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NICOTINIC ALPHA-BUNGAROTOXIN RECEPTORS IN PC12 AND H69 CELLS

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To Daniel and my family.

To laugh often and much; to win the respect of intelligent people and the affection of children; to earn the appreciation of honest critics and endure the betrayal of false friends; to appreciate beauty, to find the best in others; to leave the world a bit better, whether by a healthy child, a garden patch or a redeemed social condition; to know even one life has breathed easier because you have lived. This is to have succeeded.

-Ralph Waldo Emerson

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PREFACE

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, or those submitted or suitable for submission to learned journals. The text relating to this option is as follows:

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This thesis is divided into two experimental chapters that will be referred to as:

Chapter 2: A role for the nicotinic α-bungarotoxin receptor in neurite outgrowth in PC12 cells. (*Neuroscience* **56**, 441-451)

Chapter 3: α-Bungarotoxin blocks nicotine induced cell proliferation in a lung tumor cell line. (published in a modified form in *Brain Research* **655**, 161-167)

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LIST OF ABBREVIATIONS

5-HT	Serotonin
ANOVA	Analysis of variance
α-BGT	Alpha-bungarotoxin
B_{max}	Maximum number of binding sites
CNS	Central nervous system
ChAT	Choline acetyltransferase
DMEM	Dulbecco's Modified Eagle Medium
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis (β-aminoethyl ether) N,N,N,N-tetraacetic acid
GABA	Gamma-aminobutyrate
h	hour
HBSS	Hanks Balanced Salt Solution
K_d	Affinity constant
mAb	Monoclonal antibody
min	minute
NGF	Nerve growth factor
SCLC	Small cell lung carcinoma
S.E M.	Standard error of the mean

ABSTRACT

Current evidence indicates that there are two populations of nicotinic receptors, the α-BGT sensitive and α-BGT insensitive neuronal nicotinic receptors. The latter are known to be involved in neurotransmission. However, the physiological significance of the α-toxin sensitive population is unclear, although an involvement in trophic functions has been suggested.

The present studies were carried out to investigate the possible involvement of the nicotinic α-BGT receptor in growth related activity in neuronal PC12 cells and in H69 cells, a small cell lung carcinoma cell line. In PC12 cells, nicotine treatment resulted in a decline in neuritic outgrowth which could be prevented by treatment with α-BGT or d-tubocurarine, a nicotinic antagonist, at concentrations which correlated very well with those required to inhibit [^{125}I]α-BGT binding in PC12 cells. α-BGT had no effect on [^{3}H]noradrenaline release, a function known to be mediated through the α-toxin insensitive population of nicotinic receptors suggesting that α-BGT interacts specifically with the α-toxin sensitive population of nicotinic receptors to mediate its effects on neuritic outgrowth in PC12 cells. In H69 cells, exposure to nicotine or the nicotinic agonist, cytisine, resulted in an increase in cell number which could be blocked by α-BGT as well as by d-tubocurarine, suggesting the involvement of an α-BGT sensitive nicotinic receptor in mediating the observed effects on H69 cell number. This is supported by [^{125}I]α-BGT binding data which demonstrates that the concentrations at which these nicotinic receptor ligands exhibited their effects on H69 cell number correlate very well with the affinity of

these agents for the α-toxin receptor in H69 cells. Northern analysis demonstrated the presence of both α5 and α7 mRNA in H69 cells suggesting that as in nervous tissue, the presumed α-BGT binding neuronal nicotinic receptor subunits, α5 and/or α7, may be components of the nicotinic α-BGT receptor present in non-neuronal cells.

Taken together, these two lines of studies using both a neuronal as well as a non-neuronal cell line suggest that nicotinic α-BGT receptors may be involved in a growth related role, namely that of regulating neuritic outgrowth in PC12 cells and cell proliferation in H69 cells.

RÉSUMÉ

L'hypothèse actuelle stipule qu'il y a deux populations de récepteurs nicotiniques, soit les récepteurs nicotiniques neuronaux sensibles à l'α-BGT et ceux non-sensibles à l'α-BGT; ces derniers étant connus pour être impliqués dans la transmission nerveuse. Bien que la signification physiologique de la population de récepteurs sensibles à l'α-toxine soit ambigue, la littérature suggère leur implication dans la régulation de la croissance.

Les études présentes furent effectués afin d'étudier l'implication possible des récepteurs nicotiniques α-BGT dans l'activité de croissance des cellules neuronales PC12 et des cellules H69, une lignée cellulaire issue du carcinome pulmonaire à petites cellules. Chez les cellules PC12, un traitement à la nicotine resulta dans un déclin de l'excroissance neuritique, ceci pouvant être empêché avec un traitement à l'α-BGT ou au d-tubocurarine, un antagoniste nicotinique, à des concentrations correspondant bien à celles nécessaires pour inhiber la liaison de [^{125}I]α-BGT aux cellules PC12. L'α-BGT n'affecta pas la libération de [^{3}H]noradrénaline, un effet étant connu pour être médié par la population de récepteurs nicotiniques non-sensibles à l'α-toxine, ceci suggérant que l'α-BGT interagit de façon spécifique avec la population de récepteurs nicotiniques sensibles à l'α-toxine afin de médier son effet sur l'excroissance neuritique des cellules PC12. Chez les cellules H69, l'exposition à la nicotine ou à la cytisine, un agoniste nicotinique, resulta en l'augmentation du nombre de cellules pouvant être antagonisé par α-BGT ainsi que d-tubocurarine, ceci suggérant l'implication des récepteurs nicotiniques sensibles à l'α-BGT dans la médiation des effects observés sur le nombre de cellules H69. Ceci est supporté

par les études de liaison de [^{125}I]α-BGT qui démontrent que les concentrations auxquelles les ligands des récepteurs nicotiniques exhibent leur effets sur le nombre du cellules H69 correspondent bien à l'affinité de ces agents pour les récepteurs de l'α-toxine chez les cellules H69. Les analyses Northern démontrent la présence de ARNm α5 et α7 dans les cellules H69 suggérant que comme dans les tissus nerveux, les présumés sous-unités de liaisons à l'α-BGT des récepteurs neuronaux nicotiniques, soit l'α5 et/ou l'α7, pouriaient être les composantes des récepteurs nicotiniques α-BGT présents dans les cellules non-neurveuses.

Ensembles, ces deux plans d'étude, utilisant à la fois une lignée cellulaire neurale et non-neurale, suggèrent que les récepteurs nicotiniques α-BGT puissent être impliqués dans les activités de croissance, plus précisement dans la régulation de l'excroissance neuritique des cellules PC12 et dans la prolifération des cellules H69.

SUMMARY OF CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

In this thesis, PC12 and H69 cells in culture were used to study the role of nicotinic α-BGT receptors in neuronal and non-neuronal tissues, respectively. Studies using PC12 cells addressed the issue of the possible involvement of neuronal α-bungarotoxin receptors in neurite outgrowth. As well, the potential role of nicotinic α-BGT receptors in regulating lung tumour growth was investigated using the H69 cell line. The novel findings of this thesis are summarized below.

1. a) Nicotine exposure was found to inhibit neurite outgrowth in NGF-treated PC12 cells in culture. This effect was dose-dependent and observed at concentrations of nicotine which correlated with those required to inhibit radiolabelled toxin binding.

   b) The nicotine induced decline in neuritic outgrowth was prevented by treatment with α-BGT. This effect of α-BGT was dose-dependent and was observed at concentrations of α-BGT which correlated with those required to inhibit radiolabelled toxin binding; α-BGT alone had no effect on neurite outgrowth. This suggests that the effects of α-BGT were mediated through an interaction at the α-toxin receptor.

   c) Similar effects on the nicotine induced decline in neuritic outgrowth were also observed with the nicotinic receptor antagonist d-tubocurarine, further

indicating the nicotinic nature of the receptor.

These results are of significance since they suggest a possible functional role for the neuronal nicotinic α-BGT receptor in neurite outgrowth in nervous tissue.

2. a) Nicotine exposure results in an increase in H69 cell number. This effect was both time- and dose-dependent. A similar time- and dose-dependent effect was also observed with another nicotinic agonist cytisine. The concentrations of nicotine and cytisine required to increase cell number correlated well with those which were required to inhibit radiolabelled toxin binding.

b) The nicotine induced increase in H69 cell number was prevented by treatment with α-BGT. This effect of α-BGT was dose dependent. The concentration of α-toxin required to completely prevent the nicotine induced increase in H69 cell number correlated well with the affinity of α-BGT for its receptor; α-BGT alone had no effect.

c) α5 and α7 mRNA are present in H69 cells. This finding suggests that the molecular composition of the α-BGT receptor in non-neuronal tissues may involve these presumed neuronal nicotinic α-BGT binding subunits.

These results provide evidence that α-BGT receptors may have a role in the regulation of cell proliferation in lung tumour cell lines implicating a role for the α-toxin receptor in the pathogenesis of lung cancer.

1.0 Introduction

Nicotinic acetylcholine receptors are neurotransmitter-gated ion channels, that is, the binding of two molecules of acetylcholine to the receptor binding sites results in the opening of the receptor channel, which is an integral part of the receptor protein, and to the subsequent conduction of cations through the channel pore. As well, receptors for glutamate, serotonin (5-HT), gamma-aminobutyrate (GABA) and glycine upon binding of their appropriate ligand convert the receptor protein into an ion conducting channel. Thus, the function of this family of receptors known as the ligand-gated ion channels, is to convert a chemical signal into an electrical signal within the cell, the process of which is known as neurotransmission. Nicotinic, glutamate and 5-HT receptors are cation conducting channels which flux Na^+ and K^+ ions upon receptor activation resulting in depolarization of the cell whereas, GABA and glycine receptors are anion conducting channels which flux Cl^- ions upon receptor activation resulting in hyperpolarization of the cell. Structurally, ion channel proteins are formed by the joining of multiple, either homologous or heterologous, subunits to form a central pore. Schematically, the neurotransmitter-gated receptor subunits are thought to resemble the staves of a barrel which join together to form a channel.

Nicotinic acetylcholine receptors are the most thoroughly characterized member of this family of ligand-gated ion channels. Most of what we know today about this family of receptors is due to the large quantities of nicotinic receptors in the electric organ of *Torpedo* which allowed for the isolation and biochemical characterization of this

receptor protein. As well, the snake venom, α-bungarotoxin (α-BGT), played a significant role in the purification of the nicotinic receptor protein due to its high affinity interaction with the nicotinic acetylcholine receptors in the *Torpedo* electric organ. α-BGT also served as a probe for nicotinic type receptors in muscle since in both *Torpedo* and muscle, α-BGT binds nicotinic receptors irreversibly resulting in an inhibition of nicotinic receptor mediated functions (Changeux et al. 1970; Miledi and Potter 1971; Miledi et al. 1971; Karlin 1974; Cohen and Changeux 1975; Rang 1975; Fambrough 1979). Thus, α-BGT acts as an antagonist at *Torpedo* and muscle type nicotinic receptors and much of what is known about synaptic transmission today has been learned from studying these postsynaptic muscle type nicotinic acetylcholine receptors.

Purification studies have shown that muscle type nicotinic acetylcholine receptors are composed of 5 subunits arranged in a pseudo-symmetrical fashion with a stoichiometry of $\alpha_2\beta\gamma\delta$ in fetally derived muscle tissue (Lindstrom et al. 1979; Raftery et al. 1980; Conti-Tronconi et al. 1982; Karlin et al. 1983) whereas in the adult, the γ subunit is replaced by an ε subunit (Takai et al. 1985; Mishina et al. 1986). Photoaffinity labelling studies have defined the α subunit as the ligand binding site of the nicotinic acetylcholine receptor with a unique pair of adjacent disulfide linked cysteine residues at positions 192 and 193 contributing to the ligand binding region as well (Kao and Karlin 1986; Mosckovitz and Gershoni 1988).

Antibodies raised against purified *Torpedo* nicotinic receptor were used to immunopurify the muscle type nicotinic receptor (Tzartos and Lindstrom 1980; Stroud et al. 1990; Lingle et al. 1992; Devillers-Thiéry et al. 1993). From the deduced amino acid

sequence of the purified muscle receptor protein, the cDNA sequences encoding the muscle nicotinic receptor subunits were determined. Reconstitution of the muscle nicotinic receptor subunit cDNAs in various expression systems has provided insight into the functional role of the various nicotinic receptor subunits. For example, transfection of the muscle cDNAs in COS cells has shown that the extracellular N-terminal domains of the α, δ and γ subunits contain sequences that are responsible for receptor assembly. Injection and expression of muscle receptor subunit cDNAs in *Xenopus* oocytes has shown that the ε subunit is responsible for the short-open-time (fast-type) acetylcholine receptor channels that are seen in adult muscle (Stroud et al. 1990; Lingle et al. 1992; Devillers-Thiéry 1993).

1.1 Neuronal nicotinic receptors

Monoclonal antibodies (mAbs) to *Torpedo* nicotinic receptors were used to immunopurify neuronal acetylcholine receptors that cross-reacted with the *Torpedo* mAb (Whiting and Lindstrom 1986, Whiting et al. 1987) thereby providing a source of purified neuronal nicotinic receptor protein. From the deduced protein sequence information, the cDNAs which encode the subunits of the neuronal nicotinic acetylcholine receptors were thus determined (Whiting et al. 1987, Schoepfer et al. 1988). In an alternate approach, screening of PC12 and brain cDNA libraries with a radiolabelled muscle nicotinic acetylcholine receptor α subunit cDNA probe identified the first member of a family of homologous neuronal nicotinic acetylcholine receptor genes (Boulter et al. 1986, Boulter et al. 1987). Other members of the neuronal nicotinic acetylcholine receptor family were

identified by homology cloning (Deneris et al. 1988; Duvoisin et al. 1989; Elgoyhen et al. 1994). These genes were classified as α subunits or ß subunits. The former being characterized by 2 adjacent cysteine residues at positions homologous to 192 and 193 in the *Torpedo* α subunit sequence. Eight α subunits have been identified so far (α2 - α9). Subunits that lack these cysteines were classified as ß subunits, 3 of which have been identified so far (ß2 - ß4). Structurally, neuronal nicotinic receptors differ from muscle type nicotinic receptors in that the former appear to be composed of two (or maybe more) instead of four different subunits as in muscle. However, evidence suggests that the neuronal nicotinic receptor may also be pentameric (Anand et al. 1991, Cooper et al. 1991) as is the muscle type nicotinic receptor. Both α and ß subunits contribute to the formation of the neuronal nicotinic receptor. Co-expression of a ß subunit (ß2 or ß4) with an α subunit (α2, α3 or α4) in the oocyte expression system forms functional channels gated by nicotinic agonists, none of which however are blocked by α-BGT (Boulter et al. 1990; Luetje et al. 1990). Injection of the receptor combinations of α4ß2 and α3ß2 in *Xenopus* oocytes resulted in receptor subtypes which differed in their sensitivity to acetylcholine (Gross et al. 1991) suggesting that the α subunit is involved in determining agonist sensitivity. The ß subunits have been suggested to be involved in receptor sensitivity as well as in determining the time course of desensitization of neuronal nicotinic receptors. Injection of the receptor combinations of α3ß4 and α3ß2 in oocytes have resulted in receptor subtypes which differed in their rate of desensitization due to either DMPP or acetylcholine exposure (Cachelin and Jaggi 1991). Immunoprecipitation studies (Whiting and Lindstrom 1986, 1987) using anti-neuronal

nicotinic receptor antibodies made against rat brain have yielded both α and ß subunits suggesting that both subunits are components of the neuronal nicotinic receptor. However, it is still unclear as to the types of subunits that make up a receptor, the number of α subunits versus the number of ß subunits in a receptor and whether there are other subunits in vivo that are involved. The participation of the α6 and ß3 subunits in the formation of a functional receptor in the oocyte expression system has yet to be shown. As well, evidence suggests that the α5 gene product assembles with other α subunits to form distinctive acetylcholine receptors in chick brain (Conroy et al. 1992): however, no function has been identified so far for the α5 gene when injected into Xenopus oocytes either alone or in combination with other neuronal nicotinic acetylcholine receptor genes (Boulter et al. 1990; Couturier et al. 1990b).

Thus, molecular cloning experiments as well as functional expression studies of various subunit receptor combinations in *Xenopus* oocytes reveals the diversity in nicotinic receptor subtypes present in the central nervous system (CNS). This diversity is also reflected by differences in sensitivity of different nicotinic receptor subtypes to the snake toxin, α-bungarotoxin, which will be discussed further in section 1.2.

Radioligand binding studies have determined the localization of neuronal nicotinic receptors. Using [^{3}H]nicotine, high-affinity nicotine binding sites have been localized in regions of the rat brain (Clarke et al. 1985, Härfstrand et al. 1988) such as the medial habenula, interpeduncular nucleus, specific motor and sensory nuclei of the thalamus, substantia nigra, molecular layer of the dentate gyrus, superior colliculus and cortex. The labelling pattern for high affinity [^{3}H]acetylcholine binding sites in rat brain resembled

that for [^{3}H]nicotine suggesting that these two compounds bind to the same population of nicotinic receptors. As well, in situ hybridization methods, using [^{35}S]-labelled cRNA probes, have defined the distribution of neuronal nicotinic receptor subunit mRNAs in the CNS. Expression of the α4 and ß2 genes in overlapping and in most regions of the CNS (Wada et al. 1989) suggests that the α4ß2 nicotinic receptor subtype may be the predominant receptor subtype in brain. ß4 gene transcripts have been localized in the medial habenula, cortex, hippocampus, interpeduncular nucleus and some motor nuclei as well (Dineley-Miller and Patrick, 1992). The distribution of α5 mRNA in rat CNS differed from that of the α3 and α4 subunit transcripts (Wada et al. 1990). Using mAbs, the protein products of these genes have been localized in specific regions of the CNS (Swanson et al. 1987). Using mAb 270, which is directed against high-affinity nicotine binding sites isolated from chicken brain, a pattern of immunohistochemical labelling similar to that seen with [^{3}H]nicotine binding in the CNS was obtained and also showed that nicotinic receptors are transported along axons in the optic nerve, habenulo-interpeduncular tract and the nigrostriatal projection (Swanson et al. 1987). The localization of neuronal nicotinic receptor transcripts and protein in the CNS corresponds with areas of the CNS which have also been shown to possess functional nicotinic receptors.

Functional nicotinic receptors in the CNS have been demonstrated mainly by the technique of iontophoretic application of nicotinic agonists and antagonists and recording from single neurons. Activation of nicotinic receptors in the hippocampus resulted in a disinhibitory effect on paired pulse inhibition in pyramidal neurons which could be

prevented by nicotinic antagonists (Rovira et al. 1983). In the cerebral cortex, the nicotine induced excitatory response could be inhibited by nicotinic antagonists (Vidal and Changeux 1989). These studies suggest a role for the interaction of nicotine at neuronal nicotinic receptors in the mediation of synaptic transmission.

Nicotinic receptors have also been implicated in growth related functions in nervous tissue due to the trophic effects mediated by nicotine in the CNS. Both prenatal and neonatal exposure to nicotine, a lipophilic molecule which readily penetrates the blood-brain barrier (Schwartz and Kellar 1983), has been shown to result in alterations in CNS development as determined by changes in various neuronal markers. Navarro et al. (1989a,b) studied the effect of prenatal nicotine exposure on development of central cholinergic neurotransmitter systems by studying changes in two neuronal markers, high-affinity choline uptake and choline acetyltransferase (ChAT) activity. The former is rate-limiting to acetylcholine synthesis and subject to neuronal impulse regulation and the latter is involved in the conversion of choline to acetylcholine; thus the two markers provide indices of both synaptic proliferation and activity. Results demonstrated that prenatal exposure to nicotine had marked adverse effects on developmental patterns of choline uptake and ChAT activity suggesting that nicotine may selectively disrupt CNS development by stimulating nicotinic receptors, which are present in fetal brain prematurely, thus eliciting the developmental events ordinarily triggered postnatally by cholinergic projections. Fetal nicotine exposure also affects the function of noradrenergic

pathways in the CNS; prenatal exposure to nicotine produces a deficit in noradrenergic responsiveness postnatally (Seidler et al. 1992). These deficits may be responsible for the resulting behavioral and neuroendocrine abnormalites seen in the offspring.

