BIOCHEMICAL STUDIES ON THE NEUROTENSIN RECEPTOR

by

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ABSTRACT

Neurotensin is a tridecapeptide which appears to function as a neurotransmitter or neuromodulator in the central nervous system.

Using tritiated neurotensin as the radioligand a binding assay was developed to investigate specific binding to membranes of rat brain and bovine cortex. Subsequently the assay was adapted and used with cells of the murine neuroblastoma cell line N1E-115.

Solubilization of the specific binding activity from rat and bovine brain was approached by screening a series of detergents. An active and stable preparation was obtained from bovine cortical membranes using digitonin in the presence of magnesium. Properties of the soluble activity were examined with respect to binding and molecular characteristics.

Purification of the bovine neurotensin receptor to homogeneity was achieved in a single step using neurotensin affinity chromatography. The purified receptor was characterized in terms of ligand binding properties and subunit composition.

Begin at the beginning and go on 'til you come to the end, then stop.

Lewis Carroll, Alice Through The Looking Glass (1886).

Follow roughly outlines in outfanning movement over subject, as river rock, so mindflow over jewel-center need (run your mind over it, once) arriving at pivot, where what was dim formed "beginning" becomes sharp-necessitating "ending" and language shortens in race to wire of time-race of work, following law of Deep Form, to conclusion, last words, last trickle.

Jack Kerouac, On The Road (1957).

ACKNOWLEDGEMENTS

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Finally, and most importantly, thank you to my family for their continued faith in me.

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ABBREVIATIONS

Standard three letter symbols for amino acids (IUPAC-IUB Commision on Biochemical Nomenclature, 1967) are used throughout. Other abbreviations appear below.

Bis-Tris	Bis(2-hydroxyethyl)imino-tris(hydroxymethyl)-methane
BSA	Bovine serum albumin
CHAPS	3[(3-Cholamidopropyl)-dimethyl ammonio]-2-hydroxy-1-propane sulfonate
CNS	Central nervous system
DA	Dopamine
DTT	Dithiothreitol
EDTA	Ethylenediaminotetra-acetic acid
EGTA	Ethyleneglycol-bis(β-aminoethyl ether)tetra-acetic acid
FPLC	Fast protein liquid chromatography
GABA	4-Amino-n-butyric acid
Gpp(NH)p	Guanosine 5' (β,γ-imido)triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
HPLC	High pressure liquid chromatography
LANT-6	[Lys^{8}-Asn^{9}] Neurotensin
NMN	Neuromedin N
NT	Neurotensin
[^{3}H] NT	[3,11-tyrosyl-3,5,-^{3}H] Neurotensin
NTLI	Neurotensin-like immunoreactivity
PAGE	Polyacrylamide gel electrophoresis
PPO	2,5-Diphenyloxazole
POPOP	1,4-Bis-[2-(4-methyl-5-phenyloxazole)]-benzene

SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethyldiamine
TES	N-Tris-(hydroxymethyl)methyl-2-amino-ethane-sulfonic acid
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
T-PBS	Trypsin in phosphate buffered saline
Tris	2-Amino-2-(hydroxymethyl)propane-1,3-diol

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1. INTRODUCTION

1.INTRODUCTION

1.1 THE SYNAPSE

An important factor in the evolutionary success of multicellular organisms has been the ability to co-ordinate the activities of individual cells with the requirements of the organism as a whole. One of the means by which this is achieved is via the mediation of a nervous system.

Historically, the nervous system was perceived as a syncitium or continuous cytoplasm uninterrupted by membranes. The revolutionary Neuron Theory proposed a nervous system composed of discrete cells, termed neurons, a postulate supported by the work of Ramon y Cajal (1897). The term "synapse" was introduced to describe the hypothetical region specialized for the exchange of information between neurons.

Amongst neurons there is a large diversity of form but all share particular structural features, namely a cell body, axon and numerous dendrites (Fig. 1). At the synapse the presynaptic nerve ending contacts a specialized region on the postsynaptic nerve, a distance of approximately 20-40 nm separating the two. The area between the pre- and post-synaptic membranes, the synaptic cleft, contains the basal lamina, an oligosaccharide rich connective tissue, which serves as a support for the two interacting cells. The postsynaptic area is recognizable as a distinct membrane thickening while the presynaptic terminal is identified by

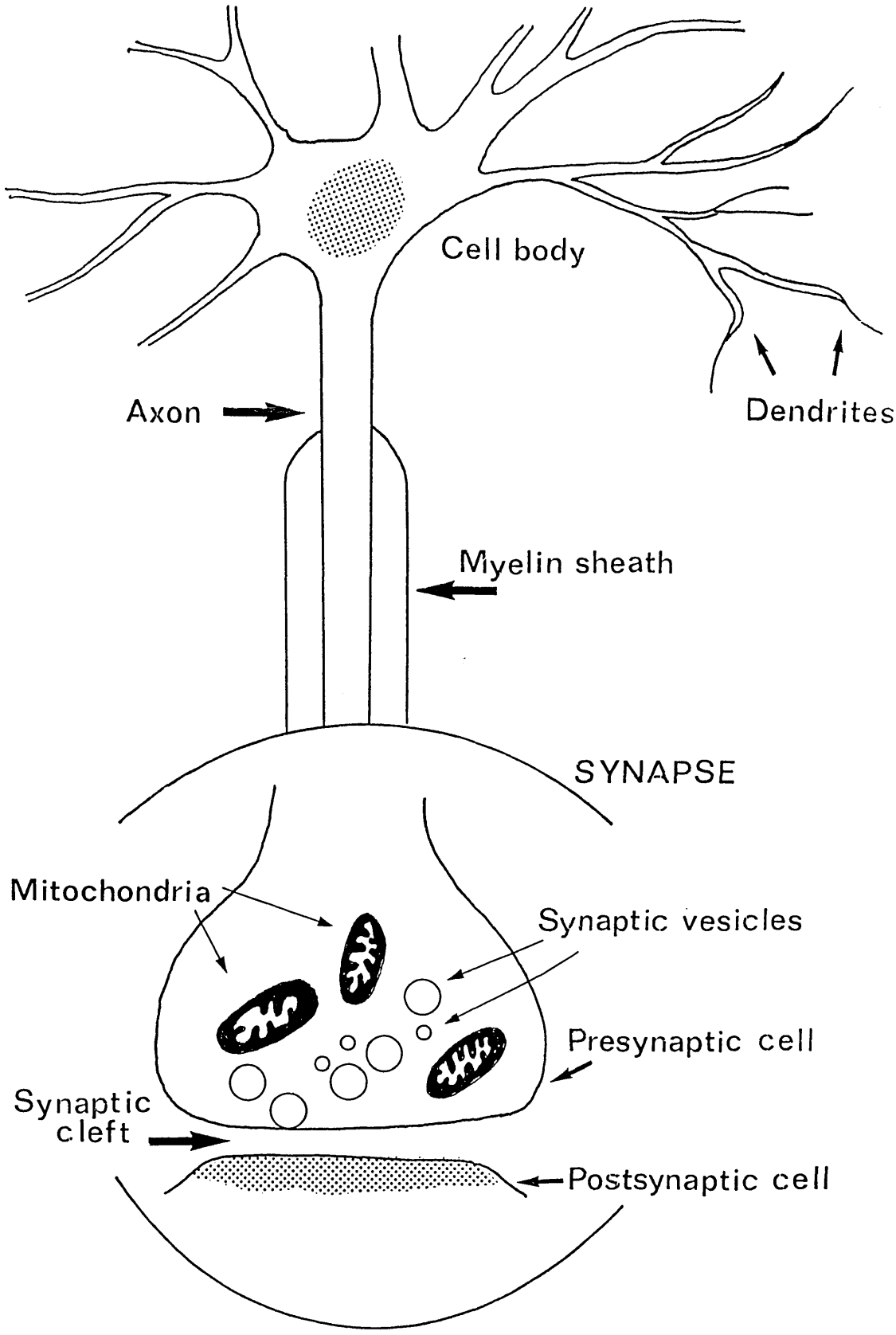


FIG. 1. Schematic illustration of a synapse.

the presence of characteristic inclusions i.e. mitochondria and neurotransmitter containing vesicles.

The electrical activity (action potential) which travels along the presynaptic nerve axon to the nerve ending can, at the synapse, be reinforced, reduced or left unaltered and transmitted to the next cell. The synapse is, therefore, not only the site of modulation of the nerve impulse but also a unidirectional valve which allows the impulse to be conducted in one direction only. At synapses the presence of the synaptic cleft would cause the rapid dissipation of any electrical signal so instead, the arrival of the action potential at the presynaptic nerve terminal results in the release of a chemical messenger or "neurotransmitter". This messenger diffuses across the synaptic cleft to interact with specific binding sites, "receptors", on the postsynaptic cell allowing, either by direct or indirect action, the opening of ion channels.

1.2 NEUROTRANSMITTERS

The earliest proof for the existence of chemical neurotransmitters was provided by Loewi (1921) who showed that vagal stimulation of a perfused frog heart *in vivo* released a substance into the perfusion medium that could, in the absence of any nerve stimulation, slow the beating frequency of a second heart. This "Vagusstoff" component was later identified as acetylcholine. Since then a large number of neuroactive substances have been positively identified as

neurotransmitters, some of which are shown in Fig. 2. These small molecules, some as small as a single amino acid, have structures which vary widely; what is it then that defines them as neurotransmitters?

1.2.1 DEFINITION OF A NEUROTRANSMITTER

A chemical is defined as a neurotransmitter (see also Hucho, 1986) if it satisfies the following criteria:

i) the chemical and its precursors must be present in the neuron,

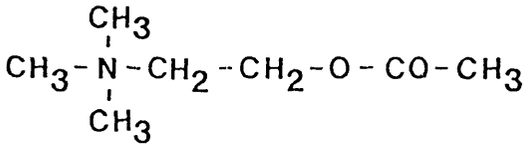
ii) the enzymes required for its synthesis and the proteins involved in the mechanism for its release must be similarly located,

iii) stimulation of the neuron must lead to the presence of the neurotransmitter in the synaptic cleft,

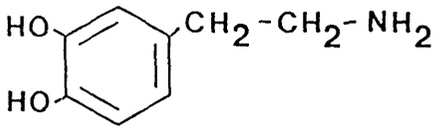
iv) after signal transmission it must be removed quickly by enzymic inactivation or uptake and,

v) when the neurotransmitter is applied externally its effect must be similar to that caused upon its release from the neuron.

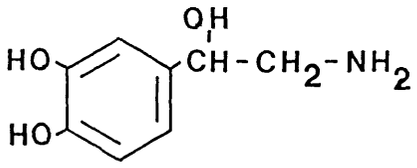
Synaptic transmitters may be inhibitory, causing hyperpolarization of the postsynaptic cell and hindering the generation of an action potential, or excitatory, depolarizing the cell and thereby generating an action potential. GABA and glycine are both inhibitory whereas glutamate is an excitatory neurotransmitter. Many neurochemicals are at present designated "putative neurotransmitters" as their exact role in the nervous system



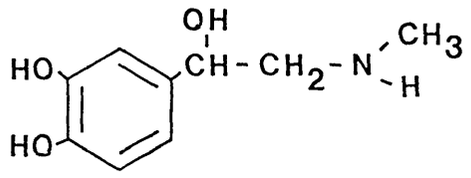
Acetylcholine



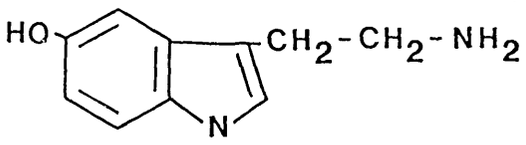
Dopamine



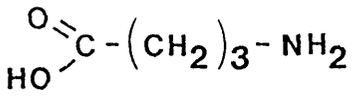
Noradrenaline



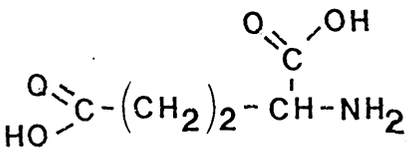
Adrenaline



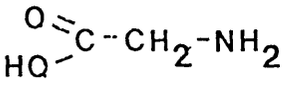
Serotonin



GABA



Glutamate



Glycine

FIG. 2. Neurotransmitters.

has yet to be established. Amongst these are members of a family of neuroactive molecules known as "neuropeptides".

1.3 NEUROPEPTIDES

For reviews see Emson, 1979; Bloom, 1981; Iversen 1983 and Bradford, 1986.

Neural tissues contain a large number of biologically active peptides (Table 1) of which substance P was the first to be identified. Von Euler and Gaddum (1931) found an unidentified substance present in alcoholic extracts of equine brain and intestine which they termed substance P (P being originally an abbreviation for powder and later for pain) which was trypsin sensitive (von Euler, 1936) and was later found to be composed of eleven amino acids (Chang and Leeman, 1970).

Other neuropeptides, including oxytocin, vasopressin and hypothalamic trophic hormone-releasing hormone, had long been known for their hormonal actions prior to their identification as neuropeptides. The time courses of hormonal and nervous system responses differ by orders of magnitude (hours/mins compared to milliseconds) and it was a well established view that, aside from some exceptions such as the presence of noradrenaline in the adrenal medulla, the two systems were entirely disparate. The advent of neuropeptides dispelled this preconception; not only were endocrine hormones found in the CNS but certain gastrointestinal hormones i.e. vasoactive intestinal peptide and cholecystokinin were also present in neurons.

Table 1. Neuropeptides present in the mammalian central nervous system.

Peptide	Peptide
Adrenocorticotropin	Kyotorphin
Angiotensins	Lipotropin
Atrial natriuretic peptides	Luteinizing hormone - releasing hormone
Bradykinins	Melanocyte stimulating - hormone
Bombesin	Motilin
Calcitonin	Neurokinins
Calcitonin-gene-related peptide	Neuromedins
Carnosine	Neuropeptide Y
Cholecystokinins	Neurotensins
Corticotropin releasing factor	Oxytocin
Delta sleep inducing peptide	Pancreatic polypeptide
Dynorphin	Proctolin
B-Endorphin	Secretin
Leu-enkephalin	Somatostatins
Met-enkephalin	Substance K
Epidermal growth factor	Substance P
Galanin	Thyroid stimulating - hormone
Gastrin releasing peptide	Thyrotropin releasing - hormone
Glucagon	Vasoactive intestinal - polypeptide
Growth hormone	Vasopressin
Growth hormone releasing - hormone	Vasotocin
Hydra head activator - peptide	
Insulin	

Material immunoreactive to antibodies raised against these substances has been found in neurons and nerve terminals of the mammalian central nervous system, other than those related to endocrine or neuroendocrine functions. The use of the plural indicates multiple molecular forms of a peptide. From Iversen (1983) and Goedert (1986).

In the case of the opioid peptides their discovery (Hughes, 1975; Simantov and Snyder, 1976; Kosterlitz, 1979) was as a result of the search for an endogenous mimic of the well documented actions of morphine.

1.3.1 SPECIAL FEATURES

Neuropeptides differ in several respects from the smaller molecules that have come to be regarded as the `classical' neurotransmitters. Firstly, neuropeptides are extremely potent and in a study (Bradford, 1986) of three major brain regions (caudate nucleus, cerebral cortex-temporal lobe and hypothalamus) neuropeptides were found to be present at appreciably lower concentrations (pmol/g) than the monoamines (nmol/g), acetylcholine (nmol/g) or GABA (umol/g). Secondly, the processing of neuropeptides tends to follow the route taken by secretory proteins in general. Briefly, a prepropeptide synthesized in the neuronal cell body is transferred to the cisternae of the endoplasmic reticulum where the pre(signal)sequence is cleaved to yield a propeptide. Further cleavages occur in the Golgi apparatus and later in the neurosecretory granules in which the peptide is transported to the nerve terminal for storage and release. In the latter a number of further modifications may also take place including C-terminal amidation, N-terminal acetylation, cyclization of glutamate to pyroglutamate, sulphation, phosphorylation and disulphide bond formation. Thus pro-opiomelanocortin may give rise to α-melanocyte stimulating hormone, adrenocorticotrophic hormone,

β -lipotropin and β-endorphins (Cooper and Martin, 1980) and β-preprotachykinin to substance P, substance K and a peptide with, as yet, unknown function (Nakanishi, 1986), depending on the enzymatic processing undergone by the prepropeptide precursor.

Many of the neuropeptides investigated appear to exist as families of related structures e.g. the cholecystokinins (Emson and Marley, 1982) that differ in size but contain a particular peptide sequence essential for activity. Furthermore, one member of a family may be enriched in a particular organ or brain region e.g. cholecystokinin-8 in the brain and cholecystokinin-33 in the gastrointestinal tract, depending on the tissue complement of processing enzymes.

Finally, an effective membrane transport system for peptides has yet to be discovered at the synapse: the mode of their inactivation appears to be that of degradation by peptidases. The implication inherent in the absence of an apparent recycling system is that the supply of any neuropeptide must be wholly dependent upon the stores of its precursor (for review see Turner, 1984).

1.3.2 COEXISTENCE STUDIES

Additional interest has been focussed on neuropeptides due to the possibility of their coexistence in neurons not only with other neuropeptides but also with other classical neurotransmitters. The majority of such studies have employed the technique of immunohistochemistry using

antibodies raised against the neurochemical in question. This leaves some results open to misinterpretation as it is possible for antibodies to cross-react with similar sequences in the family of neuropeptides present in the neuron (Hokfelt et al., 1980). Furthermore, antibodies do not penetrate intracellular storage sites; while in the cell body and axon it is probable that neuropeptides exist in low concentrations or as precursors which may not be amenable to antibody detection. However, there are a number of well documented examples of coexistence including acetylcholine and vasoactive intestinal peptide in the autonomic postganglionic neurons of cat exocrine glands and blood vessels (Hokfelt et al., 1980); adrenocoticotropin and β - endorphin in hypothalamus (Bloch et al., 1978) and serotonin and substance P in the spinal cord (Ljungdahl et al., 1978).

The phenomenon of coexistence has meant a reappraisal of the nature of intracellular storage sites. In most nerve endings there exist small (500 Å diameter) and large (1000 Å diameter) granular vesicles; immunohistochemical studies have revealed the presence of neuropeptides exclusively in the large vesicles with classical neurotransmitters in both the large and small vesicles (Goldsmith, 1977). The possibility of differential subcellular storage compartments is not totally unexpected as revealed by the observation that, in neurons containing monoamines and substance P, reserpine will deplete monoamine stores while leaving substance P levels unaffected (Hokfelt et al., 1980). The

patterns of coexistence found to date are by no means simple and in none of the cases investigated do all of the neurons which contain a particular classical neurotransmitter always contain the same neuropeptide and vice versa.

The next question to be addressed is whether these patterns of coexistence are functionally meaningful. In the case of acetylcholine and vasoactive intestinal peptide it has been shown that the former stimulates secretion by acting on secretory cells while the latter exerts an action on a separate set of cells producing concomitant vasodilation (Lundberg et al., 1982). Based upon this interaction Lundberg and Hokfelt (1983) have postulated that the individual contributions of classical neurotransmitter and colocalized neuropeptide may depend upon the amount of neuronal stimulation (Fig. 3). The peptide induced response is visualized as being slower in onset, developing gradually and of longer duration than the response attributable to the classical neurotransmitter.

The notion that two neuroactive, coreleased substances act on two different cell types is not valid for all examples of coexistence and there is evidence to suggest that substance P can block cholinergic receptors and that serotonin and substance P interact at the same receptor in the spinal cord (Hokfelt et al., 1980).

The discovery of neuropeptides and their colocalization with other neuroactive molecules implies a greater complexity in the functioning of the synapse than had previously been anticipated. From a simple model of one

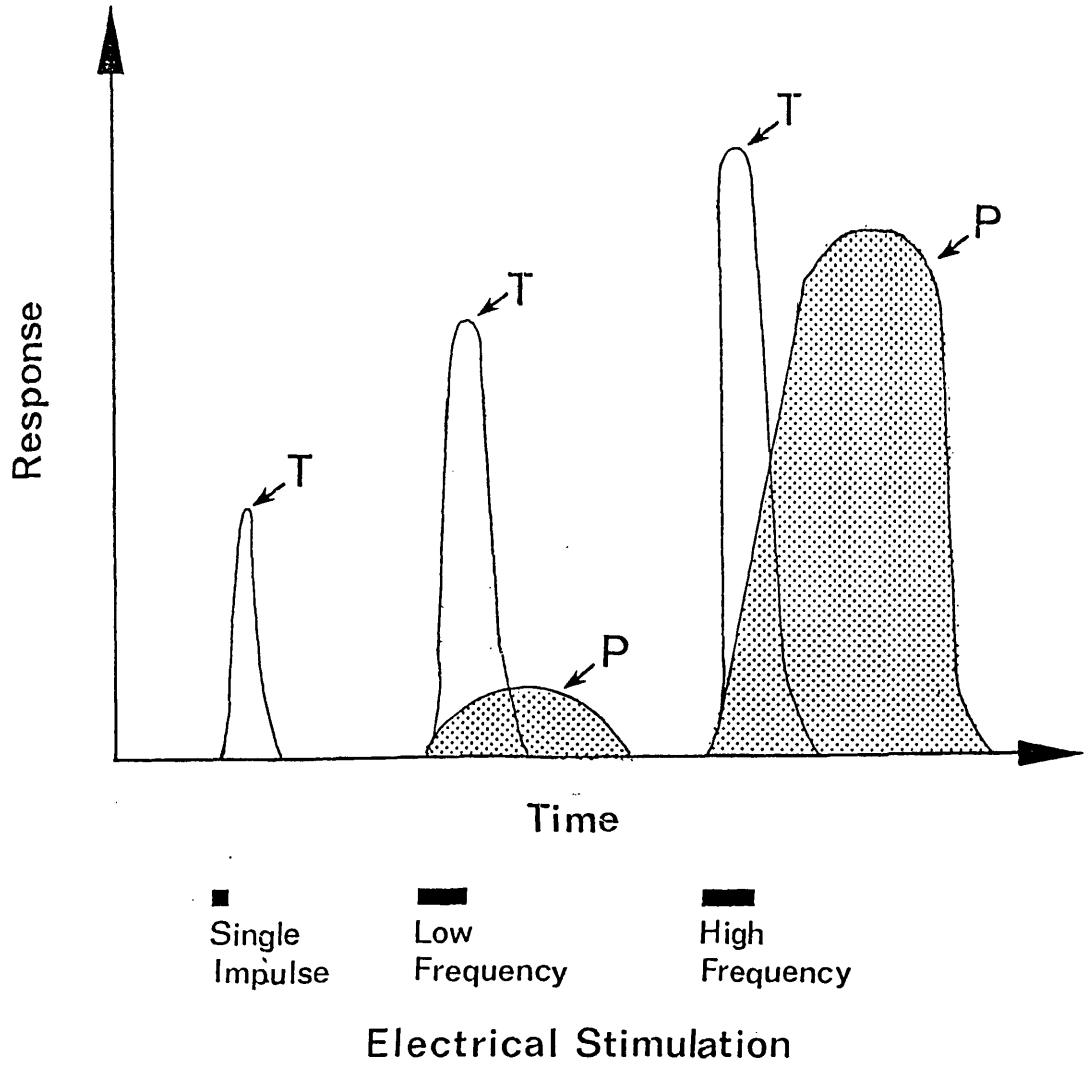


FIG. 3. Schematic illustration of the contributions of coexisting classical neurotransmitter (T) and neuropeptide (P) to a functional response. A single nerve impulse induces a response due to the release of a classical neurotransmitter; upon stimulation with higher frequencies there is an increasing functional effect caused by the peptide.

