites were also not measurable on hippocampal neurons in culture (Barrantes et al. 1995), although detectable binding was observed by Dávila-García et al. (1995). [^{3}H]Epibatidine may represent a more suitable ligand (Houghtling et al. 1994) for receptor detection.

The results here show that cell surface α-BGT binding sites are present on H19-7 cells. Previous studies show that these receptors are capable of fluxing calcium (Couturier et al. 1990; Vijayaraghavan et al. 1992; Séguéla et al. 1993; Pugh and Berg 1994; Zhang et al. 1994). To determine whether nicotinic receptor stimulation in H19-7 cells resulted in intracellular calcium mobilization, the calcium chelating dye fura-2AM was used; however, no response was observed. This may be due to insufficient numbers of receptors per cell. H19-7 cells express 7 x 10^4 binding sites/cell; in contrast, ciliary ganglion neurons in culture, which exhibit small calcium responses, express 1-2 x 10^5 sites/cell (Vijayaraghavan et al. 1992; Rathouz and Berg 1994; Zhang et al. 1994).

The present results show that the immortalized H19-7 cell line expresses α-BGT binding sites with properties similar to the rat hippocampal receptor. In view of the advantages of an immortalized hippocampal cell line, the results may suggest these cells represent a useful CNS model system for examining α-BGT nicotinic receptors.

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FIG. 2.1. Phase contrast photomicrographs of H19-7 cells in culture. A: Undifferentiated H19-7 cells, 100x magnification. B-D: Day 7 differentiated H19-7 cells, 100x magnification.

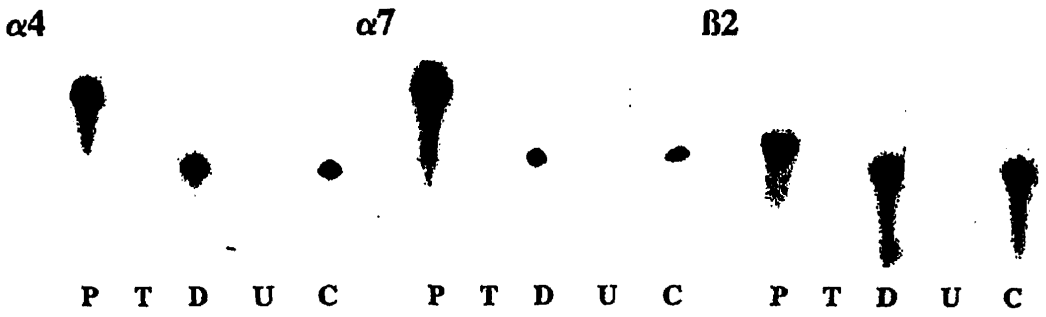
A

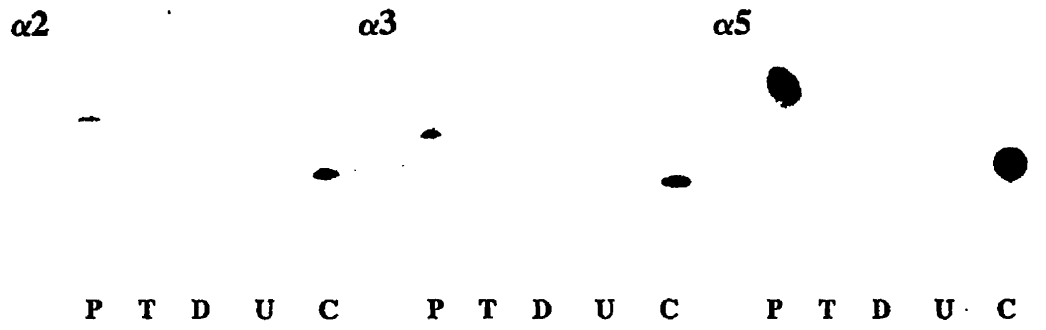
B

C

D

FIG. 2.2. RNase protection assays showing α4, α7 and ß2 transcripts in cultures of differentiated H19-7 cells as well as in rat cortex. Total RNA from H19-7 cells or rat cortex was hybridized with the indicated ^{32}P-labeled antisense probes, digested with RNase T1 treatment and analyzed for protected products. P: probe lane, T: yeast tRNA, D: differentiated H19-7 cell total RNA, U: undifferentiated H19-7 cell total RNA and C: rat cortex total RNA. Two μg total RNA was used for rat cortex RNA; 10 μg of total RNA was used for differentiated and undifferentiated H19-7 cells as well as yeast tRNA.


α4
α7
ß2
P T D U C P T D U C P T D U C


α2
α3
α5
P T D U C P T D U C P T D U C

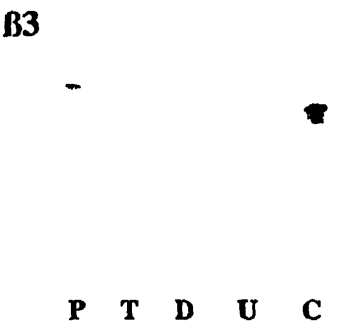

ß3
P T D U C

FIG. 2.3. Time course of development of [^{125}I]-α-BGT binding in differentiated H19-7 cells. Binding was assessed after culturing the cells for the indicated number of days in differentiation medium. Where values are depicted with an error bar, they represent the mean $\pm$ S.E.M. of 3 experiments done in triplicate. Where the S.E.M. is not depicted, the values represent the results from a single experiment, done in triplicate.

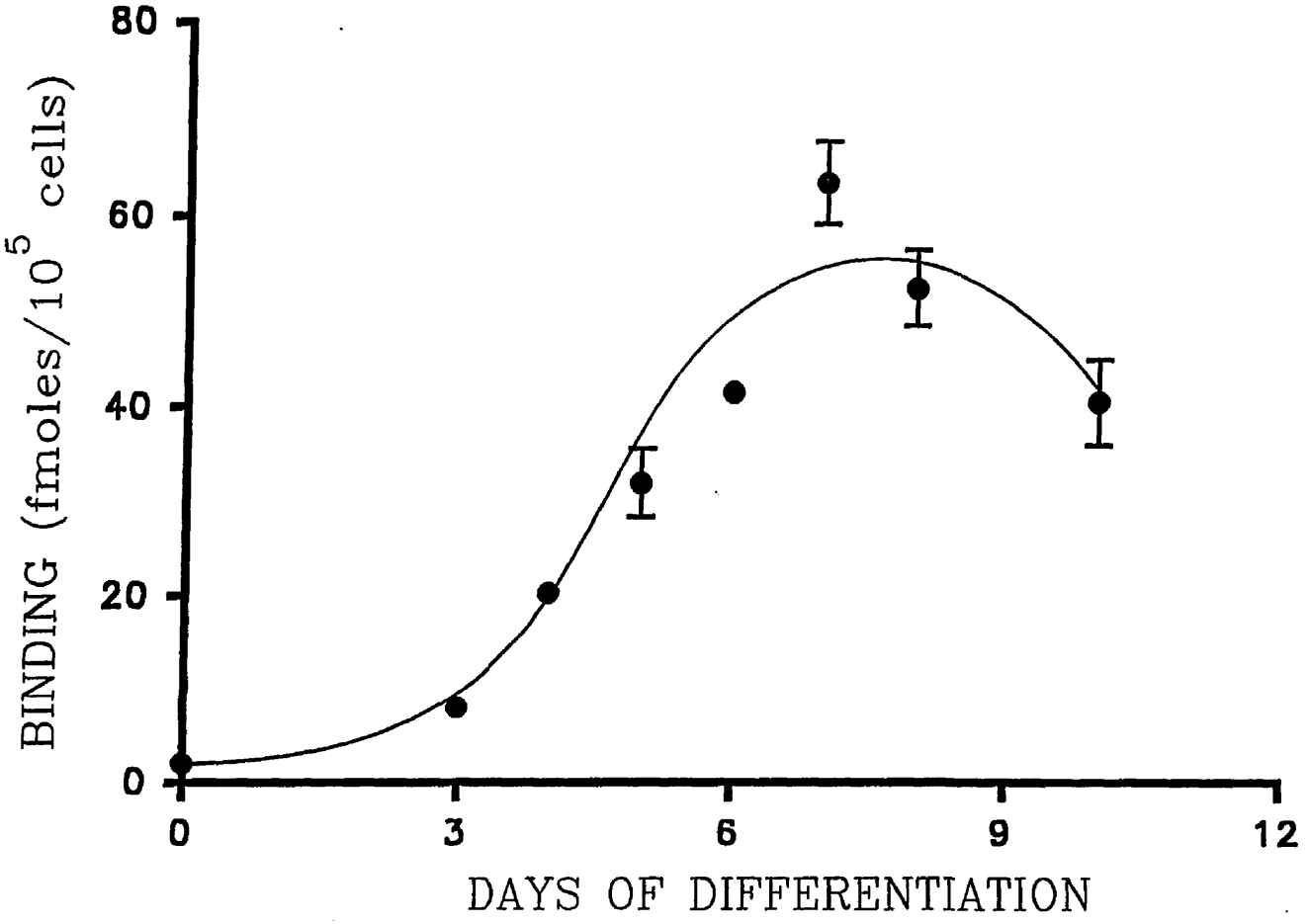

80
60
40
20
0
BINDING (fmoles/10^5 cells)
0
3
6
9
12
DAYS OF DIFFERENTIATION

FIG. 2.4. Saturation analysis of [^{125}I]-α-BGT binding to H19-7 cells differentiated for 5 to 7 days. Binding of [^{125}I]-α-BGT was measured at varying concentrations of the radioligand. Nonspecific binding was assessed in the presence of 10^{-4} M d-tubocurarine. Each value represents the mean $\pm$ S.E.M. of 3 separate experiments done in triplicate or quadruplicate. Where the S.E.M. was not depicted, it fell within the symbol. The K_d was 1.30 $\pm$ 0.05 nM (n=3) and B_{max} was 11.70 $\pm$ 1.73 fmoles/10^5 cells.