Ornithine decarboxylase catalyzes the rate-limiting step in the synthesis of polyamines and thus plays an essential role in brain growth during early periods of development (Slotkin and Bartolome 1986). To minimize the complicating factors such as maternal/placental drug effects, Beeker et al. (1992) used a non-placental chick embryo model to investigate the effect of early (76-168h) chick embryo exposure to in ovo exposure to nicotine. Results demonstrate that nicotine suppressed the peak in fetal brain ornithine decarboxylase activity that normally occurs at 120h of development.

Fetal exposure to nicotine results in offspring with altered membrane-associated adenylate cyclase activity (Slotkin et al. 1992); prenatal nicotine exposure thus disrupts a basic cellular transduction signal that is shared by various trophic factors and neurotransmitters. In mice, neonatal nicotine exposure induces permanent changes in brain nicotinic receptors and behaviour in adult mice such that neonatal nicotine treatment prevents the development of low-affinity nicotinic sites in the brain. Furthermore, mice treated with nicotine displayed a hypoactive condition as compared with nontreated mice, which displayed a hyperactive condition (Nordberg et al. 1991). Other behavioral impairments induced by prenatal nicotine include deficits in learning and memory as demonstrated by the significant impairment in performance of nicotine exposed rats in the radial-arm maze test (Sorenson et al. 1991).

Due to the potential physiological significance of nicotinic receptors in CNS

development, the issue of determining which nicotinic receptor subtypes are responsible for mediating the growth related actions of nicotine in the CNS remains of utmost interest.

1.2 Neuronal nicotinic α-BGT receptors

Radioligand binding studies using radiolabelled α-BGT demonstrated that α-BGT also bound with high affinity to neuronal membranes (Morley et al. 1979; Oswald and Freeman 1981). This binding could be competed by nicotinic but not muscarinic receptor ligands suggesting that these represented neuronal nicotinic α-BGT receptors. Thus, there is a subtype of nicotinic receptor in the CNS that binds α-BGT and will be referred to as neuronal α-BGT receptors.

These receptors have been demonstrated to be a distinct population from the rest of the neuronal nicotinic receptors. Neuronal α-BGT receptors were immunologically distinguished from neuronal nicotinic acetylcholine receptors in PC12 cells (Patrick and Stallcup 1977b). Further evidence for a distinction between neuronal α-BGT receptors and neuronal nicotinic receptors comes from observations in which α-BGT is ineffective in blocking a wide variety of nicotinic receptor mediated responses in various neuronal preparations ranging from intact tissues to cell lines. Nicotinic receptor mediated transmission in cat Renshaw cells (Duggan et al. 1976) and rat superior cervical ganglia (Brown and Fumagalli 1977) was not affected by α-BGT. Nor did the α-toxin inhibit nicotine induced sodium influx in PC12 cells (Patrick and Stallcup 1977a). α-BGT had no effect on the response to iontophoretically applied acetylcholine in chick ciliary ganglion neurons (Ravdin and Berg 1979). In adrenal chromaffin cells in culture,

carbachol induced increase in tyrosine hydroxylase activity (Quik and Trifaro 1982) was not blocked by α-BGT and in the NIE-115 neuroblastoma cell line, responses to iontophoretically applied acetylcholine were blocked by d-tubocurarine but not by α-BGT (Kato and Narahashi 1982). Despite these studies, α-BGT sensitive responses have been detected in rat cerebellum (de la Garza et al. 1987) and in dissociated hippocampal neurons in culture (Alkondon and Albuquerque 1991; Zorumski et al. 1992).

Localization studies using radiolabelled ligands and mAbs demonstrate a distinction between high affinity nicotine binding sites and α-BGT binding sites. In contrast to the similar binding pattern seen in both rat and mouse brain with [^{3}H]nicotine, [^{3}H]acetylcholine and [^{3}H]methylcarbachol, that seen with [^{125}I]α-BGT demonstrated a different regional binding pattern (Clarke et al. 1985, Boksa and Quirion 1987, Pauly et al. 1989). As well, α-BGT could not block [^{3}H]nicotine binding to rat brain nicotinic receptors (Romano and Goldstein 1980). In addition, [^{125}I]α-BGT binding was present in rat brain regions that did not receive cholinergic innervation (Hunt and Schmidt 1979). Immunohistochemical studies using mAbs made against rat and chick brain nicotinic receptors demonstrated that the labelling pattern of the mAb was similar to that obtained with [^{3}H]nicotine but different from the labelling pattern seen with [^{125}I]α-BGT (Swanson et al. 1987). Using horseradish peroxidase conjugated α-BGT, α-toxin binding sites have been localized to nonsynaptic regions such as cell bodies and on short neuritic processes on chick ciliary ganglion neurons (Jacob and Berg 1983; Loring et al. 1985). Thus, it is unlikely that these neuronal α-BGT receptors play a major role in synaptic transmission in the ganglion.

Further evidence for a distinction between the nicotinic α-BGT receptor and the nicotinic acetylcholine receptor comes from studies which look at the effects of various agents on nicotinic receptor regulation. Chronic infusion of nicotine results in a 50% increase in [^{3}H]nicotine binding sites in rat cortex whereas cortical [^{125}I]α-BGT binding sites remained unchanged (Sanderson et al. 1993). Long term exposure of PC12 cells to carbachol, a nicotinic agonist, resulted in a loss of nicotinic receptor mediated Na^{+} influx however, there was no change in [^{125}I]α-BGT binding sites (Kemp and Edge 1987). In PC12 cells cultured in serum-free medium, the release of catecholamines due to nicotinic receptor activation was abolished yet radiolabelled α-toxin binding was not affected (Mitsuka and Hatanaka 1983). These studies all suggest that α-BGT sensitive and α-BGT insensitive nicotinic receptors are regulated by different and distinct mechanisms.

Molecular cloning techniques provided new insights into the identity of the neuronal α-BGT binding protein. α-BGT binding protein was purified from chick brain using an α-BGT affinity column (Conti-Tronconi et al. 1985) from which a pure source of neuronal α-BGT receptor protein was obtained. Chick brain cDNA libraries were constructed and screened using oligonucleotides (Couturier et al. 1990a, Schoepfer et al. 1990). Two clones identified as members of the nicotinic acetylcholine family; that is they possessed 4 hydrophobic regions, 2 adjacent cysteines at positions 192 and 193 suggesting they may bind cholinergic ligands, were obtained and termed the α7 and α8 subunits (also referred to as αBGT BP1 and αBGT BP2, respectively). The rat homologue of the chick α7 gene has been cloned and expressed in the Xenopus oocyte expression system (Seguela et al. 1993).

There are several lines of evidence which support a role for the neuronal nicotinic α7 receptor subunit as being part of the α-BGT binding site in brain, with possibly other subunits also being involved (Anand et al. 1993; Gerzanich et al. 1994; Gotti et al. 1994). For example, Schoepfer et al. (1990) demonstrated that recombinant protein representing the extracellular domain of α7 bound α-BGT. In addition, expression of the chick and rat α7 in the Xenopus oocyte system results in the formation of homo-oligomeric channels that are activated by nicotinic agonists and blocked by low concentrations of α-BGT (Couturier et al. 1990a; Seguela et al. 1993). Another line of studies has also demonstrated that the pattern of α7 mRNA expression, as revealed by in situ hybridization, correlates with the pattern of radiolabelled BGT binding to rat brain sections (Clarke et al. 1985; Seguela et al. 1993).

The α5 neuronal nicotinic receptor subunit has also been suggested to be a component of the neuronal nicotinic α-BGT binding protein. Synthetic peptides corresponding to the extracellular domain of the rat α5 nicotinic receptor subunit have been shown to bind α-BGT (McLane et al. 1990). From these studies, the α-BGT binding sequence has been defined as being between amino acid residues 180 and 199 of the neuronal nicotinic subunit α5 (McLane et al. 1991). This evidence is not definitive since it is possible that even though the peptide segments were able to bind α-toxin, it is not known whether in the native α5 subunit, the toxin binding peptide segment is in the proper conformation to bind toxin. It is also possible that toxin binding sequences were not observed due to insufficient overlap or length of the peptide segments. Only when the binding of α-BGT to an α5 subunit containing receptor results in a blockade of

a nicotine induced response will the role of the α5 neuronal nicotinic receptor subunit as a component of the neuronal α-BGT binding protein be definitive.

1.3 Possible functional roles of the nicotinic α-BGT receptor

In nervous tissue, the role of the nicotinic α-BGT receptor is currently unclear: however, it appears not to play a major role in mediating synaptic transmission (Luetje et al. 1990, Deneris et al. 1991). The following sections will discuss the possible functional role of the neuronal nicotinic α-BGT receptor.

1.3.1 Role of the nicotinic α-BGT receptor in neuronal cells

There are several lines of evidence which demonstrate functional nicotinic responses in nervous tissue, which are sensitive to α-BGT, using whole-cell patch clamp technique. α-BGT sensitive nicotinic receptor mediated responses have been detected in rat cerebellum (De La Garza et al. 1987), in dissociated hippocampal neurons in culture (Alkondon and Albuquerque 1991; Zorumski et al. 1992; Alkondon and Albuquerque 1993; Alkondon et al. 1994) and in rat olfactory bulb neurons (Alkondon and Albuquerque 1994).

Vijayaraghavan et al. (1992) and Zhang et al. (1994) have shown that nicotinic activation of an α-BGT binding component in chick ciliary ganglion neurons results in an increase in Ca^{++} mobilization, which is sensitive to blockade by α-BGT, thus demonstrating a function for the α-toxin binding component. As well, the α-BGT binding subunit, α7, which forms a homo-oligomeric receptor when expressed in *Xenopus* oocytes,

has an unusually high permeability to calcium. Nicotinic activation of this homo-oligomeric receptor, which can be blocked by α-BGT, results in an influx of Ca^{++} and the subsequent activation of Ca^{++}-dependent Cl^- channels (Seguela et al. 1993). Elevation of intracellular Ca^{++} levels in the cell due to α-BGT receptor activation may serve as a second messenger to regulate a variety of cellular growth processes. Growth cone behaviour has been shown to be regulated by neurotransmitters and electrical activity by mechanisms linked to intracellular Ca^{++} (Kater and Mills 1991). Therefore Ca^{++} may act as an integrator of environmental cues that influence neurite outgrowth and synaptogenesis. In rat sensory neurons, a narrow window of intracellular calcium concentration optimal for neurite outgrowth has been described (Mohanna et al. 1992). Growth cone morphology and neurite extension are affected by intracellular Ca^{++} levels (Haydon et al. 1984; Kater and Mills 1991); if intracellular Ca^{++} falls below an optimal level, or rises significantly above it, growth cone motility and neurite outgrowth are inhibited. In rat retinal ganglion cells in culture, d-tubocurarine, a nicotinic receptor antagonist, enhanced neurite outgrowth (Lipton et al. 1988) thus implicating the nicotinic receptor in a growth related role. However, involvement of the neuronal nicotinic α-BGT sensitive versus the neuronal nicotinic α-BGT insensitive population of nicotinic receptors in process outgrowth was not determined since the effect of α-BGT on neurite outgrowth in the ganglion cells was not assessed.

Another physiological role for the BGT binding component may be in the regulation of cell death since it has been shown that elevation of Ca^{++} flux through glutamate receptors can induce cell death (Choi 1987; Choi et al. 1988). It has also been

observed that high concentrations of α-BGT in vivo prevent neuronal cell death in chick ciliary ganglia (Meriney et al. 1987). Ca^{++} entry through neuronal nicotinic α-BGT receptors may be involved in long-term phenotypic changes in neuronal properties, a role similar to that of voltage-dependent Ca^{++} channels and NMDA receptors, since increases in intracellular Ca^{++} can induce expression of certain immediate early genes such as c-fos and jun-B (Bartel et al. 1989).

The following studies suggest that the nicotinic α-BGT receptor is involved in a growth related role in CNS development due to the time course of appearance of α-BGT binding sites in developing nervous tissue. It has been demonstrated that the major increase in choline acetyltransferase activity occurs subsequent to the appearance of the α-BGT site, a finding which may suggest that the receptor plays a role in the guidance of incoming cholinergic nerve fibres (Chiappinelli and Giacobini 1978; Fiedler et al. 1987; Greene 1976; Kouvelas and Greene 1976). Further evidence for a role for α-toxin receptors in development was suggested by autoradiographic studies using radioactively labelled α-BGT as a ligand. These studies demonstrated that there is a transient and developmentally regulated expression pattern of α-BGT binding that correlates with synapse formation in the region studied. This developmental pattern of expression was seen in the developing rat hippocampus (Hunt and Schmidt 1979) as well as in the developing rat sensory cortex (Fuchs 1989). In addition, the postnatal establishment of the thalamo-cortical connections in the developing rat sensory cortex is paralleled by an increase in α-toxin staining in the corresponding cortical projection areas (Fuchs 1989). Similarily, in the toad optic tectum, the increase in α-BGT binding parallels synapse

formation between retina and optic tectum (Freeman 1977). Furthermore, evidence suggests that postsynaptic α-BGT binding sites exert a trophic influence on presynaptic terminals in the optic tectum since there was a shift in position of incoming neurons away from receptor sites blocked by the α-toxin (Freeman 1977). In line with the idea of a trophic or growth related role for the α-BGT binding receptor, mRNA for α7, the α-BGT binding neuronal nicotinic receptor subunit which forms a homo-oligomeric receptor upon expression in *Xenopus* oocytes, is transiently and developmentally expressed in developing chick optic tectum. As well, α7 mRNA expression peaks at the time when retinal axons reach the tectum (Couturier et al. 1990a) and this correlates with the developmental expression pattern described in α-BGT binding studies (Wang and Schmidt 1976). A similar correlation in α7 mRNA distribution and α-BGT binding has also been described in the developing rat cortex (Broide and Leslie, 1992).

1.3.2 Role of the nicotinic α-BGT receptor in non-neuronal cells

Studies have demonstrated the presence of α-BGT receptors in various non-neuronal cells. For example, α-BGT binding sites have been described in normal and neoplastic thymic epithelial cells (Kirchner et al. 1988), human rhabdomyosarcoma cells, small cell lung carcinoma (SCLC) cells and epithelial lung cancers of the non-SCLC type (Chini et al. 1992). α-BGT binding sites in SCLC cells were precipitated by antibodies generated against the α-BGT receptor of the IMR 32 neuroblastoma cell line (Chini et al. 1992). These results suggest that non-neuronal α-BGT receptors of unknown function present on SCLC cell lines such as the H69 cell line are immunologically similar to

neuronal α-BGT receptors.

Studies by Maneckjee and Minna (1990) demonstrated that exposure of various SCLC cell lines to opioids inhibited lung tumor cell growth and that exogenous nicotine exposure could reverse this inhibitory effect of the opioid on tumor growth. These results suggest that the nicotinic receptors present in these SCLC cell lines may be involved in mediating the effects of nicotine on tumor growth. Since the nicotinic α-BGT receptor binds nicotine, this population of nicotinic receptors may play a role in mediating these trophic effects of nicotine on SCLC cell growth. Schuller (1989) investigated the potential involvement of nicotinic cholinergic receptors in mediating the effects of nicotine and two tobacco-related nitrosamines, DEN and NNK, on human lung cell growth kinetics. Nicotine stimulated cell proliferation in H727 cells, a neuroendocrine type SCLC derived cell line. Furthermore, this effect of nicotine was blocked by the nicotinic cholinergic receptor antagonist, hexamethonium. Similar growth-stimulating effects on H727 cell proliferation were obtained with the two tobacco-related nitrosamines. These data thus suggest that nicotine as well as the nitrosamines interact at nicotinic type receptors in pulmonary neuroendocrine cells to mediate the effects observed on cell proliferation and maybe ultimately in the pathogenesis of lung cancer. From this study, evidence for the involvement of nicotinic type receptors in cell proliferation is reasonably compelling although the receptor through which the agonist mediates these effects, that is, the α-BGT sensitive or α-BGT insensitive nicotinic receptor has not been identified.

A role for the toxin receptor in cell proliferation is supported by experiments in cultured thymic epithelial cells. Acetylcholine increased cell proliferation and protein

synthesis as assessed by comparing ^{3}H-thymidine and ^{3}H-leucine incorporation in cholinergic agonist treated cells. This effect is blocked by α-BGT (Tominaga et al. 1989). These data provide evidence for a trophic role for the α-BGT receptor in regulating cell growth in thymic epithelial cells and suggest that the differentiation and maturation of thymic lymphocytes may be regulated by the nicotinic activation of thymic epithelial cells.

1.4 PC12 cells as a model for neuronal cells

PC12 cells are a clonal cell line derived from a rat adrenal pheochromocytoma (Greene and Tischler 1976) and are characteristically very similar to their parent chromaffin cells. As for adrenal chromaffin cells, in PC12 cells, the nicotinic acetylcholine receptor and the α-BGT binding sites are distinct in that cholinergic receptor dependent Na^+ fluxes are blocked by nicotinic antagonists but not by α-BGT (Patrick and Stallcup 1977a). Cells of the adrenal medulla known as chromaffin cells are derived from the neural crest and are directly innervated by sympathetic preganglionic nerve fibers of the splanchnic nerve (Weston 1970). Thus, chromaffin cells and PC12 cells, which are derived there from, are considered to be specialized postganglionic cells which share many characteristics with sympathetic neurons. For example, nerve growth factor (NGF) treated PC12 cells extend neurites in culture and thus have been used extensively as a model for neuronal cells (Greene and Tischler 1982). Furthermore, PC12 cells are excitable and they synthesize and secrete neurotransmitters such as noradrenaline, adrenaline and dopamine (Greene and Rein 1977; Greene and Tischler 1982). These neurotransmitters are stored in chromaffin granules and are released in response to

nicotinic receptor stimulation (Greene and Rein 1977). PC12 cells are an appropriate cell line for the study of nicotinic receptors and nicotinic receptor mediated responses since PC12 cells contain both the neuronal nicotinic α-BGT receptor as well as the neuronal nicotinic acetylcholine receptor. In addition, PC12 cells possess muscarinic receptors and receptors for nerve growth factor (NGF), epidermal growth factor (EGF) and adenosine (Guroff 1985).

PC12 cells are relatively easy to maintain in culture and consist of a relatively homogeneous population of pheochromocytoma cells. Furthermore, the maintenance of PC12 cells in culture allows for a controlled regulation of the environment surrounding the cells. Drugs can be added to the culture medium to determine their effects on any one of a number of parameters to be studied. Furthermore, cultured cells do not present the diffusional barriers that whole organ cultures do. PC12 pheochromocytoma cells therefore appeared to be an appropriate model system for the present studies investigating the functional role of the neuronal nicotinic α-BGT receptor.