From Lundberg and Hokfelt (1983) based on their analysis of ACh and VIP in the cat exocrine gland.

neurotransmitter one postsynaptic receptor (Fig. 4, A) to the discovery of subclasses of postsynaptic receptor (Fig. 4, B) and the presence of presynaptic receptors (Fig. 4, C) ideas have now progressed to include the higher levels of control and signalling introduced by neuropeptides (Fig. 4, D).

1.3.3 NEUROPEPTIDES - NEUROTRANSMITTERS OR NEUROMODULATORS?

An issue which has caused much debate is whether neuropeptides should be classified as neurotransmitters (1.2.1) or neuromodulators.

A neuromodulator is defined as acting at local or distant postsynaptic sites to modify the efficacy of a neurotransmitter acting at the same postsynaptic site, thus amplifying or attenuating its action. Additionally, a neuromodulator may also act presynaptically controlling the release of the neurotransmitter from its presynaptic site.

For such a diverse group of molecules as the neuropeptides it seems impossible to make any meaningful generalizations concerning their mode of action and whether they are qualitatively different from classical neurotransmitters. Substance P is a neurotransmitter in certain areas of the spinal cord (Jessell and Iversen, 1977) but its mode of action in situations where it coexists with a classical neurotransmitter is open to question.

The issue of classification has taken on an additional significance due to the controversy it has caused with respect to Dales principle. This principle, as formulated by

FIG. 4. Schematic illustration depicting the development of concepts of neurotransmission.

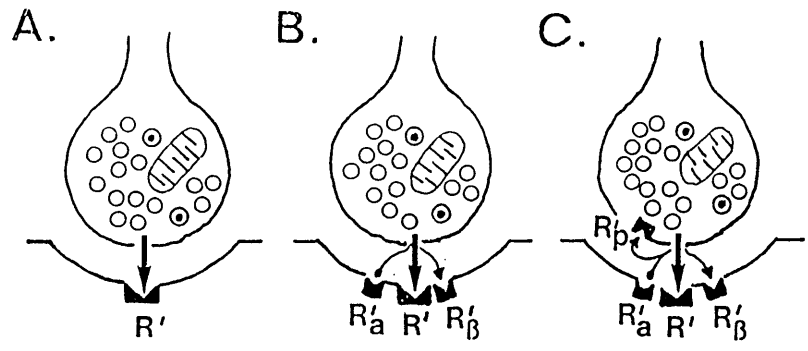
A: One transmitter acting on one postsynaptic receptor.

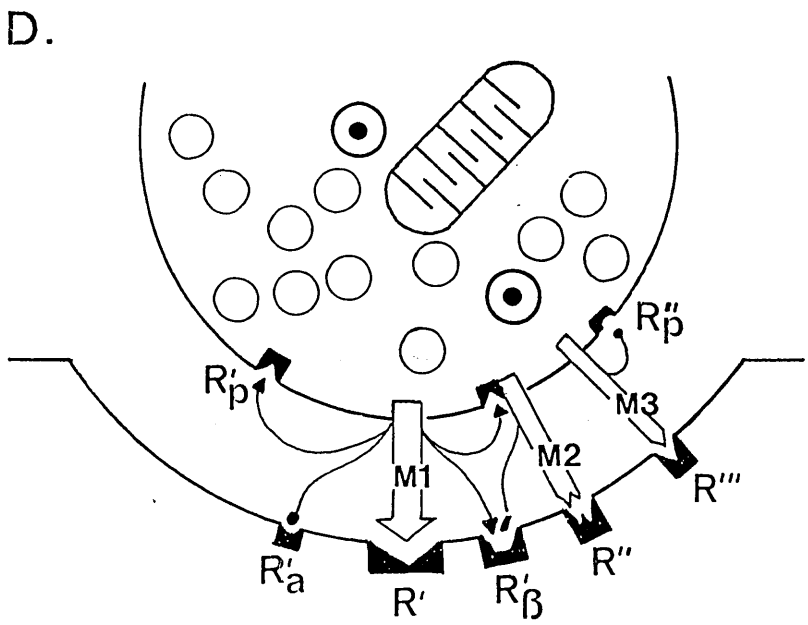
B: One transmitter acting on multiple types of post-synaptic receptor.

C: One transmitter acting on postsynaptic and pre-synaptic receptors.

D: Differentially stored multiple compounds (M1-M3) acting post- and pre-synaptically. Only some of the possible interactions are indicated.

From Lundberg and Hokfelt, 1983.


A.
B.
C.
R′
R′a R′ R′β
R′p
R′a R′ R′β


D.
R′p
R″p
M3
M1
M2
R‴
R′a
R′
R′β
R″

Eccles *et al.* (1954), states that each neuron has the ability to synthesize, store and release only one transmitter substance. If neuropeptides act as neurotransmitters when coexisting with classical neurotransmitters or other neurotransmitter-acting peptides Dales principle is compromised. Reviews on this topic, however, by Burnstock (1976) and Osborne (1979) both concluded that there is no hard and fast evidence against this theory. Until a clearer picture emerges of the precise role a neuropeptide plays in a specified location in the nervous system the importance of coexistence studies remains open to debate.

1.4 NEUROTENSIN

During the purification of substance P from extracts of bovine hypothalamus Carraway and Leeman (1973) discovered a peptide which, when injected intravenously (i.v.) into rats, caused marked hypotension, vasodilation and cyanosis. This tridecapeptide, designated neurotensin (NT), was found to have the amino acid sequence ‹Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu which was conserved in the brain and intestinal tissues of a number of mammalian species (Carraway, 1979). Chang *et al.* (1976) suggested that the glutamate^{4} residue of NT was in fact glutamine the amide side chain being lost during the acid extraction procedure. However, although [Gln^{4}] NT is a potent agonist of some actions of NT there is no evidence for its natural

occurence.

Also in 1973 a structurally related octapeptide, xenopsin, was isolated from the skin of the amphibian Xenopus laevis (Araki et al., 1973) but this peptide does not occur in mammalian species. Following these initial discoveries a `family' of NT like peptides emerged (Table 2) including avian NT (Carraway and Bhatnagar, 1980), [Ser^7] NT (Shaw et al., 1986) and two highly active hexapeptides Neuromedin N (NMN; Minoamino et al., 1984) and [Lys^8-Asn^9] NT (LANT-6; Carraway and Ferris, 1982).

Most, if not all, neuropeptides arise from prepropeptides (1.3.1) and Carraway et al. (1986) have reported that pepsin treatment of mammalian plasma generates NT like immunoreactivity (NTLI). The resultant peptides, while not identical to NT, share a number of its structural and pharmacological properties and arise from precursor-like proteins of molecular weights 65 and 350 kilodaltons. The authors proposed that NT or NT like peptides stem from a large molecular weight precursor by a process analogous to the generation of angiotensin from angiotensinogen (Valloton, 1987). Using cells from canine enteric mucosa Dobner et al. (1987) isolated a cDNA clone containing within its sequence both NT and NMN as well as an NMN like peptide. The peptides are flanked in this 170 amino acid putative precursor by paired basic amino acids which act as cleavage signals for trypsin like proteases. However, it should be noted that this precursor is not of similar structure to that described for xenopsin (Sures and Crippa, 1984) and it

Table 2. Neurotensin and related peptides

Peptide	Amino Acid Residue Number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Neurotensin (Mammalian)	‹Glu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	Tyr	Ile	Leu
Neurotensin (Chicken brain and intestine)	‹Glu	Leu	His	Val	Asn	Lys	Ala	Arg	Arg	Pro	Tyr	Ile	Leu
[Ser^{7}] Neurotensin (Guinea pig brain and intestine)	‹Glu	Leu	Tyr	Glu	Asn	Lys	Ser	Arg	Arg	Pro	Tyr	Ile	Leu
Neuromedin N (Porcine spinal cord)								Lys	Ile	Pro	Tyr	Ile	Leu
[Lys^{8}-Asn^{9}] Neurotensin (Chicken intestine)								Lys	Asn	Pro	Tyr	Ile	Leu
Xenopsin (Xenopus laevis)							‹Glu	Gly	Lys	Arg	Pro	Trp	Ile	Leu

The amino acid residue numbers given refer to the neurotensin molecule.

has yet to be shown that NMN occurs in canine mucosa.

NT is degraded at the synapse (Fig. 5) by a number of synaptically located peptidases (Camargo et al., 1983; Checler et al., 1983a, 1983b, 1984, 1985, 1986) and it is notable that all the major cleavages are of the highly conserved 8-13 region of the NT molecule (Table 2). There is no evidence to suggest that there is an uptake system for NT at the synapse (Kitabgi et al., 1977).

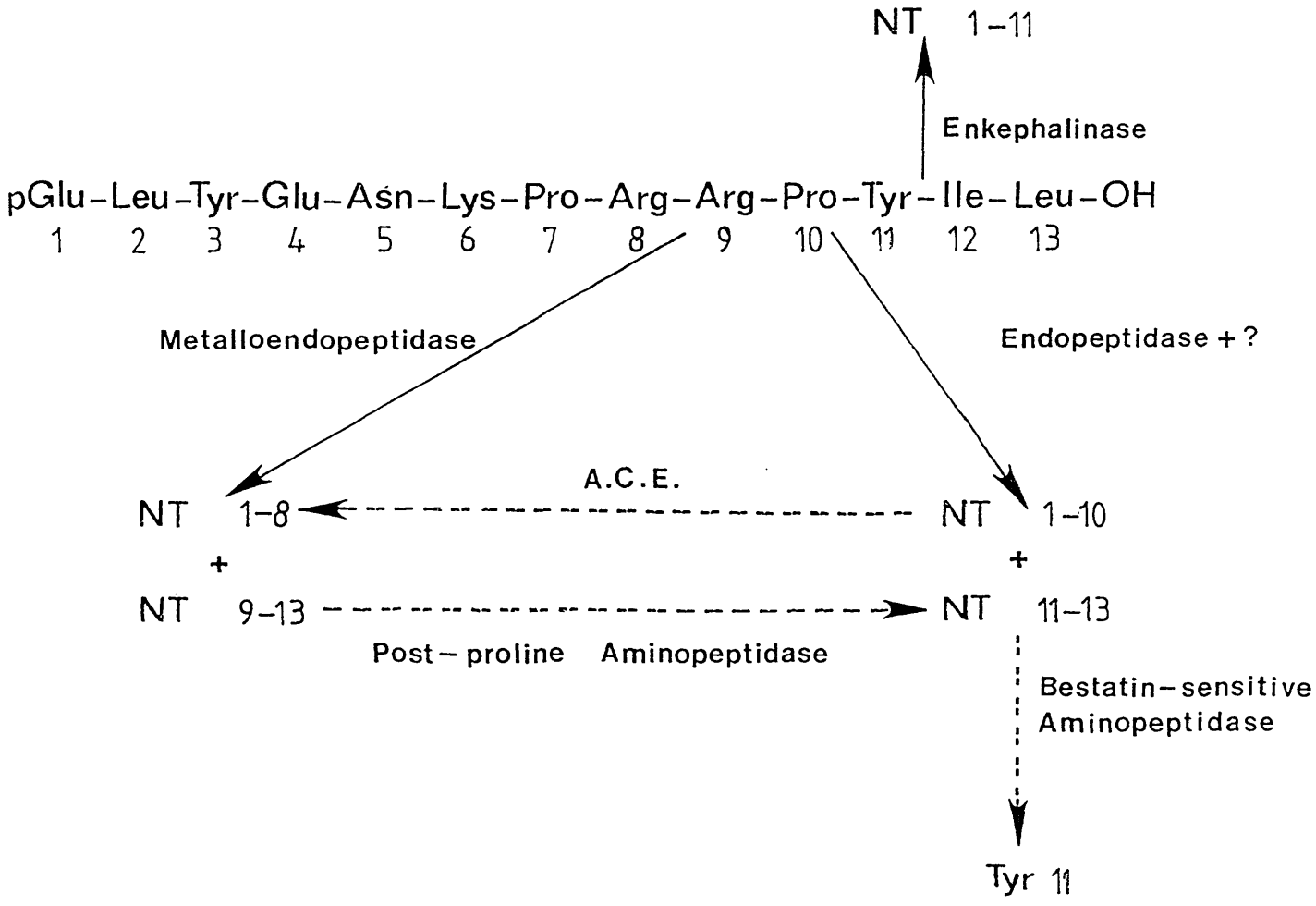
1.4.1 LOCALIZATION

Using a radioimmunoassay (RIA) Carraway and Leeman (1976) investigated the distribution of NT in one week old and adult rats. In both age groups 90% of the total body content of NT was found outside the brain and spinal cord the majority being in the mucosa and smooth muscle of the jejunum and ileum. Extensive analysis of the NTLI in calf and human intestine (Kitabgi et al., 1976; Carraway et al., 1978; Hammer et al., 1980) revealed it to be identical to NT isolated from bovine hypothalamus. Polak et al. (1977) using RIA and immunohistochemistry located NT to the N cells of the intestine, an endocrine cell type which opens out into the gut lumen (Helmstaedter et al., 1977). These observations are consistent with the suggestion that NT plays a role as a gastrointestinal hormone (vide infra).

In adult rats the hypothalamus and brain stem each contain 35% of the total brain NT the remainder being in the cerebral cortex (17%), thalamus (11%), cerebellum (1%) and pituitary (1%; Carraway and Leeman, 1976) This typical

FIG. 5. A model for the mechanism of NT inactivation by rat brain synaptic membranes. Solid arrows represent the primary cleavages of the NT molecule; dashed arrows the secondary cleavages of the NT degradation products (A.C.E. Angiotensin converting enzyme).

From Checler et al., 1985.


NT 1–11
Enkephalinase
pGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Tyr–Ile–Leu–OH
1 2 3 4 5 6 7 8 9 10 11 12 13
Metalloendopeptidase
Endopeptidase + ?
A.C.E.
NT 1–8
NT 1–10
+
+
NT 9–13
NT 11–13
Post–proline Aminopeptidase
Bestatin–sensitive
Aminopeptidase
Tyr 11

distribution of NT has also been found in rat brain by Uhl and Snyder (1977) and Kobayashi et al. (1977) and in human (Emson et al., 1985) and bovine (Uhl and Snyder, 1977) brain. It is of interest to note that high NTLI is found in areas where dopaminergic innervation is also present i.e: hypothalamus, substantia nigra, nucleus accumbens, median eminence and olfactory bulb (Jennes et al., 1982).

Subcellular fractionation studies have demonstrated the presence of NTLI in synaptosomal and microsomal fractions (Carraway and Leeman, 1975); Iversen et al. (1978) have described K^+ induced, Ca^{2+} dependent release of NTLI from hypothalamic slices in vitro.

NTLI has also been found in the pancreas (Berelowitz et al., 1980), plasma (Blackburn and Bloom, 1979) and in laminae I and II [substantia gelatinosa] of the spinal cord (Ninkovic et al., 1981).

1.4.2 PHYSIOLOGY/PHARMACOLOGY

The NT found in the gastrointestinal tract appears to act as a local hormone since plasma NTLI rises sharply following a fatty meal (Rosell and Rokaeus, 1979; Blackburn et al., 1980). NT also influences the electrolyte transport in the mucosa of the ileum (Kachur et al., 1982) and modulates intestinal peristalsis (Kitabgi and Freychet, 1978). Additionally, NT exerts an influence on carbohydrate metabolism causing an increase in blood glucose levels. In the rat NT decreases plasma glucagon levels (Brown and Vale, 1976) by stimulating directly the pancreatic α cells and

indirectly inhibiting the β cells via the adrenal medulla (Nagai and Frohman 1978). Histamine mimics the effects of NT (Nagai and Frohman, 1978) which can be blocked by a H_1, but not H_2, histamine antagonist and also by [D-Trp^{11}] NT (Ukai et al., 1982).

The contractile effect of NT on guinea pig ileum was investigated in depth by Kataoka et al. (1978) who found that the C-terminal region of NT and in particular the Arg^{8}-Arg^{9} residues were essential for this activity. Kitabgi and Freychet (1979a, b) showed this contractile activity to be mediated via the release of acetylcholine with no apparent effects on either cAMP or cGMP.

The increased inotropy caused by NT perfusion of spontaneously beating rat or guinea pig heart is antagonized by [D-Trp^{11}] NT and abolished by [Tyr-Me^{11}] NT. In this tissue the biological activity of NT resides in the Arg^{9}-Pro^{10}-Tyr^{11} sequence (Quirion et al., 1980a).

When adminstered directly into the CNS, NT produces a wide variety of effects which include hypothermia, antinociception and endocrine effects (see also Nemeroff et al., 1980a).

NT is a potent hypothermic compound in a number of vertebrate species and may play an important role in thermoregulatory physiology. The hypothermic effect of NT, first noted by Carraway and Leeman (1973), was found to be dependent on the C-terminal region of the molecule (Rivier et al., 1977, Loosen et al., 1978) and especially the Arg^{8}-

Arg^9 residues. The [D-Tyr''] and [D-Phe''] analogues of NT are potent agonists of this effect (Jolicoeur et al., 1981) which may reflect their resistance to NT degrading peptidases (Checler et al., 1983). Administration of a NT antiserum which is presumed to bind and inactivate endogenous NT (Wallace et al., 1981) produced significant hyperthermia in the rat. NT induced hypothermia can be increased by treatments which reduce the activity of the CNS dopaminergic pathways (Nemeroff et al., 1980b).

In its analgesic action intracisternally (i.c.) applied NT is more potent on a molar basis than morphine (Clineschmidt and McGuffin, 1977); [Gln ^{4}] NT is equipotent with NT (Clineschmidt et al., 1979). Pretreatment with TRH (i.c.) blocks the NT induced analgesia (Clineschmidt et al., 1979) and, together with the partial antagonism by TRH of NTs hypothermic action, suggests that the two may act as functional antagonists in the CNS. Reduction in the brain levels of serotonin after treatment with p-chlorophenylalanine potentiates the analgesic effects of NT implying that NT may supress the activity of serotonergic neurons (Long et al., 1984). NT is also present at high concentrations in the substantia gelatinosa of the spinal cord, an area implicated in the modulation of pain transmission (Ninkovic et al., 1981).

Investigations into the interplay between NT and DA in the CNS were prompted initially by the observation that centrally administered NT produced effects similar to those of neuroleptic drugs (DA antagonists; Nemeroff, 1980) and

later by their colocalization (1.4.1) in specific brain areas.

Spontaneous locomotor activity in rodents is reduced by neuroleptic drugs; a similar effect is also seen after intracerebroventricular (i.c.v.) injections of NT although both the [D-Tyr^{11}] and [D-Phe^{11}] analogues of NT increase locomotor activity (Jolicoeur, et al., 1981). Stimulation of locomotor activity by indirect DA agonists, e.g. amphetamine, which act by increasing the release of DA from presynaptic terminals can be blocked by NT; the action of direct DA agonists acting on postsynaptic receptors is NT insensitive (Nemeroff et al., 1980c). It is also possible for DA to block particular NT responses as exemplified by the D_2 receptor linked inhibition of NT induced Ca^{2+} influx into pituitary cells (Memo et al., 1986a).

The similarity in the effects of NT and neuroleptic drugs is further reinforced by the results of Garcia-Sevilla et al. (1978) who reported that i.c.v. administration of NT or [Gln^4] NT increases monoamine turnover as described for neuroleptic agents. Concentrations of NT up to 100 uM were unable to displace [^{3}H] spiperone binding and centrally injected NT did not alter the subsequent binding of this radioligand to nucleus accumbens or striatal membranes (Nemeroff et al., 1983). As yet no unifying theory has emerged to account for all the NT-DA interactions observed although the subject has been well reviewed (Nemeroff and Cain, 1985).

The localization and physiology/pharmacolgy of NT ensures its place alongside other neuropeptides such as bombesin, vasoactive intestinal peptide and the cholecystokinins which have major hormonal and CNS functions.

1.5 RECEPTORS

The term "receptor" is used by neurochemists to define, at the molecular level, the binding sites for neurotransmitters/neuromodulators. Receptors for these ligands are integral membrane proteins that fulfil a dual function in recognizing, on the cell surface, the appropriate ligand with high sensitivity and selectivity and conveying the recognition process, directly or via a transducer, to an effector, the activation of which results in the appropriate response.

1.5.1 IDENTIFICATION

A major tool for the *in vitro* study of neurotransmitter/neuromodulator receptors is the use of a radioactively labelled endogenous or exogenous ligand in binding assays. An *in vitro* binding site however, is not necessarily an *in vivo* receptor and data obtained from such assays must satisfy a number of criteria before such an identity can be established. These criteria have been extensively reviewed by Burt (1985) and can be divided into four main categories:

i) saturability - there should be a finite number of

binding sites for the ligand and saturation should occur in a physiologically significant concentration range;

ii) kinetics - the reaction of ligand and binding site should be reversible and occur with reasonable speed i.e. comparable to the time course for the biological response;

iii) distribution - binding should only occur in areas where the biological activity of the ligand is also observed and

iv) pharmacology - the ligand should be specific for the binding site and should be able to compete with other analogues which act at the same receptor.

1.5.2 MODE OF ACTION

Receptors in the nervous system employ a variety of different mechanisms in the translation of the information carried by the neurotransmitter/neuromodulator into an appropriate cellular response. Activation of the GABA, glycine or nicotinic cholinergic receptors results in the rapid and transient opening of ion channels (Na^+ for the latter, Cl^- for the other two) present in the receptor structure. However, many other receptors act by quite different mechanisms involving the generation of specific intracellular signals. These signals, which include changes in the levels of cyclic nucleotides and/or calcium, initiate a cascade of events which serve to amplify the primary signal. Many of the actions of these molecules are achieved by the activation of cAMP-dependent, cGMP-dependent and Ca^{2+}-dependent protein kinases which results in the

phosphorylation of specific substrate proteins (Nestler and Greengard, 1983). These phosphorylated proteins are involved in carrying out and regulating such diverse processes as neurotransmitter/neuromodulator biosynthesis and release; axoplasmic transport; generation of postsynaptic potentials; ion channel conductance; neuronal shape; elaboration of dendritic or axonal processes and development and maintenance of differentiated characteristics of neurons (Nestler and Greengard, 1983).