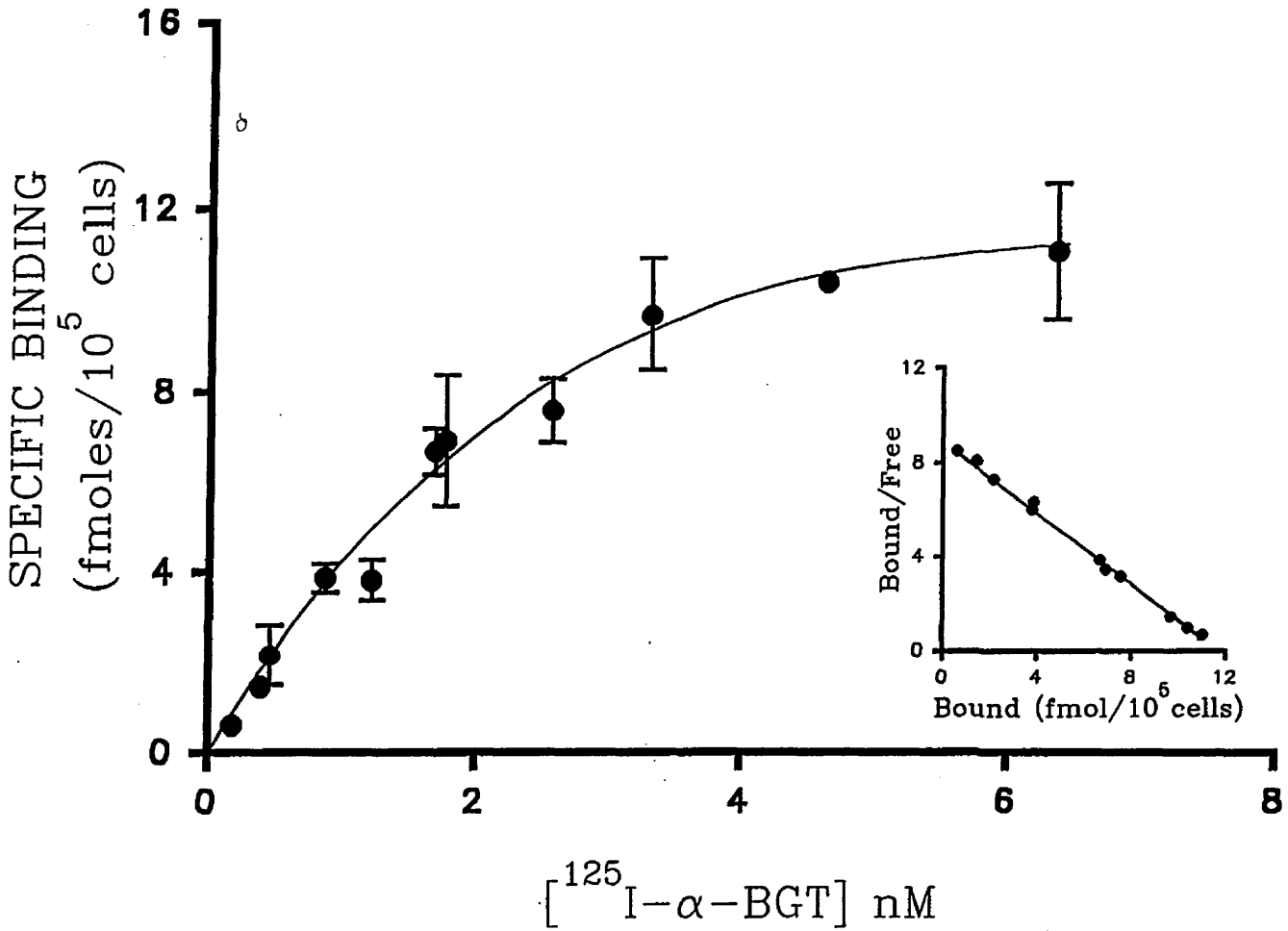
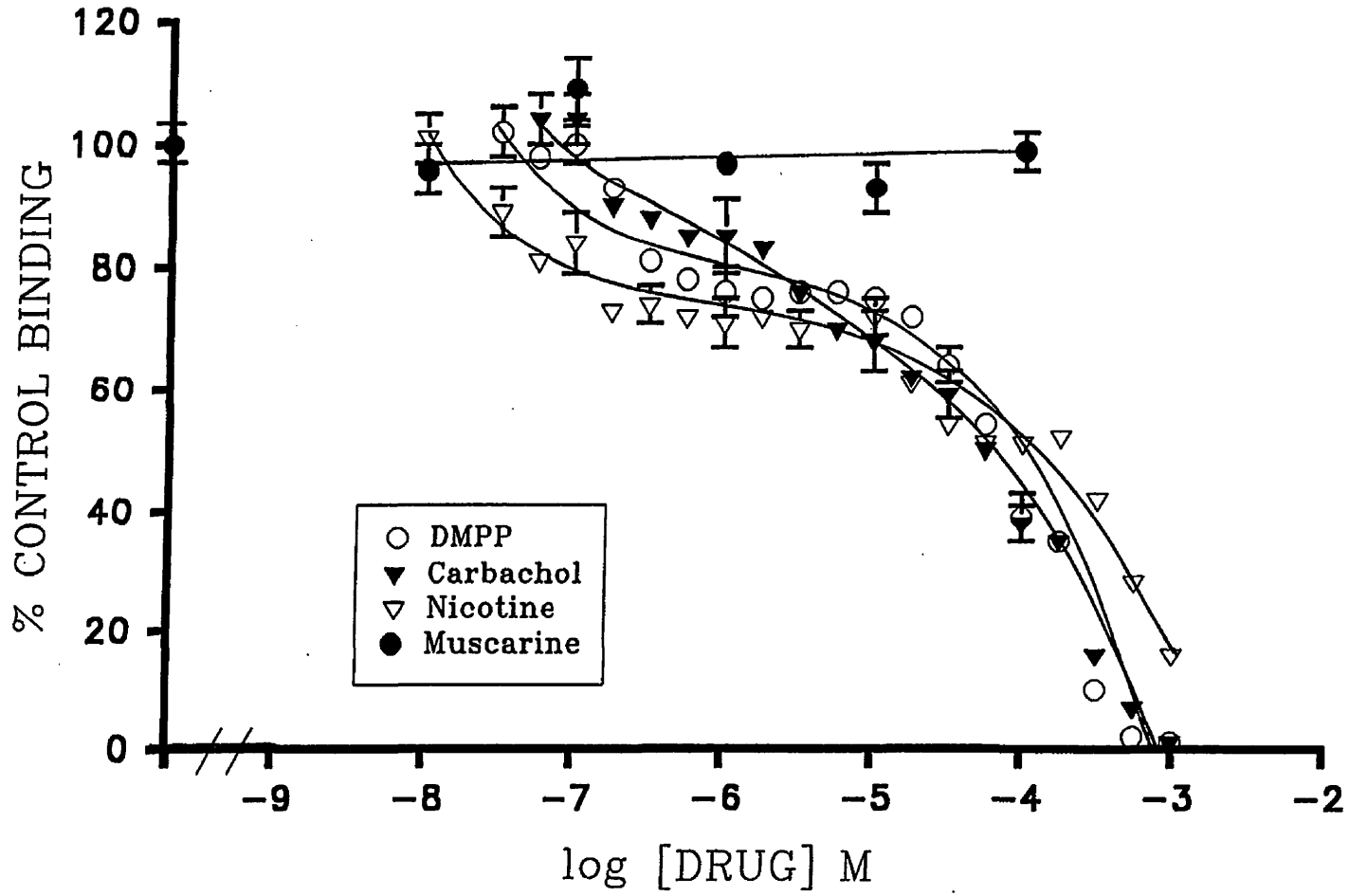

SPECIFIC BINDING
(fmoles/10^5 cells)
16
12
8
4
0
0
2
4
6
8
[125I−α−BGT] nM
Bound/Free
12
8
4
0
0
4
8
12
Bound (fmol/10^5 cells)

FIG. 2.5. The effect of (A) agonists and (B) antagonists on specific [^{125}I]-α-BGT binding to H19-7 cell membranes. Cell membranes were preincubated for 30 minutes with the indicated drug and the binding assay performed as described. Nicotinic but not muscarinic ligands inhibited the binding of radioligand to the H19-7 cells with IC_{50} values as follows: α-BGT 48 $\pm$ 3 nM (n=4), d-tubocurarine 6.4 $\pm$ 0.4 μM (n=3), carbachol 5.4 $\pm$ 0.4 μM and 180 $\pm$ 15 μM (n=3), DMPP 0.30 $\pm$ 0.04 μM and 83 $\pm$ 7 μM (n=3) and nicotine 0.32 $\pm$ 0.03 μM and 38 $\pm$ 4 μM (n=3). Values represent the mean $\pm$ S.E.M. of 3-4 experiments, done in triplicate. Where the S.E.M. was not depicted, it fell within the symbol.