1.5 H69 cells, a small cell lung carcinoma cell line: presence of nicotinic α-BGT in non-neuronal cells

Small cell lung carcinoma (SCLC), one of the most aggressive forms of cancer, accounts for approximately 25% of bronchogenic carcinomas and has distinct clinical, pathological and biological characteristics (Minna et al. 1981). SCLC cells exhibit many differentiated properties of neuroendocrine cells. These include the presence of cytoplasmic dense core neurosecretory granules, high concentrations of the key amine precursor uptake and decarboxylation cell enzymes, L-dopa-decarboxylase and neuron-

specific enolase, as well as the production of several peptides (Gazdar et al. 1985). The peptide most frequently associated with SCLC is the mammalian bombesin neuropeptide (Moody et al. 1981). As well, SCLCs have very high specific activities of the enzyme creatine kinase which is expressed in the form of the brain isoenzyme (Gazdar et al. 1981). On morphological grounds, SCLCs appear relatively undifferentiated as compared to non-SCLC. Thus, most SCLCs can be distinguished from non-SCLC lung cancers (squamous cell, large cell and adenocarcinomas) by morphology and the biochemical markers mentioned above. Radioligand binding studies using [^{125}I]α-BGT, [^{3}H]muscarine and [^{3}H]etorphine demonstrated the presence of nicotinic, muscarinic and opioid receptors in cell lines derived from SCLCs (Cunningham et al. 1985, Maneckjee and Minna 1990).

The H69 cell line is derived from a SCLC and was established and characterized by Carney et al. (1985). H69 cells possess the same biochemical and morphological characteristics as the SCLCs from which they were derived. H69 cells grow as aggregates in suspension in culture and are relatively easy to maintain. In culture, drugs could be routinely added to the culture medium to study their effects on H69 cell growth. Thus, H69 cells were chosen to study the pathological significance of nicotinic α-BGT receptors in non-neuronal tissues such as SCLCs.

1.6 Statement of the Problem

As discussed in the Introduction, numerous types of studies have distinguished the neuronal nicotinic α-BGT receptor from the neuronal nicotinic acetylcholine receptor population. This distinction was supported by localization studies which demonstrated that the tissue and cellular distribution of the two types of nicotinic receptors were different. Purification studies have determined the molecular weight of the subunits that make up the α-toxin binding protein, the structural composition of which appears to differ from that of the neuronal nicotinic acetylcholine receptor. As well, genes believed to encode α-BGT binding proteins have been isolated and cloned. Despite all this information, it is still unclear as to what the functional role of the α-toxin receptor is in neuronal and non-neuronal tissues, although it appears to be distinct from the α-BGT insensitive nicotinic receptor. Various studies in the literature suggest a role for the α-BGT receptor in development. Thus, in order to gain further insight into a possible growth related function for these receptors, studies were initiated to determine whether long term treatment with various nicotinic ligands, such as nicotine and d-tubocurarine, affected growth in the neuronal PC12 cell line and whether these observed effects on growth were mediated specifically through the neuronal α-toxin receptor population.

The recent observation that α-BGT receptors, immunologically similar to those found in nervous tissue, were also present in a variety of non-neuronal tumour cell lines led to the postulate that α-toxin receptors present in non-neuronal tissues may also be involved in growth. Thus, studies were initiated to determine the effect of long term

involved in growth. Thus, studies were initiated to determine the effect of long term exposure of nicotinic ligands on cell number of a small cell lung carcinoma cell line, as well as determining whether these effects on cell number were mediated by the α-BGT receptor population. The H69 small cell lung carcinoma cell line was chosen since evidence in the literature demonstrated the presence of α-toxin binding sites in various lung tumours. Experiments were also done to determine whether the presumed neuronal α-BGT binding subunits, α5 and/or α7, were expressed in H69 cells in order to gain further insight into the subunit composition of α-toxin receptors in non-neuronal tissue.

Thus, the main objective of my studies was to determine if the α-BGT receptor in both neuronal and non-neuronal tissues is involved in a growth related activity in order to gain a better understanding of the significance of this nicotinic receptor population.

2.0 A ROLE FOR THE NICOTINIC α-BUNGAROTOXIN RECEPTOR IN NEURITE OUTGROWTH IN PC12 CELLS

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Contributions by the authors are as follows: J. Chan performed the experiments and M. Quik provided supervisory support.

2.1 SUMMARY

The present studies show that nicotine decreased neuritic outgrowth in PC12 cells in culture. This effect occurs as early as one day after addition of nicotine to the culture medium in a concentration dependent manner. The nicotine induced decline in neurite outgrowth was prevented by d-tubocurarine (10^{-4}M) indicating that the effect was mediated through a nicotinic receptor. Interestingly, alpha-bungarotoxin (α-BGT) (10^{-8}M) was able to inhibit the nicotine induced decrease in process formation in a dose dependent manner. The concentrations of α-BGT required to affect process outgrowth correlated with those required to inhibit radiolabelled α-BGT binding. α-BGT had no effect on [^{3}H]noradrenaline release, a functional response mediated through the α-BGT insensitive neuronal nicotinic acetylcholine receptor, suggesting that α-BGT specifically interacts with the neuronal α-BGT receptor. The present results suggest a functional role for the neuronal nicotinic α-BGT receptor in neurite outgrowth.

2.2 INTRODUCTION

Two populations of nicotinic acetylcholine receptors exist in neuronal tissue (Luetje et al. 1990; Deneris et al. 1991). These include the α-BGT insensitive neuronal nicotinic receptors, which bind nicotinic receptor ligands with high affinity and do not interact with α-BGT; these receptors are thought to be involved in the mediation of synaptic transmission (Luetje et al. 1990; Deneris et al. 1991). The second population is the neuronal nicotinic α-BGT receptors , which bind α-BGT with high affinity and nicotinic ligands with a lower affinity (Morley et al. 1979; Oswald and Freeman 1981). The function of the neuronal nicotinic α-BGT receptor is currently unclear; however, it appears not to play a major role in mediating synaptic transmission. This is supported by the observation that α-BGT does not block nicotinic receptor mediated responses in most neuronal tissues (Luetje et al. 1990; Deneris et al. 1991) and also by the finding that nicotinic α-BGT binding sites are located in brain regions devoid of cholinergic innervation (Hunt and Schmidt 1978a; Hunt and Schmidt 1978b; Hunt and Schmidt 1979). On the other hand, there is correlative evidence implicating the nicotinic α-BGT receptor in a growth related or trophic role. It has been shown that the major increase in choline acetyltransferase activity occurs subsequent to the appearance of the α-BGT site, a finding which may suggest that the receptor plays a role in the guidance of incoming cholinergic nerve fibres (Greene 1976; Kouvelas and Greene 1976; Chiapinelli and Giacobini 1978; Wade and Timiras 1980; Fiedler et al. 1987). In addition, Freeman (1977) provided evidence which suggested that the postsynaptic α-BGT binding sites exert a trophic

influence on presynaptic terminals in the optic tectum since there was a shift in position of incoming neurons away from receptor sites blocked by the α-toxin. Fuchs (1989) has also suggested that the α-BGT sites may be important in the development of specific connections in rat primary sensory cortex and in the establishment of its columnar organization. Recent studies which have demonstrated the presence of α-BGT receptors in tumour cell lines, (Maneckjee and Minna 1990; Chini et al. 1992) may provide further correlative evidence for a role of the α-BGT receptor in growth related activities.

Evidence is also available which indicates that nicotine is involved in trophic function presumably through an interaction at a nicotinic cholinergic receptor population. Both prenatal and neonatal exposure to nicotine have been shown to result in alterations in nervous system development, including changes in nicotinic receptors, choline acetyltransferase activity as well as other neuronal markers (Navarro et al. 1988; Navarro et al. 1989a; Navarro et al. 1989b; Nordberg et al. 1991). Nicotine also affects tyrosine hydroxylase activity and ornithine decarboxylase activity (Slotkin et al. 1987; Smith et al. 1991a). Nicotinic stimulation of PC12 cells results in an induction of transcription of the c-fos proto-oncogene, (Greenberg et al. 1986) the expression of which has been correlated with cellular growth processes. Furthermore, in cultured retinal neurons it has been shown that nicotinic antagonists enhance process outgrowth (Lipton et al. 1988), while in lung tumor cells nicotine has been shown to have a mitogenic effect (Schuller et al. 1990). Evidence for a trophic role for nicotine is thus reasonably compelling although the receptor through which the agonist mediates these effects, that is, the α-BGT sensitive or α-BGT insensitive nicotinic receptor, was not identified.

The present experiments were done to further assess the potential involvement of nicotinic acetylcholine receptors, specifically the α-BGT receptor population, a receptor whose functional identity is as yet uncertain, in neuronal growth. This was approached by using the neuronal PC12 pheochromocytoma cell line in culture. Nerve growth factor (NGF) treated PC12 cells were chosen since these cells have been used extensively as a model for neuronal cells (Greene and Tischler 1982). Furthermore, they express both neuronal nicotinic acetylcholine receptors and neuronal α-BGT receptors (Greene and Tischler 1982). The effect of various nicotinic ligands was determined on [^{125}I]α-BGT binding to PC12 cells. Subsequently, the effects of exposure of the cells to the above mentioned agents were evaluated on neurite outgrowth and neurotransmitter release.

2.3 MATERIALS AND METHODS

2.3.1 PC12 pheochromocytoma cell culture

PC12 cells were obtained from the American Type Culture Collection, Rockville, MA. Cells were grown in collagen coated flasks in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% horse serum, 5% fetal calf serum, fungizone (amphotericin B, 0.15 μg/ml), penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37°C in humidified CO_2 and air (5:95) atmosphere. Cells were plated at the indicated densities (1-7.5x10^5 cells/dish) onto 35 mm poly-l-lysine (10 μg)-coated culture dishes (Nunc) in defined

medium composed of Dulbecco's Modified Eagle's Medium (DMEM) containing insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (5 ng/ml), progesterone (40 pg/ml), putrescine (200 ng/ml), penicillin (50 units/ml), streptomycin (50 μg/ml), and fungizone (amphotericin B, 0.15 μg/ml). 2.5S NGF (Prince Laboratories) and α-BGT (purified from *Bungarus multicinctus* venom, Miami Serpentarium Laboratory) were added to the culture medium immediately after plating; the culture medium and drugs were routinely changed every 3 days.

Experiments were done to establish the parameters which would allow for optimal NGF induced neurite outgrowth from the cells in culture. The effect of varying concentrations of NGF was evaluated; a maximal response occurred at approximately 7.5 ng/ml. This concentration of NGF was used in all subsequent experiments. The effect of varying plating density was also determined. Since optimal neurite formation was observed at a density of $1x10^5$ cells per dish, this cell density was used in the neurite outgrowth experiments. Experiments to assess the effect of the culture medium on NGF induced neurite outgrowth in PC12 cells indicated that cultures grown in a defined medium containing insulin, transferrin, sodium selenite, progesterone and putrescine, at the concentrations previously indicated, exhibited an increased neurite outgrowth compared to cultures grown in medium containing serum (10% horse serum, 5% fetal calf serum); defined medium was therefore used in all the present studies.

2.3.2 Assessment of neurite outgrowth

In the experiments in which neurite outgrowth was evaluated, cells were plated at

a density of 10^5 cells per 35 mm culture dish. Numerical analysis of PC12 cells displaying neurite outgrowth (≥80 μm in length, unless otherwise indicated) was done at various times after plating using phase contrast microscopy. The number of neurites ≥80 μm in length was quantitated in both control and drug treated cultures. The absolute number of neurites ≥80 μm in length in control cultures was expressed as 100%, while those obtained for drug treated cultures were expressed as a percent of the control culture values. Some variability between experiments was observed in the absolute numbers of neurites ≥80 μm in control cultures after similar lengths of time in culture. The reasons for variations in the number of neurites ≥80 μm in length between experiments is not clear since care was taken to ensure that similar culture conditions were maintained. However, they may relate to the variations in the different batches of reagents used. For example, cells were grown in medium containing both horse and fetal calf serum, the composition of which may have varied from batch to batch. The cells were passaged using trypsin-EDTA; the variations in activity of this enzyme between batches may have altered the integrity of the cells and thus their ability to extend neurites following passage. Furthermore, varying lots of poly-l-lysine, NGF and components in the defined medium may account for the variability observed. In general, with increasing time in culture (7 days), a decrease in the number of longer neurites was observed. However, in some experiments no decline was observed. This may relate to the fact that those experiments were done over only a 5 day period. This reduction in the number of longer neurites appeared to be due to a loss of viability of the cells with time in culture. For this reason, most experiments were terminated after 7 to 8 days in culture. The number of cells that

were assessed for neurite outgrowth were quantitated as follows. A diametric strip representing approximately 20 fields was counted at x100 magnification for each culture dish. There were approximately 30 to 50 cells per field, thus on average 600 to 1000 cells were assessed for each culture dish. Each culture condition was tested in triplicate or quadruplicate.

2.3.3 [^{3}H]Noradrenaline release studies

Basal and nicotinic receptor evoked release of [^{3}H]noradrenaline (specific activity 56.9 Ci/mmol, New England Nuclear) was evaluated as previously described (Greene and Rein 1977). PC12 cells were plated at a density of 7.5×10^5 cells per dish. On the day of the experiment, cells were washed 3 times with DMEM over a 30 minute (min) period at 37°C. Cells were then exposed to 800 µl of amino-acid free DMEM containing 10^{-7} M [^{3}H]noradrenaline and incubated for 30 min at 37°C. After this loading phase, each dish was washed 6 times with 1 ml DMEM over a 60 min period. Basal release of [^{3}H]noradrenaline was determined over a 2 min period; stimulated [^{3}H]noradrenaline release was then assessed in the presence of 10^{-4} M nicotine for a 2 min period. Cells were subsequently lysed with perchloric acid (0.4 N) to determine intracellular [^{3}H]noradrenaline content. Radioactivity in the fractions (1 ml) were measured in a liquid scintillation spectrometer. Total uptake of [^{3}H]noradrenaline was the sum of the radioactivity measured in the perchloric acid extract and the radioactivity secreted under basal and stimulating conditions. [^{3}H]noradrenaline release was expressed as a percentage of total [^{3}H]noradrenaline originally taken up.

2.3.4 [^{125}I]α-BGT binding to PC12 cells in culture

After 4 days in culture, cells (plated at a density of $3.0x10^5$ cells per dish) were washed with DMEM containing bovine serum albumin over a 60 min period prior to the binding assay. Cultures were then pre-incubated with varying concentrations of the indicated drugs for 60 min at 37°C, followed by a 90 min incubation with 1.5 nM [^{125}I]α-BGT (10-20 μCi/μg, New England Nuclear). To remove unbound radiolabelled toxin, cultures were washed 4 times with DMEM over a 40 min period. Specific binding of [^{125}I]α-BGT was defined as the total binding minus the binding occurring in the presence of $3x10^{-4}$ M d-tubocurarine; non-specific binding, using 1.5 nM [^{125}I]α-BGT represented approximately 5% of the total binding. The cells were then scraped from the culture dish with a rubber policeman using two 500 μl aliquots of water and the radioactivity counted using a gamma counter.

2.3.5 Cell counts

PC12 cell counts of control and drug treated cultures were carried out as described. Following neurite outgrowth assessment on Day 7, medium was removed and cells were washed twice with Ca^{2+}- and Mg^{2+}- free Hank's Balanced Salt Solution (HBSS). Cells were then incubated for 15 min in 0.01% trypsin, followed by 2 washes with HBSS and centrifugation. The pellet was resuspended in HBSS and trypan blue was added. Cell counts were done using a haemocytometer.

2.3.6 Statistics

Statistical comparisons were done using the Student's *t*-test or a one way analysis of variance (ANOVA) followed by the Scheffé test as indicated.

2.4 RESULTS

2.4.1 Effect of nicotine on neurite outgrowth in PC12 cells in culture

PC12 cells were exposed to varying concentrations of nicotine (10^{-6} M, 10^{-5} M, 10^{-4} M) as depicted in Fig. 2.1 to 2.4 (control condition). Exposure to 10^{-5} M and 10^{-4} M nicotine resulted in a significant decrease in the number of neurites ≥80 μm in length as compared to cultures not treated with agonist. This effect was observed as early as 1 day after nicotine exposure (Fig. 2.3 and 2.4); similar results were seen after 2 days and persisted up to 7 days in culture (Fig. 2.1 and 2.2). The concentrations of nicotine necessary to produce a significant decline in process outgrowth corresponded well with those required to inhibit [^{125}I]α-BGT binding to the cells in culture (Fig. 2.5); 10^{-5} M nicotine inhibited binding approximately 80 - 90%; with maximal inhibition occurring at 10^{-4} M nicotine. Similarily, the nicotine induced decline in neuritic outgrowth appeared to plateau at 10^{-4} M with an almost complete inhibition at 10^{-5} M. Exposure of PC12 cells to 10^{-6} M nicotine, had no significant effect on neurite outgrowth; as well, this concentration of agonist did not alter radiolabelled toxin binding.

Cell counts (Table 2.1) were done to determine the number of PC12 cells per dish after 7 days exposure to nicotine: the results show that the cell numbers were similar under all conditions tested. Thus the observed alterations in process outgrowth under the different experimental conditions were not the result of changes in cell number.

2.4.2 Effect of α-BGT on the nicotine induced decline in neurite outgrowth

Although the functional role of the α-BGT site is currently not known, previous studies have implicated this nicotinic receptor population in growth and development. We therefore tested the effect of α-BGT on PC12 cell neurite outgrowth both alone and in combination with nicotine. As can be seen in Fig. 2.1, 10^{-8} M α-BGT completely prevented the nicotine induced decline in neurite outgrowth at all days tested. The α-toxin did not appear to alter neurite length, number or morphology on its own. Fig. 2.2 demonstrates the concentration dependence of the ability of α-BGT to prevent the nicotine induced decline in neurite outgrowth after various days in culture. A complete reversal of the nicotine induced effect is observed at 10^{-8} M α-BGT. This concentration correlates very well with that which results in an essentially complete block of radiolabelled toxin binding to PC12 cells in the receptor assay (Fig. 2.5).

Cell counts (Table 2.1) showed that exposure to α-BGT either in the absence or presence of varying concentrations of nicotine had no effect on PC12 cell number. Thus, the return of process length to control values in the presence of both nicotine and α-BGT was not a function of increased cellular proliferation in the presence of the α-toxin.

2.4.3 Effect of the nicotinic antagonist, d-tubocurarine, on neurite outgrowth

D-tubocurarine, a nicotinic blocking drug effective at both neuronal and neuromuscular nicotinic receptors, was also tested on neurite outgrowth in PC12 cells. The antagonist had no observable effects when tested alone; however, when nicotine was added to the cultures in combination with d-tubocurarine, the nicotine induced decline in neurite outgrowth was prevented at all time periods evaluated (Fig. 2.3). This ability of d-tubocurarine to prevent the diminished neurite outgrowth elicited by nicotine exposure was concentration dependent as shown in Fig. 2.4. The observed concentration dependence of d-tubocurarine to reverse the nicotine induced decline in neuritic outgrowth is consistent with its ability to interact at the α-BGT receptor as determined from [^{125}I]α-BGT binding to PC12 cells. D-tubocurarine inhibited binding approximately 50% at $3x10^{-6}$ M, with a maximal inhibition of binding at 10^{-4} M of the antagonist (Fig. 2.5). A similar dose response was observed in the experiments to assess alterations in neurite outgrowth.