The question then arises as to how receptor occupancy is linked to the production of an intracellular signal? Activation of adenylate cyclase, the enzyme that produces cAMP, requires the interaction of three different and separate components: at the outer membrane surface the neurotransmitter/neuromodulator receptor (R) and at the inner membrane face a catalytic (C) and a guanine nucleotide (G) regulation unit. The latter contains a site for binding GTP and is responsible for regulating the activity of C. A number of G-proteins have been described in the literature some of which have an, as yet, unknown function; of these the best characterized are G_s and G_i which stimulate and inhibit adenylate cyclase activity respectively (for reviews see Rodbell, 1980, 1985; Gilman, 1984). Each regulatory unit is an oligomer composed of three distinct protein subunits, α , β and γ ; the GTP binding site is located on the α subunit. The β and γ subunits (molecular weights 35,000 and 8-10,000 Daltons) are highly conserved and are shared by both G_s and G_i whereas the α subunits differ.

Thus $G_{s\alpha}$ has a molecular weight of 45,000 and $G_{i\alpha}$ 41,000 and the former can be ADP-ribosylated by Cholera toxin only and latter by Pertussis toxin only. Rodbell (1980) has proposed that agonist binding to a receptor coupled to a G-protein oligomer, in the presence of GTP, results in the dissociation of the G-protein-receptor complex and the dissociation of the $G\alpha$ from the β and γ subunits. Following dissociation the receptor is desensitized and has a low affinity for agonist binding while the released G-protein can regulate the activity of C. The G-protein is inactivated by virtue of an inherent GTPase activity thereby allowing the re-formation of the $G\alpha\beta\gamma$ complex. This pattern of events appears to be valid for most, if not all, receptor-adenylate cyclase coupled systems although many of the precise details e.g. the nature of the R-G coupling have yet to be elucidated. It has been recently shown that the G_i of cardiac muscle is involved in the coupling of the muscarinic aceylcholine receptor to a K^+ channel (review Noma, 1986) and this is consistent with the idea of a family of G-proteins coupled to other effector systems besides adenylate cyclase (Rodbell, 1985).

If the receptor systems which regulate the levels of cAMP via adenylate cyclase can be considered as a group then a second group encompasses those that use Ca^{2+} as an intracellular signal (for reviews see Downes, 1983; Berridge and Irvine, 1984). Briefly, the occupancy of Ca^{2+} mobilizing receptors by agonist results in the hydrolysis of

phosphatidyl inositol, a membrane phospholipid, to inositol 1,4,5 trisphosphate and diacylglycerol by a phospholipase C. The former mobilizes Ca^{2+} by acting at mitochondrial and endoplasmic reticulum storage sites while the latter activates protein kinase C. This particular protein kinase is referred to as being Ca^{2+}/phosphatidylserine dependent and is stimulated by trace quantities of diacylglycerol (review Bell, 1986). In addition to its effect on protein kinase C, Ca^{2+} also activates guanylate cyclase which produces cGMP although increased guanylate cyclase activity does not invariably require phosphoinositol turnover (Kendall, 1986). Membrane phospholipids also act a storage sites for arachidonic acid which is released via the action of diacylglycerol lipase on diacylglycerol. The pharmacologically active agents prostaglandins, thromboxanes and leukotrienes are all derived from arachidonic acid thereby conferring additional complexity on this transducing system. As in the case of adenylate cyclase the mechanism for receptor-enzyme coupling is not fully understood although it has been proposed that agonist-receptor binding causes perturbation of the membrane structure allowing closer orientation of enzyme and substrate (Irvine et al., 1984). However, data is accumulating which suggests that inositol phosphate hydrolysis may also be under the control of a G-protein (Cockcroft and Gomperts, 1985; Kikuchi et al., 1986).

1.6 NEUROTENSIN RECEPTORS

The existence of specific NT binding sites has been demonstrated by the use of radiolabelled NT or NT analogues in autoradiography and radioligand binding studies. Both tritiated and iodinated NT have been employed and while the former has the advantage that it is identical to native NT the specific radioactivity incorporated per NT molecule is considerably lower than that attainable with ^{125}I. Iodinated NT used in the early studies of Uhl *et al.* (1977) was a mixture of peptides in which one or both tyrosine molecules had incorporated ^{125}I making the analysis of binding data difficult. Subsequent refinements have led to the development of monoiodo [Trp^{11}] NT (Mazella *et al.*, 1983) and [^{125}I Tyr^{3}] NT (Sadoul *et al.*, 1984a) although the [Trp^{11}] analogue of NT is not active in all tissues (Sadoul *et al.*, 1984a).

1.6.1 PERIPHERAL TISSUES

When considering the fact that 90% of the total body content of NT is found in the periphery (Carraway and Leeman, 1976) surprisingly little is known about NT receptors in peripheral tissues.

In the guinea pig ileum the majority of [^{3}H] NT binding sites, as determined by autoradiography, are located in the circular smooth muscle layer with lower levels in the longitudinal muscle (Goedert *et al.*, 1984a). Saturation binding studies in ileal homogenates indicated a single population of sites with an equilibrium dissociation

constant (K_D) of 3-4 nM and a maximum number of binding sites (B_{max}) of 14 fmol/mg protein (Goedert et al., 1984a). Additionally, NT (8-13) and NT (9-13) were able to inhibit the binding of [^{3}H] NT with potencies that correlated well with their effectiveness in the ileum contraction assay. A similar dissociation constant, 4 nM, was obtained in the earlier studies of Kitabgi and Freychet (1979a) who used the longitudinal muscle of guinea pig ileum. These authors also confirmed the importance of the Arg^8-Arg^9 residues of NT in this system by ligand competition studies as indicated by Kataoka et al. (1979). This is consistent with the proposal by Leeman and Carraway (1982) that the interaction of NT with its receptors is through the ionic interactions with the pair of arginine residues together with the weaker, hydrophobic interactions at the Tyr^{11}-Ile^{12}-Leu^{13} region. In plasma membranes derived from rat fundus (Kitabgi et al., 1984) or canine intestinal circular smooth muscle (Ahmad et al., 1987) the binding of iodinated NT, monoiodo [Trp^{11}] NT and [^{125}I Tyr^3] NT respectively, revealed the presence of two populations of binding site with high and low affinity for the radioligand. In both cases the affinity of the two sites differed by about twenty fold; the K_D of the low affinity site was 2-3 nM which is similar to the value obtained in intestinal tissue using [^{3}H] NT. The binding of iodinated NT to canine intestinal membranes was reduced by physiological concentrations of Na^+ and divalent cations (Ahmad et al., 1987); from this it was inferred that the two

sites represent two forms of a single receptor population with the low affinity form being operative *in vivo*. Further evidence for the ubiquity of a `low affinity' site ,i.e. K_D 2-4 nM, and its physiological importance in intestinal tissues comes from studies on the HT 29 cell line; binding of [^{3}H] NT to this human colon carcinoma has a K_D of ~2 nM (Kitabgi *et al.*, 1980).

In the rat adrenal gland [^{3}H] NT binding sites are present in the cortex with lower levels in the medulla (Goedert *et al.*, 1984b) where NT is known to inhibit B-cells (Nagai and Frohman, 1978). In homogenates of whole rat adrenal gland the binding of [^{3}H] NT was found to be of low capacity. However, since 75% of the [^{3}H] NT binding sites were found by, autoradiography, in a single layer of the cortex, a localized high concentration of sites is suggested (Goedert *et al.*, 1984b). The dissociation constant for the binding of [^{3}H] NT in homogenates was 3.5 nM comparable to that found in the gastrointestinal tract (vide supra).

The NT receptors present in rat mast cells (Lazarus *et al.*, 1977a) and mouse macrophages (Goldman and Bar-Shavit, 1983) are clearly distinct from those found in the gastrointestinal tract and adrenal gland with K_Ds for [^{3}H] NT binding of 154 and 28 nM, respectively. In both cell types NT analogues shorter than the pentapeptide NT (9-13) were unable to displace the [^{3}H] NT binding in competition studies, further confirming the Leeman and Carraway (1982) theory of NT - NT receptor interaction. In mouse macrophages

the hierachy of binding potencies of NT analogues was in agreement with their ability to enhance phagocytotic activity (Goldman and Bar-Shavit, 1983).

While the NT receptors in rat heart and portal vein have not been characterized in terms of radioligand binding parameters their pharmacology presents the first evidence for NT receptor subtypes. Two subtypes have been proposed by Regoli (1984) based on the premise that receptors can be classified by measuring the relative affinity of agonists and/or by estimating the apparent affinities of competitve antagonists. The pharmacological preparations important in this context are the rat portal vein, heart and stomach strip where NT causes vasoconstriction, increased inotropy and contraction, respectively. In the portal vein and heart the agonists [Trp^{11}] NT and NT (8-13) are equipotent with NT while in the stomach [Trp^{11}] NT is 30% more active than NT itself whereas NT (8-13) is 30% less active (Rioux et al., 1980). This differentiation was reinforced when the antagonists [D-Trp^{11}] NT and [Tyr(Me)^{11}] NT were investigated as these are active in the vasculature and heart but not in the stomach (Rioux et al., 1980; Quirion et al., 1980b). Using this data Regoli (1984) has proposed that there at least two subtypes of NT receptor: NT_1 and NT_2 where NT_1 is defined as that present in the portal vein and heart. The possibility of a NT receptor heterogeneity in other tissues has yet to be investigated, a process which is hampered by the commercial unavailability of the majority of

the agonists and antagonists required.

1.6.2 CENTRAL NERVOUS SYSTEM

1.6.2.1 DISTRIBUTION

Neurotensin receptors are widely and unevenly distributed in the mammalian central nervous system. In a definitive study of rat brain Goedert *et al.* (1984c) compared the distribution of [^{3}H] NT binding and NTLI in homogenates of different brain regions (Table 3). The levels of [^{3}H] NT binding were highest in the hypothalamus and frontal cortex followed by the midbrain and striatum; NTLI was at its most abundant in the hypothalamus and midbrain and at low levels in the striatum and frontal cortex. This mismatch of receptor density and ligand is not uncommon and has been described for noradrenaline and β-adrenergic receptors in rat striatum (Alexander *et al.*, 1975) and substance P and its receptors in the rat substantia nigra (Mantyh *et al.*, 1983). The reason for the inverse relationship between NTLI and NT receptors is unclear but may be due to the differential turnover rates for the peptide and its receptor. It is also possible that there are low affinity binding sites present that are not detectable at nM concentrations of radioligand or by radioimmunoassay or immunohistochemistry. Similar patterns of NT receptor density were also found in studies of rat brain by Lazurus *et al.* (1977b) and Uhl *et al.* (1977) with high concentrations found in the substantia nigra/midbrain which,

Table 3. Distribution of [^{3}H] neurotensin binding and neurotensin-like immunoreactivity in rat brain.

Brain Region	[^{3}H] neurotensin binding (%)	neurotensin-like immunoreactivity (%)
Hypothalamus	100	100
Frontal cortex	93	2
Midbrain	79	60
Striatum	74	25
Thalamus	69	33
Hippocampus	6	11
Olfactory bulb	65	5
Cerebellum	38	1
Pons-medulla	36	30

From Goedert et al. (1984c); values are expressed as a percentage of the corresponding value found in the hypothalamus.

as suggested by Palacios and Kuhar (1981), are on putative dopaminergic neurons. In this area destruction of dopaminergic cell groups by 6-hydroxydopamine resulted in a decreased number of NT receptors (Palacios and Kuhar, 1981; Herve et al., 1986) while an increase was apparent following chronic neuroleptic administration (Herve et al., 1986).

Although the hypothalamus of the rat was found to contain the highest concentration of NT receptors the same was not true for either calf or human brain. Greater densities of NT receptor were found in the thalamus of the former (Uhl et al., 1977) and the substantia nigra/midbrain of the latter (Kanba et al., 1986; Sadoul et al., 1984a; Sarrieau et al., 1985). Interestingly the NT receptor content in the human midbrain/substantia nigra was significantly reduced in patients with Parkinsons disease (Sadoul et al., 1984b; Uhl et al., 1984). These studies suggest that there are species variations in the distribution of NT receptors in the brain as also seen for the histamine H_1 receptor (Kanba and Richelson, 1984).

From subcellular fractionation studies of rat brain NT binding was found to be highest in the synaptosomal (P_2) fraction (Lazurus et al., 1977b).

Finally, the distribution of NT receptors in the rat spinal cord parallels that of NTLI i.e. in layers I and II of the substantia gelatinosa (Ninkovic et al., 1981).

1.6.2.2 BINDING STUDIES

Specific NT binding sites in the mammalian CNS were

first demonstrated by Uhl and Snyder (1977) in homogenates of whole rat brain. Using [^{125}I] NT, a mixture of iodinated peptides, they reported a single class of binding sites with a dissociation constant of 3 nM and a maximal binding capacity of 3.1 pmol/g tissue. Subsequent investigations, however, in mammalian brain and neuronal cell lines have failed to yield a consensus with regard to the binding parameters of this receptor. The K_D values obtained from NT receptor binding studies in brain tissue and neuronal cell lines are summarized in Table 4 . In rat and human brain there exists some controversy, as seen in the periphery, as to whether there are one or two populations of NT binding site. To account for this Kanba et al. (1986) have proposed that the identification of two rather than one binding site for NT is a function of the specific activity of the radioligand used. Sadoul et al. (1984c) in their study on human brain using [^{125}I Tyr^3] NT with specific activities of 2000 and 100 Ci/mmol, the latter being comparable to that of [^{3}H] NT, only detected two sites when the radioligand of higher specific activity was used. However, this line of argument is not supported by the results of Nakagawa et al. (1984) and Schotte et al. (1986) who reported the presence of high and low affinity sites in rat brain using [^{3}H] NT. Additionally, Sadoul et al. (1984c) and Sarrieau et al. (1985) investigated the binding of [^{125}I Tyr^3] NT binding to human brain and found two classes and one class of site, respectively.

Whatever the cause of the discrepancy in these

Table 4. Summary of NT binding studies involving brain tissue or neuronal cell lines.

Species	Radioligand	Sites	K_D (nM)	Reference
rat	[^{125}I]	1 (a)	3	Uhl et al. (1977).
rat	[^{125}I]	1 (a)	8	Lazurus et al. (1977,b).
rat	[^{3}H]	1 (b)	2	Kitabgi et al. (1977).
rat	[^{3}H]	1 (a)	5.7	Young & Kuhar (1981).
rat	[^{3}H]	1 (a)	2.85	Goedert et al. (1984,c).
rat	[^{3}H]	2 (b)	0.44, 19	Nakagawa et al. (1984).
rat	[^{125}I Trp^{11}]	2 (b)	0.1, 4.7	Mazella et al. (1983).
rat	[^{125}I Tyr^{3}]	2 (b)	0.18, 5.1	Sadoul et al. (1984,a).
rat	[^{3}H]	1 (c)	1.4	Memo et al. (1986,b).
rat	[^{3}H]	2 (a)	0.20, 7.9	Schotte et al. (1986).
mouse	[^{3}H]	2 (b)	0.86, 13	Nakagawa et al. (1984).
guinea pig	[^{125}I Tyr^{3}]	2 (b)	0.16, 6.3	Sadoul et al. (1984,a).
cat	[^{3}H]	1 (d)	1.54	Goedert et al. (1984,d).
human	[^{3}H]	1 (a)	2.0	Kanba et al. (1986).
human	[^{125}I Tyr^{3}]	2 (e)	0.26, 4.3	Sadoul et al. (1984,c).
human	[^{125}I Tyr^{3}]	1 (f)	4.8	Sarrieau et al. (1985).
NIE-115	[^{125}I Trp^{11}]	1	0.15	Poustis et al. (1984).
NIE-115	[^{3}H]	1	11.0	Gilbert et al. (1986).
NIE-115	[^{125}I Tyr^{3}]	1	0.75	Amar et al. (1985).
NIE-115	[^{125}I Tyr^{3}]	1	0.056	Bozou et al. (1986).
NG108-15	[^{3}H]	1	0.86	Nakagawa et al. (1984).
mouse	[^{125}I Tyr^{3}]	1 (g)	0.3	Checler et al. (1986,b).

(a) Whole brain homogenates
(b) Whole brain synaptic membranes
(c) Pituitary homogenate
(d) Striatum homogenate
(e) Substantia nigra homogenate
(f) Brain slices
(g) Primary cultured neurons

results, it is unlikely to be attributable to the nature of the brain preparation employed since in the majority of studies homogenates or synaptic membranes prepared from whole brain were used (Table 4). Furthermore, Goedert et al. (1984c) have shown in a study of rat brain regions that there is little or no difference in the K_D values obtained.

The significance of the putative high affinity NT binding site is unknown and while Mazella et al. (1983) and Schotte et al. (1986) have interpreted their data in terms of two different and independent classes of binding site Sadoul et al. (1984a) postulated two different states of a single class of binding sites. In support of the former, Schotte et al. (1986) described the displacement of [^{3}H] NT by levocabastine, a histamine H_1 antagonist, from the low affinity (K_D 7.9 nM) binding sites but not the high affinity sites (K_D 0.2 nM) of rat brain. However, Mazella et al. (1983) noted that both classes of sites possess the same first order rate constant of dissociation, a finding which would seem to imply the presence of a single receptor type.

A large variation is also seen in the maximal binding capacities reported in parallel studies i.e. studies in which identical brain preparations and radiolabelled forms of NT were used. An excellent example of this is the binding of [^{3}H] NT to rat whole brain homogenates where B_{max} values as diverse as 430 (Goedert et al., 1984c) and 74 (Nakagawa et al., 1984) fmol/mg protein have been reported. In synaptosomal preparations of whole rat brain this

discrepancy is even more pronounced the B_{max} obtained by Nakagawa et al. (1984) of 1250 fmol/mg protein being approximately ten fold higher than that of 135 fmol/mg protein as reported by Kitabgi et al. (1977).

Binding of iodinated, [^{125}I Tyr^{3}] or [^{125}I Trp^{11}], NT to cells of the murine neuroblastoma cell line N1E-115 was shown in three separate studies to be to a single class of sites with a K_D of < 1 nM (Table 4) and B_{max} values of 9 (Poustis et al., 1984), 45 (Amar et al., 1985) and 30 fmol/mg protein (Bozou et al., 1986). In each case the cells had been differentiated for 48-72 hours in culture medium containing 1.5% (v/v) DMSO - a procedure established by Kimhi et al. (1976) and used by Poustis et al. (1984) who reported the complete lack of NT binding sites in non-differentiated cells. Work by Gilbert et al. (1986) however, directly contradicted this: they reported [^{3}H] NT binding to non-differentiated cells which was of high capacity, 180-250 fmol/mg protein, but of comparatively low affinity, K_D 11 nM. Second messenger activity linked to the NT receptor (vide infra) was also found in non-differentiated cells by Gilbert and Richelson (1984), Gilbert et al. (1986), Snider et al. (1986) and Kanba and Richelson (1987). It is perhaps significant that the K_D values for the high affinity sites of differentiated cells and the low affinity sites in non-differentiated cells are comparable to those reported for the 2 classes of binding site in mammalian brain (Table 4). However, until a study is made of these cells in their differentiated and non-differentiated states using a single

form of radiolabelled NT, it would be presumptious to draw any conclusions from this.

Structure-activity studies on the inhibition of radiolabelled NT binding by NT fragments have indicated, as seen in the periphery, that the pentapeptide NT (9-13) is the shortest fragment with this capability. In N1E-115 cells (Gilbert et al.,1986); rat (Mazella et al., 1983; Goedert et al., 1984c; Kitabgi et al., 1977); cat (Goedert et al., 1984d) and human (Kanba et al., 1986) brain the pentapeptide had < 5% of the activity of NT. Species differences however emerged when the hexapeptide NT (8-13) and the NT analogue [Trp^{11}] NT were investigated. In rat brain (Mazella et al., 1983; Sadoul et al., 1984a) and N1E-115 cells (Poustis et al., 1984) [Trp^{11}] NT is equipotent with NT but has a complete lack of activity in human (Sadoul et al., 1984c) and guinea pig (Sadoul et al., 1984a) brain. In its inhibition of radiolabelled NT binding NT (8-13) is more potent than NT in N1E-115 cells (Gilbert et al.,1986); rat (Mazella et al., 1983; Goedert et al., 1984c) and cat (Goedert et al., 1984d) brain and is less active (Sadoul et al., 1984c; Sarrieau et al., 1985) or equipotent (Kanba et al., 1986) in human brain. On the basis of the activity of these two compounds the NT receptors in mammalian species may be provisionally classified into two groups: one present in the rat, cat and mouse (N1E-115 cells) and the other in guinea pig and human. Further investigation using other compounds active at the NT receptor i.e. LANT-6 and NMN will

be needed before this classification can be firmly established.

1.6.3 SECOND MESSENGERS

Occupation of the NT receptor by ligand has been associated with PI turnover, Ca^{2+} influx, cGMP synthesis and inhibition of adenylate cyclase (Table 5).

Stimulation of PI turnover by NT has been reported in rat brain (Goedert et al., 1984e); N1E-115 cells (Snider et al., 1986; Kanba and Richelson, 1987) and HT 29 cells (Amar et al., 1986). In rat brain an excellent correlation was found between the number of [^{3}H] NT binding sites in a given area and the magnitude of NT stimulated PI turnover (Goedert et al., 1984e). Maximal stimulation in N1E-115 and HT 29 cells was 100-200% and 200-500% respectively, above the basal level. Increased PI metabolism has been linked to the mobilization of Ca^{2+} and Snider et al. (1986) found identical dose response profiles in N1E-115 cells for NT stimulated PI changes and Ca^{2+} influx.

It is now well established that in rat pituitary NT induces PI metabolism (Canonico et al., 1985) and influx of Ca^{2+} leading to prolactin release (Memo et al., 1986a). In this tissue the rise in intracellular Ca^{2+} was not affected by the Ca^{2+} channel antagonist verapamil or the Na^+ channel blocker tetrodotoxin (Memo et al., 1986a). However, in the rat fundus the contractile activity of NT was antagonized by verapamil and potentiated by the Ca^{2+} channel agonist Bay K 8644 (Donoso et al., 1986). These conflicting results would

Table 5. Summary of NT receptor linked second messenger activity.

Source	Associated Activity	EC_{50} NT (nM)	Reference
rat brain	Phosphoinositol turnover	ND	Goedert et al. (1984,e).
rat pituitary	Ca^{2+} influx	4.2	Memo et al. (1986,a).
rat fundus	Ca^{2+} influx	ND	Donoso et al. (1986).
NIE-115 cells, differentiated	cGMP synthesis	2.0	Amar et al. (1985).
	adenylate cyclase inhibition	2.0	Bozou et al. (1986).
NIE-115 cells	cGMP synthesis	13.0	Gilbert et al. (1984).
	cGMP synthesis	1.5	Gilbert & Richelson (1986).
	cGMP synthesis	1.5	Gilbert et al. (1986).
	Ca^{2+} influx	4.0	Snider et al. (1986).
	Phosphoinositol turnover	0.9	
	cGMP synthesis	1.2	Kanba & Richelson (1987).
	Phosphoinositol turnover	0.9	
HT 29 cells	Phosphoinositol turnover	50-100	Amar et al. (1986).