120
100
80
60
40
20
0
% CONTROL BINDING
DMPP
Carbachol
Nicotine
Muscarine
−9
−8
−7
−6
−5
−4
−3
−2
log [DRUG] M

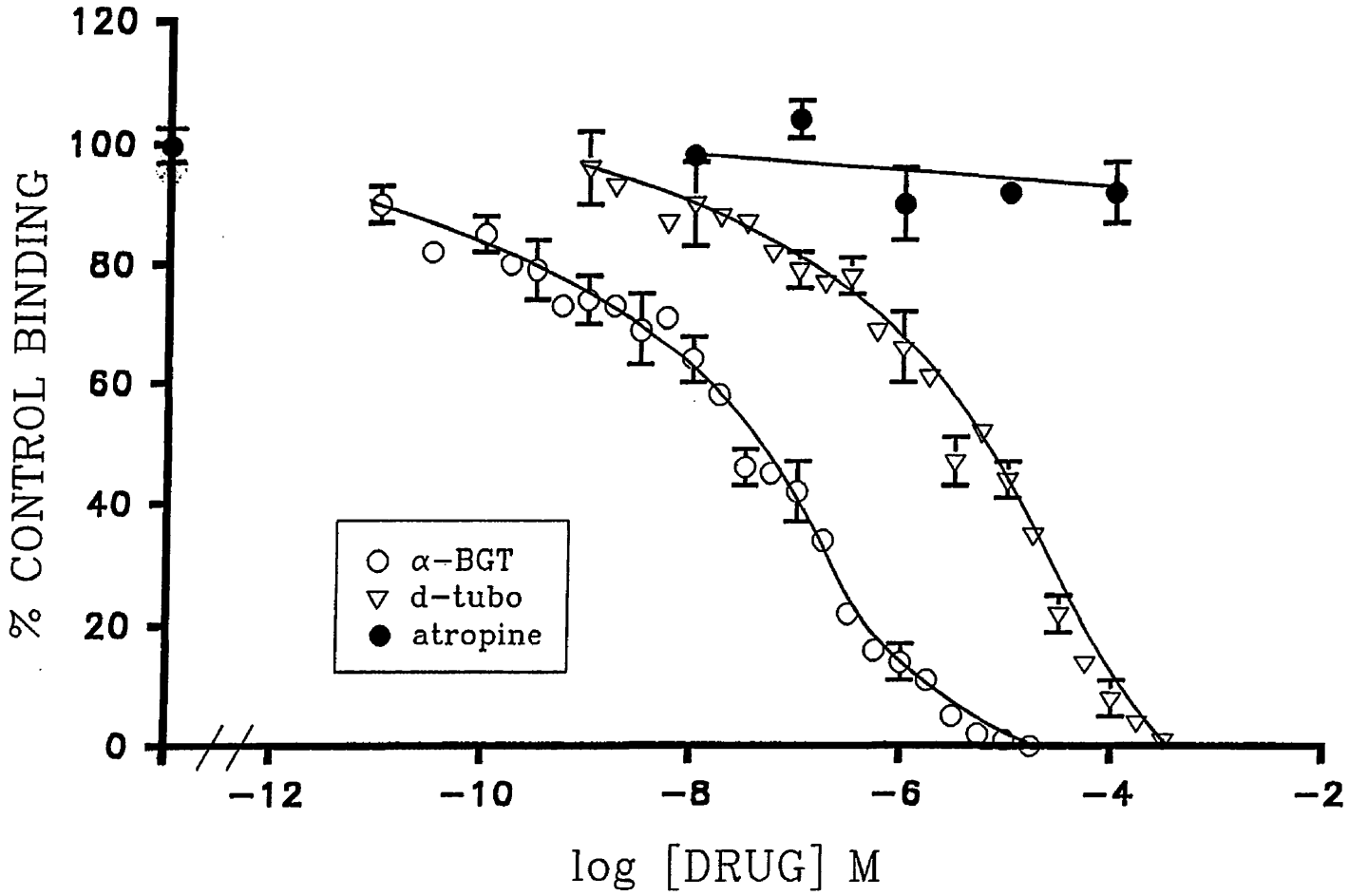
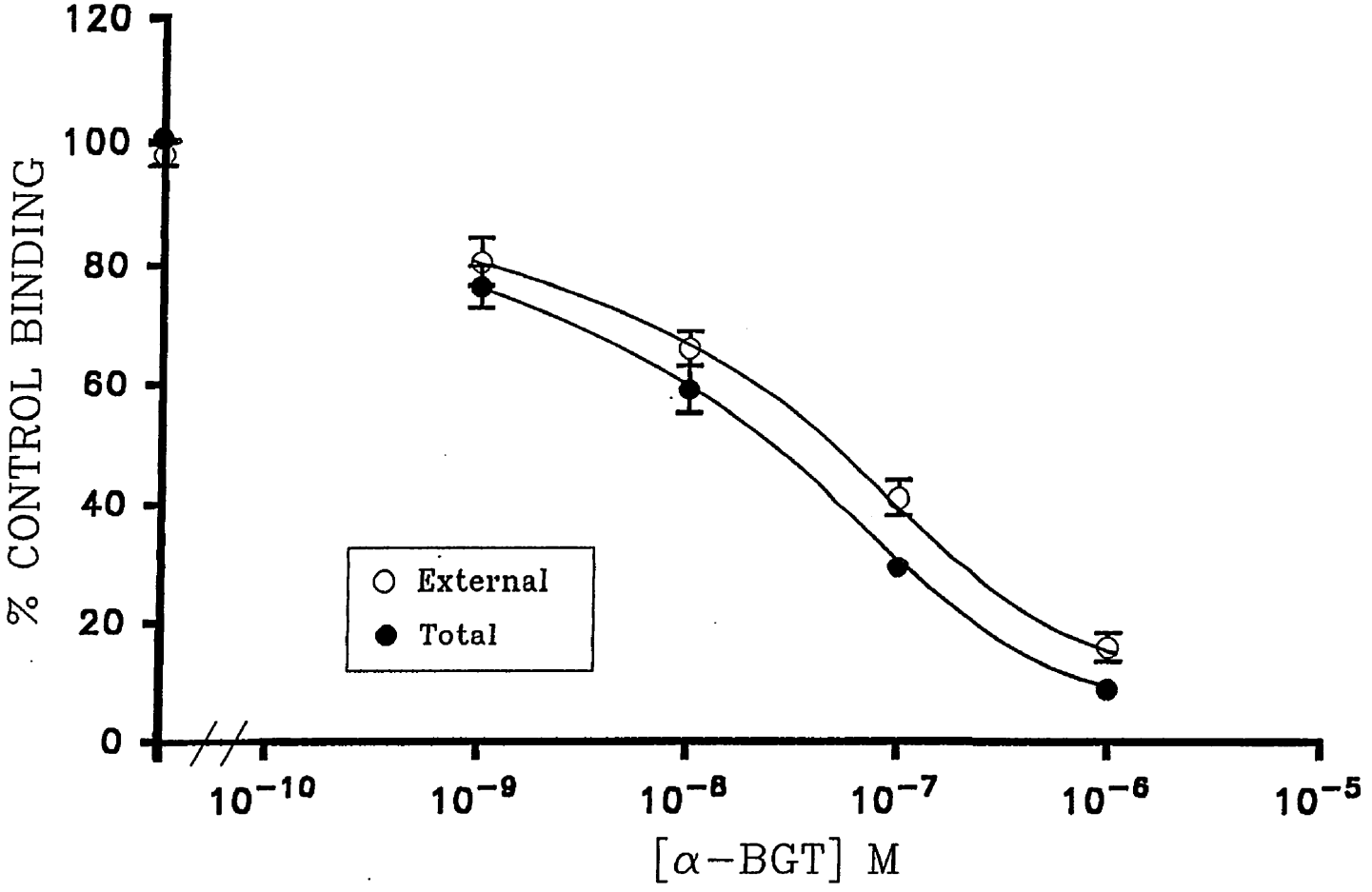
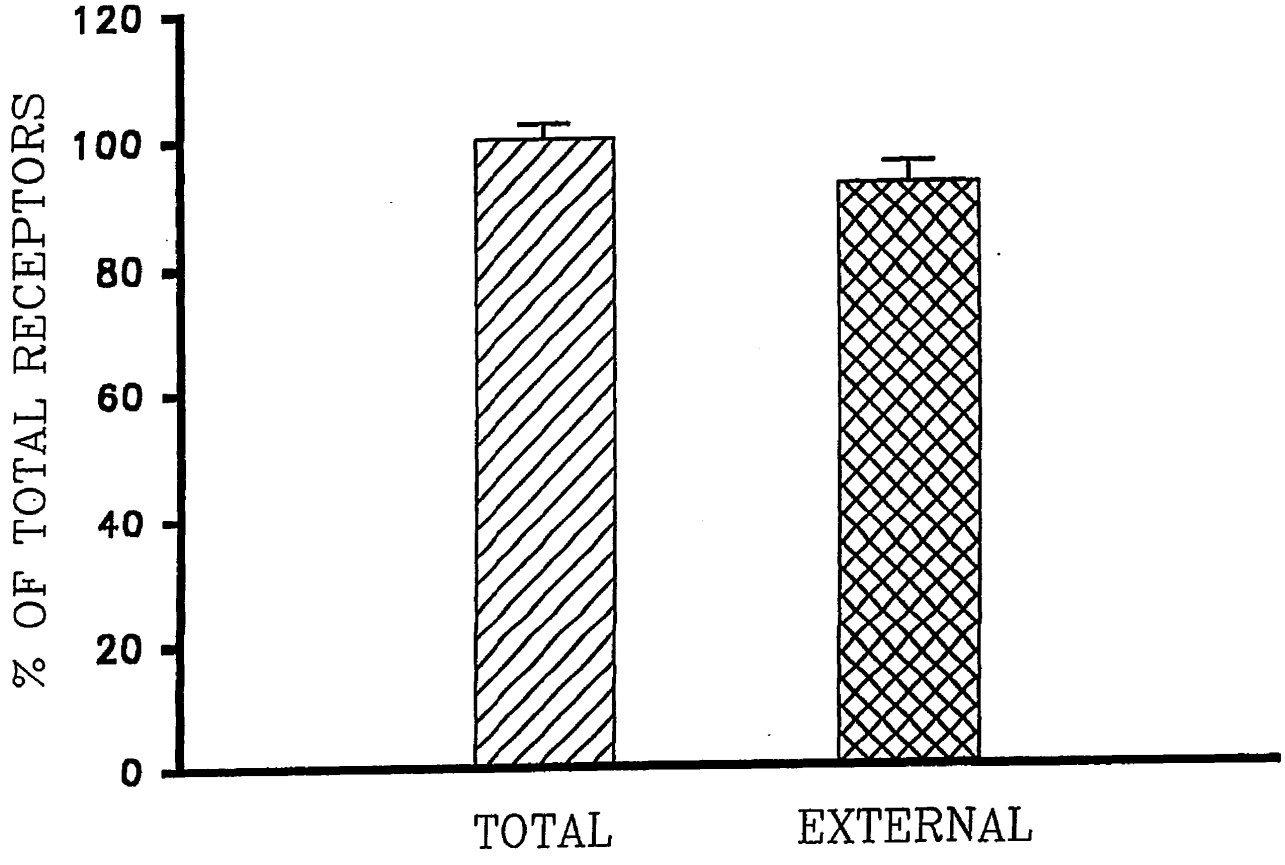