2.4.4 Effect of α-BGT on neurotransmitter release

The effect of exposure of PC12 cells to α-BGT was determined on basal and nicotine (10^{-4}M) evoked [^{3}H]noradrenaline release from PC12 cells. In the short term (acute) experiments, cells were pretreated with either α-BGT (10^{-8} M), d-tubocurarine (10^{-4} M) or no drug (control) for 45 min. The results in Table 2.2 show that nicotine evoked release of [^{3}H]noradrenaline is blocked by d-tubocurarine in line with previous results (Greene and Rein 1977). On the other hand, α-BGT did not significantly alter either basal or nicotine stimulated neurotransmitter release. Furthermore, exposure to α-BGT did

not affect the release process when acetylcholine (10^{-4} M) was used as the secretagogue (data not shown).

In the long term experiments, cells were exposed to α-BGT (10^{-8}M) immediately after plating. After 6 days, basal and nicotine stimulated release of [^{3}H]noradrenaline were determined for control and α-BGT pre-treated cultures (Table 2.2); however, neither resting nor nicotine evoked radiolabelled catecholamine release were altered.

2.5 DISCUSSION

The present results show that exposure of PC12 cells to nicotine results in a decline in neuritic outgrowth. The concentration of agonist required for inhibition of neurite outgrowth in PC12 cells correlated well with the affinity of nicotine for the α-BGT receptor suggesting that nicotinic activation of α-BGT receptors is involved in the inhibition of neurite outgrowth. The observation that nicotine has effects on trophic functions is in line with previous studies using retinal ganglion cells in culture. Lipton et al. (1988) demonstrated that the nicotinic blocker d-tubocurarine resulted in an enhanced neurite outgrowth, a finding which suggested that the nicotinic cholinergic system exerted a tonic inhibitory influence on process formation in these cells. The hypothesis that the nicotinic cholinergic system exerts a negative modulatory control is further supported by *in vivo* studies involving nicotine administration, which showed that both pre and postnatal exposure resulted in nervous system impairment (Navarro et al.

1989b; Nordberg et al. 1991; Smith et al. 1991a; Smith et al. 1991b; Sorenson et al. 1991). The functional role of the nicotinic α-BGT receptor is currently unclear, although correlative evidence exists implicating this site in trophic and/or growth related activities. Based on this rationale, the effect of α-BGT was evaluated on neurite morphology in PC12 cells. Interestingly, α-BGT prevented the nicotine induced decline in neuritic outgrowth in PC12 cells in culture, although the toxin alone had no observable effects. The relative potency of α-BGT to reverse the nicotine elicited effects on process formation in PC12 cells was very similar to the potency of the α-toxin to inhibit ^{125}I-α-BGT binding; this close agreement suggests that the snake toxin is exerting its effect on neurite formation through an interaction at the nicotinic α-BGT receptor. This hypothesis is further supported from the results of experiments which show that d-tubocurarine, a nicotinic receptor antagonist which interacts at both α-BGT insensitive and α-BGT sensitive nicotinic receptors, also prevents nicotine from eliciting its effects on neurite formation.

In the present work using NGF treated PC12 cells, α-BGT (10^{-8} M) altered nicotine induced trophic effects, while the toxin alone had no effect. In a previous study involving PC12 cells not treated with NGF, α-BGT on its own at 10^{-8} M had very little effect in agreement with the present study, although 10^{-6} M of the toxin had elicited neurite outgrowth (Quik et al. 1990). The significance of the effect of 10^{-6} M α-BGT was uncertain because 10^{-8} M of the toxin had resulted in a maximal inhibition of α-BGT binding. Because of the discrepancy between the binding and functional data in the previous experiments, the effect of concentrations of α-BGT greater that 10^{-8} M were not

tested on neuritic outgrowth in the present study.

Evidence for the specificity of the α-toxin's interaction at the nicotinic α-BGT receptor population is demonstrated by the lack of effect of the toxin on nicotinic receptor evoked neurotransmitter release. This is in agreement with previous studies which had shown that α-BGT did not inhibit a wide variety of nicotinic receptor mediated responses related to neurotransmission (Morley et al. 1979; Oswald and Freeman 1981; Quik et al. 1987). As expected, d-tubocurarine did block neurotransmitter release providing evidence that the observed release was indeed due to nicotinic receptor stimulation. The fact that both neurotransmitter release and the nicotine induced decline in neuritic outgrowth were prevented by d-tubocurarine indicated that this antagonist does not discriminate between the α-BGT sensitive and α-BGT insensitive nicotinic receptors in line with previous data.

The results presented in this paper provide evidence for a functional role of the α-BGT receptor in neuritic outgrowth in PC12 cells. They suggest that *in vivo*, the α-BGT receptor may be involved in aspects of neuronal function such as growth and development. Recent studies have demonstrated that purified chick α-BGT receptors reconstituted in a lipid bilayer form functional cation channels (Gotti et al. 1991; Gotti et al. 1992) and the α-BGT sensitive homo-oligomeric α7 receptor from chick and rat brain also exhibits properties of a cation-gated ion channel (Couturier et al. 1990; Seguela et al. 1993). Although the nature of the ion(s) which flux(es) through these channnels is as yet uncertain, studies have demonstrated that activation of the α-BGT receptor in chick ciliary ganglion neurons leads to an influx of calcium (Vijayaraghavan et al. 1992). Interestingly, neurite outgrowth has also been shown to be regulated by the levels of intracellular calcium (Kater and Mills 1991).

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Table 2.1. Effect of Nicotine and α-BGT on PC12 Cell Number

Condition	[Nicotine] (M)	Number of cells per dish (x 10^5)
Control	0	1.1 ± 0.1
	10^{-6}	1.2 ± 0.1
	10^{-5}	1.3 ± 0.1
	10^{-4}	1.1 ± 0.1
α-BGT	0	1.1 ± 0.1
	10^{-6}	1.2 ± 0.2
	10^{-5}	1.3 ± 0.1
	10^{-4}	1.2 ± 0.2

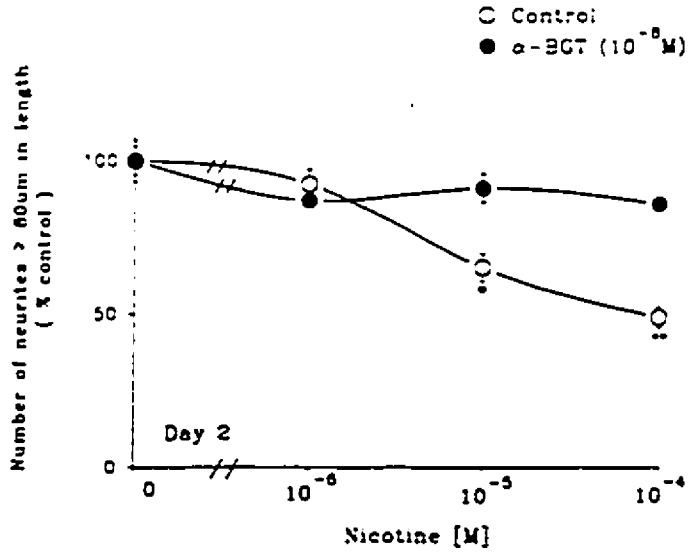
α-BGT (10^{-8} M) in the absence or presence of varying concentrations of nicotine was added to PC12 cells in culture (10^5 cells/dish) immediately after plating. The number of cells per culture dish was determined following neurite outgrowth assessments on day 7. Each value represents the mean ± S.E.M. of 3-4 culture dishes. The results are representative of 3 separate experiments.

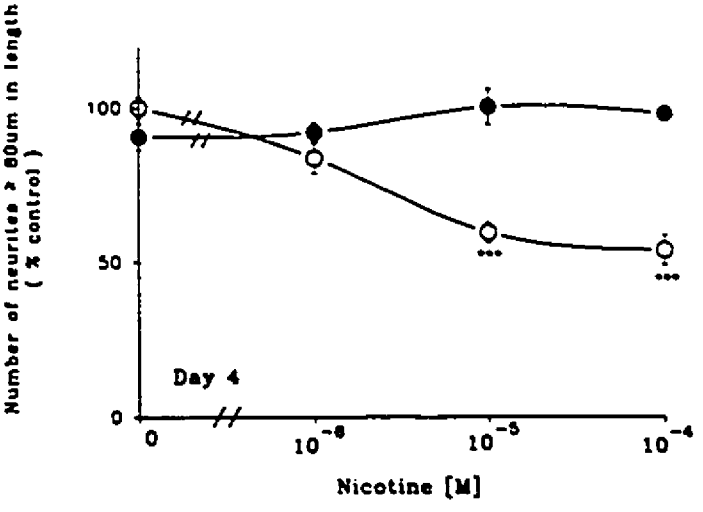
Table 2.2 The Effect of Acute and Long Term α-BGT Exposure on ^{3}H-Noradrenaline Release From PC12 Cells

Exposure Period	Treatment	[Drug] (M)	% Total ^{3}H-Noradrenaline Released: Basal	Nicotine Evoked
Acute	Control	None	1.99 ± 0.22	5.52 ± 0.44*
	α-BGT	10^{-8}	2.33 ± 0.17	6.08 ± 0.40*
	D-Tubocurarine	10^{-4}	2.05 ± 0.31	2.44 ± 0.43
Long term	Control	None	1.52 ± 0.12	3.89 ± 0.25*
	α-BGT	10^{-8}	1.57 ± 0.13	3.57 ± 0.11*

PC12 cells were cultured and plated ($7.5x10^5$ cells/dish) as described in Experimental Procedures. After 6 days in culture, the cells were washed and the basal and nicotine (10^{-4}M) evoked release of ^{3}H-noradrenaline determined over a 2 min collection period each. In the acute experiments, cells were incubated in the absence or presence of 10^{-8} M α-BGT or 10^{-4}M d-tubocurarine 45 min prior to, as well as during the 2 min basal release period and the 2 min nicotine stimulation period. In the long term studies, cells were incubated in the absence or presence of 10^{-8}M α-BGT for 6 days. On the day of assay, the α-toxin was removed prior to the determination of basal and nicotine evoked release. Each value represents the mean ± S.E.M. of 10 to 12 culture dishes pooled from three separate experiments. *$P < 0.05$ indicates a significant difference between basal and nicotine evoked release of ^{3}H-noradrenaline as determined using the Student's *t*-test.

Fig. 2.1. Effect of α-BGT on the nicotine induced decline in neurite outgrowth. Varying concentrations of nicotine were added to PC12 cells in culture (10^5 cells/dish) in the absence or presence of α-BGT (10^{-8} M) immediately after plating. The number of processes ≥ 80 μm in length was assessed after 2, 4 and 7 days in culture. Each symbol represents the mean ± S.E.M. of twelve culture dishes and is the pooled result of three separate experiments. The number of neurites ≥ 80 μm in length under control conditions (that is, in the absence of nicotine and α-BGT) were 177 ± 20, 225 ± 10 and 115 ± 4 neurites on days 2, 4 and 7 in culture, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control cultures (no nicotine) after a one way ANOVA followed by the Scheffé test.


○ Control
● α-BGT (10^{-8}M)
Number of neurites ≥ 80um in length
(% control)
100
50
0
Day 2
0
10^{-6}
10^{-5}
10^{-4}
Nicotine [M]


Number of neurites ≥ 80um in length
(% control)
100
50
0
Day 4
0
10^{-6}
10^{-5}
10^{-4}
Nicotine [M]

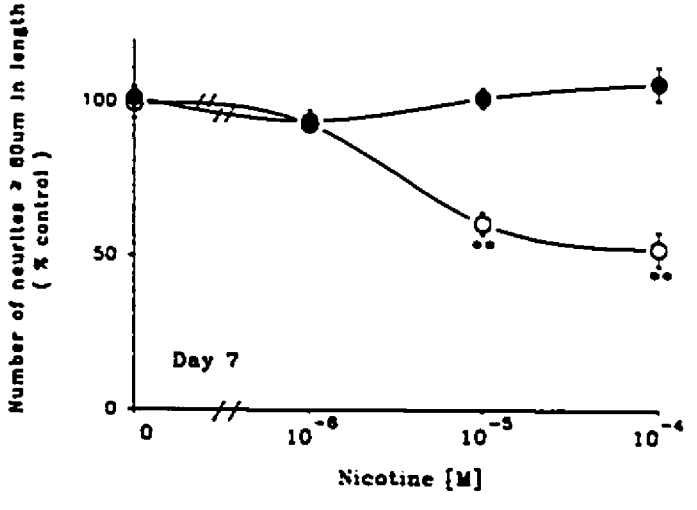
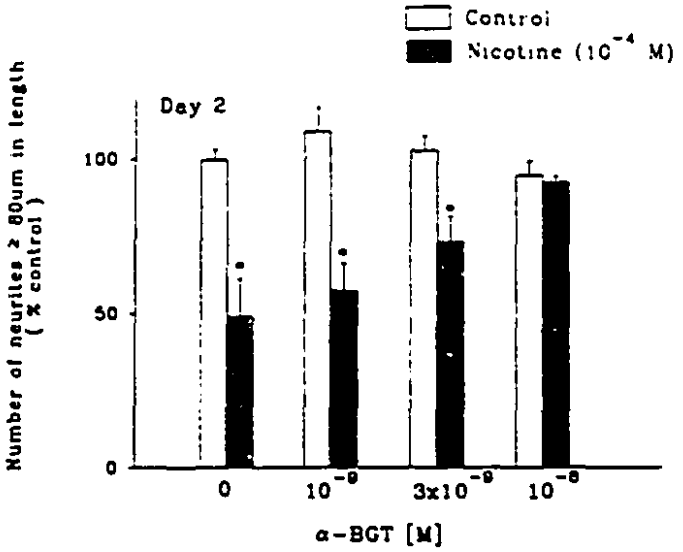
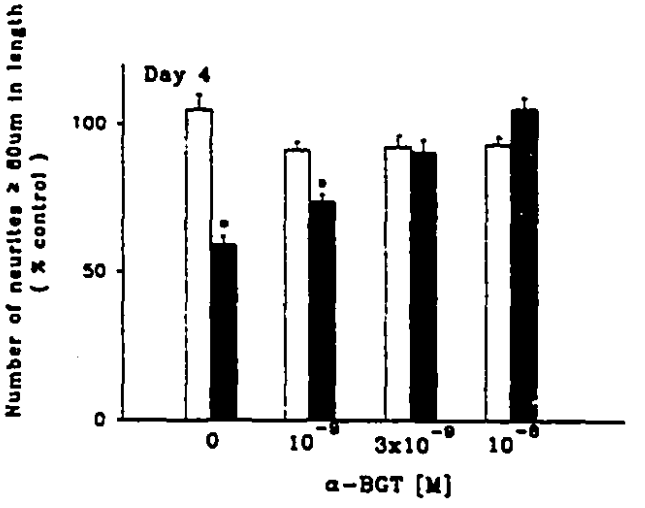

Number of neurites ≥ 80um in length
(% control)
100
50
0
Day 7
0
10^{-6}
10^{-5}
10^{-4}
Nicotine [M]

Fig. 2.2. Concentration dependence of α-BGT in preventing the nicotine induced decline in neuritic outgrowth. Nicotine (10^{-4} M) and varying concentrations of α-BGT were added to PC12 cells in culture (10^5 cells/dish) immediately after plating. The number of processes ≥ 80 μm in length was determined after 2, 4 and 7 days in culture. Each bar represents the mean ± S.E.M. of twelve culture dishes and is the pooled result of three separate experiments. The number of neurites ≥ 80 μm in length under control conditions (that is, in the absence of nicotine and α-BGT) were 241 ± 16, 189 ± 22 and 120 ± 7 neurites on days 2, 4 and 7 in culture, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control cultures (no α-BGT) after a one way ANOVA followed by the Scheffé test.


Control
Nicotine (10^{-4} M)
Day 2
Number of neurites ≥ 80um in length
(% control)
100
50
0
0
10^{-9}
$3x10^{-9}$
10^{-8}
α-BGT [M]


Day 4
Number of neurites ≥ 80um in length
(% control)
100
50
0
0
10^{-9}
$3x10^{-9}$
10^{-8}
α-BGT [M]

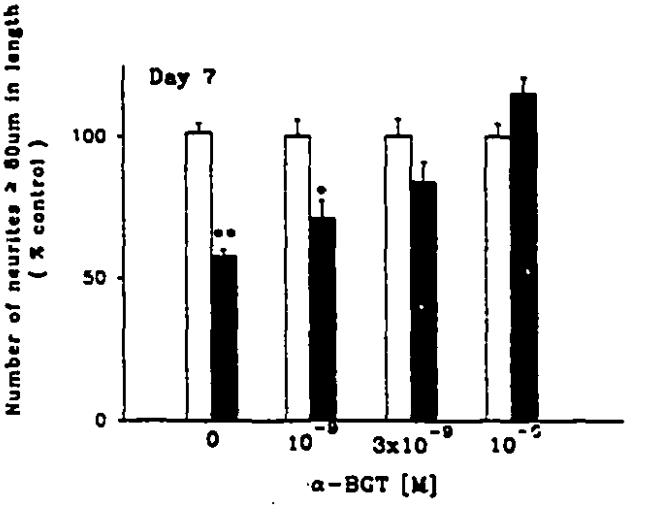

Day 7
Number of neurites ≥ 80um in length
(% control)
100
50
0
0
10^{-9}
$3x10^{-9}$
α-BGT [M]

Fig. 2.3. Effect of d-tubocurarine on the nicotine induced decline in neurite outgrowth. Varying concentrations of nicotine were added to PC12 cells in culture (10^5 cells/dish) in the absence and presence of d-tubocurarine (10^{-4}M) immediately after plating. The number of processes ≥ 80 μm in length was assessed after 1, 3 and 5 days in culture. Each symbol represents the mean ± S.E.M. of twelve culture dishes and is the pooled result of three separate experiments. The number of neurites ≥ 80 μm in length under control conditions (that is, in the absence of nicotine and d-tubocurarine) were 48 ± 3, 181 ± 12 and 196 ± 10 neurites on days 1, 3 and 5 in culture, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control cultures (no nicotine) after a one way ANOVA followed by the Scheffé test.