Unless stated otherwise the cell lines studied were not differentiated; ND not determined.

seem to suggest that the NT receptor is linked to different Ca^{2+} channels according to the tissue specificity. In both cases the effect of NT is lost in the absence of extracellular Ca^{2+} indicating a movement of Ca^{2+} into the cells rather than the mobilization of intracellular stores.

Increased synthesis of cGMP brought about by NT has been investigated in N1E-115 cells where the controversy persists as to whether the cells require differentiation before they can express NT receptor activity. Amar et al. (1985) found no increase in cGMP synthesis upon application of NT to non-differentiated cells, a result not confirmed by other groups (Table 5). The EC_{50} values determined for NT and second messenger activities are almost identical in differentiated and non-differentiated cells (Table 5) aside from an early study by Gilbert and Richelson (1984) which was later amended (Gilbert and Richelson, 1986). The increase in cGMP levels was to approximately 10 fold above basal levels (Gilbert and Richelson, 1984; Amar et al., 1985; Kanba and Richelson, 1987): the rank order of potency of NT and related compounds in promoting this was NT (8-13) > NT > NMN > LANT-6 which correlated with their established K_D values (Gilbert et al., 1985; Amar et al., 1985; Gilbert and Richelson, 1986). These compounds also had closely matched EC_{50}s for PI metabolism and stimulation of cGMP synthesis (Kanba and Richelson, 1987) suggesting that these two responses arise from the activation of a single type of NT binding site. N1E-115 cells also contain receptors for

thrombin and bradykinin which produce a cGMP response with the same time course and Ca^{2+} dependency as that observed for NT yet there is no competition, in terms of binding assays, between the ligands (Gilbert and Richelson, 1984). The NT induced elevation of cGMP levels was Ca^{2+} dependent but Ca^{2+} itself was unable to mimic the action of NT (Gilbert and Richelson, 1984; Amar et al., 1985) indicating that the two processes may not be directly linked. Further evidence for this hypothesis comes from desensitization studies - in terms of the movement of Ca^{2+} the NT receptors of N1E-115 cells are desensitized at NT concentrations greater than 100 nM (Snider et al., 1986) while for cGMP synthesis this occurs much earlier at approximately 30 nM NT (Amar et al., 1985). Snider et al. (1986) also found that eicosatetraynoic acid (ETYA) a lipoxygenase inhibitor blocked NT induced cGMP synthesis.

Distinct from its effects on PI turnover, Ca^{2+} mobilization and cGMP synthesis NT is also able to promote, in N1E-115 cells, the inhibition of adenylate cyclase (Amar et al., 1985; Bozou et al., 1986). In a comprehensive study on this aspect of NT receptor action, Bozou et al. (1986) showed that NT inhibited prostaglandin E_1 stimulated cAMP production by up to 55% with an EC_{50} of 2 nM. This inhibitory effect could be blocked by pretreatment of the cells with pertussis toxin - a protocol which did not affect NT stimulated cGMP production. The affinity of binding of [^{125}I Tyr^3] NT to these cells was decreased in the presence of Na^+ and guanyl nucleotides, a feature of adenylate

cyclase modulating receptors (Rodbell, 1980); pertussis toxin selectively ADP-ribosylated a single protein with the same molecular weight (41,000) as the α subunit of G_i.

It is noteworthy that in N1E-115 cells NT inhibits cAMP production and at the same time stimulates cGMP production through apparently different transduction mechanisms. The question then arises as to whether these two responses occur through the same or distinct populations of NT receptors. Before attempting to answer this question the following experimental evidence should be considered: i) Scatchard analysis of binding to N1E-115 cells has always revealed a single population of non-interacting sites (Table 4); ii) EC_{50} values for the ability of NT to trigger the various second messenger responses are similar (Table 5) and iii) structure-activity studies show that it is the carboxy-terminal hexapeptide, NT (8-13), which is responsible for binding and the induction of the second messenger response (vide supra; Bozou et al., 1986).

Bozou et al. (1986) have proposed two hypotheses which would answer the above question and account for the experimental data. In the first hypothesis a single, unique population of NT receptors would mediate both the cAMP and cGMP responses. This would imply that the same NT receptor molecule has two distinct transduction domains, one interacting with G_i and the other with a cGMP generating system. In the second, alternative hypothesis two separate populations of NT receptors with similar recognition sites

and affinities for NT and related peptides are proposed having different transduction domains, each mediating a different response to NT in the N1E-115 cells. As yet there is no definitive answer to this question but it is clear that the cAMP and cGMP responses involve different transduction mechanisms and that the latter is also linked to PI turnover.

1.6.4 MOLECULAR CHARACTERIZATION

Using the radioactive NT analogue [^{125}I Trp^{11}] NT, Mazella et al. (1985) have covalently labelled the NT binding sites in rat brain by two methods. In the first a photoreactive analogue, [^{125}I-azidobenzoyl-Trp^{11}] NT, was used to photoaffinity label the receptor while, in the second the reversible interaction between [^{125}I Trp^{11}] NT and its receptor was made irreversible by the use of a bifunctional crosslinking reagent. Two specifically labelled proteins of M_r 49,000 and 51,000 were identified in both cases after SDS-PAGE; the size of the two proteins was the same when electrophoresed in the presence or absence of reducing agent. Variations in the radioligand concentration did not alter the relative labelling intensities of the two bands and this has been interpreted as indicating similar molecular structures for the high and low affinity [^{125}I Trp^{11}] NT binding sites of rat brain previously characterized by this group (Mazella et al., 1983). The model proposed by the authors for the structure of the NT receptor is therefore that of a non-covalently linked

heterodimer.

Unfortunately, the limited species reactivity of [Trp^{11}] NT (Sadoul et al., 1984a) means that this procedure is not applicable to all NT receptors and, to date, the validity of this structural model has not been confirmed.

1.7 OBJECTIVES OF THE PRESENT STUDY

Although the structure of the rat brain NT receptor has been inferred from covalent labelling studies the labelled receptor can no longer bind NT and the studies for which this preparation can be used are, therefore, limited. In order to circumvent this problem it is necessary to solubilize and purify the receptor in a functional state. The methodology involved in such an undertaking is complex and has been extensively reviewed for other receptors (Gavish et al., 1979; Laduron and Ilien, 1982; Hjelmeland and Chrambach, 1984; Lunt, 1987; Jones et al., 1987). The solubilization and purification of the NT receptor in an active state has not been reported until now.

The first and second parts of this thesis, therefore, describe the detailed characterization of [^{3}H] NT binding to rat and bovine brain membranes and to the neuroblastoma cell line N1E-115; in particular the characterization of these sites as receptors and their suitability for solubilization studies is assessed.

In the third part the detergent solubilization of rat and bovine NT receptors is investigated and the [^{3}H] NT binding parameters and molecular properties of the bovine NT

receptor in detergent solution are reported.

Finally, the purification to homogeneity of the bovine NT receptor is described together with the [^{3}H] NT binding properties and molecular composition of the purified receptor.

2. MEMBRANE STUDIES

2.1 INTRODUCTION

Prompted by the numerous inconsistencies existing in the literature regarding NT receptors this chapter details the development of a [^{3}H] NT binding assay and its use in the characterization of the NT binding sites in rat and bovine brain.

The binding sites identified were examined with respect to their possible identification as NT receptors and suitablity for solubilization and purification studies.

2.2 METHODS

2.2.1 TISSUE PREPARATION

2.2.1.1 RAT

Male Sprague-Dawley rats weighing 150-200 g were killed by cervical dislocation. The brains minus cerebellum were rapidly removed onto ice and used immediately for membrane preparation (2.2.2).

2.2.1.2 BOVINE

Fresh bovine cortex, obtained from a local slaughterhouse, was roughly dissected to remove the white matter and frozen on dry ice within one hour of excision. Storage thereafter was at -20°C.

2.2.2 MEMBRANE PREPARATION

Rat brain (~5 g) or thawed bovine cortex (10-15 g) was minced with a pair of scissors in 15 ml ice-cold Buffer A [10 mM TES KOH, pH 7.5, 0.3 M sucrose, 1 mM EDTA (Na^+ salt), 1 mM benzamidine HCl, 1 mM DTT, 0.1 mM PMSF, 0.01% (w/v) bacitracin and 0.002% (w/v) soybean trypsin inhibitor]. The volume was adjusted to 50 ml with Buffer A and the tissue homogenized at 4°C: twice for 15 s at medium speed and once for 10 s at high speed using an Ultra-Turrax homogenizer (model TP 18/2N with Thyristor speed control). Buffer A was added to 100 ml and the brain homogenate centrifuged at 1,500 x g, 4°C for 10 min. The supernatants obtained were transferred on ice and the pellets

rehomogenized and centrifuged as described above. Following centrifugation of the pooled supernatants at 45,000 x g, 4°C for 30 min, the pelleted membranes were resuspended in 50 ml ice-cold Buffer B [Buffer A minus sucrose] by six strokes of a glass-teflon homogenizer and stirred magnetically at 4°C for 30 min in a total volume of 150 ml Buffer B.

The membrane pellets obtained after centrifugation at 45,000 x g, 4°C for 30 min were resuspended in Buffer B to 15-20 mg protein/ml using a 25-gauge needle, distributed into 1 ml aliquots and immediately frozen in liquid nitrogen. Membranes were stored at -20°C, the preparation yielding approximately 40-50 mg protein/g wet weight of brain in both cases.

2.2.3 BINDING ASSAYS

2.2.3.1 STANDARD ASSAY

Frozen membranes to be assayed for ligand binding were thawed, washed with 40 volumes of ice-cold 10 mM TES KOH, pH 7.5, centrifuged (45,000 x g, 4°C, 30 min) and resuspended in the same buffer at 1-2 mg protein/ml using a 25-gauge needle.

The ligand binding assays were carried out in 10 mM TES KOH, pH 7.5, containing 1 mM EGTA-K^+, 0.02% (w/v) bacitracin, 1 mM benzamidine HCl, 0.002% (w/v) soybean trypsin inhibitor, 10 uM 1,10-phenanthroline, 1 mg/ml BSA [Binding Assay Buffer], 0.1-0.4 mg membrane protein/ml and tritiated NT at the required concentration in a total volume

of 500 ul. Non-specific binding was defined as that obtained under the same conditions but in the presence of 10^{-6} M unlabelled NT. After 1 hour incubation at room temperature, bound and free ligand were separated by filtering under vacuum 400 ul through Whatman GF/B glass fibre filters pre-soaked in 0.3% (v/v) polyethylenimine solution. Each filter was immediately washed with 3 x 4 ml ice-cold Tris HCl, pH 7.4, dried under an infra-red lamp and liquid scintillation counted at an average efficiency of 40%. All measurements were as independent triplicates.

2.2.3.2 MODIFICATIONS OF STANDARD ASSAY

DETERMINATION OF OPTIMUM PROTEIN CONCENTRATION

The protein concentration used in binding assays was chosen by investigating the range of protein concentrations at which the specific binding of [^{3}H] NT increased linearly. Briefly, the specific binding of 3 nM [^{3}H] NT to membranes was assayed (2.2.3.1) at increasing protein concentrations to a maximum of 0.5 mg protein/ml assay. The experiment was repeated three times for each tissue.

THE EFFECT OF PH ON [^{3}H] NT BINDING

The optimum pH for specific [^{3}H] NT binding was determined at 3 nM [^{3}H] NT. TES KOH, 10 mM, was adjusted to pHs in the range 6-9 (0.5 pH unit increments) and used in the Binding Assay Buffer (2.2.3.1). Protein concentration and all other procedures were as described (2.2.3.1) and the experiment performed twice for each tissue.

COMPETITION STUDIES

NT (1-13), NT analogues: NT (1-12); NT (1-10); NT (1-8); n-acetyl NT (8-13); NT (9-13); [D-Trp^{11}] NT; [D-Phe^{11}] NT and NT-like peptides: tuftsin; bombesin; NMN were assayed for their ability to inhibit specific [^{3}H] NT binding. At [^{3}H] NT concentrations of 10 nM and 3 nM, rat and bovine membranes, respectively, putative inhibitors were present in the Standard Assay (2.2.3.1) at final concentrations of 10^{-12}-10^{-5} M in the Binding Assay Buffer (2.2.3.1). Between 1 and 7 independent experiments, measurements as independent triplicates, were performed for each inhibitor.

THE EFFECTS OF CATIONS AND GUANYL NUCLEOTIDE

The effect of increasing concentrations of cation on the specific binding of [^{3}H] NT was examined at 6 nM (rat membranes) and 3 nM (bovine membranes) [^{3}H] NT. Cations: Na^+; Mg^{2+}; Mn^{2+}; Ca^{2+}; Li^+ and K^+ were included as their chloride salts (Mg^{2+} as its sulphate) at concentrations of 1-100 mM in the Binding Assay Buffer of the Standard Assay (2.2.3.1). For the analysis of saturation isotherms in the presence of cations a single concentration of cation was chosen and the concentration of [^{3}H] NT varied between 1 and 20 nM.

Specific binding of [^{3}H] NT in the presence of the non-hydrolyzable GTP analogue guanosine 5'-(β,γ -imido) triphosphate [Gpp(NH)p] was measured at 100 uM Gpp(NH)p,

10mM Mg^{2+} and 1-50 nM [^{3}H] NT.

All other procedures were as detailed for the Standard Assay (2.2.3.1) and between 1 and 5 independent experiments performed for each experimental condition.

KINETIC STUDIES

Membranes (0.3-1.0 mg protein/ml) were incubated under Standard Assay conditions (2.2.3.1) with [^{3}H] NT (10 nM-rat membranes, 5 nM-bovine membranes) in a final volume of 25 ml.

For ligand association studies duplicate aliquots, 400 ul, were filtered immediately after the final addition to the assay (zero time point), at 1-2 min intervals for the initial 20 min and thereafter at 10-20 min intervals up to 1 hour.

Ligand dissociation studies were performed by equilibrating membranes with [^{3}H] NT (concentrations as previously) for 60 min at room temperature. Unlabelled NT was then added to a final concentration of 10^{-6} M and aliquots, 400ul, filtered at time intervals as described above.

Dissociation of ligand by dilution was investigated by pre-equilibrating membranes with [^{3}H] NT as detailed above. After 60 min incubation the membranes were diluted 1:60 in Binding Assay Buffer (2.2.3.1) and duplicate aliquots of 1 ml filtered as before.

2.2.4 STABILITY OF [^{3}H] NT

[^{3}H] NT was checked routinely for purity and stability using both thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC).

The stability of NT in the presence of membranes was investigated by incubating membranes and [^{3}H] NT (5 nM) in a final volume of 1ml. Incubation was for 90 min under the standard conditions for ligand binding (2.2.3.1) and the membranes were subsequently pelleted by centrifugation in an Eppendorf centrifuge at 10,000 x g for 10 min at 4°C. The supernatant was removed, acidified with trifluoracetic acid (TFA) to a final concentration of 0.2% (v/v) and loaded onto a 1 ml C_{18} reverse phase Sep-Pak column pre-washed with methanol and equilibrated with 5 column volumes of 0.2% (v/v) TFA in 10 mM TES KOH. The column was washed with 2 ml 0.2% (v/v) TFA in water and NT and any degradation products eluted with 1 ml methanol and lyophilized overnight. The lyophilized samples were then resuspended in 20 ul ethanol containing 1 mg/ml unlabelled NT for TLC analysis or 20 ul 82% (v/v) methanol, 0.2% (v/v) TFA for HPLC investigations. Control samples of [^{3}H] NT (5 nM) were incubated and treated as described above but in the absence of membranes.

2.2.4.1 THIN LAYER CHROMATOGRAPHY

Untreated [^{3}H] NT (5 ul, 20 nM), control samples (10 ul) and [^{3}H] NT obtained after preincubation with

membranes (10 ul) were spotted with 15 ul 1N HCl onto Avicel Cel 400 cellulose plates (5 cm x 15 cm), dried and transferred to a TLC tank pre-equilibrated with n-butanol:acetic acid:water (25:4:10). The plates were removed after the solvent front had travelled 10-12 cm and dried. Peptides were visualized by spraying with ninhydrin/cadmium reagent (100:15, 1% (w/v) ninhydrin in acetone: 7% (w/v) cadmium acetate in 2:1 acetic acid:water) and radioactivity determined by counting 1 cm x 0.25 cm strips from the plates in 5 ml Toluene/Soluene scintillant (91:9 toluene:soluene, 7% (w/v) PPO, 0.6% (w/v) POPOP) for 5 min. Intact NT had an R_f value of 0.5.

2.2.4.2 HIGH PRESSURE LIQUID CHROMATOGRAPHY

[^{3}H] NT (20 ul treated, control or untreated) was chromatographed on a Techsphere reverse phase C_{18} (5 um) column using an isocratic solvent system of 82% (v/v) methanol, 0.2% (v/v) TFA at a flow rate of 2 ml/min. Unlabelled standards (20 ul) of NT, NT (1-11), acetyl NT (8-13) and NT (1-8) at concentrations of 1 mg/ml gave retention times (t_r) of 22, 16.5, 8.5 and 5.5 min, respectively. The distribution of radioactivity was measured by counting 1 ml fractions in Toluene/Soluene scintillant (2.2.4.1) for 5 min.

2.2.5 PROTEIN DETERMINATION

Protein determination was by a modification of the Lowry procedure (Hess et al., 1978). The following stock

solutions were prepared:

A: 2% (w/v) Na-carbonate, 0.4% (w/v) NaOH, 1% (w/v) SDS

B: As A including 0.16% (w/v) Na-tartrate

C: 4% (w/v) $CuSO_4$

D: 100 volumes A plus 1 volume C

E: Folin-Ciocalteaus phenol reagant diluted 1:1 in water.

Solutions D and E were made immediately prior to use.

Radioimmunoassay grade BSA, 1 mg/ml in water, was diluted to 1 ml with solution A to give a series of standards in the range 10-50 ug/ml; membrane samples were similarly diluted. Aliquots of 200 ul, in triplicate, were each mixed with 800 ul solution D and left to incubate for 30 min at room temperature. Solution E, 60 ul, was then added, the samples vortexed immediately and left to stand at room temperature for an additional 45 min. At the end of this time absorbance was measured at a wavelength of 750 nm.

2.2.6 DATA ANALYSIS

Equilibrium and competition data were analysed using the LIGAND program of Munson and Rodbard (1980). The relevant equations and their derivations for these and kinetic analyses are detailed below.

2.2.6.1 EQUILIBRIUM BINDING STUDIES

The equilibrium binding data were transformed using

Scatchard (Scatchard, 1949) and Hill (Hill, 1910) plots.

In a simple model of ligand - receptor interaction (Clark, 1933) a homogenous species of ligand reacts with a single, non-interacting population of binding sites or receptors.

$$[R] + [L] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [RL]$$

where [R] = concentration of free receptor

[L] = concentration of free ligand

[RL] = concentration of receptor - ligand complex

= bound ligand

k_1 = association rate constant

k_{-1} = dissociation rate constant.

Two equations can be used to describe this system:

i) Equilibrium equation

$$\frac{[R]\,[L]}{[RL]} = K_d = \frac{k_{-1}}{k_1} = \frac{1}{K_a}$$

where K_d = equilibrium dissociation constant

K_a = equilibrium association constant

ii) Conservation equation

$$[R_T] = [R] + [RL]$$

where $[R_T]$ = total concentration of receptor

From i) and ii)

$$K_d = \frac{[L]([R_T] - [RL])}{[RL]}$$

$$\text{and } \frac{[RL]}{[R_T]} = \frac{[L]}{K_d + [L]} \quad \text{Clark's equation.}$$

Both the Scatchard and Hill plots linearize Clark's equation and allow an evaluation of K_d and $[R_T]$.

Scatchard plot: [RL]/[L] versus [B]

Using the rearranged equation:

$$\frac{[RL]}{[L]} = -\frac{[RL]}{K_d} + \frac{[R_T]}{K_d}$$

a plot of [RL]/[L] versus [B] has a slope of $-1/K_d$ and an intercept on the x - axis of $[R_T]$ or B_{max}. This assumes that there is only one homogenous set of binding sites without cooperative interaction between them.

Once $[R_T]$ or B_{max} is known the data may be plotted as a Hill plot.

Hill plot: log ([RL]/$[R_T]$ - [RL]) versus log [L]

For a receptor with interacting sites the Hill equation may be applied,

$$\frac{[RL]}{[R_T]} = \frac{[L]\ n_H}{K + [L]\ n_H}$$

where n_H = Hill coefficient representing the number of interacting binding sites per molecule of receptor and K = an equilibrium dissociation constant.

For a receptor with non interacting sites $n_H = 1$, the Hill equation simplifies to Clark's equation and can be rearranged as follows:

$$\log \frac{[RL]}{[R_T] - [RL]} = n_H \log [L] - n_H \log K_{0.5}$$

where $K_{0.5}$ = concentration of ligand giving 50% receptor occupancy.

A plot of log ([RL]/[R_T] - [RL]) versus log [L] has a slope of n_H and an intercept on the abscissa of $K_{0.5}$. If the reaction follows mass action principles n_H = 1.0 and $K_{0.5} = K_d$.

2.2.6.2 COMPETITION STUDIES

Assuming that an unlabelled analogue/compound, I, is a competitive inhibitor of radioligand, L, the following equilibria can be described:

$$[R] + [L] \longrightarrow [RL]$$

$$[R] + [I] \longrightarrow [RI]$$

where [R] = concentration of free receptor

[L] = concentration of free ligand

[I] = concentration of free inhibitor

K_d = equilibrium dissociation constant for ligand

$$= \frac{[R][L]}{[RL]}$$

and K_d^I = equilibrium dissociation constant for inhibitor

$$= \frac{[R][I]}{[RI]}$$

The result of these two equilibria in terms of [RL] may be written as:

$$[RL] = \frac{[L][R_T]}{K_d(1 + [I]/K_d^I) + [L]}$$

where [R_T] = total concentration of receptor.

When I is added at a concentration, [I_{50}], that

reduces [RL] to 50% of it's value in the absence of I, $[RL_{so}]$, we get:

$$[RL_{so}] = \frac{[L][R_T]}{K_d(1 + [I_{so}]/K_d^I) + [L]}$$

$$[RL] = \frac{2[L][R_T]}{K_d(1 + [I_{so}]/K_d^I) + [L]}$$

and $$K_d^I = \frac{[I_{so}]}{1 + [L]/K_d}$$ (Cheng and Prusoff, 1973).

For an accurate determination of K_d^I an indirect Hill plot (logit-log plot) was used.

$$\log \frac{[RL]_I}{[RL] - [RL]_I} = -n \log [I] + n \log [I_{so}]$$

where [RL] = radioligand bound in absence of I

$[RL]_I$ = radioligand bound in the presence of I

and n = apparent Hill coefficient.

A plot of log ($[RL]_I/[RL] - [RL]_I$) versus log I has a slope of -n and an intercept on the abscissa of $[I_{so}]$.