120
100
80
60
40
20
0
% CONTROL BINDING
α-BGT
d-tubo
atropine
−12
−10
−8
−6
−4
−2
log [DRUG] M

FIG. 2.6. Cellular localization of α-BGT binding sites in H19-7 cells. Intact H19-7 cells in culture were exposed to the indicated concentrations of non-radioactive α-BGT for 90 min prior to the binding. Binding assays were then carried out using both intact cells and lysed cell preparations as described in *Materials and methods*. A: Inhibition curves for cell surface receptors and total receptors. B: Comparison of total binding sites *vs.* binding sites located on the cell surface of H19-7 cells. All values represent the mean $\pm$ S.E.M of 3 experiments done in triplicate. Where the S.E.M. was not depicted, it fell within the symbol.


120
100
80
60
40
20
0
% CONTROL BINDING
External
Total
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
[α-BGT] M


% OF TOTAL RECEPTORS
120
100
80
60
40
20
0
TOTAL
EXTERNAL

3.0. STABLE TRANSFECTION OF NICOTINIC RECEPTOR SUBUNIT $\alpha 7$ IN RAT PITUITARY GH_4C_1 CELLS

This chapter contains Northern analysis and RNase protection assays done by J. Komourian; it represents part of a manuscript in print in *Journal of Neurochemistry* **67**, 145-154 (1996) by M. Quik, J. Choremis, J. Komourian, R. J. Lukas and E. Puchacz.

The preceding experimental results suggest that the H19-7 immortalized hippocampal cell line represents a useful CNS model system for examining α-BGT sensitive nicotinic receptors. An alternative approach for investigating the structure, function and regulation of the α-BGT receptor is to stably transfect the α7 nicotinic receptor subunit (the α-BGT binding subunit) in a model system not endogenously expressing the α7 nicotinic subunit. The following chapter describes the first reported stable transfection of the rat α7 nicotinic subunit in a rat clonal cell line (GH_4C_1) which does not endogenously express any nicotinic receptor subunits. The α7 transfected GH_4C_1 cell line may also represent a unique and useful model system for investigating α-BGT sensitive nicotinic receptors.

3.1. SUMMARY

The present results demonstrate stable expression of the α-bungarotoxin (α-BGT) binding neuronal nicotinic receptor subunit α7 by cells of the GH_4C_1 rat pituitary clonal line. RNase protection assays demonstrated that wild-type GH_4C_1 cells do not express detectable mRNA for the neuronal nicotinic receptor subunits α2, α3, α4, α5, α7, ß2 or ß3. In contrast, GH_4C_1 cells stably transfected with rat nicotinic receptor α7 cDNA (α7/GH_4C_1 cells) express the transgene abundantly as mRNA. Furthermore, Northern analysis confirmed that the α7 transcript being expressed in α7/GH_4C_1 cells is of the predicted size. The present results suggest that α7/GH_4C_1 cells may serve as a suitable model for examining α7/α-BGT neuronal nicotinic receptor expression.

3.2. INTRODUCTION

Neuronal nicotinic receptors exhibit an extensive diversity. To date eleven neuronal nicotinic receptor subunits have been isolated from avian, rodent or human tissues, including α2-α9 which encode the ligand binding subunits and ß2-ß4 which may represent structural subunits (Luetje et al. 1990; Deneris et al.1991; Clarke 1992; Role 1992; Papke 1993; Sargent 1993; Elgoyhen et al. 1994). Although it has been demonstrated that α2-α4 in some combination with the ß subunits (ß2-ß4) may form functional ion channels (Luetje et al. 1990; Deneris et al.1991; Role 1992; Papke 1993; Sargent 1993) and that α7-α9 subunits are capable of independently forming functional homooligomeric ion channels (Couturier et al. 1990; Schoepfer et al. 1990; Gotti et al. 1991; Bertrand et al. 1992; Séguéla et al. 1993; Elgoyhen et al. 1994; Gotti et al. 1994) in *Xenopus* oocyte expression systems, the molecular compositions of neuronal receptors and the functional identity of distinct receptor subtypes *in vivo* has not been fully elucidated.

Expression of receptors in heterologous systems has been a widely employed experimental system to address this issue. Transient expression models such as *Xenopus* oocytes have been a powerful tool in understanding receptor structure and function. Injection of different nicotinic receptor subunit cDNAs or cRNAs, either alone or in combination, has helped answer questions regarding receptor composition and structure, as well as agonist/antagonist sensitivities and desensitization (Couturier et al. 1990; Role 1992; Papke 1993; Sargent 1993; Séguéla et al. 1993). While transient expression may

be very useful for certain studies, there are also potential drawbacks to this approach: oocytes must be painstakingly and individually injected with genetic material and the window of time for receptor expression is only a few days long (Bertrand et al. 1991).

As an alternate approach, stable receptor expression in somatic cells has the advantage of yielding larger quantities of cells expressing the desired receptor which are required for biochemical analyses. Furthermore, these cells continually express the receptor. Whiting et al. (1991) transfected M10 fibroblast cells with α4 and ß2 subunits, which represent the predominant type of (-)[^{3}H]nicotine binding, α-BGT insensitive neuronal nicotinic receptor.

In addition to transfection of cells with α-BGT insensitive subunits, stable expression of the α7 subunit, which comprises the predominant type of α-BGT sensitive binding site, has also been achieved. Puchacz et al. (1994) described the overexpression of rat α7 cDNA in a human neuroblastoma cell line, SH-SY5Y. However, since wild-type (untransfected) SH-SY5Y cells endogenously express the nicotinic receptor subunit α7 (Puchacz et al. 1994) in addition to subunits α3 and ß4 (Lukas et al. 1993), studies with these cells may not clearly define the effects of cell transfection. More recently, Gopalakrishnan et al. (1995) have transfected a human embryonic kidney cell line HEK-293 with the human α7 cDNA, which resulted in functional α-BGT receptors which are not detected in the wild-type cells. Rat α7 cDNA was selected for transfection in the present studies since the rat nicotinic α-BGT receptor is extensively characterized and would thus allow for easier comparisons between *in vitro* results and the native receptor *in vivo*.

The present results show the stable heterologous expression of α7 mRNA as a result of transfecting a clonal rat pituitary cell line GH_4C_1 with the rat α7 cDNA. The cell line does not endogenously express any detectable message for any nicotinic receptor subunit tested; however, the transfected cells (α7/GH_4C_1 cells) abundantly express the transgene as mRNA of the predicted size.

3.3. MATERIALS AND METHODS

3.3.1. Materials

The rat pituitary cell line GH_4C_1 was generously provided by Dr. P. R. Albert (Department of Pharmacology, McGill University). [α-^{32}P]dCTP (3 000 Ci/mmol) and [α-^{32}P]UTP (800 Ci/mmol) were acquired from DuPont/NEN (Boston, MA). RNA polymerase SP6 and all restriction endonucleases were purchased from New England Biolabs (Mississauga, ON), random primed DNA labelling kit from Boehringer Mannheim (Laval, QC), nucleic acid transfer membrane (Hybond-N+) from Amersham (Oakville, ON). Hybridization buffer (High Efficiency Hybridization System) was bought from Molecular Research Center, Inc. (Cincinnati, OH). Supplies and chemicals for cell culture were purchased from Gibco/BRL (Montreal, QC).