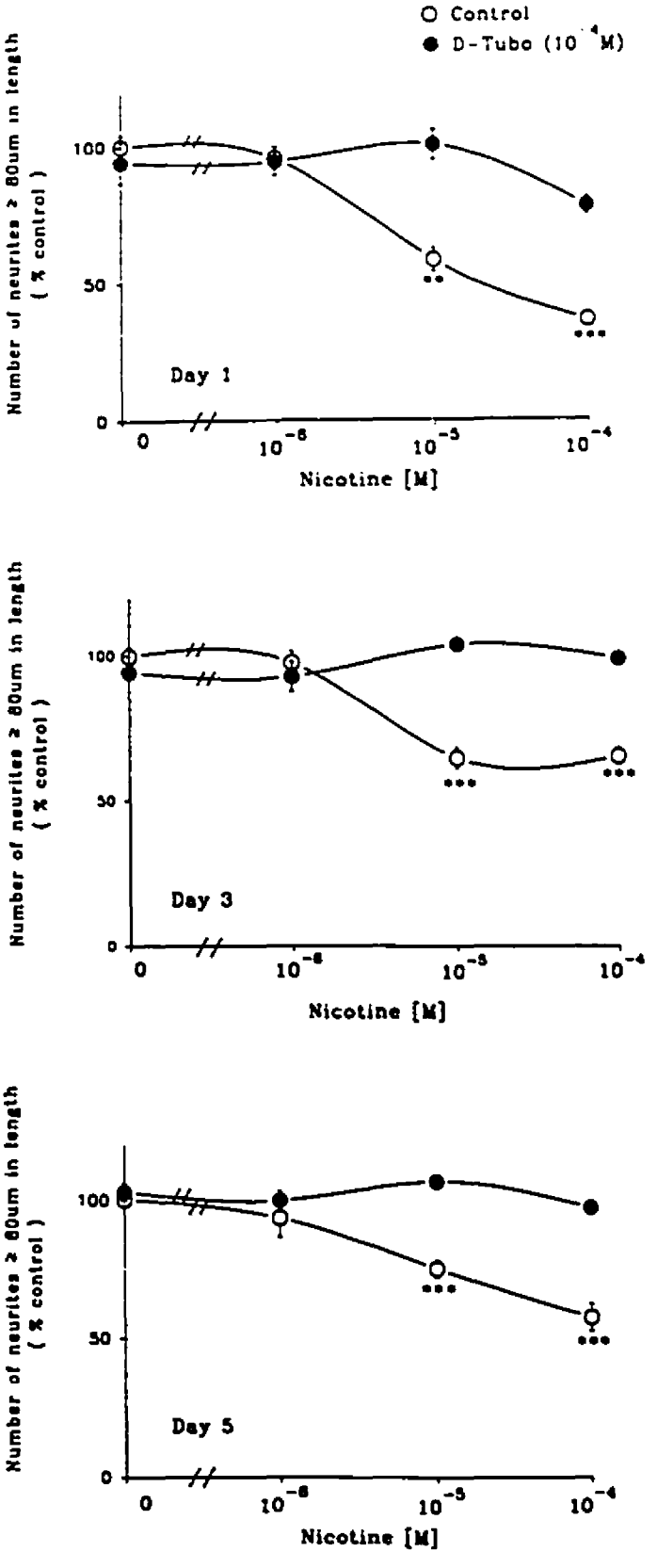
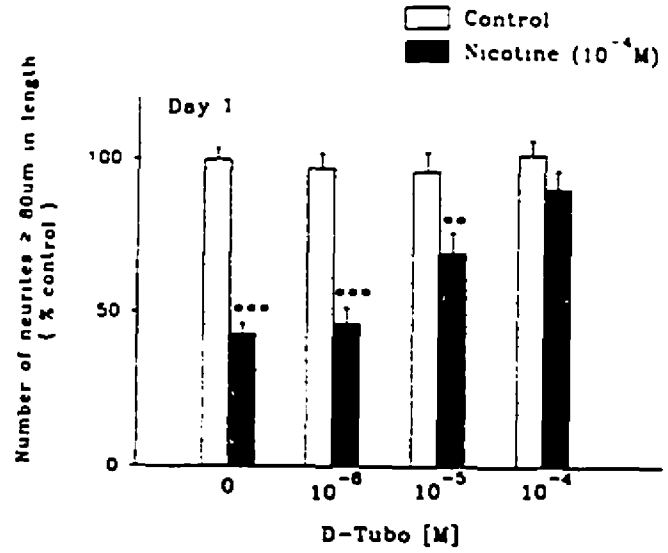
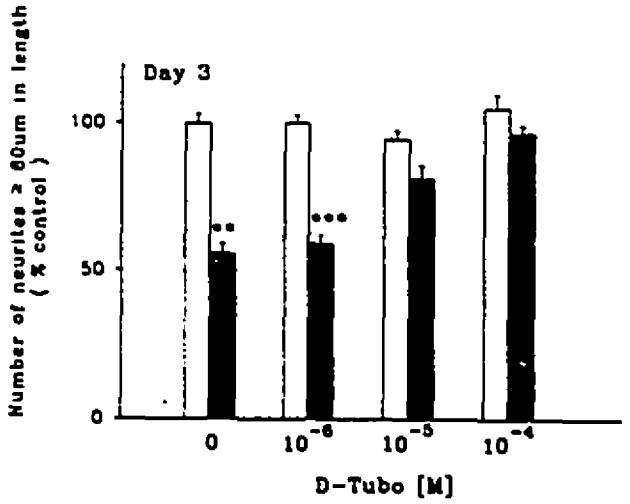

○ Control
● D-Tubo (10^{-4}M)
Number of neurites ≥ 80um in length (% control)
100
50
0
Day 1
**
***
0
10^{-6}
10^{-5}
10^{-4}
Nicotine [M]
Number of neurites ≥ 80um in length (% control)
100
50
0
Day 3
***
***
0
10^{-6}
10^{-5}
10^{-4}
Nicotine [M]
Number of neurites ≥ 80um in length (% control)
100
50
0
Day 5
***
***
0
10^{-6}
10^{-5}
10^{-4}
Nicotine [M]

Fig. 2.4. Concentration dependence of d-tubocurarine in preventing the nicotine induced decline in neuritic outgrowth. Nicotine (10^{-4} M) and varying concentrations of d-tubocurarine were added to PC12 cells in culture (10^5 cells/dish) immediately after plating. The number of processes ≥ 80 μm in length was determined after 1, 3, and 5 days in culture. Each bar represents the mean ± S.E.M. of twelve culture dishes and is the pooled result of three separate experiments. The number of neurites ≥ 80 μm in length under control conditions (that is, in the absence of nicotine and d-tubocurarine) were 43 ± 4, 175 ± 20 and 246 ± 21 neurites on days 1, 3 and 5 in culture, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control cultures (no d-tubocurarine) after a one way ANOVA followed by the Scheffé test.


Control
Nicotine (10^{-4}M)
Day 1
Number of neurites ≥ 80um in length
(% control)
100
50
0
***
***
**
0
10^{-6}
10^{-5}
10^{-4}
D-Tubo [M]


Day 3
Number of neurites ≥ 80um in length
(% control)
100
50
0
**
***
0
10^{-6}
10^{-5}
10^{-4}
D-Tubo [M]

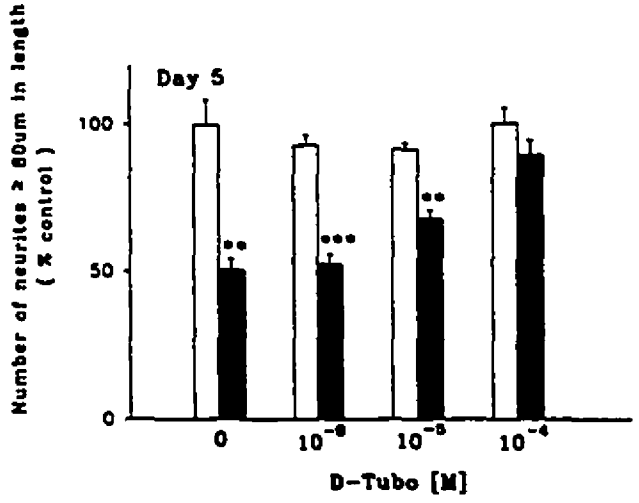
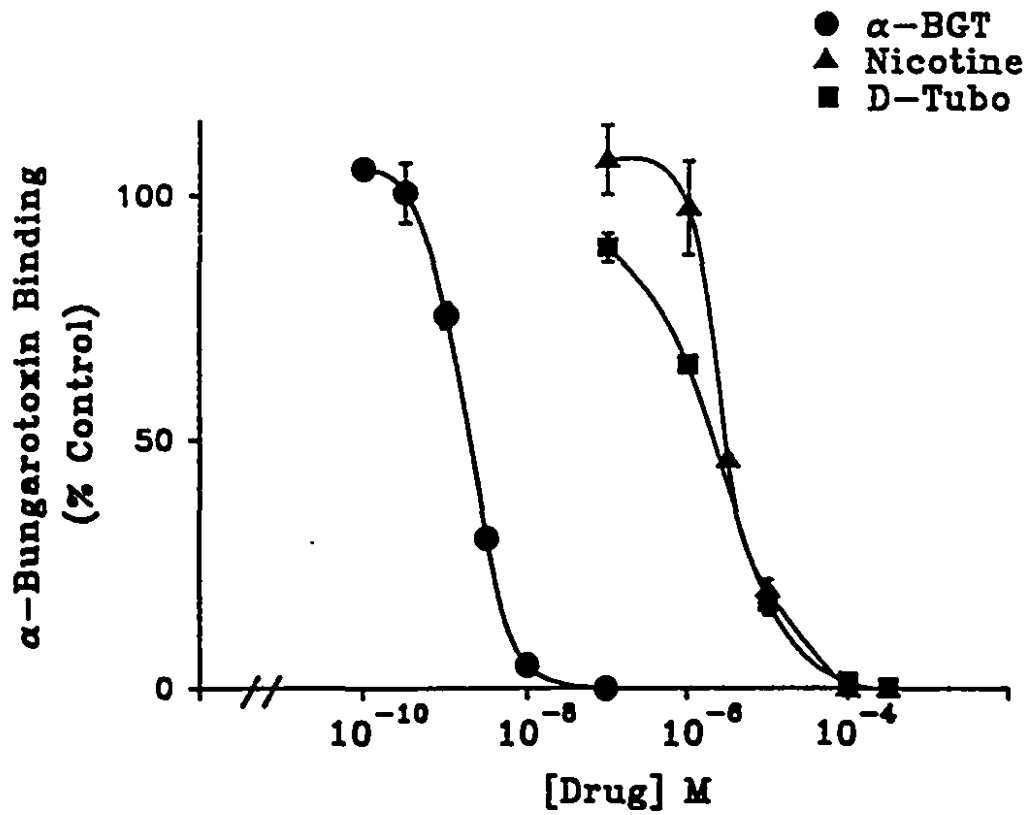

Day 5
Number of neurites ≥ 80um in length
(% control)
100
50
0
**
***
**
0
10^{-6}
10^{-5}
10^{-4}
D-Tubo [M]

Fig. 2.5. Effect of cholinergic ligands on [^{125}I]α-BGT binding to PC12 cells in culture. [^{125}I]α-BGT binding to PC12 cells in culture ($3x10^5$ cells/dish) was assessed 4 days after plating. Cells were preincubated for 60 min in the absence or presence of the indicated concentration of α-BGT, nicotine or d-tubocurarine (D-Tubo) and the binding assay done as described in Experimental Procedures. Each symbol represents the mean ± S.E.M. of five culture dishes. Where the S.E.M. is not depicted, it fell within the symbol. Results are representative of three separate experiments.


α–BGT
Nicotine
D–Tubo
α–Bungarotoxin Binding
(% Control)
100
50
0
10^{-10}
10^{-8}
10^{-6}
10^{-4}
[Drug] M

3.0 α-BUNGAROTOXIN BLOCKS NICOTINE INDUCED CELL PROLIFERATION IN A LUNG TUMOR CELL LINE

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3.1 SUMMARY

The function of the neuronal nicotinic α-bungarotoxin (α-BGT) receptor is currently unclear; however, the α-toxin site has been implicated in growth related activities in both neuronal and non-neuronal cells. The following experiments demonstrate that in H69 cells, a lung tumor cell line, nicotine (10^{-5}M) exposure results in a 50% increase in cell number after 9 days in culture. This effect of nicotine was both dose and time dependent. Similar results were observed with another nicotinic agonist, cytisine. Interestingly, α-BGT (10^{-8}M) was able to prevent this increase in H69 cell number which occured after nicotine exposure. This effect of α-BGT exhibited a dose response. α-Toxin alone in the absence of nicotine had no effect on H69 cell number. [^{125}I]α-BGT binding demonstrated the presence of high affinity α-BGT binding sites (K_d= 25 nM, B_{max}= 10.4 fmol/10^6 cells) which could be competed for by ligands such as nicotine, cytisine and d-tubocurarine thus demonstrating the nicotinic nature of these α-BGT binding sites in H69 cells. Furthermore, Northern analysis showed that mRNA for α7, a putative nicotinic α-bungarotoxin binding subunit, and for α5 were present in H69 cells. Taken together, these results suggest that the α-BGT site may be involved in modulating the proliferative responses in neuroendocrine derived small cell lung carcinoma cells.

3.2 INTRODUCTION

Evidence suggests that two distinct populations of nicotinic acetylcholine receptors, that is α-BGT sensitive and insensitive, are present in both the central and peripheral nervous system (Deneris et al 1991: Luetje et al. 1990). The snake venom α-bungarotoxin (α-BGT) binds to the muscle type nicotinic receptor with high affinity and blocks neurotransmission (Berg et al. 1972). High affinity, saturable toxin binding sites are also present in brain (Morley et al. 1979; Oswald and Freeman 1981). The function of toxin binding sites in nervous tissue is unclear; studies suggest the involvement of α-BGT receptors in a growth related role (Freeman 1977; Fuchs 1989; Vijayaraghavan et al. 1992). In line with these studies, the presence of toxin sensitive responses in nervous tissue have been described. For example, the neuronal nicotinic acetylcholine receptor subunit, α7, forms a homo-oligomeric cation channel blocked by α-BGT (Couturier et al. 1990). Using whole-cell voltage clamp, nicotinic acetylcholine currents sensitive to and inhibited by α-BGT were detected in cultured postnatal rat hippocampal neurons (Zorumski et al. 1992). Nicotinic responses sensitive to α-BGT were also observed in rat cerebellum (de la Garza et al. 1987). In addition, high affinity α-BGT binding sites not involved in the formation of acetylcholine-gated channels have also been described in a variety of neuronal preparations (Patrick and Stallcup 1977a,b; Clementi et al. 1986; Quik and Geertsen 1988).

The subunit composition of these α-BGT receptors is still unclear. Peptide binding studies suggest that the α5 neuronal nicotinic acetylcholine receptor subunit binds α-BGT (McLane et al. 1990). In chick brain and ciliary ganglia, the α5 gene product has been

is involved in the formation of an α-BGT binding receptor (Couturier et al. 1990; Seguela et al. 1993). In situ hybridization data for the α7 neuronal nicotinic receptor subunit has demonstrated the localization of α7 mRNA to the same regions of ^{125}I-α-BGT binding in rat brain (Seguela et al. 1993). This also suggests that the α7 receptor subunit is a component of the α-BGT binding protein.

To date, all the nicotinic acetylcholine receptor subunits that have been cloned have been shown to be expressed either in muscle or in nervous tissue. Studies suggest that α-BGT binding sites are also present in non-neuronal tissue such as thymic epithelial cells (Tominaga et al. 1989), epidermoid carcinoma cells, hepatoma cells and small cell lung carcinoma cells (SCLC) (Chini et al 1992). The functional significance of toxin binding sites in these cells is unknown.

SCLC is derived from a very agressive type of cancer and accounts for 25% of all cases of lung cancer and has a high correlation with smoking (Teeling et al. 1988; Gazdar et al. 1985). SCLC cells produce and secrete various polypeptide hormones such as calcitonin and bombesin and are characterized by a variety of neuronal markers such as the brain isozyme of creatinine kinase, l-dopa decarboxylase, and neuron-specific enolase (Carney et al. 1985). Muscarinic, nicotinic and opioid receptors as well as voltage operated calcium channels have been demonstrated to be present (Roth and Barchas 1986, Maneckjee and Minna 1990; Chini et al. 1992)

There is a strong association between cigarette smoking, nicotine addiction and the development of lung cancer; however, the role of nicotine therein is unknown (Hecht and Hoffman 1988; Schuller et al. 1990). In the CNS and PNS, nicotine exerts a variety

of behavioral, trophic, endocrine and neuromuscular effects via activation of nicotinic type receptors (Fiedler et al. 1987, Quik and Geertsen 1988, Fuchs 1989, Navarro et al. 1989 a,b; Nordberg et al. 1991; Chan and Quik 1993; Pugh and Berg 1994). Nicotine has been shown to increase SCLC cell number via a nicotinic type receptor (Schuller 1989). However, the type of nicotinic receptor through which this occurs has not been studied.

Due to possible involvement of neuronal nicotinic α-BGT receptors in a growth related function (Chan and Quik 1993), we have investigated the possible trophic role of nicotine and α-BGT receptors in H69 cells, a human SCLC cell line (Gazdar et al. 1985), in culture. α-Toxin binding sites in H69 cells were characterized via ^{125}I-α-BGT binding. Subsequently, the effects of long term exposure of the cells to α-BGT and various nicotinic receptor ligands were evaluated on cell number. Furthermore, Northern analysis was used to determine if α5 or α7 mRNA is expressed in H69 cells.

3.3 MATERIALS AND METHODS

3.3.1 H69 cell culture

H69 cells were obtained from the American Type Culture Collection, Rockville, MA. Cells were grown in RPMI-1640 Medium containing 10% fetal calf serum (heat inactivated), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in humidified CO_2 and air (7:93) atmosphere. Cells were plated at the indicated densities (2.5 x 10^4 or

0.5 x 10^6 cells/well) into 24-well multiwell culture plates (Costar). Drugs were added to the culture medium immediately after plating: the culture medium and drugs were routinely changed every 3 days. Nicotine ((-)nicotine di(+) tartrate salt) was obtained from Sigma. α-Bungarotoxin was obtained from Biotoxins Inc. (Florida).

3.3.2 XPC12 cell culture

XPC12 cells were obtained from Dr. J. Patrick (Baylor College of Medicine). Cells were grown in RPMI-1640 Medium containing 10% fetal calf serum, 5% horse serum (heat inactivated), penicillin (100 units/ml) and streptomycin (100 ug/ml) at 37°C in humidified CO_2 and air (7:93) atmosphere. Medium was changed every 2 days.

3.3.3 H69 cell counts

H69 cell counts of control and drug treated cultures were carried out as described. Cells were pelleted, culture medium was removed and cells were washed twice with Ca^{2+}- and Mg^{2}- free Hank's Balanced Salt Solution (HBSS). Cells were then incubated for 5 min in 0.05% trypsin to obtain a single cell suspension, followed by 2 washes with HBSS and centrifugation. The pellet was resuspended in HBSS and trypan blue-stained cells were counted using a haemocytometer.

3.3.4 [^{125}I]α-BGT binding to H69 cells in culture

On the day of assay, cells were removed from the culture flask, placed in an eppendorf tube (0.5 x 10^6 cells/tube) and pelleted. The pellet was washed three times

with RPMI medium containing bovine serum albumin over a 30 min period. Cells were preincubated with α-BGT (1μM) for 60 min (saturation binding experiments) or varying concentrations of the indicated drugs (competition binding experiments) at 37°C, followed by a 90 min incubation with varying concentrations of ^{125}I-α-BGT (10-20 μCi/μg, NEN) (saturation binding experiments) or 25nM ^{125}I-α-BGT (competition binding experiments) at 37°C. To remove unbound radiolabelled toxin, cells were pelleted and washed 4 times with RPMI containing bovine serum albumin over a 20 min period. Pellets were placed into new eppendorf tubes and counted using a gamma counter. Specific binding of ^{125}I-α-BGT was defined as the total binding minus the binding occuring in the presence of 1 μM α-BGT; non-specific binding represented approximately 50% of the total binding.

3.3.5 Northern Blot Analysis

Poly A$^+$ RNA was isolated using the Fast Track Poly A$^+$ mRNA Isolation Kit (Invitrogen Corp.) according to manufacturer's instructions. RNA was electrophoresed in a 2.2M formaldehyde/1% agarose gel. RNA was transferred onto a Hybond N membrane (Amersham) according to manufacturer's instructions. Membrane was stained with methyllene blue and then prehybridized in 6X SSC / 5X Denhardt's / 0.5% SDS / 50% formamide / 100 μg/ml denatured salmon sperm DNA for 2 h at 42°C. The full-length α5 and α7 cDNA clones were obtained from Dr. J. Patrick (Baylor College of Medicine, Houston, Texas). The *Hind*III-*BSSH*II fragment of the α5 cDNA clone and the *AVA*I-*PVU*II fragment of the α7 cDNA clone were gel purified and used as cDNA probes for random primed labelling. cDNA probes were labelled with ^{32}P-dCTP (3000 Ci/mmol,

NEN) using the Random Primed DNA Labelling Kit (Boehringer Mannheim) to a specific activity of 1.0 x 10^9 dpm/μg and added to the hybridization solution at a final concentration of 2-5 x 10^6 cpm/ml solution. Membranes were hybridized for 16-24 h at 42°C. Membranes were then washed for four 10 min periods at room temperature in 2X SSC / 0.1% SDS, followed by a 10 min wash at room temperature and a 10 min wash at 50°C in 1X SSC / 0.1% SDS. Membranes were exposed to Kodak XAR film with an intensifying screen at -80°C for 1 -10 days.