2.2.6.3 KINETIC STUDIES

$$[R] + [L] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [RL]$$

At time t the formation of [RL] may be written as:

$$[RL] = k_1 [R][L]$$

and the formation of [R] + [L] as:

$$[R] + [L] = k_{-1} [RL]$$

The observed association rate, k_{obs}, is a function of

the two reactions:

$$k_{obs} = k_1 [R][L] - k_{-1} [RL]$$

Assuming that the effective concentration of ligand remains unchanged $k_1 [R][L]$ may be written as $k_1'[R]$,

$$k_{obs} = k_1' [R] - k_{-1} [RL]$$

$$= (k_1' + k_{-1})([RL_{eq}] - [RL])$$

$$\text{Ln } ([RL_{eq}]/[RL_{eq}] - [RL]) = (k_1' + k_{-1})t$$

where $[RL_{eq}]$ = radioligand bound at equilibrium.

A plot of Ln $([RL_{eq}]/[RL_{eq}] - [RL])$ versus time has a slope of $k_1' + k_{-1}$ and k_1 may be calculated from the equation $k_1 = \frac{k_{obs} - k_{-1}}{[L]}$.

The dissociation rate constant, k_{-1}, is determined under conditions such that $[R] + [L] \longrightarrow [RL]$ does not proceed. The rate constant can then be measured as the gradient of a graph of Ln $([RL]/[RL_{eq}])$ versus time.

2.2.6.4 STATISTICAL ANALYSIS

Statistical comparisons of results were made using the Students t-test.

2.3 RESULTS AND DISCUSSION

2.3.1 PURITY OF [^{3}H] NT

Prior to use in a binding assay each batch of [^{3}H] NT was assessed for purity using TLC and HPLC analysis.

In a typical TLC experiment (Fig. 6 ,A) 90-92% of the radioactivity recovered from the plate migrated as authentic NT i.e. had the same R_f value (0.5) as unlabelled NT stained with ninhydrin/cadmium reagent. Similarly, using the higher resolving power afforded by HPLC, 90-96% (representative experiment) of the eluted radioactivity (Fig. 7 ,B) had a t_r of 22 min (elution volume 44 ml) identical to that of NT (Fig. 7, A). In all cases the purity of each batch of [^{3}H] NT as determined by these two methods was > 90%.

2.3.2 BINDING ASSAYS - PRELIMINARY INVESTIGATIONS

In initial experiments it was found that [^{3}H] NT, in the absence of any membrane protein, bound to Whatman GF/B glass fibre filters in a manner displaceable by unlabelled NT (data not shown). This binding was abolished by pretreating the filters with 0.3% (w/v) polyethylenimine for at least 30 min prior to use. NT is a highly basic peptide, positively charged at physiological pH, and polyethylenimine is believed to act by neutralizing the negative charges present on glass fibre filters (Bruns *et al.*, 1983).

The suitability of the protease inhibitors used in the Standard Assay (2.2.3.1) was confirmed by the integrity of [^{3}H] NT after exposure to brain membranes under assay

FIG. 6. TLC analysis of [^{3}H] NT. Untreated [^{3}H] NT (A) and [^{3}H] NT obtained after preincubation with brain membranes (B) were chromatographed on Avicel Cel 400 TLC plates as described (2.2.4.1). The heavy bar indicates the migration position of unlabelled NT as determined by ninhydrin/cadmium reagent staining.

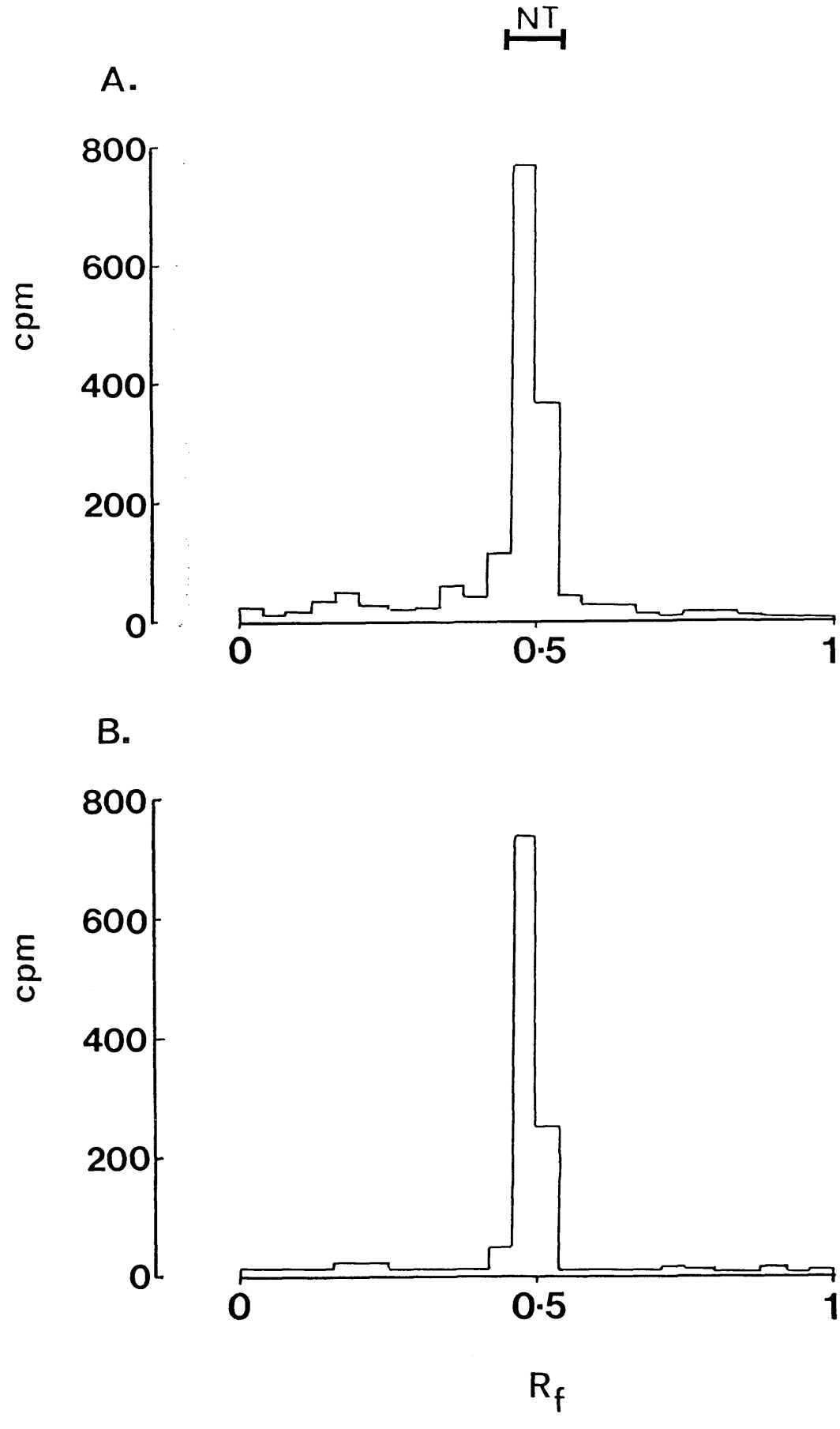
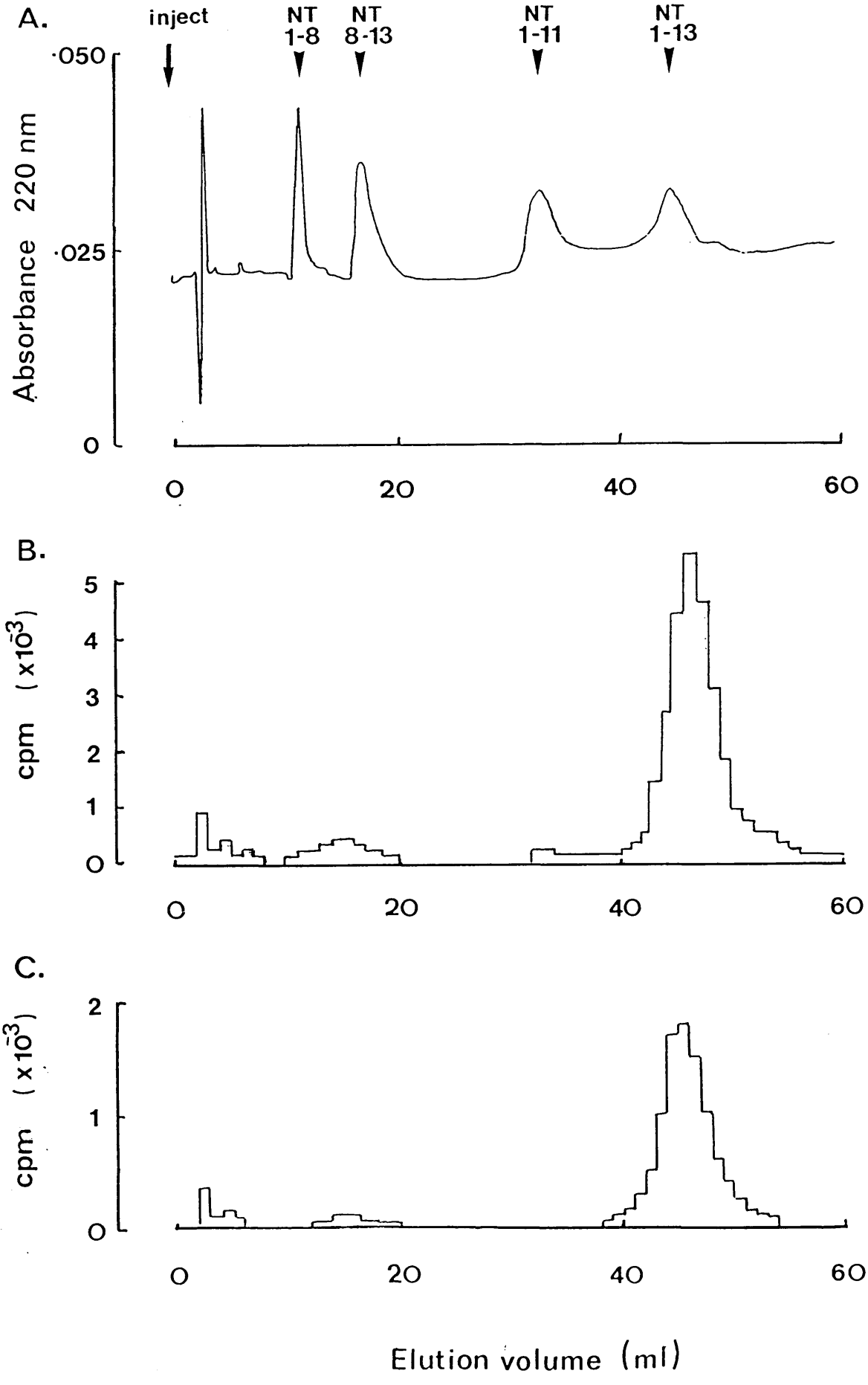

NT
A.
800
600
400
200
0
cpm
0
0·5
1
B.
800
600
400
200
0
cpm
0
0·5
1
R_f

FIG. 7. HPLC analysis of [^{3}H] NT. Unlabelled NT and NT fragments (A), untreated [^{3}H] NT (B) and [^{3}H] NT obtained after preincubation with brain membranes (C) were analysed on a C_{18} reverse phase HPLC column as described (2.2.4.2).


A.
inject
NT 1-8
NT 8-13
NT 1-11
NT 1-13
·050
·025
0
Absorbance 220 nm
0
20
40
60
B.
cpm (x10⁻³)
5
4
3
2
1
0
0
20
40
60
C.
cpm (x10⁻³)
2
1
0
0
20
40
60
Elution volume (ml)

conditions. Using both TLC (Fig. 6 ,B) and HPLC (Fig. 7 ,C) the resultant radioactivity profiles were identical to that of [^{3}H] NT incubated under assay conditions in the absence of membranes, the major peak of radioactivity migrating at the same R_f or t_r value as NT.

When assayed at 3 nM [^{3}H] NT the relationship between specific binding and assay protein concentration (rat or bovine membranes) was linear at values < 0.2 mg protein/ml assay (Fig. 8) and concentrations of 0.1-0.2 mg protein/ml assay were used thereafter in the Standard Assay.

The pH optimum for specific [^{3}H] NT binding was also determined at a radioligand concentration of 3 nM: in both rat (Fig. 9 ,A) and bovine (Fig. 9 ,B) membranes highest binding occurred at pH 8.0 a result consistent with that of Goedert et al. (1984c) who investigated [^{3}H] NT binding to rat brain homogenates. Also in common with the results of Goedert et al. (1984c) the pH profile of specific binding was not especially pronounced and the `physiological' pH of 7.5 was therefore used in all subsequent assays.

2.3.3 EQUILIBRIUM BINDING OF [^{3}H] NT

The specific binding of [^{3}H] NT to membranes prepared from rat brain (Fig. 10) or bovine cortex (Fig. 11) was concentration dependent and saturable with SEMs of < 10% of the mean. In both cases non-specific binding increased linearly with increasing ligand concentration (Figs.10, 11); at the highest [^{3}H] NT concentration used routinely

FIG. 8. Values are the mean of triplicate determinations; SEMs were less than 10%. The variation in the mean values between experiments (n = 6) was 10% or less.

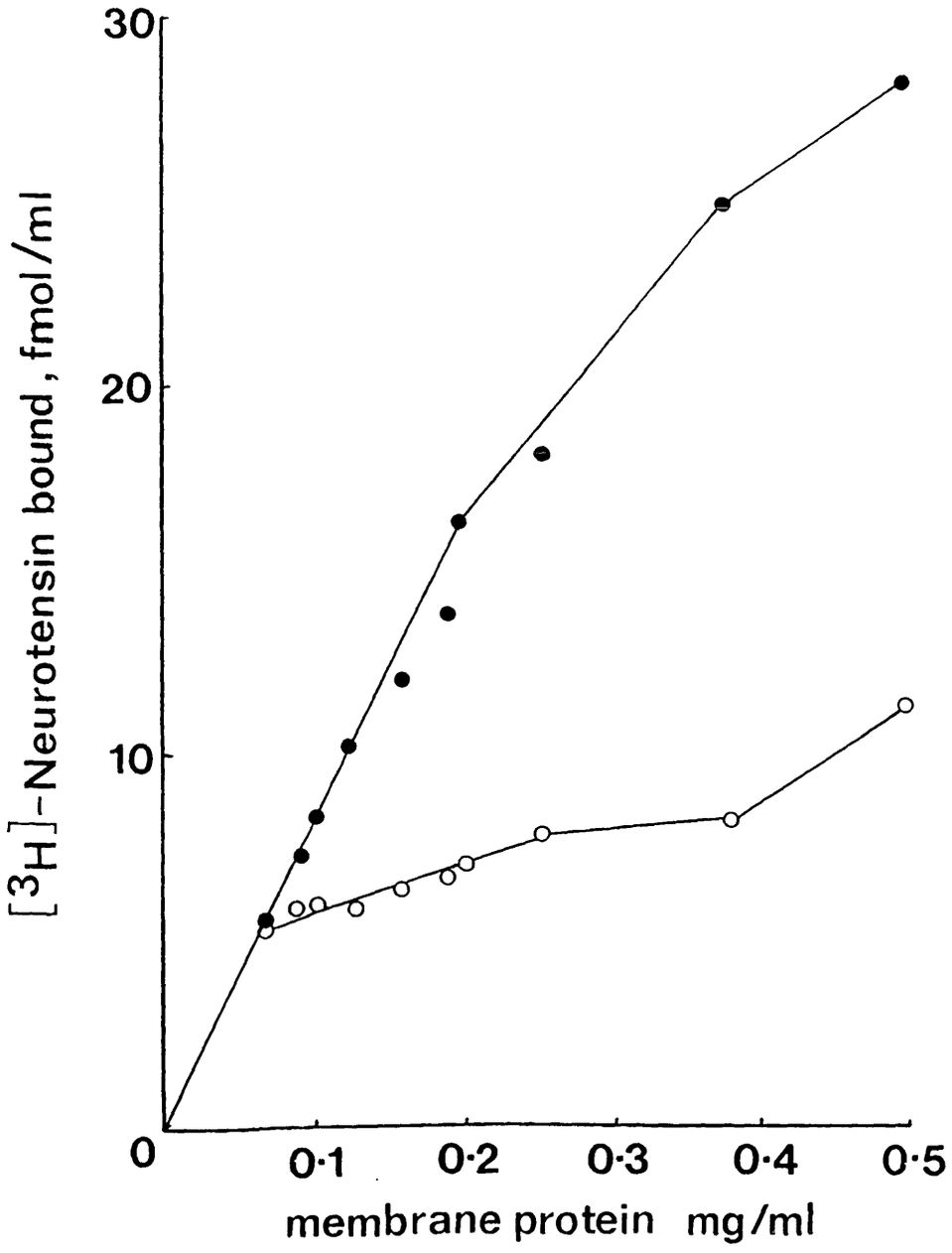
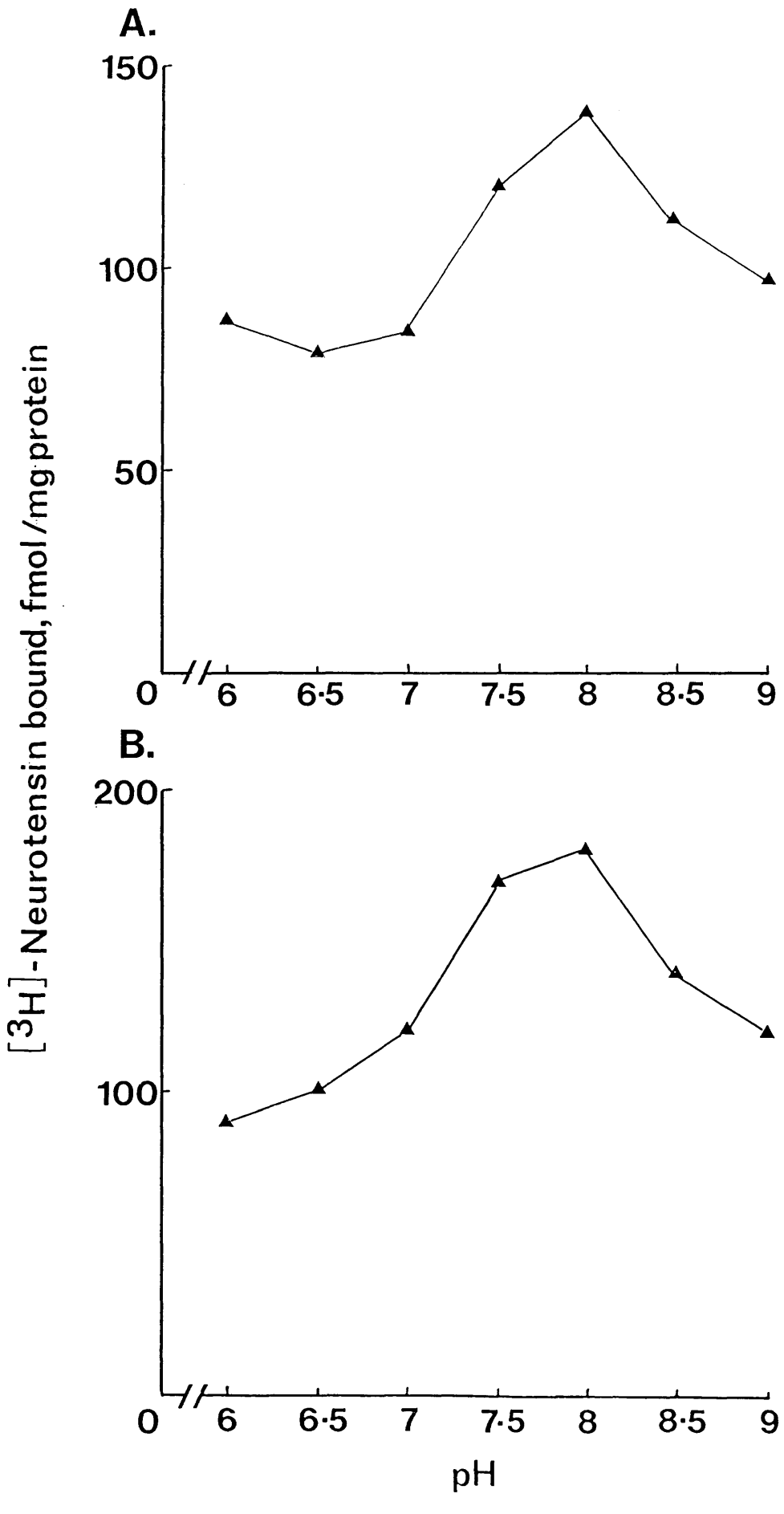


FIG. 8. Binding of [^{3}H] NT as a function of membrane protein concentration. Increasing concentrations of rat brain membranes were incubated with 3 nM [^{3}H] NT for 1 h at 20°C. Non-specific binding (o) was defined as binding in the presence of 1 uM NT and specific binding (●) obtained by subtracting non-specific from total binding. The experiment shown is typical of three independent experiments; similar results were obtained for bovine membranes.

FIG. 9. Specific [^{3}H] NT binding as a function of the pH of the incubation medium. [^{3}H] NT (3 nM) was incubated with rat brain (A) or bovine cortex (B) membranes for 1 h at 20°C at pH values ranging from 6.0 to 9.0. A typical experiment is shown and similar results were obtained in three independent experiments.

FIG. 9. Values are the mean of triplicate determinations; SEMs were less than 10%. The variation in the mean values between experiments (n = 3) was 12% or less.