3.3.2. GH_4C_1 Cell Transfection

The neuroendocrine pituitary cell line GH_4C_1 (Tashjian 1979) was grown in F10 medium supplemented with 8% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% O_2 / 5% CO_2. GH_4C_1 cells (2 x 10^6) were plated in medium on a 100-mm-diameter dish 24 h before transfection. The vector α7/pCEP4 (Puchacz et al. 1994) or the corresponding vector containing no α7 insert was introduced (20 μg/ml) into the target cells using a calcium phosphate protocol (Sambrook et al. 1989). Selection was initiated after 24 h by adding 0.2 mg/ml of Hygromycin B to the cells transfected with α7/pCEP4 vector. For colony selection, cells stably transfected with the α7 cDNA were plated at a density of 100 cells per 100-mm-diameter dish in growth medium containing the selection agent (Hygromycin B). After 2-3 weeks, resistant colonies representing the original cells were transferred to 20-mm-diameter wells for further growth. When the cells reached confluency, they were passaged to consecutively larger plates to achieve higher cell titers as required for the RNase protection assays and Northern analysis.

3.3.3. RNA Isolation

Total RNA was extracted from control or α7/GH_4C_1 cells, rat cortex or hippocampus using a guanidinium isothiocyanate-phenol-chloroform mixture as previously described (Chomcynski and Sacchi 1987) except that a second phenol-chloroform extraction was performed. RNA concentrations were determined by OD_{260}. The integrity of the isolated RNA was assessed by ethidium bromide staining in agarose.

3.3.4. RNA Probes

Riboprobes were generated for rat nicotinic subunit α2 - α5, α7, ß2 and ß3 mRNAs by runoff transcription using RNA polymerase SP6 and appropriate DNA constructs. Probes were transcribed from the pcDNAI/Neo plasmid which contained the full length coding sequence encoding the genes. The α2 probe, generated by digestion of the cDNA containing plasmid with *Stu*I, corresponds to the region between base pairs 1141 and 1546 (Wada et al. 1988). The α3 probe was produced by digestion with *Hinc*II and represents the region between base pairs 1188 and 1707 (Boulter et al. 1986). The α4 probe, generated by digestion with *Bsa*H1, corresponds to the interval between base pairs 1726 and 2111 (Goldman et al. 1987). The α5 probe was produced by digesting the cDNA with *Hind*III; the probe represents the span from base pair 945 to 1424 (Boulter et al. 1990). *Pvu*II was used to digest the α7 cDNA and the resulting probe corresponds to the region between base pairs 1617 and 1974 (Séguéla et al. 1993). The ß2 probe was also produced using *Pvu*II digestion and corresponds to base pairs 1206 to 2017 (Deneris et al. 1988). ß3 probe was formed by digestion with *Sph*I and it corresponds to base pairs 1377 to 2207 (Deneris et al. 1989). Antisense probes were transcribed using carrier free [α-^{32}P]UTP (800 Ci/mmol). Unprotected probe size and protected size, respectively, are as follows: α2, 453 and 405 bp; α3, 567 and 519 bp; α4, 433 and 385 bp; α5, 527 and 479 bp; α7, 405 and 357 bp; ß2, 859 and 811 bp; and ß3, 878 and 830 bp.

3.3.5. RNase Protection

RNase protection assays were performed as described (Krieg and Melton 1987). Briefly, 2 (only in the case of α7-transfected cells being probed for the α7 message) or 10 μg of total RNA were allowed to hybridize with a molar excess (2.50 x 10^5 cpm) of ^{32}P-labeled antisense RNA probe for 5 min at 85°C in 80% formamide, 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.4), 0.4 M NaCl and 1 mM ethylenediaminetetra-acetic acid (EDTA). Hybridization was continued at 60°C overnight (14-18 h). Single stranded RNA was subsequently digested over a 30 min period at 37°C by adding 2 units RNase T1. The RNase was then inactivated by incubation with 10 mg/ml proteinase K and 10% sodium dodecyl sulfate for 15 min at 37°C. Any protected RNA duplexes present were subsequently extracted with phenol/chloroform/isoamyl alcohol (25:24:1 by volume), precipitated with absolute ethanol at -20°C and dissolved in 30 μl RNA loading buffer (80% formamide, 1 mM EDTA, 0.01% xylene cyanol and 0.01% bromophenol blue). Samples were then electrophoresed at 230 V for 3.5-4 h in a 5% acrylamide gel containing 8 M urea. The gel was dried on Whatman 3 MM paper and exposed to Hyperfilm ECL film for 36-72 h at -80°C, using an intensifying screen.

3.3.6. Northern Analysis

Total RNA (10-50 μg) from either rat hippocampus or α7/GH_4C_1 cells was loaded onto a 1.3% agarose/formaldehyde gel, resolved by electrophoresis and transferred to a nylon nucleic acid membrane. The α7 cDNA probe was generated by digestion of a

pcDNAI/Neo plasmid containing the full-length coding cDNA for α7 with *Bst*XI to yield a 921 bp cDNA probe (Séguéla et al. 1993). The probe was subsequently labelled with [α-^{32}P]dCTP using a random primed DNA labelling protocol. The blots were then hybridized to the ^{32}P-labelled α7 cDNA probe at 42°C for 14-18 h in High Efficiency Hybridization System as per manufacturer's instructions. Blots were subsequently washed in 0.2 x saline-sodium citrate/ 0.5% sodium dodecyl sulfate at 68°C and exposed to film for 1-5 days at -80°C using an intensifying screen.

3.4. RESULTS

3.4.1. RNase Protection Assay

To determine which nicotinic receptor subunit RNAs are expressed in wild-type and transfected GH_4C_1 cells, RNase protection assay was used (Fig. 3.1). Wild-type (untransfected) cells did not contain transcripts for any of the nicotinic receptor subunit mRNAs tested (α2, α3, α4, α5, α7, ß2 and ß3). Transfected cells expressed mRNA only for the α7 subunit. As previously shown by various other groups, rat brain cortex contains α2, α3, α4, α5, α7, ß2 and ß3 subunit mRNA (Deneris et al. 1989; Wada et al. 1989; Boulter et al. 1990; Séguéla et al. 1993), and was therefore used as a positive control. As is evident from Fig. 3.1, cortex RNA protected all of the probes used. Yeast tRNA, which was used as a negative control, failed to protect any of the probes

(data not shown), thus eliminating the possibility of non-specific probe self-protection. The difference between the probe lengths and the protected band sizes is 48 bp, which represents the distance from the SP6 initiation site to the 3' end of the subunit cDNAs. This segment does not hybridize with the target RNA and is therefore not protected from subsequent RNase digestion.

Thus, rat brain cortex contains transcripts for all of the mRNA examined (α2, α3, α4, α5, α7, ß2 and ß3). Transfected GH_4C_1 cells displayed a signal only α7 subunit while untransfected cells did not show a positive signal for any subunits tested.

3.4.2. Northern Analysis

To determine whether the α7 mRNA transcript detected in α7/GH_4C_1 cells by RNase protection assay is of the size predicted based on the cDNA used for transfection, Northern analysis was performed. Fig. 3.2 demonstrates that the size of α7 mRNA isolated from the cells is 2.1 kb, which is only slightly larger than the coding cDNA length of 1965 bp. This difference may be attributed to a 3' poly A tail contributed by the SV40 polyadenylation signal from the pCEP4 vector. Thus, Northern analysis demonstrates that a correct size message is produced as a result of transfection.

3.5. DISCUSSION

The present findings demonstrate that the transfection of a rat pituitary cell line

GH_4C_1 with α7/pCEP4 DNA results in the stable expression of α7 mRNA. In addition, Northern analysis confirms that the transfected cells express the correct size α7 receptor subunit message as predicted by the clone employed for transfection. Neither α7 transfected nor control untransfected cells express α2, α3, α4, α5, ß2 and ß3 transcripts.

Alpha7 gene transcript distribution in rodent brain overlaps with the pattern of [^{125}I]-α-bungarotoxin (BGT) binding sites (Clarke et al. 1985; Séguéla et al. 1993) suggesting that the α7 subunit comprises most, if not all, of the high affinity [^{125}I]-α-bungarotoxin sensitive nicotinic receptor subtype. In line with this interpretation, further work with this model system (Quik et al. 1996) has demonstrated that α7 transfected GH_4C_1 cells express saturable, high affinity α-BGT binding sites and that the pharmacological profile of these sites are similar in membranes prepared either form rat brain or α7/GH_4C_1 cells. Furthermore, K_d and K_i values for [^{125}I]-α-BGT binding sites on intact α7/GH_4C_1 cells are similar to those for hippocampal neurons in culture (Barrantes et al. 1995). In addition, sucrose density gradient analysis demonstrated that the size of the α-BGT binding site being expressed in α7/GH_4C_1 cells is similar to native brain α-BGT receptors. Taken together, these findings suggest that stably transfected α7/GH_4C_1 cells may represent a unique and useful *in vitro* model system for examing α7/α-BGT receptors.