3.3.6 Statistics

Statistical comparisons were done using a one way analysis of variance (ANOVA).

3.4 RESULTS

3.4.1 Effect of nicotine on H69 cell number

H69 cells were exposed to varying concentrations of nicotine (10^{-8}M to 10^{-4}M) as depicted in Figure 3.1. There was a significant increase in H69 cell number in nicotine treated cultures as compared to control (no nicotine) cultures, which is expressed as a % increase in cell number/well. After 6 days of exposure to nicotine, there was a maximal 25% increase in H69 cell number in the cultures treated with 10^{-4}M nicotine compared

to non-treated cultures. After 9 days in culture, exposure to a nicotine concentration as low as 10^{-8}M resulted in a significant increase in H69 cell number compared to control cultures with a maximal increase (approximately 50%) in cell number observed with exposure to 10^{-5}M nicotine. A similar trend was observed after 12 days in culture. The concentration of nicotine required to produce a maximal increase in H69 cell number correlates well with that required to inhibit [^{125}I]α-BGT binding to these cells (Figure 3.7): 10^{-5}M nicotine inhibited approximately 90-95% of binding; with a maximal inhibition occuring at 10^{-4}M nicotine. As well, the maximal increase in H69 cell number seen on days 9 and 12 appeared to plateau at 10^{-5}M nicotine with no further increase in H69 cell number with 10^{-4}M nicotine. The fact that 10^{-7}M and 10^{-8}M nicotine had an effect on H69 cell number whereas these concentrations of agonist had no effect of [^{125}I]-α-BGT binding (Figure 3.7) may suggest that after several days exposure to agonist in culture, the cells become more sensitive to nicotine.

3.4.2 Effect of a nicotinic agonist, cytisine, on H69 cell number

H69 cells were exposed to increasing concentrations of cytisine (10^{-8}M to 10^{-4}M), a nicotinic agonist. As seen in Figure 3.2, after 9 days in culture, exposure to cytisine resulted in a dose dependent increase in H69 cell number with a significant increase observed with 10^{-6}M nicotine and a maximal effect at 10^{-4}M cytisine. A similar trend was observed after 12 days in culture with a maximal increase observed with 10^{-5}M cytisine.

3.4.3 Effect of α-BGT on the nicotine induced increase in H69 cell number

Since previous studies in brain and PC12 cells have implicated the nicotinic α-BGT receptor population in a trophic role, we tested the effect of α-BGT on H69 cell proliferation both alone and in combination with nicotine. As can be seen in Figure 3.3, 10^{-8}M α-BGT completely prevented the nicotine (10^{-4}M) induced increase in H69 cell number at all time points evaluated. The ability of α-BGT to prevent the nicotine induced increase in H69 cell proliferation exhibits a concentration dependence. Furthermore, the concentration of α-toxin required to block the nicotine induced increase in cell number correlates well with the affinity of α-BGT for its receptor as determined from an [^{125}I]-α-BGT saturation binding assay where a K_d of approximately 25nM was obtained (Figure 3.6). The α-toxin on its own (Figure 3.3, control condition) had no significant effect on H69 cell proliferation at the concentrations tested.

In addition, α-BGT was able to block the cytisine (10^{-4}M) induced increase in H69 cell number and exhibited a dose response (Figure 3.4). This effect of the α-toxin was maximal at 10^{-8}M, a concentration which is consistent with the K_d of 25nM for α-BGT binding to its receptor in H69 cells (Figure 3.7).

3.4.4 Effect of d-tubocurarine on the nicotine induced increase in H69 cell number

The effect of d-tubocurarine, a nicotinic receptor antagonist, was tested on H69 cell number as depicted in Figure 3.5. After 9 days of exposure in culture to the antagonist alone, there was no effect on H69 cell number at the concentrations tested (10^{-7}M to 10^{-4}M). However, d-tubocurarine was able to prevent the nicotine induced

(10^{-4}M) increase in H69 cell number in a concentration dependent manner with a complete block observed with 10^{-4}M d-tubocurarine. Similar results were observed after 6 and 12 days of exposure to these agents in culture (data not shown). In addition, the concentration at which these effects were observed correlates well with that which is required to completely inhibit [^{125}I]-α-BGT binding to H69 cells (Figure 3.7).

3.4.5 [^{125}I]α-BGT binding sites in H69 cells

To characterize the α-BGT binding sites in H69 cells, saturation binding assays (Figure 3.6) and competition binding assays (Figure 3.7) were done. From the saturation binding assay, high affinity (K_d= 25nM), saturable [^{125}]I-α-BGT sites (B_{max}= 10.4 fmol/10^6 cells) have been demonstrated to be present in H69 cells in culture. The competition binding assay (Figure 3.7) demonstrates the ability of the nicotinic ligands, nicotine, cytisine and d-tubocurarine to completely inhibit radiolabelled toxin binding to the α-toxin receptor at a concentration of 10^{-4}M which correlates with the concentration in which the effects of these ligands on modulating H69 cell number were observed. This latter binding assay demonstrates that the α-BGT receptor in H69 cells exhibits the characteristics of a nicotinic receptor.

3.4.6 Presence of α5 and α7 mRNA in H69 cells

In order to determine if the mRNA for the presumed α-BGT binding neuronal nicotinic receptor subunits, α5 and α7, are expressed in H69 cells, cDNA probes were used in a Northern analysis of H69 polyA+ mRNA (Figure 3.8). A 6.6kb transcript for

the α-toxin binding subunit, α7, was detected in both positive control tissues, XPC12 cells and rat brain, as expected. Expression of α7mRNA in H69 cells however, was more difficult to detect. A weak hybridization signal detected at 6.6 kb suggests that α7mRNA is present in H69 cells in low abundance. Three transcripts (7.5kb, 4.5kb and 2.5kb) for the neuronal nicotinic receptor subunit, α5, were detected in both positive controls, XPC12 cells and rat midbrain, as expected. Transcripts for α5 were also detected in H69 cells with hybridization signals at 2.7kb, 2.0kb and 1.1kb (Figure 3.8).

3.5 DISCUSSION

The present results demonstrate that exposure of H69 cells, a small cell lung carcinoma cell line, to nicotine results in an increase in H69 cell number. The concentration of nicotine required to result in a maximal increase in H69 cell number correlates well with the affinity of the agonist for the α-bungarotoxin receptor implicating the involvement of α-BGT type nicotinic receptors in the regulation of H69 cell proliferation. The observation that nicotine increases cell proliferation in H69 cells is in line with previous studies using a neuroendocrine cell line derived from a hamster lung tumour (Schuller et al. 1990) which demonstrated that nicotine exposure in culture results in a 100% increase in cell number. Studies in a human neuroendocrine lung cancer cell line (Schuller 1989) demonstrated that exposure to nicotine, as well as two tobacco-related

metabolites of nicotine known as nitrosamines, also increased cell number thus suggesting a role for nicotine in affecting the development of lung cancer. Nicotine, a component of tobacco smoke, interacts at nicotinic receptors to mediate its effects; however, the nature of the nicotinic receptors involved that is, α-BGT sensitive and/or insensitive, is still unknown.

The functional role of the nicotinic α-BGT receptor is currently unclear, although evidence suggests a trophic and/or growth related role for the α-toxin binding site in neuronal tissue. Previous studies in our laboratory have suggested a role for the neuronal α-BGT receptor in neurite outgrowth in PC12 cells (Chan and Quik 1993). In addition, others have shown that nicoinic α-BGT receptor activation modulates neurite outgrowth in ciliary ganglion neurons in culture (Pugh and Berg 1994). Based on this rationale, the effect of α-BGT was evaluated on cell proliferation in H69 cells. Interestingly, α-BGT prevented the nicotine induced increase in H69 cell number. The concentration of α-toxin required to prevent the increase in cell number correlated well with the affinity of α-BGT for the toxin binding site in H69 cells which suggests that the effects of the snake toxin on cell number is being mediated by an α-BGT sensitive site. The α-toxin alone had no effect on H69 cell proliferation.

Experiments using various nicotinic agonists and antagonists further support the hypothesis for the involvement of nicotinic type receptors in tumour cell proliferation. D-tubocurarine, a nicotinic receptor antagonist at both α-BGT sensitive and α-BGT insensitive type nicotinic receptors, also prevented the nicotine induced increase in H69 cell number in a dose dependent manner at concentrations which correlated with those

required to inhibit α-BGT binding to its receptor suggesting the involvement of α-toxin receptors. Cytisine, a nicotinic agonist, resulted in a dose dependent increase in H69 cell number at concentrations which correlated well with the affinity of the agonist for the α-BGT binding site in H69 cells. The cytisine induced increase in H69 cell number was blocked by α-BGT providing further support for the involvement of a specific type, namely, the α-BGT sensitive type of nicotinic receptor in lung tumour cell growth.

In the present studies, α5 and α7 cDNA probes were used for Northern blot analysis. Peptide binding studies (McLane et al. 1991) which demonstrated that peptides generated from the α5 cDNA sequence could bind α-BGT with high affinity suggest that α5 may be an α-BGT binding subunit. Functional expression studies in which α7 cDNA was injected into *Xenopus* oocytes resulting in the formation of homo-oligomeric receptor proteins with an α-BGT sensitive nicotinic receptor mediated response indicates that α7 forms an α-BGT receptor (Couturier et al. 1990; Seguela et al. 1993). The present results demonstrate the presence of both α5 and α7 mRNA in H69 cells wherein three transcripts were obtained for α5 and a single transcript for α7. Results from the Northern analysis for α5 mRNA corroborate those obtained by Chini et al. (1992) who demonstrated the presence of α5 mRNA in various non-neuronal cell lines derived from lung adenocarcinomas, epithelioid carcinomas of the cervix and skin, adenocarcinomas of the rectum as well as from hepatomas, all of which also expressed α-BGT binding sites. From their results, Chini et al. (1992) correlated the presence of mRNA for the neuronal nicotinic α-BGT binding subunit, α5, with the possibility that nicotinic α-BGT receptors in these various non-neuronal cell lines contained the α5 subunit. From the Northern

analysis in H69 cells, the presence of α5 mRNA in this non-neuronal SCLC cell line is in line with the results of Chini et al. (1992); however, it is not clear whether the neuronal nicotinic α5 receptor subunit is a component of the α-BGT receptor. In addition, results obtained from the Northern analysis demonstrate that the size of the α7 transcript in brain and in H69 cells is the same. This provides the first evidence which demonstrates that the α-BGT binding neuronal nicotinic receptor subunit α7 is expressed in non-neuronal tissue.

In conclusion, results from these studies demonstrate that nicotine induces an increase in H69 cell number. Furthermore, they suggest that this effect is mediated through the nicotinic α-BGT receptor population. Taken together, results from these studies suggest that the nicotinic α-BGT receptor may be involved in the proliferative responses of neuroendocrine tumors.

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Fig. 3.1. Effect of nicotine on H69 cell number. Varying concentrations of nicotine were added to H69 cells in culture (2.5×10^4 cells/well) immediately after plating. The number of cells per culture well was determined after 6, 9 and 12 days in culture and expressed as % increase in cell number as compared to control (no nicotine) cultures. Each value represents the mean ± S.E.M. of 8 culture wells. The results are pooled from 2 separate experiments. The number of cells/well under control conditions (that is, in the absence of nicotine) were $41\pm2 \times 10^3$, $72\pm1 \times 10^3$ and $107\pm3 \times 10^3$ on days 6, 9 and 12 in culture, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control cultures (no nicotine) after a one way ANOVA.

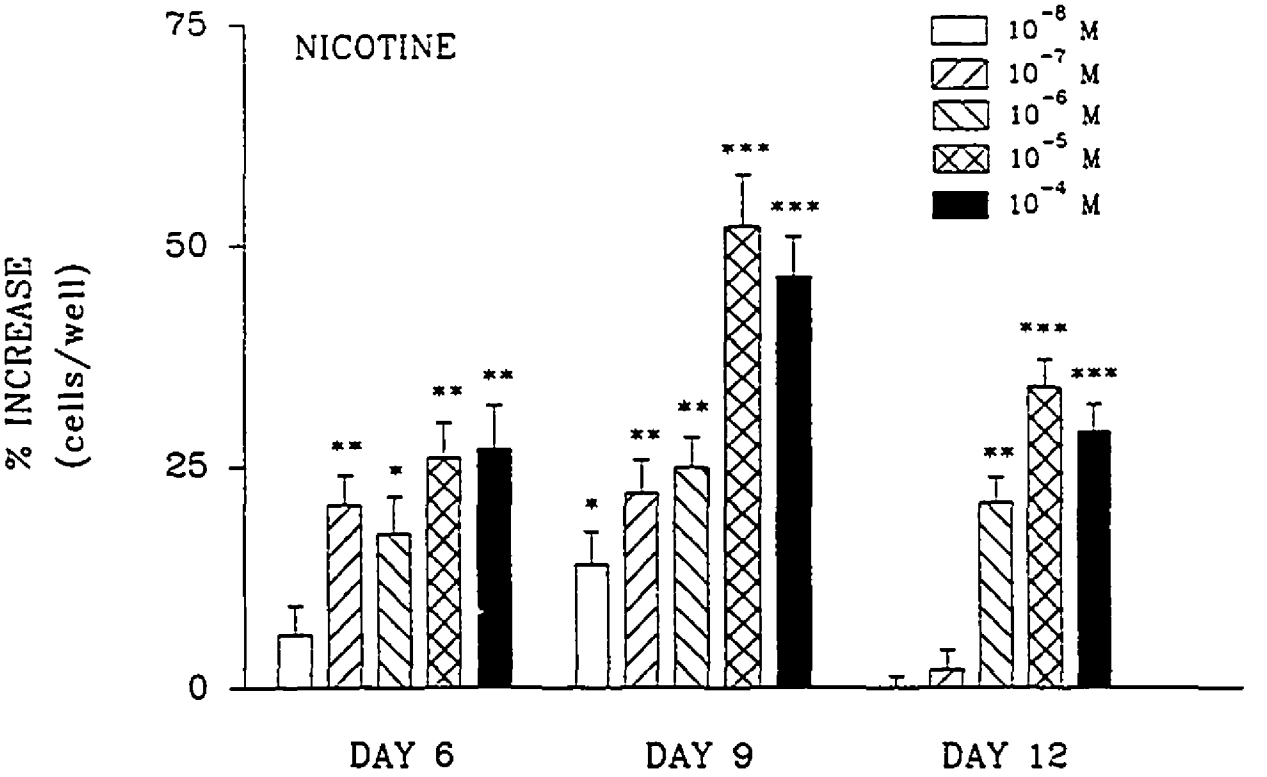
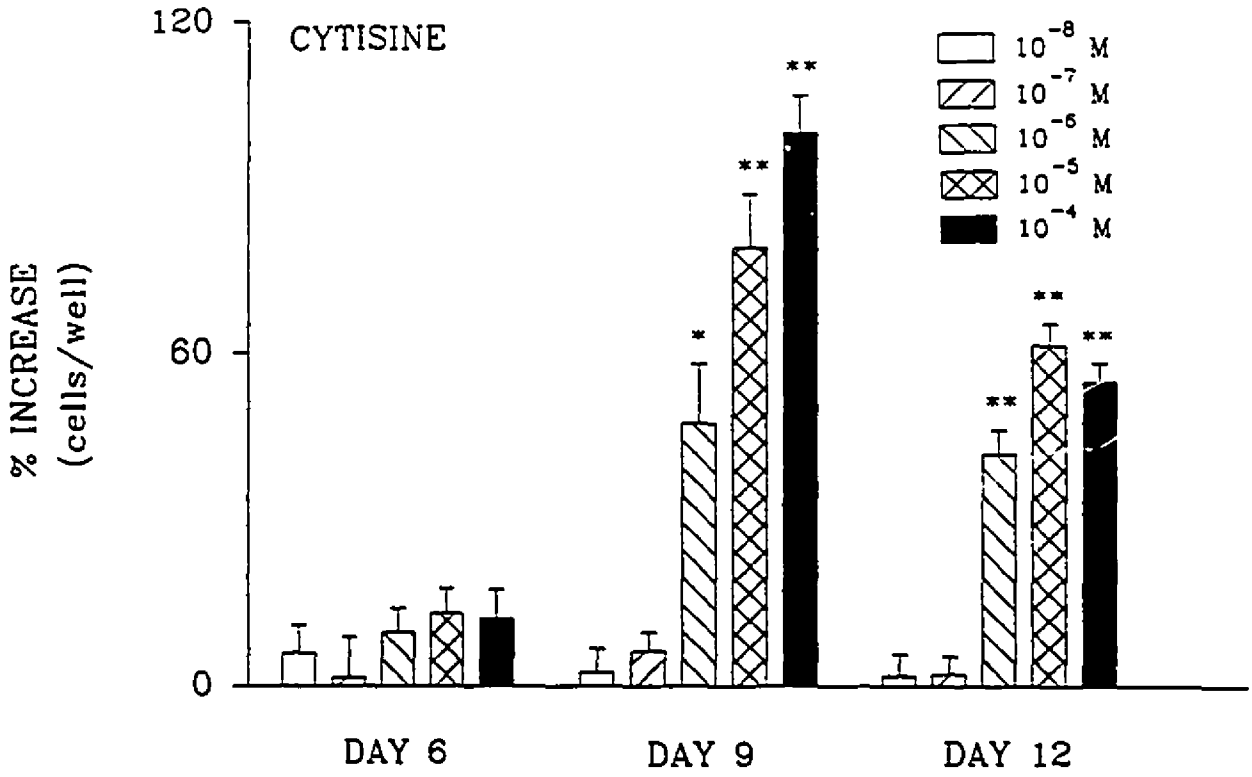

NICOTINE
% INCREASE
(cells/well)
75
50
25
0
10^{-8} M
10^{-7} M
10^{-6} M
10^{-5} M
10^{-4} M
DAY 6
DAY 9
DAY 12

Fig. 3.2. Effect of the nicotinic agonist cytisine on H69 cell number. Varying concentrations of cytisine were added to H69 cells in culture (2.5 x 10^4 cells/well) immediately after plating. The number of cells per culture well was determined after 6, 9 and 12 days in culture. Each bar represents the mean ± S.E.M. of 4-8 culture wells. The results are pooled from two separate experiments. The number of cells/well under control conditions (that is, in the absence of cytisine) were 47 ± 4 x 10^3, 65 ± 3 x 10^3 and 118 ± 4 x 10^3 on days 6, 9 and 12 in culture, respectively. $^*P< 0.01$ and $^{**}P< 0.001$ compared with control cultures (no cytisine) after a one way ANOVA.