A.
150
100
50
0
6
6·5
7
7·5
8
8·5
9
B.
200
100
0
6
6·5
7
7·5
8
8·5
9
pH
[3H]-Neurotensin bound, fmol/mg protein

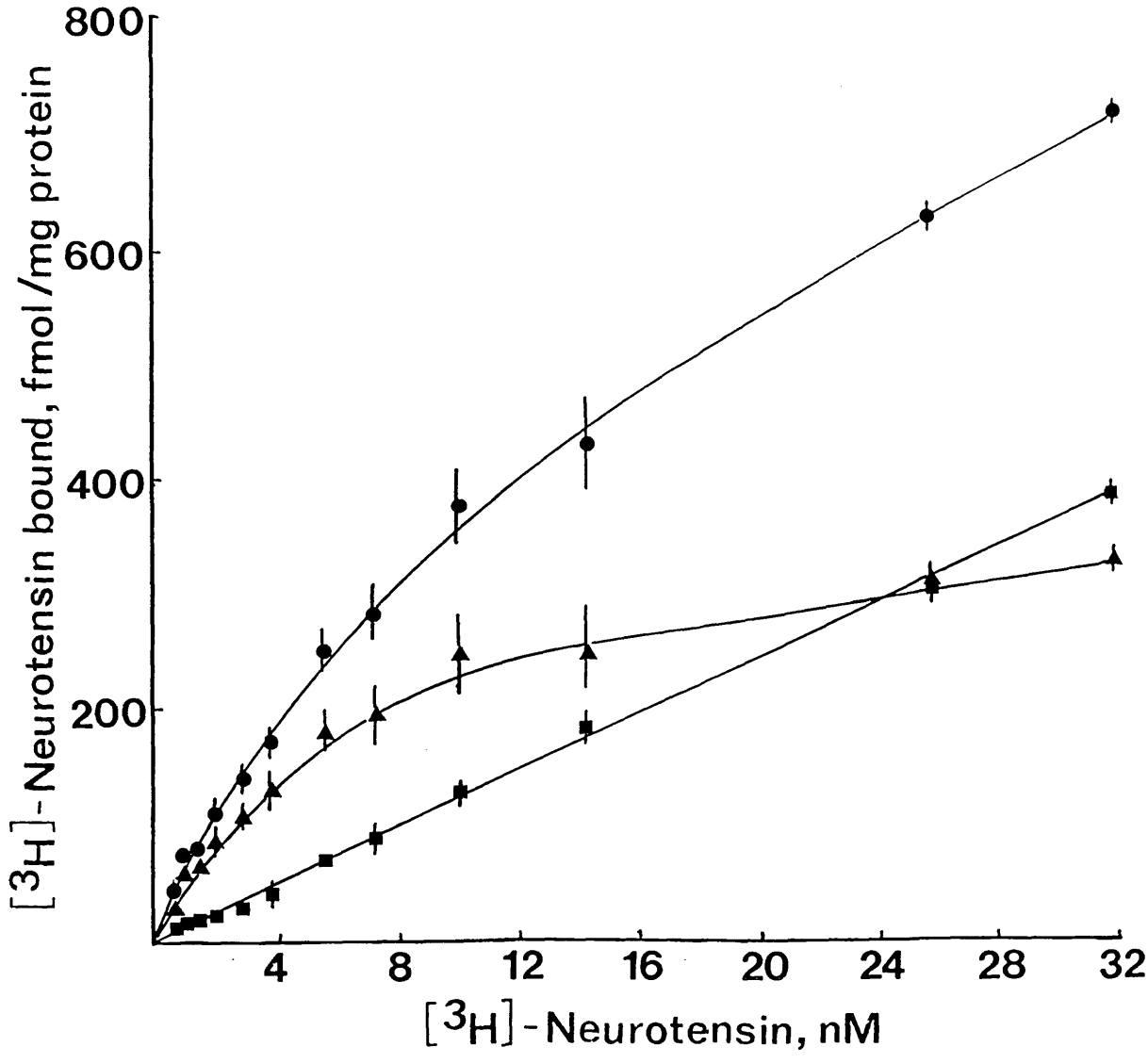


FIG. 10. Concentration dependence of [^{3}H] NT binding to rat brain membranes. Increasing concentrations of [^{3}H] NT were incubated with rat brain membranes for 1 h at 20°C. Non-specific binding (■) was defined as binding in the presence of 1 uM NT and specific binding (▲) obtained by subtracting the non-specific from the total (●) binding. A typical experiment is shown. Similar results were obtained in 7 separate experiments.

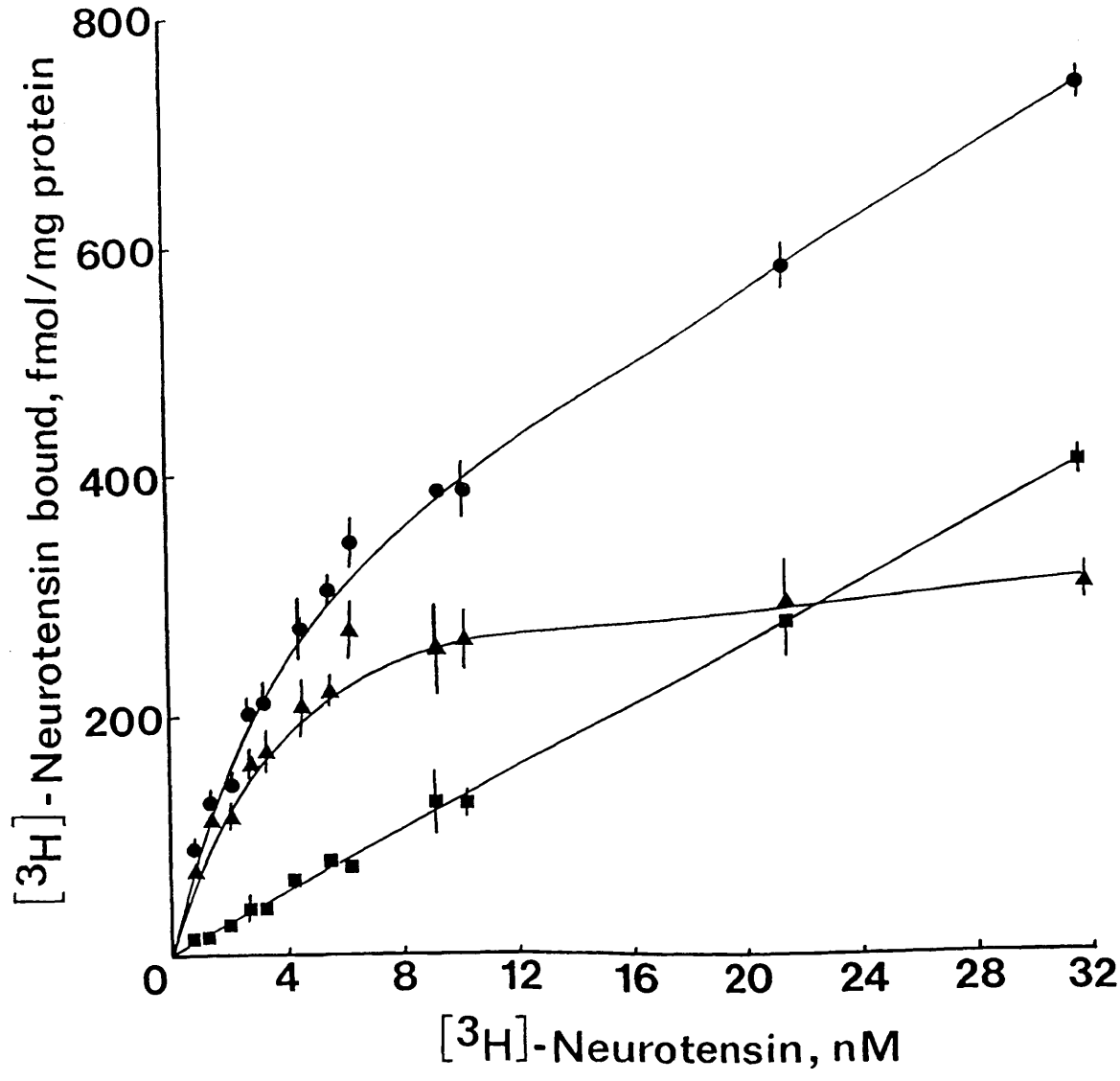


FIG. 11. Concentration dependence of [^{3}H] NT binding to bovine cortex membranes. Increasing concentrations of [^{3}H] NT were incubated with bovine cortex membranes for 1 h at 20°C. Non-specific binding (■) was defined as binding in the presence of 1 uM NT and specific binding (▲) obtained by subtracting the non-specific from the total (●) binding. A typical experiment is shown. Similar results were obtained in 5 separate experiments.

(~30 nM) this represented 40-50% of the total radiolabelled ligand bound.

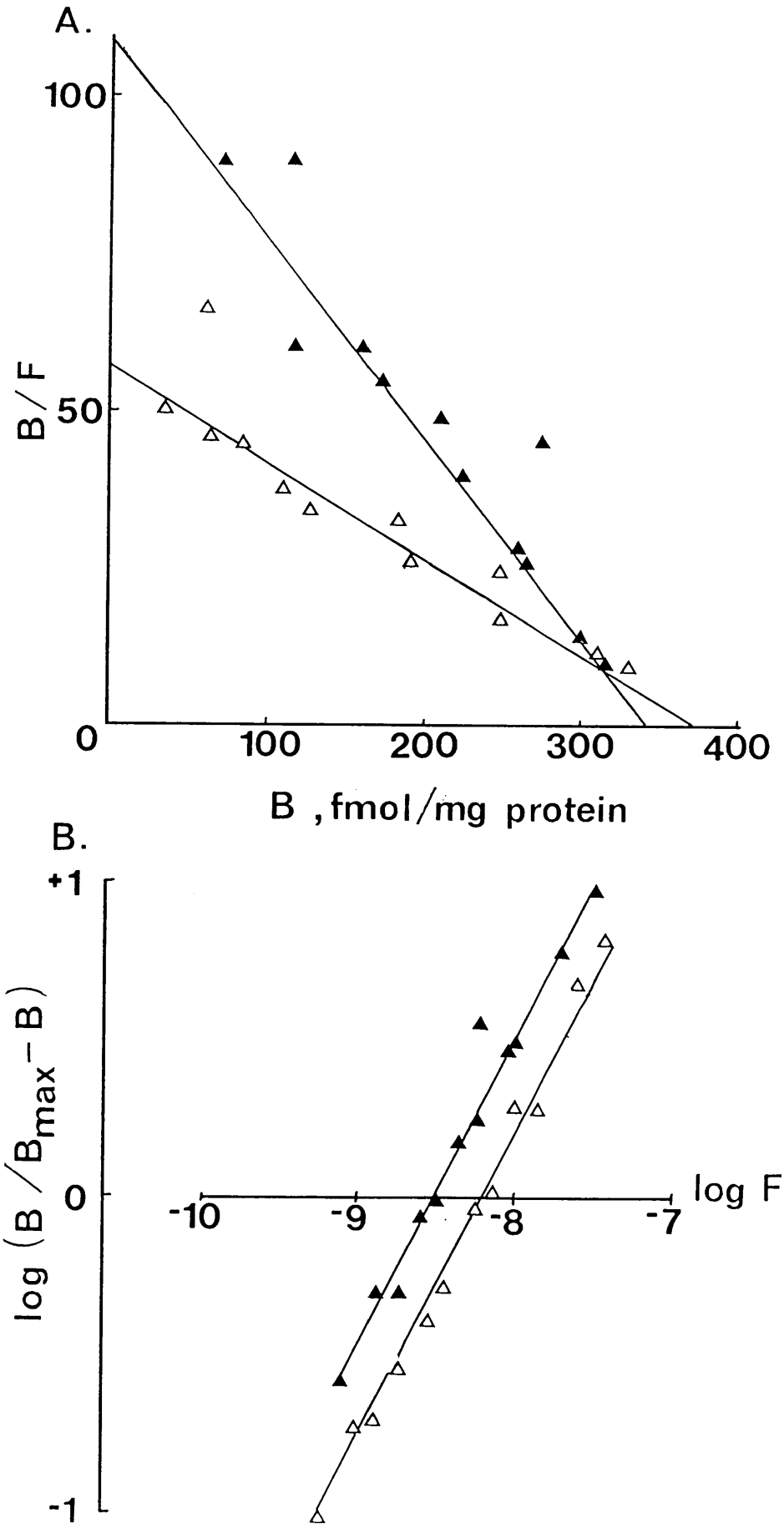
Analysis of the equilibrium binding data, using the LIGAND computer program, gave the best fit for a single class of non-interacting sites in the concentration range used (Figs. 10, 11); determinations at higher [^{3}H] NT concentrations (40 and 50 nM) did not show any further increase in binding (data not shown) suggesting the absence of any additional binding sites.

The equilibrium dissociation constants obtained were 6.3 $\pm$ 0.3 nM (n = 7) and 3.3 $\pm$ 0.2 nM (n = 5) for rat and bovine membranes, respectively, with corresponding B_{max} values of 400 $\pm$ 8 and 350 $\pm$ 10 fmol/mg protein. The interaction of [^{3}H] NT with one class of sites is consistent with the linear Scatchard plots obtained from these data (Fig. 12, A) and the Hill coefficients (Fig. 12, B): rat n_H = 1.08 $\pm$ 0.04, bovine n_H = 0.97 $\pm$ 0.08, which were close to unity.

The differences in the [^{3}H] NT binding parameters determined for these two tissues were statistically significant ($p < 0.005$) and the NT binding sites in rat brain can therefore be considered to have lower affinity but higher capacity than those found in bovine cortex.

A comparison of the K_D values obtained here with those reported in the literature revealed that the K_D value for [^{3}H] NT in rat brain is in close agreement with that found in the same tissue by Young and Kuhar (1981). The dissociation constant for [^{3}H] NT binding to bovine cortex,

FIG. 12. Scatchard (A) and Hill plot (B) of the specific binding of [^{3}H] NT to rat brain (△) and bovine cortex (▲) membranes. Binding (B) is expressed as fmol/mg protein, free ligand (F) as nanomolar (nM) and B/F as fmol/mg protein.nM. Similar results were obtained in 7 and 5 independent experiments, rat and bovine membranes, respectively.


A.
100
50
0
B/F
100
200
300
400
B , fmol/mg protein
B.
+1
0
-1
log (B /B$_{max}$ - B)
-10
-9
-8
-7
log F

however, was approximate to values obtained in similar studies with rat (Uhl et al., 1977; Kitabgi et al., 1977; Goedert et al., 1984c) and human (Kanba et al., 1986) brain.

The total number of [^{3}H] NT binding sites reported here for both brain preparations were in the same range as observed in rat brain by Goedert et al. (1984c).

There was no evidence from equilibrium binding studies for a `high' affinity binding site in either rat or bovine brain but as discussed previously (1.6.2.2) this may reflect upon the comparatively low specific activity of [^{3}H] NT (40-70 Ci/mmol in these studies).

2.3.4 KINETIC STUDIES

The specific binding of [^{3}H] NT at 5 nM to brain membranes, rat or bovine, reached a steady state in less than 20 min (Figs. 13, A; 14, A). Measurements of this binding after 60 min incubation showed no decrease indicating that there had been no degradation of the [^{3}H] NT binding sites.

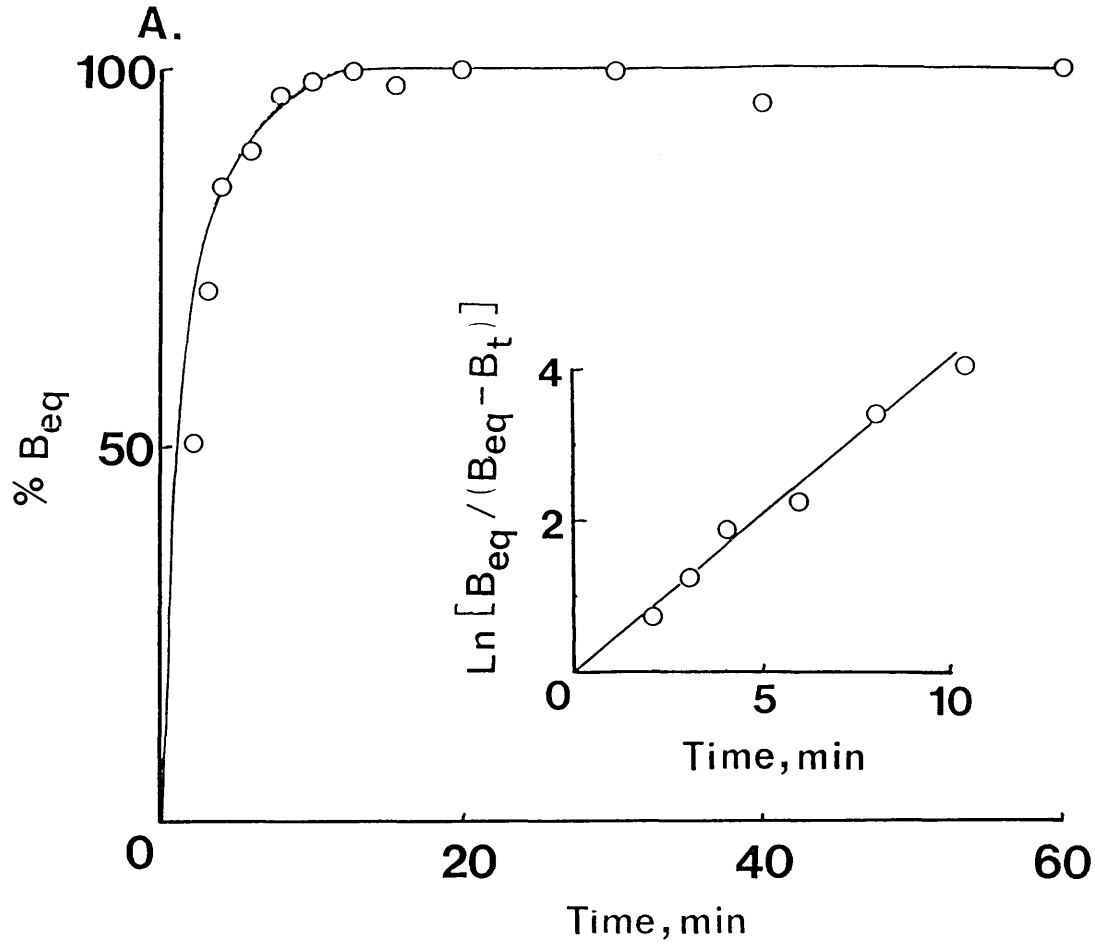
Calculation of the observed association rate, k_{obs}, from a first-order plot (Fig. 13, A, inset; Fig. 14, A, inset) gave values of 6.8×10^{-3} s^{-1} and 7.2×10^{-3} s^{-1} for rat and bovine membranes, respectively, with $t_{1/2}$ values of approximately 2 min in both cases.

The time-dependent dissociation of specifically bound [^{3}H] NT, 5 nM, was determined upon 50 fold dilution or addition of unlabelled (10^{-6} M) NT (Figs. 13, B; 14, B).

FIG. 13. Association (A) and dissociation (B) kinetics of [^{3}H] NT binding to rat brain membranes. At each time point the values represent the mean specific binding of triplicate samples, as a percentage of B_{eq} the value at equilibrium. Dissociation was measured by 50-fold dilution with the medium (□) or after the addition of excess (1 uM) cold ligand (o). The results are from one of three independent experiments each of which gave similar data.

Inset: Semi-log plot.

The variation in the mean values between experiments (n = 3) was 8% or less.