The physiological role of α-BGT sensitive α7 nicotinic acetylcholine receptors is not fully understood. The very ancient lineage of these receptors and its conservation throughout the course of evolution may suggest that it plays a significant role in neuronal function (Ballivet et al. 1996). Reports have implicated this receptor in numerous

different functions including a role in CNS development (Quik and Geertsen 1988; Fuchs 1989; Clarke 1992). α-BGT receptors are also thought to have a trophic role; there is evidence to suggest that they regulate neurite outgrowth (Chan and Quik 1993; Pugh and Berg 1994). In line with a trophic role for the α-BGT receptors, α-BGT can block the nicotine receptor mediated increase in cell numbers in neuroendocrine small cell lung carcinoma (SCLC) cell lines (Codignola et al. 1994; Quik et al. 1994). As well, α-BGT receptors have been associated with the modulation of synaptic transmission (McGehee et al. 1995). The observation that α-bungarotoxin can regulate the levels of neurotrophic factors such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in rat hippocampus (Freedman et al. 1993) provides evidence of a neuroprotective role for α-bungarotoxin receptors. A possible involvement of α-BGT sensitive receptors in epileptic seizures may be indicated by the higher levles of [^{125}I]-α-BGT binding sites in a mouse strain with a hightened sensitivity to nicotine induced seizures (Miner et al. 1986). In addition, evidence exists linking α-BGT recptors to auditory sensory gating deficit (Adler et al. 1992; Luntz-Leybman et al. 1992; Adler et al. 1993; Freedman et al. 1995), a psychophysiological deficit found in schizophrenia. This may explain why changes in the expression of α7 receptors have been associated with the pathology of schizophrenia (Alder et al. 1993; Freedman et al. 1995). However, the mechanisms of receptor regulation in these disease states have not been elucidated. The availability of an *in vitro* model such as stably transfected α7/GH_4C_1 cells presents an opportunity for investigating the structure, function and regulation of this enigmatic receptor subtype.

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FIG. 3.1. Identification of nicotinic receptor subunit mRNAs in untransfected and $\alpha 7$ transfected GH_4C_1 cells. RNase protection assays with the $\alpha 7$ probe indicate the presence of the transcript in $\alpha 7/GH_4C_1$ cells (Tr) but not in control untransfected GH_4C_1 cells (C). P indicates undigested probe and CX, rat cortex RNA. RNase protection experiments did not detect transcripts for $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, ß2 and ß3 subunits in either control or $\alpha 7/GH_4C_1$ cells. Cortex RNA served as a positive control and consistently protected all of the probes, while yeast tRNA served as a negative control and never managed to protect any of the probes used (data not shown). All experiments were done twice.

α7

P Tr C Cx

α2

P Tr C Cx

α3

P Tr C Cx

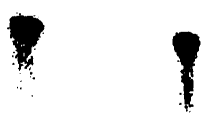
α4

P Tr C Cx

α5

P Tr C Cx

ß2

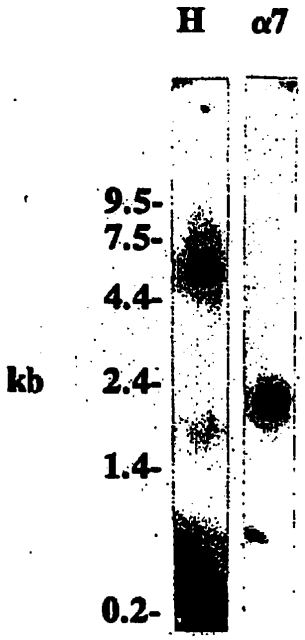


P Tr C Cx

ß3

P Tr C Cx

FIG. 3.2. Northern blot analysis of total RNA from rat hippocampus (H) and $\alpha 7/GH_4C_1$ cells ($\alpha 7$) using a 921 bp *Bst*XI fragment from a full length $\alpha 7$ coding cDNA. The positions of the RNA size markers are shown to the left. Transfected cells exhibited a 2.1 kb message while rat brain $\alpha 7$ mRNA was 5.6 kb as previously reported. Intervening lanes were removed for clarity.


H
α7
9.5-
7.5-
4.4-
kb
2.4-
1.4-
0.2-

4.0. c-*fos* mRNA LEVELS IN PC12 CELLS FOLLOWING NERVE GROWTH FACTOR AND/OR NICOTINE EXPOSURE

The main purpose for developing various model systems to study the α-BGT sensitive nicotinic receptor is to investigate the structure, function and regulation of this receptor subtype. Through the examination of such models, the α-BGT nicotinic receptor has been implicated in a trophic/developmental role, for example, neurite extension. Nicotinic stimulation of PC12 cells has been shown to transiently increase the level of the proto-oncogene c-*fos*, which also mediates cell growth and is involved in cellular differentiation and neuronal physiology. As it has not been demonstrated whether α-BGT sensitive or insensitive nicotinic receptors are involved in upregulating c-*fos* levels in PC12 cells, it is possible that the α-BGT receptor exerts its trophic effects on the cell via the activation of c-*fos*. The following experiments seek to elucidate the functional significance of α-BGT receptors, specifically their role in the regulation of c-*fos* proto-oncogene expression.

4.1. SUMMARY

Exposure of undifferentiated pheochromocytoma cells (PC12) to nerve growth factor (NGF) or exposure of neuronally differentiated PC12 cells to nicotine have both been reported to result in a rapid and transient induction of transcription of the c-*fos* proto-oncogene. In the present study, Northern analysis of mRNA isolated from either undifferentiated PC12 cells exposed to NGF or differentiated PC12 cells exposed to nicotine both failed to demonstrate an induction of c-*fos* transcription under all conditions tested. This may be due to differences between the PC12 cells used in this study as compared to those used in previous reports in terms of the ability to couple cellular stimulation to c-*fos* mRNA transcription. Alternatively, levels of c-*fos* mRNA may have increased but may have been below the level of detection of the present assay. Other possibilities are also discussed.

4.2. INTRODUCTION

Many types of stimuli, some relating to the process of differentiation and others linked to neuronal excitation, lead to a transient induction of c-*fos* mRNA and protein (Greenberg and Ziff 1984; Morgan and Curran 1986). mRNA levels peak at 10-15 min post-stimulation and then decline back to basal levels, with the transcripts having a relatively short half-life of about 10 to 15 min (Greenberg and Ziff 1984; Greenberg et al. 1985; Greenberg et al. 1986). Synthesis of c-*fos* protein follows mRNA expression, and the protein is turned over with a half life of about 2 hours (Koistinaho 1991; Ren and Sagar 1992; Koistinaho et al. 1993; Matta et al. 1993). In PC12 cells, examples of stimuli that induce c-*fos* expression include growth factors such as NGF and epidermal growth factor (EGF) (Greenberg et al. 1985). In addition, depolarizing events such as calcium influx through voltage-gated channels brought about by stimuli such as high extracelluar K^+ (Greenberg et al. 1986; Morgan and Curran 1986) or nicotine (Greenberg et al. 1986), which acts on its receptor and subsequently results in an opening of voltage gated Ca^{+2} channels as well, also induce c-*fos* expression in PC12 cells. In addition to the exposure of rat cell cultures to nicotine, direct administration of nicotine to rats also results in an increase in c-*fos* protein levels in brain 1-3 h post-injection as detected by immunohistochemistry (Koistinaho 1991; Ren and Sagar 1992; Koistinaho et al. 1993; Matta et al. 1993).

It has been demonstrated that α-bungarotoxin (α-BGT) sensitive nicotinic receptors are capable of raising intracellular calcium levels by fluxing calcium directly (Séguéla

et al. 1993) and by activating voltage-gated calcium channels (Vijayaraghavan et al. 1992; Rathouz and Berg 1994; Zhang et al. 1994). This calcium influx may be related to a growth/developmental role for the α-BGT receptor, such as neurite extension (Chan and Quik 1993; Pugh and Berg 1994). Calcium influx mediated by nicotinic receptors is known to induce c-*fos* expression (Greenberg et al. 1985; Greenberg et al. 1986; Koistinaho 1991; Ren and Sagar 1992; Koistinaho et al. 1993; Matta et al. 1993), which also has a growth/development related role, although it has not been determined whether the nicotinic receptors involved in c-*fos* activation are the α-BGT sensitive nicotinic receptors or not. In the present study we sought to delineate the role of nicotinic receptors in c-*fos* induction using a PC12 cell model system.

4.3. MATERIALS AND METHODS

4.3.1. Materials

PC12 cells, the c-*fos* probe and the ß-actin probe were all purchased from American Type Culture Collection (Rockville, MD). Restriction enzymes were obtained from New England Biolabs (Mississauga, ON), random primed DNA labelling kit from Boehringer Mannheim (Laval, QC), [α-^{32}P]dCTP (3 000 Ci/mmol) from DuPont/NEN (Boston, MA), nucleic acid transfer membrane (Hybond-N+) from Amersham (Oakville, ON), oligo (dT)-cellulose columns and hybridization buffer for Northern blots from Molecular

Research Center, Inc. (Cincinnati, OH). Nicotine and NGF-2.5S were acquired from Sigma Chemical Co. (St. Louis, MO). Supplies and chemicals for culture were purchased from Gibco/BRL (Montreal, QC).