CYTISINE
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60
0
% INCREASE
(cells/well)
10^{-8} M
10^{-7} M
10^{-6} M
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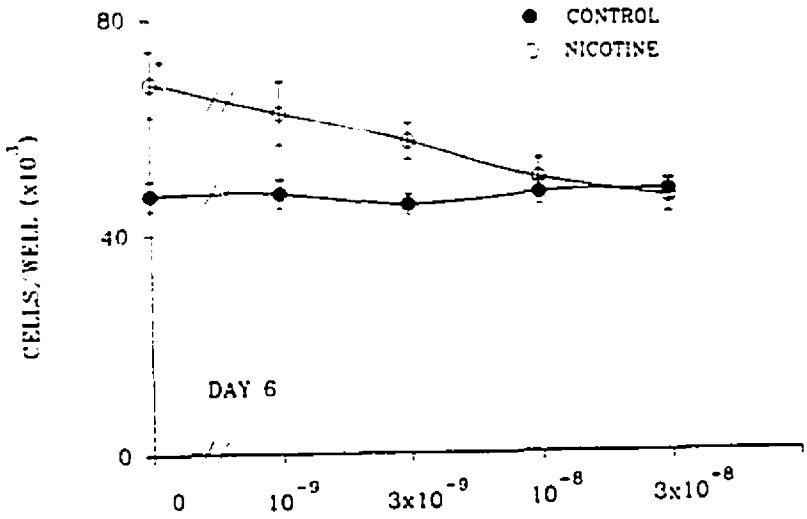
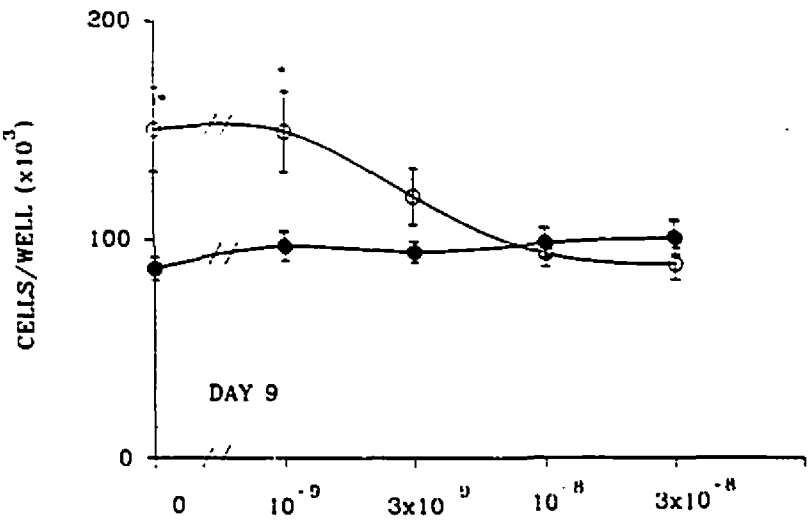
DAY 6
DAY 9
DAY 12

Fig. 3.3. Effect of α-BGT on the nicotine induced increase in H69 cell number. Nicotine (10^{-4}M) in the absence or presence of varying concentrations of α-BGT was added to H69 cells in culture (2.5×10^4 cells/well) immediately after plating. The number of cells per culture well was determined after 6 (A), 9 (B) and 12 (C) days in culture. Each value represents the mean ± S.E.M. of 8-12 culture wells, pooled from 2-3 separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control cultures (no nicotine or α-BGT) after a one way ANOVA.

A


CONTROL
NICOTINE
80
40
0
CELLS/WELL (x10^3)
DAY 6
0
10^{-9}
$3x10^{-9}$
10^{-8}
$3x10^{-8}$

B


200
100
0
CELLS/WELL (x10^3)
DAY 9
0
10^{-9}
$3x10^{-9}$
10^{-8}
$3x10^{-8}$

C

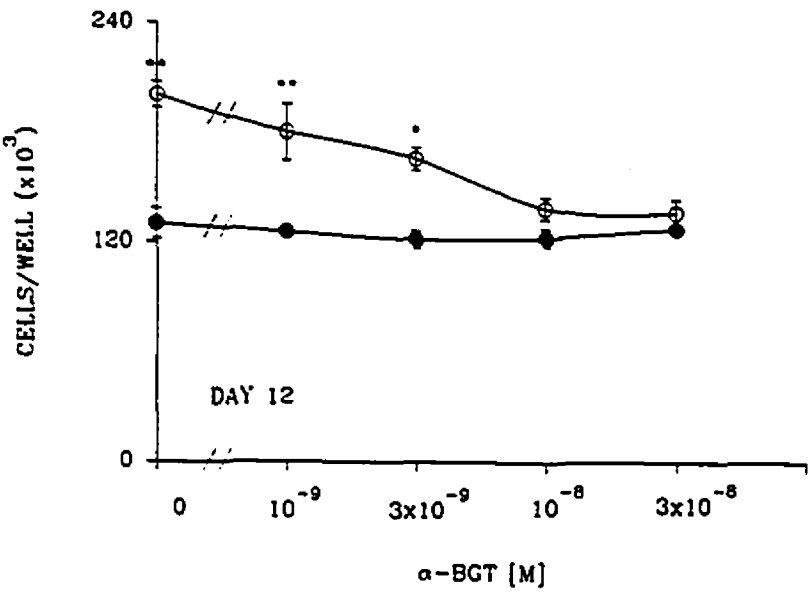

240
120
0
CELLS/WELL (x10^3)
DAY 12
0
10^{-9}
$3x10^{-9}$
10^{-8}
$3x10^{-8}$
α-BGT [M]

Fig. 3.4. Effect of α-BGT on the cytisine induced increase in H69 cell number. Cytisine (10^{-4}M) in the absence or presence of varying concentrations of α-BGT was added to H69 cells in culture (2.5 x 10^4 cells/well) immediately after plating. The number of cells per well was determined after 9 days in culture. Each bar represents the mean ± S.E.M. of 8 culture wells, pooled from 2 separate experiments. $^{***}P<0.001$ compared with control cultures (no drug) after a one way ANOVA.

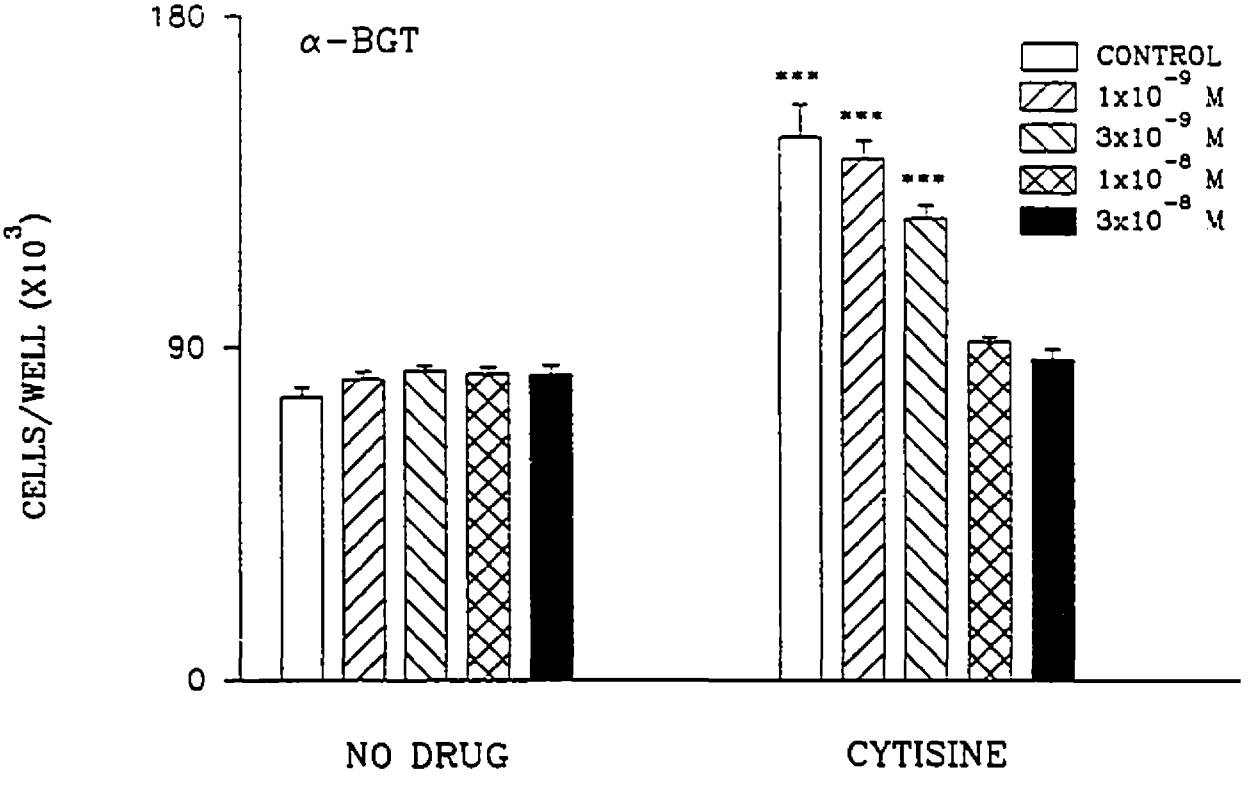

180
90
0
α-BGT
CELLS/WELL (X10^3)
CONTROL
1x10^-9 M
3x10^-9 M
1x10^-8 M
3x10^-8 M
***
***
***
NO DRUG
CYTISINE

Fig. 3.5. Effect of d-tubocurarine on the nicotine-induced increase in H69 cell number. Nicotine (10^{-4}M) in the absence and presence of varying concentrations of d-tubocurarine was added to H69 cells in culture (2.5 x 10^4 cells/well) immediately after plating. The number of cells per culture well was determined after 9 days in culture. Each bar represents the mean ± S.E.M. of 8 culture wells. The results are pooled from 2 separate experiments. $^{**}P< 0.01$ compared with control cultures (no drug) after a one way ANOVA.

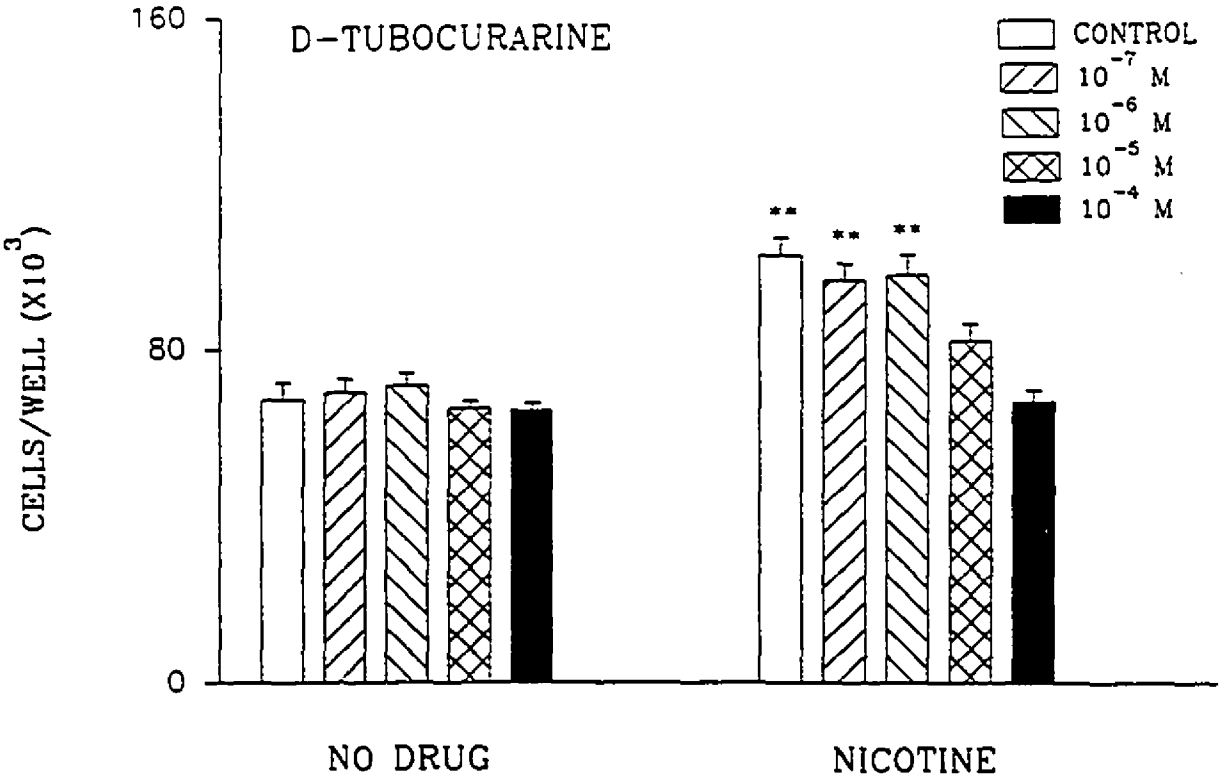

D-TUBOCURARINE
CONTROL
10^-7 M
10^-6 M
10^-5 M
10^-4 M
160
80
0
CELLS/WELL (X10^3)
**
**
**
NO DRUG
NICOTINE

Fig. 3.6. ^{125}I-α-BGT binding to H69 cells in culture. Cells (0.5 x 10^6 cells/well) were preincubated for 60 min in the absence or presence of cold α-BGT (1μM) and the binding assay done as described in Materials and Methods. Each symbol represents the mean ± S.E.M. of 4 culture wells. Results are representative of 3 separate experiments. Where the S.E.M. is not depicted, it fell within the symbol.

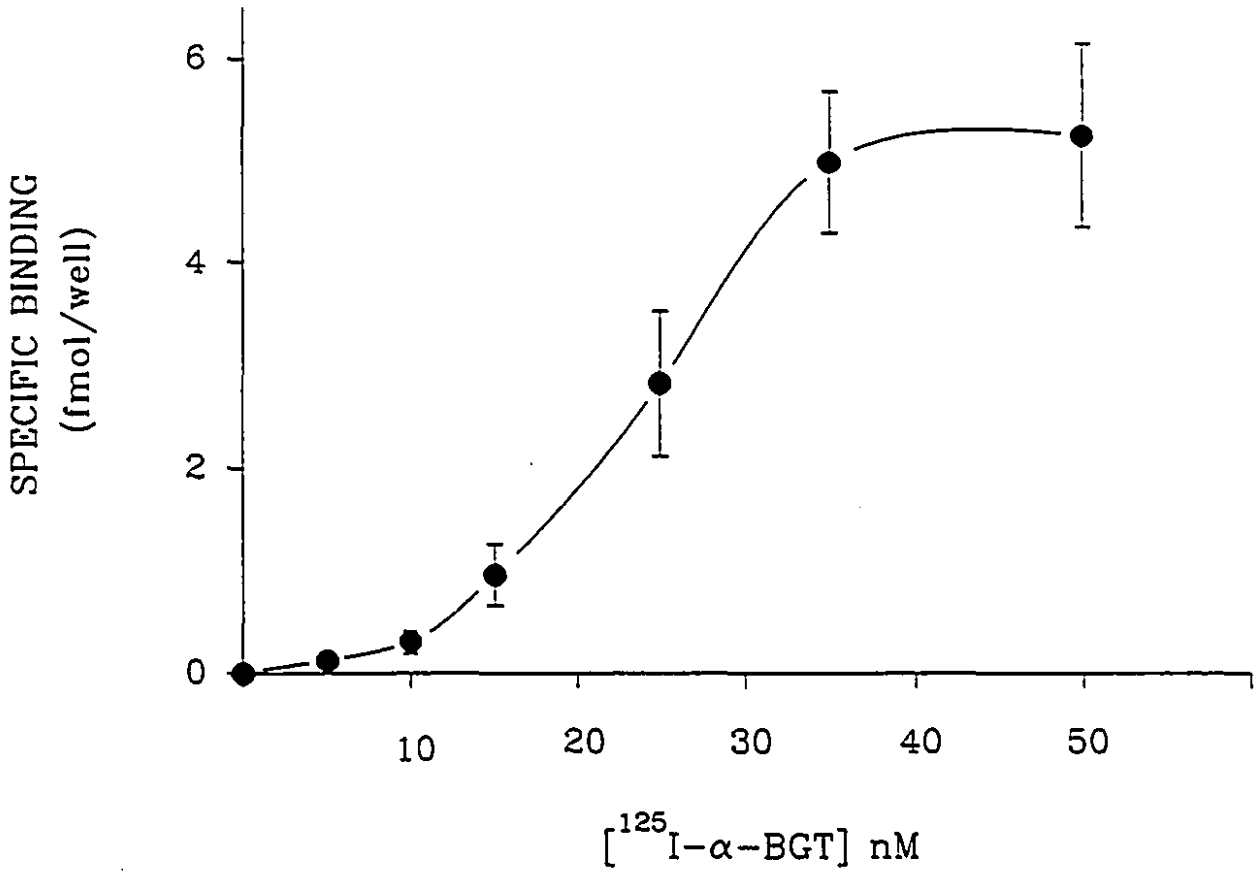

SPECIFIC BINDING
(fmol/well)
6
4
2
0
10
20
30
40
50
[125I-α-BGT] nM

Fig. 3.7. Effect of nicotinic cholinergic ligands on [^{125}I]α-BGT binding to H69 cells in culture. Cells (0.5 x 10^5 cells/well) were preincubated for 60 min in the absence or presence of the indicated concentration of nicotine, cytisine or d-tubocurarine and the binding assay done as described in Materials and Methods. Each symbol represents the mean of 4 culture wells. Results are representative of 4 separate experiments.