A.
100
50
0
% Beq
0
20
40
60
Time, min
Ln [Beq / (Beq - Bt)]
4
2
0
5
10
Time, min

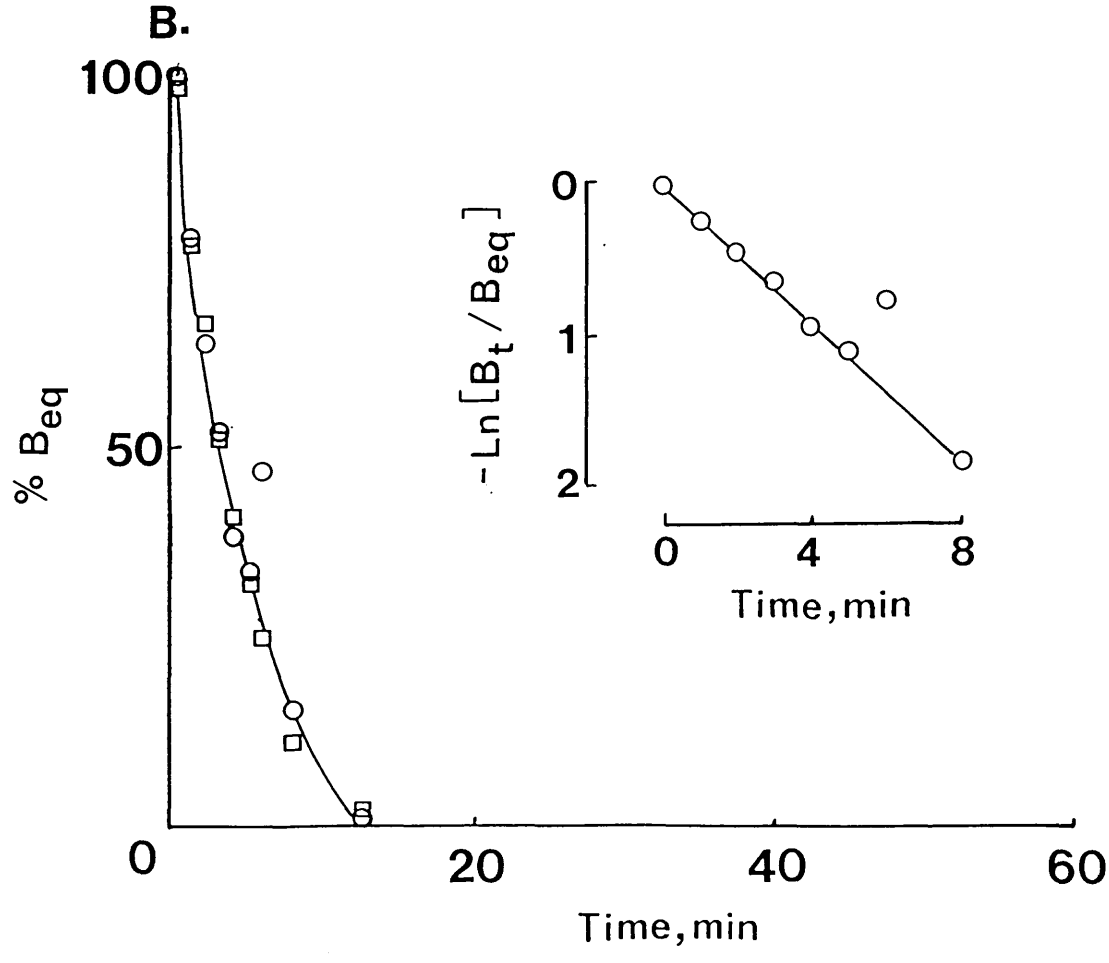
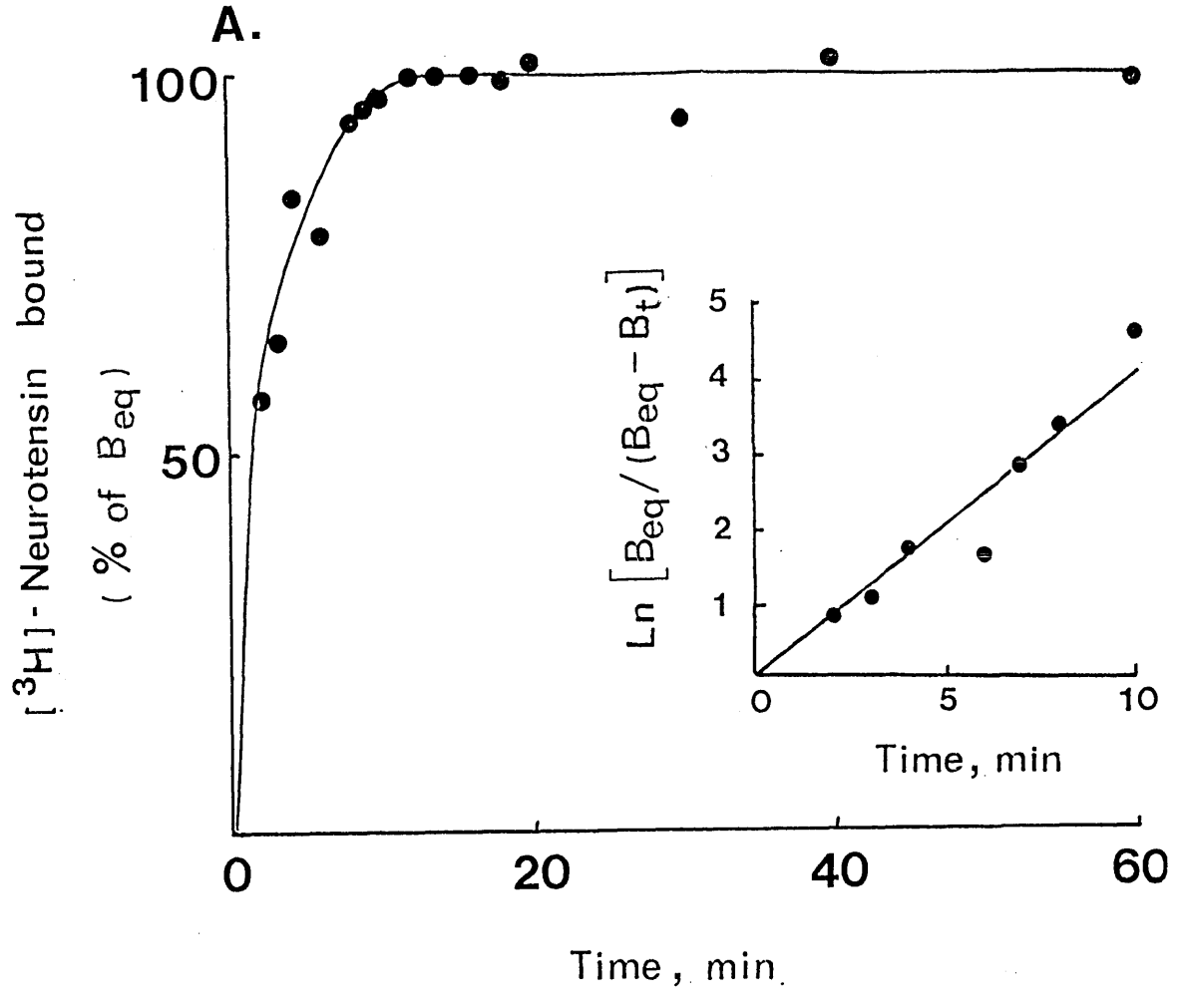
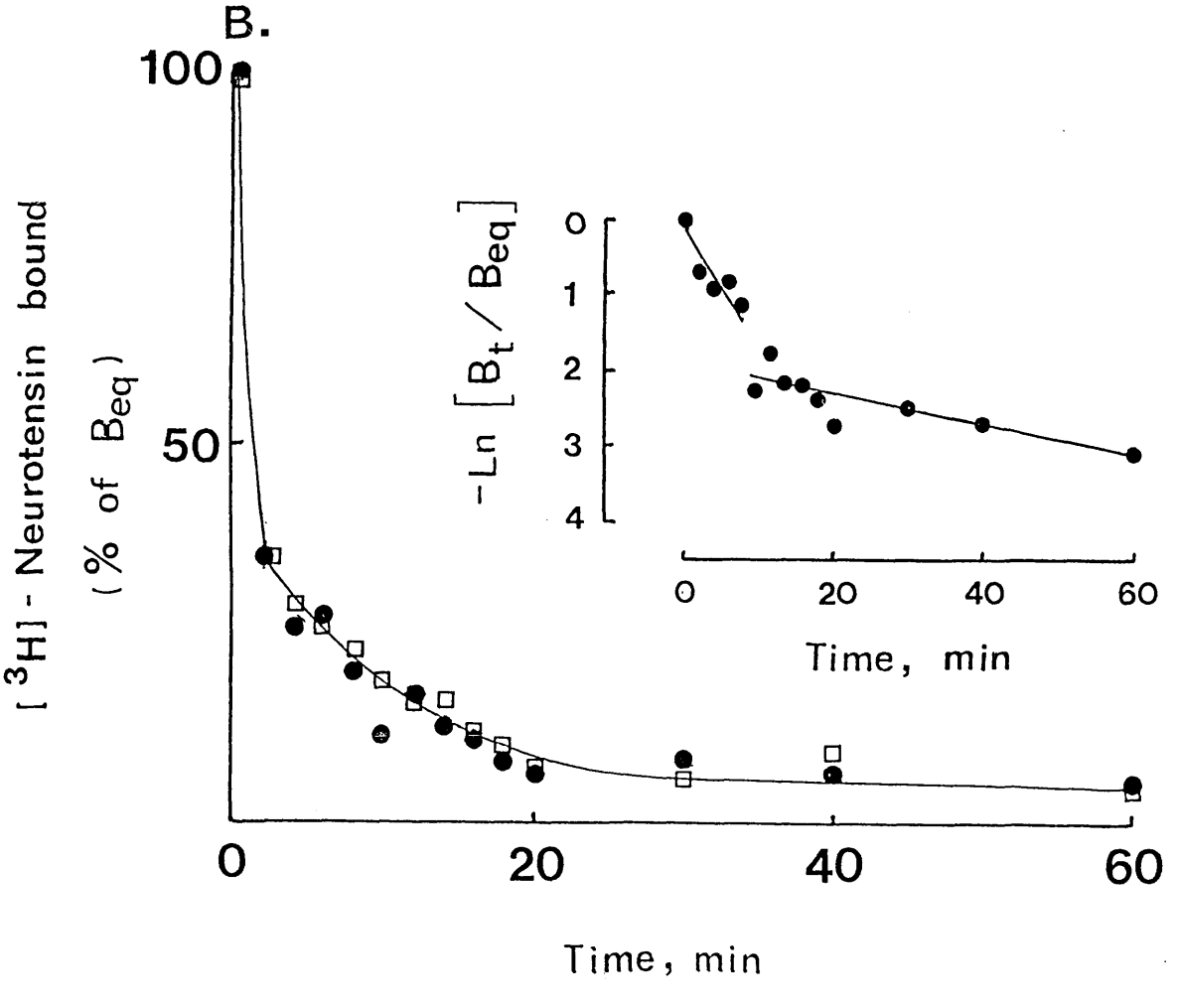

B.
100
50
0
% Beq
0
20
40
60
Time, min
- Ln [Bt / Beq]
0
1
2
0
4
8
Time, min

FIG. 14. Association (A) and dissociation (B) kinetics of [^{3}H] NT binding to bovine cortex membranes. At each time point the values represent the mean specific binding of triplicate samples, as a percentage of B_{eq} the value at equilibrium. Dissociation was measured by 50-fold dilution with the medium (□) or after the addition of excess (1 uM) cold ligand (●). The results are from one of three independent experiments each of which gave similar data. Inset: Semi-log plot.

The variation in the mean values between experiments (n = 3) was 13% or less.


A.
100
50
0
20
40
60
Time, min
[3H]- Neurotensin bound
(% of Beq)
Ln [Beq/(Beq − Bt)]
5
4
3
2
1
0
5
10
Time, min


B.
100
50
0
20
40
60
Time, min
[3H]- Neurotensin bound
(% of Beq)
-Ln [Bt / Beq]
0
1
2
3
4
0
20
40
60
Time, min

The semi-logarithmic first-order dissociation plot for rat membranes was a straight line with a $t_{1/2}$ of 3.5 min and a slope equivalent to k_{-1} of 3.3 x 10^{-3} s^{-1} (Fig. 13, B, inset). Using the equation $(k_{obs} - k_{-1})/[L] = k_1$ a value of 7.0 x 10^5 M^{-1} s^{-1} was obtained for the association rate constant. This was used in the equation $k_{-1}/k_1 = K_D$ to calculate the equilibrium dissociation constant which then had a value of 4.7 nM.

For bovine membranes, however, the semi-logarithmic first-order dissociation plot (Fig. 14, B, inset) was biphasic. The [^{3}H] NT bound dissociated from the majority of sites with a fast rate ($t_{1/2}$ = 1.3 min) and from the remaining sites with a much slower rate ($t_{1/2}$ = 36 min). The same curve was obtained whether the dissociation measurements were made after dilution of the [^{3}H] NT or upon addition of excess unlabelled ligand. This showed that co-operatively interacting sites on the receptor molecule were not present. The dissociation rate constants (k_{-1}) obtained after correcting for the contribution of each phase were 2.01 x 10^{-3} s^{-1} and 3.32 x 10^{-4} s^{-1} for these two components. Assuming the same association constant for both sites the equilibrium dissociation constants calculated were K_{D1} = 0.3 nM and K_{D2} = 2.0 nM for the higher and lower affinity sites, respectively.

In rat membranes the dissociation constant obtained, K_D = 4.7 nM, from kinetic studies was close to that obtained from equilibrium measurements, 6.3 nM, and neither study indicated the presence of a second `high' affinity class of

sites. The binding parameters, however, obtained from the kinetic studies of bovine brain suggested the presence of a second population of sites with a high affinity for [^{3}H] NT. The K_D value, 2.0 nM, for the `low' affinity sites correlates well with that measured in equilibrium studies (3.3 nM). The results from equilibrium studies (2.3.3) do not rule out the possibility of a small population of very high affinity sites. Since the high affinity sites seen kinetically represent < 10% of the total binding sites it is possible that these sites were not differentiated within the ligand concentrations used. It is also possible that the NT binding sites in bovine brain may be linked to a multiplicity of second messengers as seen in the N1E-115 cell line (1.6.3). The two populations of site may thus be linked to different second messengers as postulated for NT binding to N1E-115 cells by Bozou et al. (1986). Alternatively, these sites of low fractional occupancy may represent NT binding sites coupled to effector molecules. A different conformational state of effector coupled receptor molecules could account for the differences in the rates of ligand dissociation.

2.3.5 STRUCTURE - ACTIVITY STUDIES

The apparent dissociation constants obtained for the various NT fragments and analogues in competition studies (Table 6) indicate the importance of the carboxyl terminus amino acid residues in binding. The NT fragments NT (1-6),

Table 6. Inhibition of the specific binding of [^{3}H] NT to rat brain and bovine cortical membranes by NT fragments and analogues.

Peptide	K_D^I (nM)	
	Rat	Bovine
NT	6.1 ± 0.3 (7)	3.3 ± 0.2 (5)
[Gln^4] NT	3.5 ± 0.3 (3)	0.13 (2)
NT (8-13)	3.3 ± 0.3 (3)	0.71 ± 0.03 (3)
NMN	ND	3.7 ± 0.2 (3)
NT (1-12)	NC (2)	> 10^{-5} (2)
[D-Trp^{11}] NT	NC (4)	NC (3)
[D-Phe^{11}] NT	NC (2)	NC (2)
NT (1-6)	NC (2)	NC (2)
NT (1-8)	NC (2)	NC (2)
NT (1-10)	NC (1)	NC (2)
Bombesin	NC (2)	NC (2)
Tuftsin	NC (1)	NC (1)

Membranes were incubated with [^{3}H] NT (6 nM or 3 nM rat and bovine membranes, respectively) and varying concentrations of NT or its analogues. Data are mean values of two experiments or mean ± SEM values of several independent experiments (number in parentheses). NC indicates no competition detectable at 10^{-5} M peptide, ND not determined.

(1-8) and (1-10) did not compete with [^{3}H] NT binding, in either rat or bovine brain, at concentrations as high as 10^{-5} M. Similarly, an extremely low affinity ($K_D^I > 10^{-5}$ M) was obtained for NT (1-12) in bovine brain, while at the same concentration NT (1-12) was unable to displace the binding of [^{3}H] NT in rat brain. The significance of the Tyr^{11} residue in the interaction of NT with its binding site (Leeman and Carraway, 1982) was confirmed by the inability of [D-Trp^{11}] NT and [D-Phe^{11}] NT to compete with [^{3}H] NT in either tissue.

In both tissues the hexapeptide NT (8-13) had a higher affinity for the [^{3}H] NT binding sites than NT itself. This was ~2 and 5 fold higher in the rat and bovine membranes, respectively. This phenomenon has been reported for other species (1.6.2.2) and is one of the criteria upon which a tentative NT receptor classification has been based (1.6.2.2). Thus, the ligand specificity of the NT binding sites described here for rat and bovine brain appear to resemble those present in rat, cat, mouse and N1E-115 cells (1.6.2.2).

A species difference, however, was observed for [Gln^4] NT which was found to be equipotent with NT (8-13) in rat brain but ~5 times more potent in bovine brain (~25 fold more active than NT). [Gln^4] NT is equipotent with NT (8-13) in competition studies performed on N1E-115 cells (Gilbert et al., 1986) suggesting a similarity with the binding sites in rat brain.

The hexapeptide NMN, identical in its last five amino

acid residues to NT (9-13), has been shown in N1E-115 cells to compete for [^{3}H] NT binding sites (Gilbert et al., 1986) and to stimulate cGMP synthesis (Gilbert and Richelson, 1986; Gilbert et al., 1986; Kanba and Richelson, 1987) and phosphoinositol release (Kanba and Richelson, 1987). The K_D^I and EC_{50} values for these effects were ~4 fold higher than those obtained with NT yet, in competition studies in bovine brain NMN is equipotent with NT.

Bombesin, a peptide formerly thought to be functionally related to NT (Nemeroff and Prange, 1982) and tuftsin were unable to compete for the NT binding sites in either rat or bovine brain.

2.3.6 CATION EFFECTS

Cations and guanine nucleotides are known to regulate the affinity of receptors coupled to GTP binding proteins (Rodbell, 1980): the effect of these modulators on ligand binding activity provides indirect evidence for such a link.

The specific binding of [^{3}H] NT (6 nM) to rat brain membranes was investigated in the presence of monovalent and divalent cations at a single physiological concentration namely: 10 mM Mg^{2+}; 0.1 M Na^+; 2mM Ca^{2+} or 0.5 mM Mn^{2+} (Fig. 15). In each case binding was significantly lower ($p < 0.005$) than that observed in control experiments performed in the absence of ions, the most marked inhibition being seen in the presence of Na^+ or Mg^{2+} (63% and 77% of control levels, respectively). The effects of Na^+ and Mg^{2+} were not

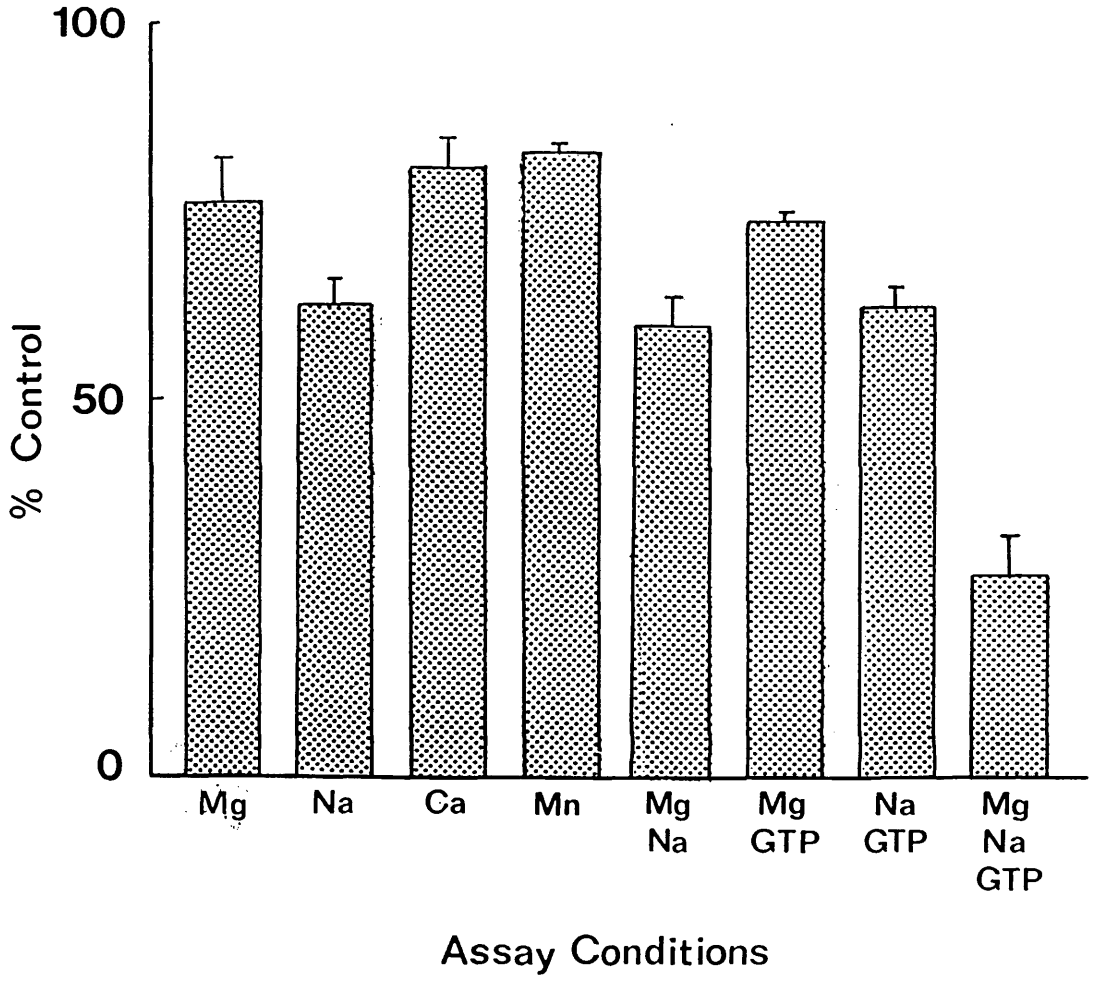


FIG. 15. The effects of cations and guanine nucleotide on specific [^{3}H] NT (6 nM) binding to rat brain membranes. Membranes were incubated for 1 h at 20°C in the presence of cations (Na^+ 0.1 M, Mg^{2+} 10 mM, Ca^{2+} 2 mM or Mn^{2+} 0.5 mM) and/or Gpp(NH)p (0.1 mM). Binding is expressed as a percentage of that obtained under the same conditions but in the absence of cations and Gpp(NH)p. Results are the mean ± SEM values of triplicate determinations from a single experiment; similar results were obtained in three independent experiments.

additive (Fig. 15); a combination of the two ions gave the same percent inhibition as seen for Na^+ alone. Data in the literature regarding this aspect of NT binding is limited, Uhl et al. (1977) have described a decrease in specific [^{125}I] NT binding to rat brain membranes in the presence of 0.1 M Na^+ to 47% of control levels. Similarly, specific binding of [^{125}I Tyr^3] NT to N1E-115 cells under the same conditions was only 25% of that present in the absence of ions (Bozou et al., 1986).

The non-hydrolyzable GTP analogue Gpp(NH)p did not potentiate the inhibition of specific binding by Na^+ or Mg^{2+} alone however, in the presence of both cations Gpp(NH)p reduced binding to 28% of control levels (Fig. 15).

Scatchard analysis of [^{3}H] NT binding to rat brain membranes in the presence of cations and/or Gpp(NH)p (Table 7) revealed a decrease in affinity with no significant change in the total number of binding sites. As expected the shift in K_D was more pronounced when Na^+ (K_D = 14.6 $\pm$ 2.86 nM) rather than Mg^{2+} (K_D = 9.4 $\pm$ 1.48 nM) was included in the assay and the K_D obtained in the presence of Na^+ and Mg^{2+} (13.86 $\pm$ 1.5 nM) was comparable to that measured in the presence of Na^+ alone. Goedert et al. (1984c) in their study of [^{3}H] NT binding to rat brain observed that 150 mM Na^+ caused a three fold increase in K_D with a corresponding loss of 50% of the binding sites. In N1E-115 cells however, the K_D was elevated five fold by 0.1 M Na^+ with no significant reduction in the number of binding sites (Bozou et al., 1986). These two groups also disagree on the effect of

Table 7. Binding parameters of the NT receptor of rat brain in the presence of cations and guanine nucleotide.

Assay Conditions	K_D (nM)	B_{max} (fmol/mg protein)	n
No ions	6.3 ± 0.3	400 ± 8	7
Mg^{2+}	9.4 ± 1.5*	382 ± 10	4
Na^{+}	14.6 ± 2.9*	390 ± 5	4
Mg^{2+}/Na^{+}	13.8 ± 1.5*	397 ± 3	3
Mg^{2+}/Na^{+}/Gpp(NH)p	39.9 ± 2.7*	410 ± 15	3

*p <0.005 when compared to binding in the absence of ions.

Specific [^{3}H] NT to rat brain membranes was determined in the presence of 10 mM Mg^{2+}, 0.1 M Na^{+}, 0.1 mM Gpp(NH)p or combinations of these. Maximum number of binding sites (B_{max}) and dissociation constants (K_D) were calculated from Scatchard plots. Results are expressed as the mean ± SEM; n denotes the number of experiments.

Gpp(NH)p on ligand binding: Goedert et al. (1984c) found a complete lack of effect whilst Bozou et al. (1986) reported a decrease in affinity in the presence of this nucleotide. The requirement for both Na^+ and Mg^{2+} for the supression of specific [^{3}H] NT binding by Gpp(NH)p has also been observed for the opioid receptor of both rat brain (Demoliou-Mason and Barnard, 1986) and the NG108-15 neuroblastoma x glioma cell line (Blume, 1978). Unfortunately, any similarity between the two receptor systems would seem to end there as opioid receptor binding affinity is increased in the presence of Mg^{2+}.

The effects of cations, at increasing concentrations, on the specific binding of [^{3}H] NT (3 nM) to bovine cortical membranes was investigated (Fig. 16). Na^+ was found to be the most potent in decreasing the binding of [^{3}H] NT with an IC_{50} of 35 mM. A much smaller inhibition was obtained with K^+ or Li^+ suggesting a degree of selectivity since at concentrations as high as 100 mM the [^{3}H] NT binding had only been reduced to 40% of control values. There was a similar differentiation in the effects of the divalent cations Mg^{2+} and Mn^{2+}: Mg^{2+} had an IC_{50} of 60-65 mM in contrast to Mn^{2+} which supressed binding activity only at concentrations > 60 mM. There was, however, no significant effect on the specific binding of [^{3}H] NT at Mg^{2+} and Mn^{2+} concentrations close to physiological (10 mM and 0.5 mM, respectively).