4.3.2. PC12 Cell Culture

PC12 cells were grown on collagen coated flasks and maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% horse serum, 5% fetal bovine serum (FBS), penicillin 50 units/ml, streptomycin 50 μg/ml and 1% fungizone (anti-fungal agent), at 37°C in a humidified atmosphere of 95% O_2 / 5% CO_2. Medium was routinely changed every 2-3 days and cells were used at low (<20) passage numbers. Cells were divided into two groups: one group to study the effects of NGF stimulation on undifferentiated PC12 cells and the second to study the effects of nicotine on NGF treated, neuronally differentiated PC12 cells. NGF-2.5S (50 ng/ml) was added 0 minutes (control) to 30 minutes prior to initiating the total RNA isolation protocol for group 1. For group 2, nicotine (100 μM) was added to PC12 cells that had been exposed to NGF for 1 week (to promote neuronal differentiation) 0 (control) to 30 minutes prior to the isolation of RNA.

4.3.3. RNA Isolation

Total RNA was extracted from PC12 cells using a guanidinium isothiocyanate-phenol-chloroform mixture as previously described (Chomcynski and Sacchi 1987) except that a second phenol-chloroform extraction was performed. RNA concentrations were

determined by OD_{260} and the integrity of the isolated RNA was assessed by ethidium bromide staining in agarose. To isolate the poly $(A)^+$ RNA fraction from total RNA, oligo (dT)-cellulose columns were used in accordance with the manufacturer's instructions.

4.3.4. cDNA Probes

The c-*fos* probe was generated by digestion of a pSP65 plasmid containing the cDNA insert with *Hind*III and *Eco*R1 to yield a 1.1 kb cDNA probe. Similarly, the ß-actin probe was isolated from a pBluescript KS- vector by digestion with *Pst*I and resulted in a 1.4 kb ß-actin probe. Probes were labelled with [^{32}P]dCTP using a random primed DNA labelling protocol.

4.3.5. Northern Blots

Varying amounts of total RNA (30 or 60 μg) or poly $(A)^+$ RNA ($\approx$2 μg) were resolved by electrophoresis through 1.3% agarose/formaldehyde gels and transferred to nylon nucleic acid membranes. The blots were hybridized to the ^{32}P-labelled probes in High Efficiency Hybridization System buffer as per manufacturer's instructions at 42°C for 14-18 h. Blots were subsequently washed in 0.2x SSC/ 0.5% SDS at 68°C and exposed to film for 1-7 days at -80°C using an intensifying screen.

4.4. RESULTS

4.4.1. Northern Blots

An initial experiment using 30 μg of total RNA per lane from either control PC12 cells (either differentiated cells not exposed to nicotine or undifferentiated cells not exposed to NGF), nicotine treated differentiated PC12 cells (100 μM nicotine exposure for 10, 15 or 25 minutes prior to RNA isolation) or NGF treated undifferentiated cells (50 ng/ml NGF exposure for 10, 15 or 25 minutes) failed to show hybridization of cellular RNA message with the c-*fos* probe for all the conditions analyzed, presumably due to a lower level of this particular message in the RNA preparations than the Northern analysis can detect. The blot was subsequently stripped by treatment with boiling 0.1% SDS and reprobed with the ß-actin probe, which resulted in a visible band for all lanes except 7, corresponding to a size of approximately 1.8 - 2.0 kb (Fig. 4.1). The low intensity of staining for ß-actin was due to the fact that the exposure period was very brief (36 h); however, the presence of bands for ß-actin confirmed the integrity of the RNA preparations, the efficiency of the gel to membrane RNA transfer protocol, the efficacy of the random primed DNA labelling procedure as well as the efficiency of the hybridization and wash procedures. A second experiment was then performed using twice the amount of RNA (60 μg) per lane. Again, despite the fact that ß-actin probe hybridization was visible as a 1.8 - 2.0 kb band in all lanes after a 36 h exposure of the blot to film, c-*fos* probe hybridization was not observed in any lane, even after a prolonged (7 day) exposure (data not shown). In a third experiment, gel lanes were

loaded with the poly $(A)^+$ RNA fraction isolated from 200 μg of total RNA from PC12 cells which had been subjected to each experimental condition. For this experiment, PC12 cells from 2 different sources (both at a low number of passages) were used and an extra time point (5, 10, 20 and 30 minute exposure) was included; c-*fos* hybridization was not observed despite the very high level of cellular RNA used (data not shown).

4.5. DISCUSSION

Two previous reports had suggested that the transcription rate as well as the mRNA level of c-*fos* are transiently increased after exposure of PC12 cells to either NGF or nicotine, up to 100 times their basal levels (Greenberg et al. 1985; Greenberg et al. 1986). c-*fos* mRNA levels were observed to rise from baseline (0 min exposure), reach a peak at 10-15 min of exposure and then return to basal levels within a period of 20-30 min of exposure. In contrast to these previous findings, elevated levels of c-*fos* message were not seen in this study following either of the two treatments. Previous reports demonstrate that basal (unstimulated) c-*fos* mRNA levels are below the level of detection of Northern analysis; however, after stimulation of cells with either NGF or nicotine, the c-*fos* band is readily apparent and has an intensity that is two to three times greater than the ß-actin band (Greenberg and Ziff 1984; Greenberg et al. 1985; Greenberg et al. 1986). As ß-actin bands were observed in the present report after only a 36 h exposure of the blot to film, then c-*fos* bands should have also been observed if the expression of

c-*fos* mRNA had increased over the basal level, since these bands would be even more intense than ß-actin. Despite exposure to film for up to 7 days, c-*fos* bands were never observed in the present report, which may suggest that stimulation of the PC12 cells used in this study did not result in an elevation of c-*fos* mRNA levels. This discrepancy between the previous reports and the present study may be due to several reasons.

In both previous reports by Greenberg et al. (1985; 1986), the PC12 cells were grown in RPMI 1640 medium, whereas DMEM medium was used in the present study. As medium composition is an important factor for the growth/development of cells and c-*fos* is a regulator of cell growth/differentiation/development it is possible that the medium used by Greenberg et al. (1985; 1986) affected the response of the PC12 cells to the different extracellular stimuli applied.

Also, in both of the treatment groups (NGF and nicotine) only the effects of a single concentration were examined (50 ng/ml and 100 μM, respectively), selected from dose-response curves established by Greenberg et al. (1985; 1986). However, the cells used in this study may express a different dose-response relationship as a consequence of differences in either the number or the affinity of the NGF and nicotinic receptors as compared to the PC12 cells used in Greenberg's reports.

A third possibility relates to the nature of the PC12 cells used. Mutant PC12 cells are known to exist, such as the PC12-U2 cell line (Lilien and Claude 1985), which have lost the ability to fully respond to NGF stimulation. Other stimuli which have been reported to elevate c-*fos* levels in PC12 cells, such as high extracellular K^+ (Greenberg et al. 1986; Morgan and Curran 1986) or EGF (Greenberg et al. 1985) could be used to

examine stimulus-transcription coupling in our PC12 cells. As well, other proto-oncogenes could be looked at, for example, c-*myc* mRNA levels are also known to rise in PC12 cells following either NGF or nicotine exposure (Greenberg et al. 1985; Greenberg et al 1986). Lack of responses in these situations would lead to the conclusion that the stimulus-transcription coupling ability of the cells used is compromised.

Another possibility is that due to the rapid response kinetics reported for c-*fos* transcription induction, the spike phase was missed in the time points tested. To remedy this more time points could be selected, for example every 2-3 min during the exposure period. In addition to Northern analysis, the more sensitive nuclear run-off transcription assay (Greenberg and Ziff 1984; Greenberg et al. 1985; Greenberg et al. 1986) could be used to examine increases in the transcription rate of c-*fos*. If there was a problem with the c-*fos* cDNA probe employed in these studies, an alternate approach to measure c-*fos* induction would be immunohistochemistry, as reports show that c-*fos* protein levels following nicotine administration in rats closely mirror the results obtained by measuring transcript levels (Koistinaho 1991; Ren and Sagar 1992; Koistinaho et al. 1993; Matta et al. 1993).

In conclusion, levels of c-*fos* mRNA may have risen in the PC12 cells but were not detected. Alternatively, the lack of an observed result may be due to a mutation in the cells used in this study. If the latter is the case, the PC12 cells used here may be an interesting and useful model system for studying signal transduction and stimulation-transcription coupling.

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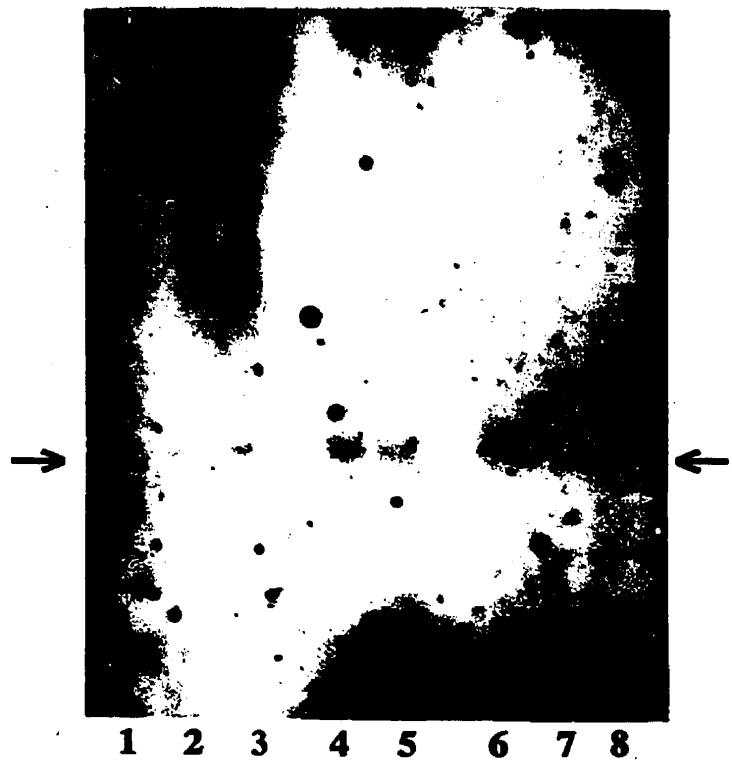
FIG. 4.1. Northern analysis demonstrating the presence of a 1.8 - 2.0 kb ß-actin transcript in PC12 cells. 30 μg of total RNA was used in all 8 lanes. Lanes 1-3: NGF treatment. Undifferentiated PC12 cells were exposed to 50 ng/ml NGF for 10 min (Lane 1), 15 min (Lane 2) or 25 min (Lane 3). Lanes 4-5: controls. Undifferentiated PC12 cells not exposed to NGF (Lane 4) and differentiated PC12 cells not exposed to nicotine (Lane 5). Lanes 6-8: nicotine treatment. PC12 cells were exposed to NGF to promote differentiation during a two week period. Cells were then exposed to 100 μM nicotine for 10 min (Lane 6), 15 min (Lane 7) or 25 min (Lane 8).