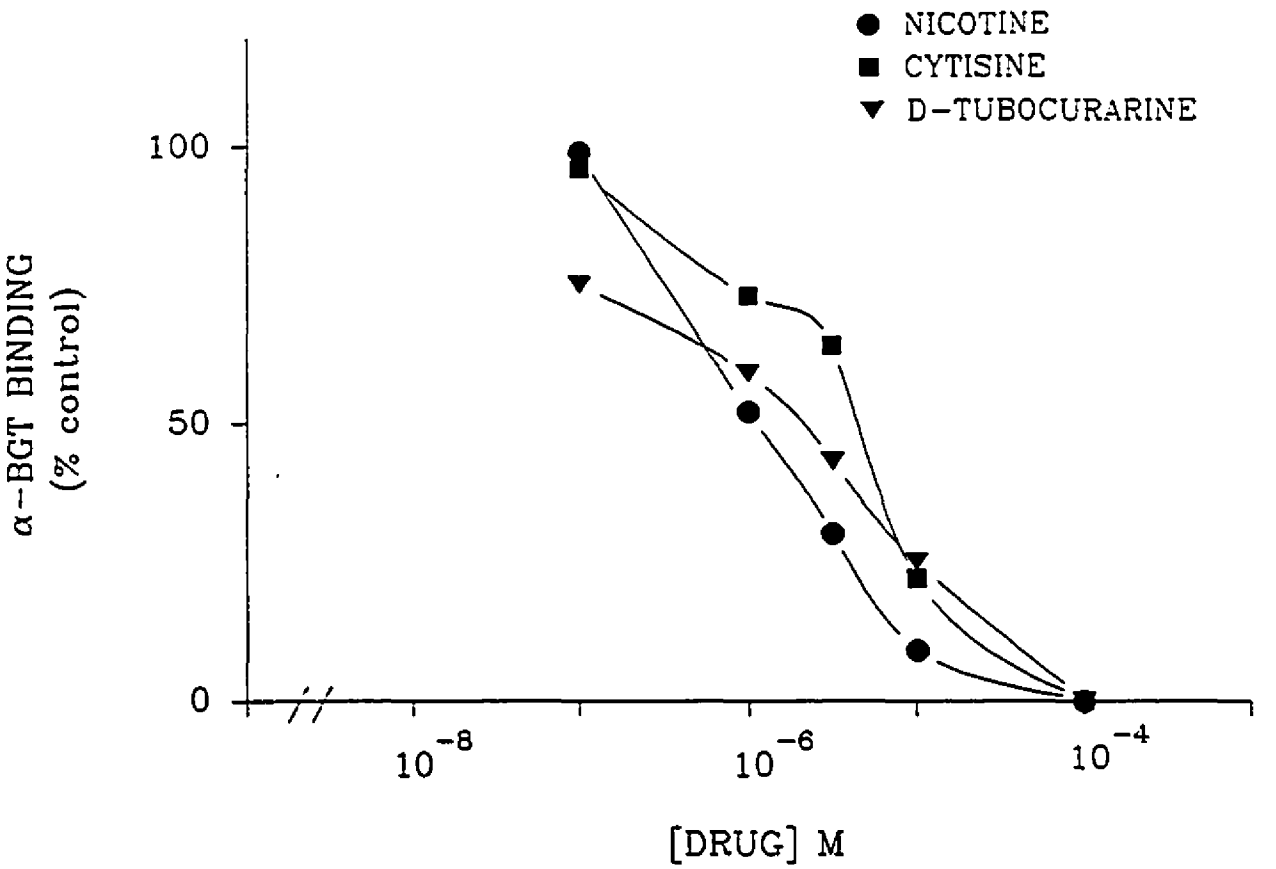
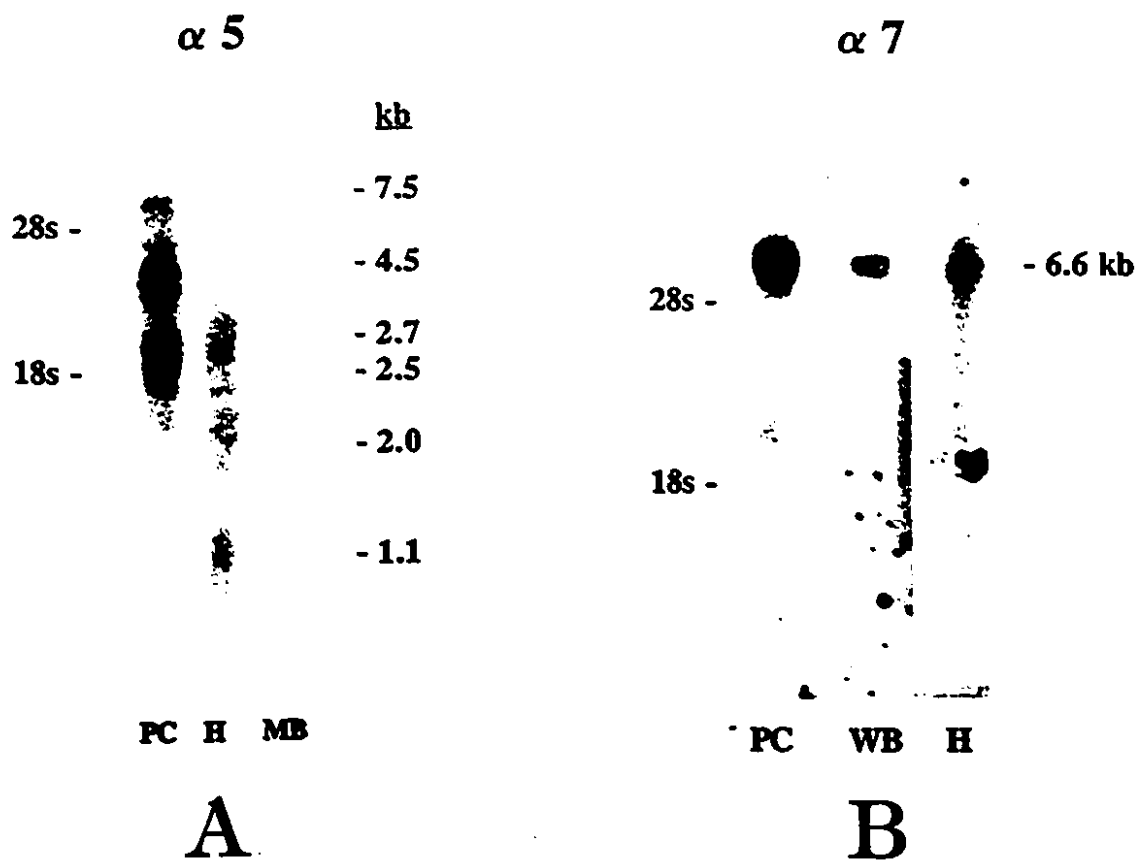

NICOTINE
CYTISINE
D-TUBOCURARINE
100
50
0
α-BGT BINDING
(% control)
10^-8
10^-6
10^-4
[DRUG] M

Fig. 3.8. Northern blot analysis of α5 (A) and α7 (B) mRNA in PC12(PC) and H69(H) cells and in rat mid-brain(MB) or whole brain(WB). In (A), 5μg of poly A^+ mRNA isolated from PC12 cells, rat mid-brain tissue and 5.5μg of poly A^+ mRNA isolated from H69 cells were electrophoresed, blotted and probed with a ^{32}P-labelled α5 cDNA probe (*Hind*III - *BSSH*II fragment of the α5 clone). In (B), 10μg of poly A^+ mRNA isolated from PC12 cells, rat whole brain tissue and 20μg of poly A^+ mRNA isolated from H69 cells were electrophoresed, blotted and probed with a ^{32}P-labelled α7 cDNA probe (*AVA*I - *PVU*II fragment of the α7 clone). In (A) and (B), the position of the 28s and 18s rRNA are indicated on the left.


α 5
kb
- 7.5
28s -
- 4.5
- 2.7
18s -
- 2.5
- 2.0
- 1.1
PC H MB
A
α 7
- 6.6 kb
28s -
18s -
PC WB H
B

4.0 GENERAL DISCUSSION

As discussed in the Introduction, the application of molecular, immunological and physiological techniques has led to the characterization of the nicotinic α-BGT receptor as a distinct member of the nicotinic acetylcholine receptor family (for reviews see Clarke 1992; Role 1992; Papke 1993; Sargent 1993). Functional and localization studies have distinguished the neuronal nicotinic α-BGT receptor from the α-BGT insensitive nicotinic acetylcholine receptor population. In addition, molecular biology approaches have led to the isolation and cloning of the genes for the α7 and α8 neuronal nicotinic receptor subunits that encode α-BGT binding proteins (Couturier et al. 1990a; Schoepfer et al. 1990), although the subunit composition of the neuronal toxin binding receptor channel *in vivo* remains unclear. Toxin sensitive responses have also been observed *in vitro* by various laboratories in a number of neuronal preparations (DeLa Garza et al. 1987; Alkondon and Albuquerque 1991; Zorumski et al. 1992; Alkondon and Albuquerque 1993, 1994; Alkondon et al. 1994; Pugh and Berg 1994). Our results in PC12 and H69 cells and others (Pugh and Berg 1994) suggest that one role in which these α-BGT nicotinic receptors may be involved is in growth related processes both in neuronal and non-neuronal cells.

4.1 Role for the nicotinic α-BGT receptor in neuronal development

Numerous studies using a variety of neuronal and non-neuronal preparations have

demonstrated a potential role for neurotransmitters such as dopamine, 5-HT, glutamate, purine analogs, opioids and their receptors in the regulation of neuronal growth and developmental processes (Lankford et al. 1988; McCobb et al. 1988; Mattson et al. 1989; Greene et al. 1990; Maneckjee and Minna 1990, 1992). For example, in morphogenesis monoamines and acetylcholine have been shown to have an effect on the morphogenetic cell movements during the development of early embryos (Lauder 1993). In terms of neuronal development, studies have demonstrated that neurotransmitters may be involved in the regulation of neurogenesis by either enhancing or inhibiting the development of the intricate neuronal architecture through the interaction of the neurotransmitter at it's receptor.

In line with the hypothesis presented above, the involvement of the toxin binding receptor in neuritic outgrowth was investigated. The results presented in Chapter 2 show that nicotine decreased neurite outgrowth in PC12 cells. This data provides further support for the idea that neurotransmitters and their receptors are not solely involved in neuronal communication but may also play a role in developing neuronal connections. Another interesting finding is that this nicotine induced inhibition in neuritic outgrowth was blocked by α-BGT. This suggests that the α-toxin receptor is involved in a trophic role, that is, in neurite outgrowth.

Other laboratories have also obtained findings which suggest a role for the nicotinic regulation of neurite outgrowth in a variety of neuronal preparations (Zheng et al. 1994; Small et al. 1995). Furthermore, Pugh and Berg (1994) showed that the nicotinic receptor modulated changes in neurite outgrowth in chick ciliary ganglion

neurons were inhibited by α-BGT.

In addition, as mentioned in the Introduction (page 15), the correlation of the time course of appearance of neurotransmitter receptors with developmental processes may suggest a trophic role for these receptors in neuronal tissues. For example, Chun et al. (1987) have demonstrated that the appearance of neuropeptide receptors for cholecystokinin and somatostatin correlated with the processes of neurite outgrowth, halting and re-growth in the immature cat telencephalon thus implicating the neuropeptide receptors in neuronal development. Interestingly, α-BGT receptors also exhibit a time course of appearance that can be correlated with developmental processes (Greene 1976; Kouvelas and Greene 1976; Wang and Schmidt 1976; Freeman 1977; Chiappinelli and Giacobini 1978; Hunt and Schmidt 1979; Couturier et al. 1990a). In neuronal tissue, it has been shown that there is an initial increase in α-BGT receptors during development, which peaks at about day 13 to 19 depending on tissue and species. Furthermore, this increase in receptor number often occurs prior to the major increase in choline acetyltransferase activity, an observation which has led to the suggestion that the α-BGT receptors and/or the postsynaptic element may be involved in neuronal development by guiding the presynaptic terminal (Fiedler et al. 1987; Quik and Geertsen 1988). In another line of studies by Fuchs (1989), it has been shown that α-BGT receptors are arranged in a unique columnar pattern in the somatosensory cortex. Taken together, results observed on neuritic outgrowth in PC12 cells combined with the experimental evidence from other laboratories provide further support for the trophic role of the nicotinic α-BGT receptor in neuronal development *in vivo*.

4.2 Role for α-BGT receptors in the modulation of cell proliferation in non-neuronal cells

As discussed in the Introduction (page 17), Schuller et al. (1989) had demonstrated that exposure of lung tumour cells to nicotine resulted in an increase in cell proliferation; however, these authors did not determine whether these effects were mediated through α-BGT sensitive or insensitive type nicotinic receptors. In order to address this issue, experiments in the H69 lung tumour cell line were done.

Results from the cell proliferation experiments (Chapter 3) implicate the α-toxin binding nicotinic receptors in the modulation of H69 cell proliferation. Nicotine exposure resulted in an increase in cell number, which was blocked by α-BGT. These results are in line with those of Codignola et al. (1994) who also showed that stimulation of the nicotinic α-BGT receptor increased SCLC cell number.

The mechanisms by which the activation of α-BGT receptors may regulate growth related processes is not known. The increase in cell numbers that was observed in the nicotine exposed H69 cells may be explained as follows: nicotinic activation of toxin binding nicotinic receptors may result in a decrease in cell division time and/or an increase in the number of cell divisions. Results from the present studies in H69 cells show that the nicotine induced increase in cell number occurs after approximately 6 days of nicotine exposure which corresponds to the cell doubling time of 5 to 7 days. Thus, an increase in cell proliferation may be suggested as a potential mechanism. Experimental evidence is available from other laboratories which is consistent with this hypothesis. Catteneo et al. (1993) have shown that nicotine results in an increase in [^{3}H]thymidine incorporation in H69 cells as compared to controls, an effect which is blocked by the

nicotinic antagonist mecamylamine. Nicotine has also been shown to inhibit cell death or apoptosis in several non-SCLC cell lines; however, this appears to occur through a non-receptor mediated mechanism (Wright et al. 1993).

4.3 Subunit composition of the nicotinic α-BGT receptor

As mentioned in the Introduction (page 11), a variety of experimental approaches now suggest the involvement of the α7 and α8 neuronal nicotinic receptor subunits in the formation of the nicotinic α-BGT receptor in nervous tissue (Anand et al. 1993; Gerzanich et al. 1994; Gotti et al. 1994). In addition, peptide binding studies demonstrated that peptides generated from the α5 cDNA sequence bound α-BGT with high affinity (McLane et al. 1990, 1991), raising the possibility that this neuronal nicotinic receptor subunit may also be involved in the formation of the nicotinic α-toxin receptor in neuronal tissue.

In the present experiments, Northern blot analysis for the α5 and α7 neuronal nicotinic α-toxin binding subunit mRNAs in neuronal PC12 cells and non-neuronal H69 SCLC cells was performed. Results from these Northern analysis demonstrated that both α5 and α7 neuronal nicotinic receptor mRNAs were present in neuronal PC12 cells, findings consistent with those in the literature (Henderson et al. 1994). In the non-neuronal H69 cell line, α5 mRNA was detected; these observations are in line with those obtained by Chini et al. (1992) who demonstrated the presence of α5 mRNA in a variety of non-neuronal cell lines. In addition, results from these Northern

analysis demonstrated that α7 mRNA was present in H69 cells and that furthermore, in this non-neuronal cell line the size of the α7 transcript resembles the one detected in rat brain. The identification of mRNA for a neurotransmitter receptor in SCLC cells can probably be explained on the basis of the neural crest origin of these cells. These results are in line with those from other laboratories which have demonstrated the presence of neuronal markers such as the brain isozyme of creatinine kinase, neuron-specific enolase, L-dopa decarboxylase and various neuropeptides and their receptors in non-neuronal SCLC cells (Gazdar et al. 1985, Roth and Barchas 1986).

The present results show that α5 and α7 mRNA are present in both PC12 and H69 cells. The question now remains whether these subunits are indeed expressed and whether they are involved in the composition of the α-BGT receptor. In order to determine if the α5 and/or α7 toxin binding subunits are a functional component of the α-toxin receptor, further work needs to be done. One approach may include the use of antisense oligonucleotides against the α5 and α7 neuronal nicotinic receptor subunits. Experiments could be done to determine whether the nicotine induced inhibition in neuritic outgrowth in PC12 cells or the nicotine induced increase in SCLC proliferation in the H69 cell line is still observed after exposure of the cells to antisense oligonucleotides. This rationale is supported by results from Listerud et al. (1991) who studied the contribution of individual subunits to neuronal nicotinic receptor subtypes in sympathetic neurons by selectively deleting subunits with antisense oligonucleotides and subsequently observing the effect on the channel properties of the nicotinic receptor.

Alternatively, mAbs generated against the α5 and α7 toxin binding proteins may

be used to determine if the α-toxin sensitive responses seen on neurite outgrowth in PC12 cells and on cell proliferation in H69 cells can be altered. This immunological approach has been used by other groups who have generated mAbs against the α7 and α8 α-BGT binding subunits in order to immunoisolate α-BGT receptor subtypes from chick optic lobe and retina (Anand R. et al. 1993; Gotti et al. 1994).

4.4 Role for c-fos activation and calcium in mediating the trophic effects of nicotine at the nicotinic α-BGT receptor

The observation, that α-BGT can block the effect of nicotinic agonists on neurite outgrowth in PC12 cells (Chapter 2) and on H69 cell proliferation (Chapter 3), raises the question concerning the mechanism by which nicotine interacting at the α-toxin site induces these trophic effects.

Experimental evidence is available from several laboratories which demonstrates that nicotinic receptor stimulation results in the modulation of c-fos expression in a variety of neuronal tissues. For example, studies in rat superior cervical ganglion neurons showed that nicotine exposure resulted in the differential expression of immediate early genes such as c-fos. c-Fos has been implicated in the regulation of a variety of biological processes including cell proliferation and differentiation (Angel and Karin 1991; Koistinaho et al. 1993). Following systemic administration of nicotine, an increase in c-fos immunostaining has also been observed in rat brain (Ren and Sagar 1992; Matta et al. 1993). Greenberg et al. (1986) demonstrated that exposure of PC12 cells to nicotine resulted in a calcium dependent increase in c-fos mRNA. However, the population of

nicotinic receptors which mediates this effect, that is α-BGT sensitive or insensitive, was not tested. Further studies are necessary to investigate the potential involvement of c-fos in mediating α-BGT sensitive responses. If it can be demonstrated that exposure of PC12 cells to α-toxin can block the nicotine induced increase in c-fos transcription, this would provide evidence for a role for this immediate early gene in regulating neurite outgrowth.

Studies from other laboratories indicate that α-BGT nicotinic receptors mediate their effects in a calcium dependent manner. Both molecular and immunological approaches *in vitro* have demonstrated that the α-BGT sensitive homo-oligomeric α7 receptor is a cation-gated ion channel. Injection of α7 cDNA into oocytes resulted in expression of a nicotinic cation channel that was highly permeable to calcium (Couturier et al. 1990a; Bertrand et al. 1992; Seguela et al. 1993; Zhang et al. 1994). In addition, studies in chick ciliary ganglion neurons have demonstrated that activation of the neuronal nicotinic receptors led to an influx of calcium which was sensitive to blockade by α-BGT (Vijayaraghavan et al. 1992; Zhang et al. 1994).

Thus, the second messenger systems involved in mediating the toxin sensitive nicotine induced responses in PC12 cell neurite outgrowth and H69 cell proliferation are still under investigation, although evidence now supports an involvement of calcium and possibly c-fos. The possible involvement of these intermediates in mediating the effect of α-BGT receptor activation is particularly interesting in view of the role of both c-fos and calcium in growth related processes.

5.0 REFERENCES FOR INTRODUCTION AND GENERAL DISCUSSION

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APPENDIX A

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ADDENDUM

Re: Statistical Analysis

Addendum Regarding Statistical Tests

The following addendum addresses the statistical tests that were performed in this thesis. Following examination by Dr. Paul Clarke (Internal Examiner), the following points should be clarified with examples of reanalysis to follow.

The first comment applies to Figure 2.1 from Chapter 2 of this thesis. As indicated in the figure legend (page 46), a one-way ANOVA for each of the two α-BGT conditions (with and without nicotine) was performed followed by the Scheffé test. Please note that these statistical tests were inappropriate for the reasons as described here. Firstly, this figure represents a 2 x 4 experimental design with nicotine and α-BGT being the factors. Thus, a two-way ANOVA as opposed to a one-way ANOVA would have been appropriate. In addition, since all nicotine concentrations were being compared to one control, Dunnett's test should have been done instead of the Scheffé test which compares all data points with each other.

The second comment applies to Figure 2.2 from Chapter 2 of this thesis. As indicated in the figure legend (page 48), a one-way ANOVA followed by the Scheffé test was performed. Clarification of the actual statistical test that should have been used is as follows. The asterisks in the figures show significant differences between the nicotine and control (no nicotine) conditions suggesting that they were probably obtained through performing multiple unprotected t-tests as opposed to an ANOVA. With this being the case, the Bonferroni correction should also have been done in order to protect these multiple t-tests from resulting in potentially misleading false positive results.

The third comment applies to Figures 3.1 and 3.5 from Chapter 3 of this thesis. The figure legends (pages 77 and 85) indicate that a one-way ANOVA was performed. However, similarly to the second comment, the asterisks suggest that multiple unprotected t-tests were done which thus should have been protected by the Bonferroni correction.

The fourth comment applies to Figures 3.3 and 3.4 from Chapter 3. Note that a one-way ANOVA was inappropriate since there are two independent factors (nicotine and α-BGT for Figure 3.3 and cytisine and α-BGT for Figure 3.4) and thus a two-way ANOVA would have been appropriate.

The fifth comment applies to Figure 3.6 which demonstrates an S-shaped binding curve. As the x-axis was plotted in absolute units, the resulting Scatchard plot would have resulted in a curve as opposed to a straight line. A curved Scatchard plot would suggest that there is more than one binding site. Thus, the single K_D and B_{max} that was reported in this paper is potentially misleading.