1 2 3 4 5 6 7 8

5.0. GENERAL DISCUSSION

As discussed in the Introduction, the use of molecular, immunological as well as physiological techniques has led to our greater understanding of the structure, function and regulation of neuronal nicotinic acetylcholine receptors in general and the α-bungarotoxin sensitive receptor in particular (Deneris et al. 1991; Clarke 1992; Role 1992; Papke 1993; Sargent 1993). Functional and localization studies have distinguished the toxin sensitive receptor population from its α-bungarotoxin insensitive neuronal nicotinic acetylcholine receptor counterparts. Furthermore, with the advent of molecular biology techniques, the genes encoding the α7, α8 and α9 nicotinic receptor subunits (i.e., the α-BGT sensitive subunits) were isolated and cloned (Schoepfer et al. 1990; Séguéla et al. 1993; Elgoyhen et al. 1994). Although there is evidence to suggest that the toxin insensitive neuronal receptors may mediate the effects of acetylcholine activation and may be involved in synaptic transmission (Luetje et al. 1990; Deneris et al. 1991; Galzi et al. 1991; Role 1992; Papke 1993; Sargent 1993), the physiological role of the α-BGT sensitive receptor remains more elusive and serves as the focus of much current research.

5.1. Model Systems for Examining α-BGT Sensitive Receptors

As indicated in the Introduction, two of the more common model systems which are used to study α-BGT receptors involve either primary rat cell cultures (Barrantes et al. 1995; Dávila-García et al. 1995; Didier et al. 1995) or malignant (neuroblastoma) cell

lines (Lukas 1993; Lukas et al. 1933; Puchacz et al. 1994) which endogenously express the receptor of interest. While both of these systems have advantages and have laid a solid foundation for the study of the enigmatic toxin sensitive receptor, there are also potential drawbacks. Primary cells in culture may be difficult to maintain and do not readily yield sufficient material for biochemical analyses, while tumor cell line usage is limited by its malignant nature. Thus, the central goal of the present studies was to develop and validate novel and unique model systems for studying the α-BGT receptor in order to gain further insight into the structure, function and regulation of this receptor subtype. In keeping with this aim, two such models are presented in the current work: the immortalized rat hippocampal cell line H19-7 (discussed in Chapter 2) and the first reported stable transfection of the rat α7 nicotinic receptor subunit in a rat clonal cell line (GH_4C_1) which does not otherwise express any nicotinic receptor subunits (discussed in Chapter 3).

As described in Chapter 2, RNase protection assays demonstrated that the immortalized rat hippocampal cell line (H19-7) expresses a similar set of mRNA encoding for nicotinic receptor subunits as adult rat hippocampus (Deneris et al. 1989; Duvoisin et al. 1989; Wada et al. 1989; Boutler et al. 1990; Séguéla et al. 1993). Furthermore, binding assay analysis using the H19-7 cells demonstrated that receptors expressed by this system are similar to the α-BGT sites described both in rat brain (Morley et al. 1997; Marks and Collins 1982) as well as to the toxin sensitive sites in primary cultures (Barrantes et al. 1995; Didier et al. 1995) in terms of both K_d and B_{max}. Furthermore, IC_{50} (or K_i) values obtained for H19-7 cells using the nicotinic ligands

α-BGT, d-tubocurarine, carbachol, DMPP and nicotine were similar to those found for rat brain in the literature.

The second model system developed and validated in these studies was the stably expressing α7 rat pituitary clonal line GH_4C_1. As described in Chapter 3, RNase protection assays demonstrated that untransfected cells express no mRNA for any nicotinic receptor subunits while the transfected cells express mRNA for the α7 nicotinic receptor subunit exclusively. Further, Northern analysis confirmed the correct size for the α7 message as predicted by the clone employed for transfection. Further work with this system (Quik et al. 1996) demonstrated that the transfected cells express saturable, high affinity α-BGT binding sites and that the pharmacological profile of these sites are similar in membranes prepared either from rat brain or α7/GH_4C_1 cells. Also, K_d and K_i values for the [^{125}I]-α-BGT binding sites on intact α7/GH_4C_1 cells are similar to those for hippocampal cells in culture. Furthermore, sucrose gradient analysis demonstrated that the size of the α-BGT binding site being expressed in α7/GH_4C_1 cells is similar to native brain receptors. Lastly, fura-2AM calcium assays demonstrated an α-BGT sensitive nicotinic receptor mediated increase in free intracellular calcium in α7/GH_4C_1 cells as a result of exposure to nicotinic ligands, a finding in agreement with those of other investigators (Vijayaraghavan et al. 1992; Séguéla et al. 1993; Rathouz and Berg 1994; Zhang et al. 1994). Thus, the two model systems described here seem to represent relevant, novel and unique model systems to further investigate the α-BGT receptor.

5.2. Functional Role of the α-BGT Receptor

As stated previously, the purpose in having model systems expressing the α-BGT receptor is to enable the study of its structure, function and regulation. Through the examination of various model systems, evidence has been collected which suggests a possible involvement of the α-BGT receptor in trophic, developmental or growth related processes (Greene 1976; Fielder et al. 1987; Fuchs 1989; Chan and Quik 1993; Codignola et al. 1994; Pugh and Berg 1994; Quik et al. 1994; Renshaw 1994). This then raises questions concerning the mechanism by which the α-toxin site exerts these trophic effects. It has been demonstrated that α-BGT receptors are capable of increasing intracellular calcium directly (Séguéla et al. 1993) and by activating voltage-gated calcium channels (Vijayaraghavan et al. 1992; Rathouz and Berg 1994; Zhang et al. 1994). When taken together with the finding that nicotinic receptor mediated calcium influx induces c-*fos* expression (Greenberg et al. 1985; Greenberg et al. 1986a), this suggests that a possible mechanism by which the α-BGT receptor mediates its trophic role is via the activation of immediate early genes such as c-*fos* (Freedman et al. 1993). As discussed in Chapter 4, nicotinic stimulation of PC12 cells has been shown to transiently increase the level of the proto-oncogene c-*fos*, which also mediates cell growth and is involved in cellular differentiation and neuronal physiology. Since it is not known whether the nicotinic receptors involved in c-*fos* activation are exclusively the α-BGT sensitive variety or not, the goals of the studies presented in Chapter 4 were to replicate previous studies involving nicotinic receptor mediated c-*fos* activation and to further elucidate the possible role of the α-BGT receptor in immediate early gene activation and

ultimately in cellular growth and differentiation.

In our studies with PC12 cells (described in Chapter 4), nicotinic receptor mediated c-*fos* induction was not observed, for reasons which were not completely clear to us. Possibly the two novel model systems developed, validated and presented in the present studies may serve as an impetus for future studies to further investigate the role of c-*fos* to mediate the function of the enigmatic α-bungarotoxin sensitive neuronal nicotinic acetylcholine receptor.

6.0. REFERENCES FOR INTRODUCTION AND GENERAL DISCUSSION

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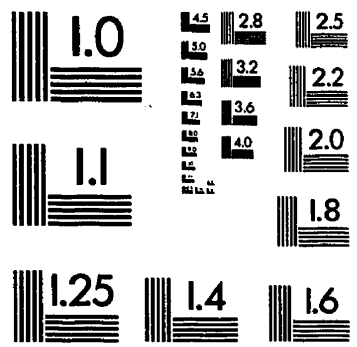

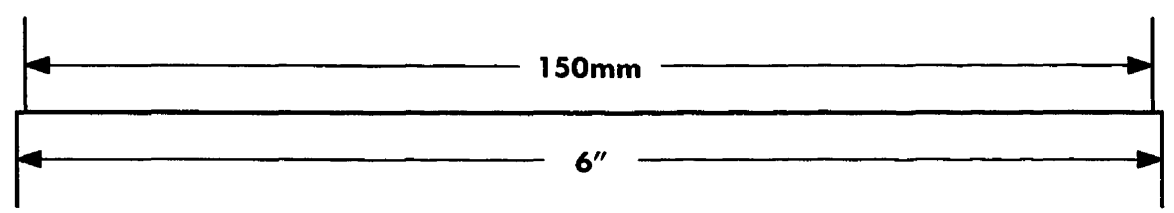

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