At the `physiological' concentrations of cation used

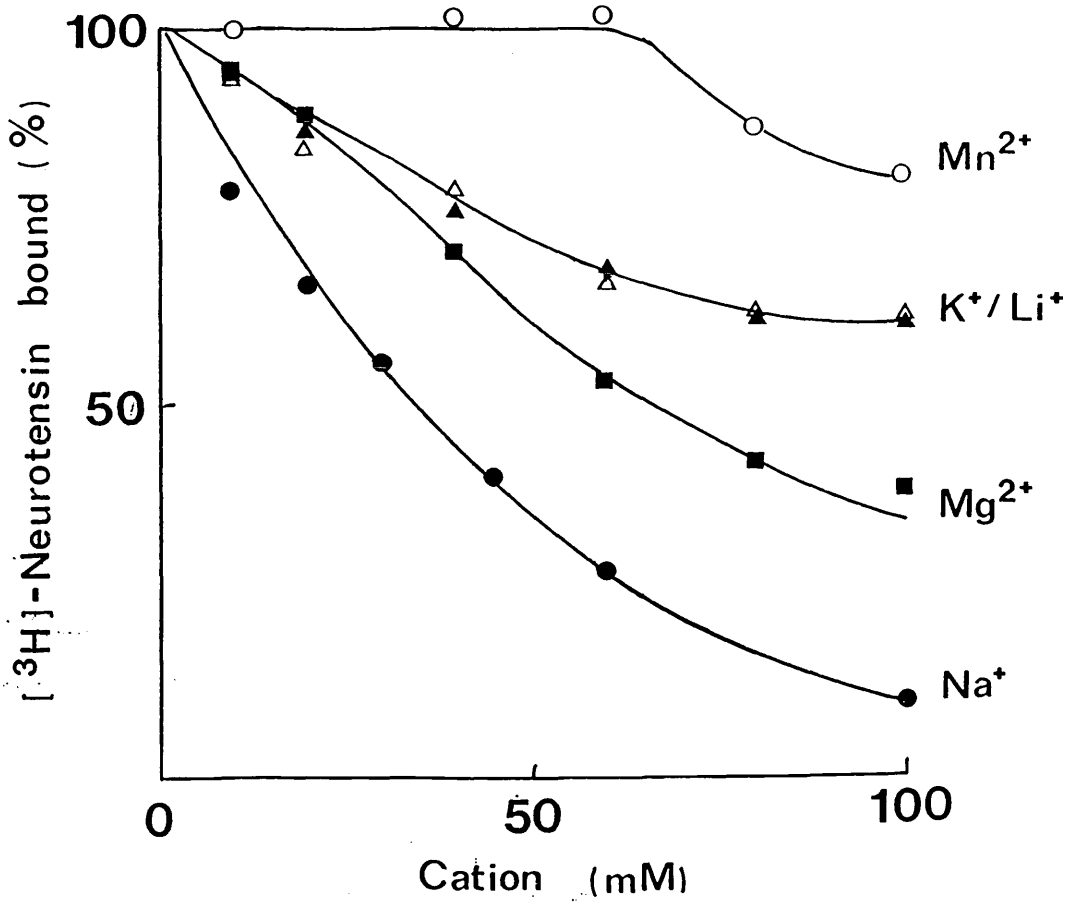


FIG. 16. Effects of cations on the specific binding of [^{3}H] NT (3 nM) to bovine cortex membranes. Membranes were incubated for 1 h at 20°C in the presence of cations at the concentrations shown. The results are expressed as the percentage of specific binding in the absence of cations. Each point represents the mean of triplicate samples; similar results were obtained in two independent experiments. the mean values of which did not differ by more than 10%.

in the study with rat brain (vide supra), the effect of Na^+ was more pronounced in bovine brain, the reverse being true for Mg^{2+} and Mn^{2+}. Scatchard analysis of the saturation isotherms obtained for the bovine NT receptor in the presence of cations revealed, in all cases, no changes in the total number of binding sites (Table 8). At a concentration of 35 mM Na^+ (IC_{50}) the K_D value was 3-4 fold higher than in its absence; the K_D value for Mg^{2+} was not significantly different from that obtained in the absence of ions.

In direct contrast to the results from rat brain reduction in [^{3}H] NT binding to bovine cortex in the presence of Gpp(NH)p occurred in the presence of Mg^{2+} and absence of Na^+. This effect was not potentiated by the addition of Na^+ and the shift in the K_D induced by Na^+ was significantly smaller ($p < 0.005$) than that caused by Mg^{2+}/Gpp(NH)p. The same phenomenon has also been seen for the binding of [^{3}H] ligand to α-noradrenergic receptors in calf brain although, in this case, Na^+ decreased both the affinity and the maximum number of binding sites (U'Prichard & Snyder, 1978).

Rodbell (1980) in his review of GTP regulatory proteins noted that in many cases divalent cations and especially Mg^{2+} promoted agonist binding. In this study [^{3}H] NT binding to both rat and bovine brain was significantly reduced in the presence of divalent cations. This difference in effect, however, does not preclude the interaction of the NT binding site with a G protein as Mg^{2+} is also without

Table 8. Binding parameters of the NT receptor of bovine brain in the presence of cations and guanine nucleotide.

Assay Conditions	K_D (nM)	B_{max} (fmol/mg protein)	n
No ions	3.3 ± 0.3	350 ± 10	5
Mg^{2+}	3.9 ± 0.3	365 ± 12	3
Na^{+}	11.0 ± 0.1*	370 ± 25	4
Mg^{2+}/Gpp(NH)p	15.0 ± 0.5*	350 ± 15	3

* $p < 0.005$ when compared to binding in the absence of ions.

Specific [^{3}H] NT binding to bovine cortical membranes was determined in the presence of 10 mM Mg^{2+}, 0.1 M Na^{+}, or 10 mM Mg^{2+}/0.1 mM Gpp(NH)p. Maximum number of binding sites (B_{max}) and dissociation constants (K_D) were calculated from Scatchard plots. Results are expressed as the mean ± SEM; n denotes the number of experiments.

effect on ligand binding to the glucagon receptor which is linked to G_s (Rojas and Birnbaumer, 1985).

In the presence of cations the Hill coefficient for opioid ligand binding in rat brain is significantly altered (Demoliou-Mason and Barnard, 1986): the effect of cations on [^{3}H] NT binding to rat and bovine brain membranes did not alter this parameter which remained close to unity.

2.4 SUMMARY

The filtration assay described in this chapter for the measurement of [^{3}H] NT binding to rat and bovine brain membranes was optimized: specific radioligand binding to the glass fibre filters was abolished; the integrity of [^{3}H] NT and its binding site were preserved during the course of the assay by use of a combination of protease inhibitors; the relationship between specific binding and assay protein concentration was linear. Since the highest specific binding occurred at pHs 7.5/8.0 the `physiological' pH of 7.5 was used in all subsequent measurements.

Under these assay conditions the binding of [^{3}H] NT to brain membranes derived from the two mammalian species investigated was found to be specific, saturable, time-dependent and reversible.

Equilibrium binding studies indicated, in both cases, the presence of 1 class of non-interacting sites with K_D and B_{max} values comparable to those reported in the literature.

Kinetic analysis of the [^{3}H] NT binding to rat brain membranes was in agreement with the equilibrium binding studies for the presence of a single class of sites in this species. However, in bovine cortex the presence of a second `high affinity' population of sites was detected by kinetic measurements. The significance of the `high affinity' binding site is, as yet, unknown. The kinetic studies also excluded the possibility of a co-operative system. Two populations of NT binding site have been reported in

mammalian brain tissue by a number of groups (Table 4), the proportion of `high affinity' sites ranging from values as low as 4% (Nakagawa et al., 1984) to as high as 50% (Sadoul et al., 1984a) of the total number of binding sites. In bovine cortex in this study this value is approximately 10% which is close to that reported by Mazella et al. (1983) for rat brain.

Whether the [^{3}H] NT binding sites observed here represent true NT receptors can best be answered by examining whether the binding characteristics conform to the criteria described by Burt (1985) and detailed earlier (1.5.1). The criteria of saturability (i) and kinetics (ii) are satisfied as is that of pharmacology (iv): the fragments of NT that are able to compete with [^{3}H] NT binding are those which are active in biological assays (1.4.2). The differences in binding potencies seen with [Gln^4] NT and NMN are likely to be related to species differences.

The distribution of [^{3}H] NT binding sites in the various brain areas has not been investigated in this study and thus the third condition, that of binding occurring only where the biological activity of the ligand is also observed, cannot be fulfilled. The effects of cations and Gpp(NH)p on [^{3}H] NT binding, however, have provided indirect evidence for an associated G protein activity suggesting a functional role for this binding site. It would therefore seem reasonable to identify the [^{3}H] NT binding sites of rat and bovine brain as putative NT receptors. On the basis of

this and the high density of [^{3}H] NT binding sites observed both tissues were considered suitable for the solubilization of NT receptors.

3. CELL LINE STUDIES

3.1 INTRODUCTION

In any studies involving brain tissue it is accepted that the cell population is heterogenous. Using immortal cell lines of neuronal origin, however, it is possible to investigate a homogenous and better defined population of cells.

This chapter describes the measurement of [^{3}H] NT binding activity in neuronal and non neuronal cell lines and the further characterization of these binding sites in the neuroblastoma clone N1E-115. The binding parameters obtained are discussed with respect to NT receptor characterization and their potential for further investigations.

3.2 METHODS

3.2.1 CELL CULTURE

3.2.1.1 NIE-115 CELLS

Murine neuroblastoma NIE-115 cells (passage number 17-36) were propagated as a monolayer in 24 well plates or 90 mm petri dishes. The cells were cultured in Dulbeccos Modified Eagles Medium (DMEM) containing 10% (v/v) foetal calf serum (FCS), 20 ug/ml Gentamycin and 0.3 g/l glutamine ,referred to hereafter as Culture Medium, at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Cells grown in petri dishes were passaged every 5-6 days when they had reached approximately 80% confluency; those grown in 24 well plates were used for Solid Phase Binding Assays (3.2.3.1). The cells were detached from the dish by incubating for 3 min at 37°C in 5 ml phosphate buffered saline [3.5 mM KH_2PO_4, 160 mM K_2HPO_2, 140 mM NaCl, 2.5 mM KCl, 0.85 mM EDTA, 8 g/l glucose] containing 0.05% (w/v) trypsin (T-PBS). At the end of this time the cells were triturated off and transferred to a 50 ml Falcon tube containing 1 ml Culture Medium per ml of T-PBS used. Following centrifugation (150-200 x g; 5 min) the supernatant was removed and the cells were resuspended in 5 ml Culture Medium/original dish. Petri dishes (90 mm) were inoculated with 1 ml cell suspension/dish to give a seeding density of approximately 10^6 cells/dish. For passaging to 24 well plates an aliquot of the cell suspension was counted in

a haemocytometer, the cells subsequently diluted to 10^5 cells/ml with Culture Medium and aliquoted out at 1 ml/well.

At intervals of 5-6 passages a fraction of the growing cells was frozen in order to preserve a stock. Using the procedure for passaging to 24 well plates the cell suspension was counted, recentrifuged (150-200 x g; 5 min) and resuspended at 5 x 10^6-15 x 10^6 cells/ml in ice-cold 8% (v/v) DMSO in FCS. Portions of 200 ul were transferred to 2 ml freezing vials, stored overnight at -70°C and thereafter in liquid nitrogen. Cells were recovered from storage by thawing rapidly and diluting each 200 ul aliquot into 10 ml Culture Medium/2 ml FCS in a 90 mm petri dish at 37°C. All other procedures were then as described above.

3.2.1.2 OTHER CELL LINES

Rat glioma (C6-Bu1) and mouse fibroblast (Swiss 3T3) cells were grown by Dr. M. Hanley. The protocol used was essentially that described for the mouse neuroblastoma (3.2.1.1).

3.2.2 DIFFERENTIATION PROCEDURE

Cells were passaged and grown for 24 hours as described (3.2.1.1). The Culture Medium was replaced with 0.5% (v/v) FCS/1.5% (v/v) DMSO in DMEM or Culture Medium containing 1 mM dibutyryl cyclic AMP , 5 ug/ml insulin, 35 ug/ml transferrin, 10 ug/ml selenium, 20 uM ethanolamine and the cells left to incubate for a further 48 hours. At

the end of this time the fully differentiated cells, as determined by morphology, were used in binding assays (3.2.3) as described.

3.2.3 BINDING ASSAYS

3.2.3.1 SOLID PHASE ASSAY

Cells propagated in 24 well plates for 5-6 days post passage (5-10 x 10^5 cells/well) were washed *in situ* with 3 x 1 ml modified Hanks buffer [1.3 mM $CaCl_2.2H_2O$, 5.4 mM KCl, 0.5 mM $MgSO_4$, 137 mM NaCl, 4.2 mM $NaHCO_3$, 3 mM $NaH_2PO_4.2H_2O$, 8 g/l glucose, pH 7.2] per well. Binding assays were performed by the addition to each well of 1 ml modified Hanks buffer containing 0.5 mg/ml BSA. 0.002% (w/v) soybean trypsin inhibitor, 0.02% (w/v) bacitracin, 0.015% (w/v) benzamidine HCl, 10 uM 1,10-phenanthroline and 1-10nM [^{3}H] NT. Non specific binding was determined in the presence of 10^{-6} M unlabelled NT; all determinations were as independent triplicates.

The binding reaction was terminated by removing the buffer and washing each well with 3 x 1 ml ice-cold modified Hanks buffer. Cells and bound [^{3}H] NT were recovered by the addition of 1 ml 1% (v/v) Triton X-100/1 mg/ml BSA per well and transferred to scintillation vials. Each well was washed with an additional 0.5 ml of Triton X-100 solution and the total extract counted for 5 min in 20 ml of water compatible scintillation fluid. Cell numbers per well (triplicate determinations) were estimated after extraction using a

haemocytometer.

3.2.3.2 Cell Suspension Assay

Cells grown for 5-6 days post passage in petri dishes were harvested by trituration and transferred to a 50 ml Falcon tube. After centrifugation (150-200 x g; 5 min) the supernatant was removed and the cells resuspended gently in modified Hanks buffer (2 ml/original plate) using a plastic pipette. This suspension was then used to measure [^{3}H] NT binding using the filtration method as described for brain membranes (2.2.3) except that modified Hanks buffer plus inhibitors (3.2.3.1) was used as the incubation medium, the assay tubes were mixed every 5-10 min to prevent settling of the cells and each filter was washed with 3 x 4 ml ice-cold modified Hanks buffer.

Protein estimation was as described for brain membranes (2.2.5).

3.3 RESULTS AND DISCUSSION

3.3.1 CHOICE OF CELL LINE - SOLID PHASE ASSAYS

A systematic search for [^{3}H] NT binding sites in different cell cultures was undertaken using a solid phase binding assay. The cell lines chosen were of both neuronal (murine neuroblastoma N1E-115) and non-neuronal (rat glioma C6-Bu1; murine fibroblast Swiss 3T3) origin. The N1E-115 cell line is a well characterized adrenergic cell type (Amano *et al.*, 1972) which possesses, in addition, muscarinic acetylcholine receptors (Matsuzawa and Nirenberg, 1975). These cells can be differentiated easily (Kimhi *et al.*, 1976) and the binding of [^{3}H] NT was investigated in both non-differentiated (Fig. 17) and differentiated cells (Fig. 18).

All of the cell lines tested exhibited the ability to bind [^{3}H] NT specifically (Fig. 19, A). The highest density of binding sites was seen in the non-differentiated N1E-115 cells with a B_{max} of 69.0 $\pm$ 2.6 fmol/10^6 cells (57.5 $\pm$ 2.1 fmol/mg protein). This value was approximately 3 fold higher than that found in any of the other cell lines (Fig. 19, B). Scatchard transformation of the [^{3}H] NT binding data for this cell line (Fig. 19 ,B) was linear with a K_D of 5.09 $\pm$ 0.26 nM (n = 3); the Hill coefficient obtained from the same data was 1.1 $\pm$ 0.2. The specific [^{3}H] NT binding sites in this case would appear to comprise a single class of non interacting binding sites , a result consistent with other studies of NT binding to this cell line (Table 4).

FIG. 17. Non-differentiated neuroblastoma N1E-115 cells. Neuroblastoma cells of the cell line N1E-115 were cultured as described (3.2.1.1). The scale bar represents 100 uM.

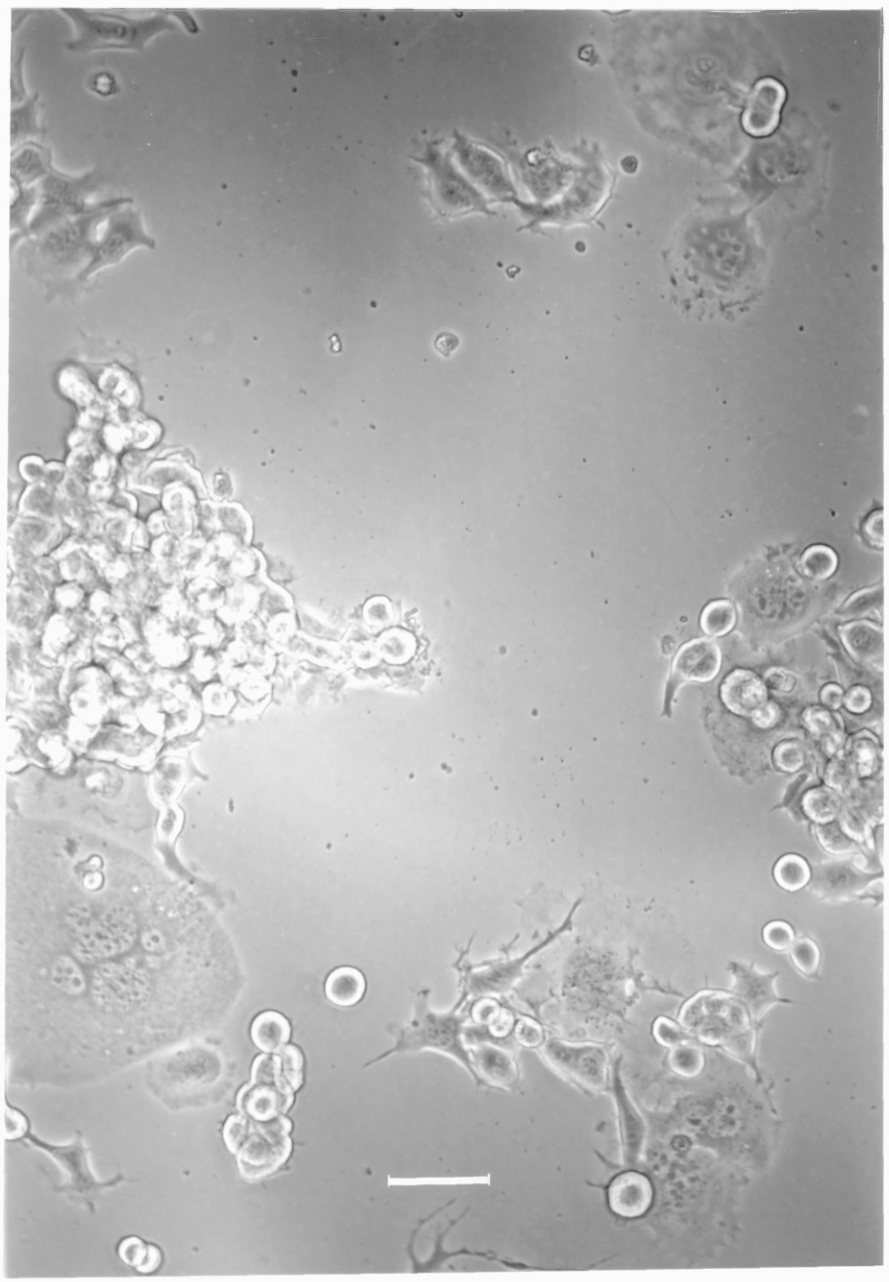


FIG. 18. Differentiated neuroblastoma N1E-115 cells. Neuroblastoma N1E-115 cells were differentiated by the addition of 1.5% (v/v) DMSO to the culture medium (3.2.2). An identical morphology was seen for N1E-115 cells differentiated by the presence of dibutyryl cAMP (3.2.2). The scale bar represents 100 uM.

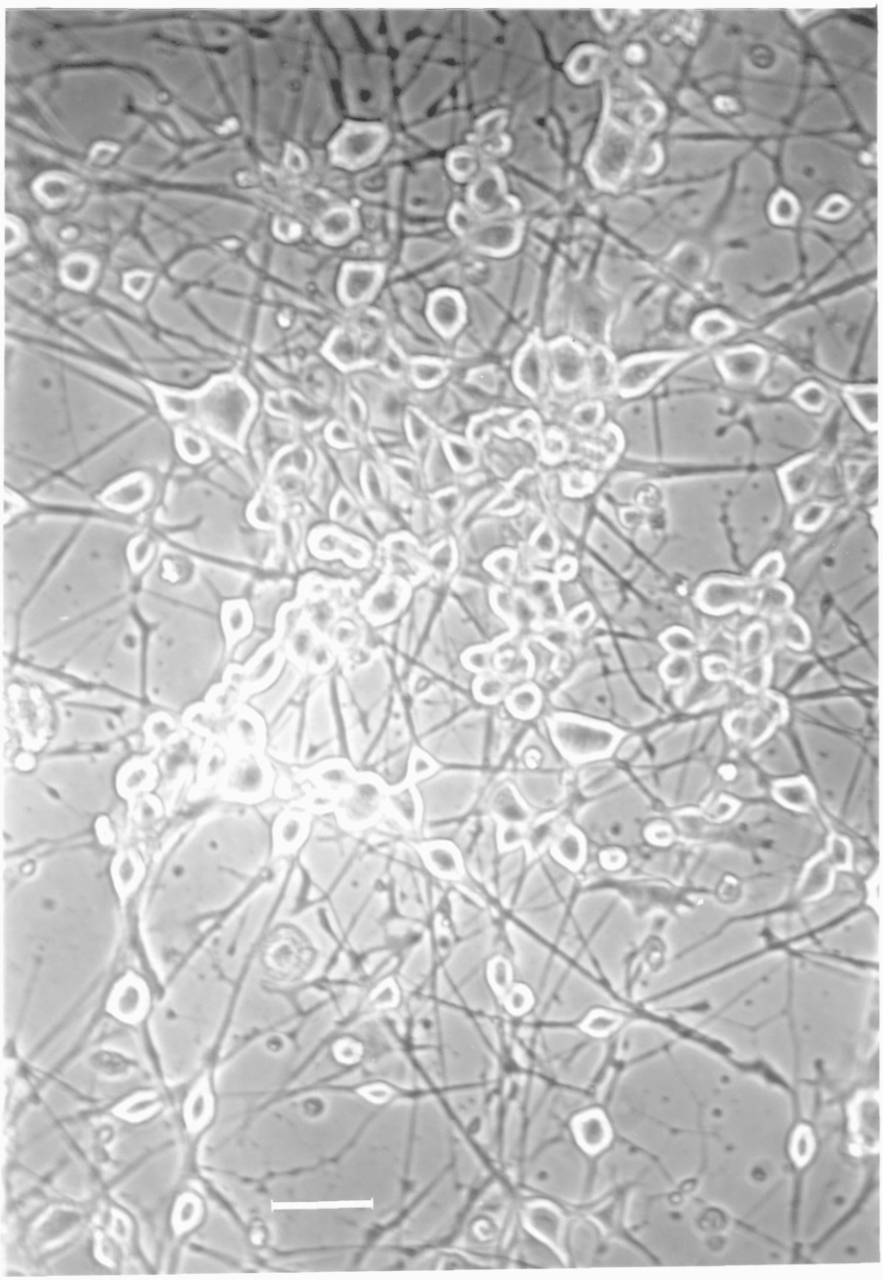
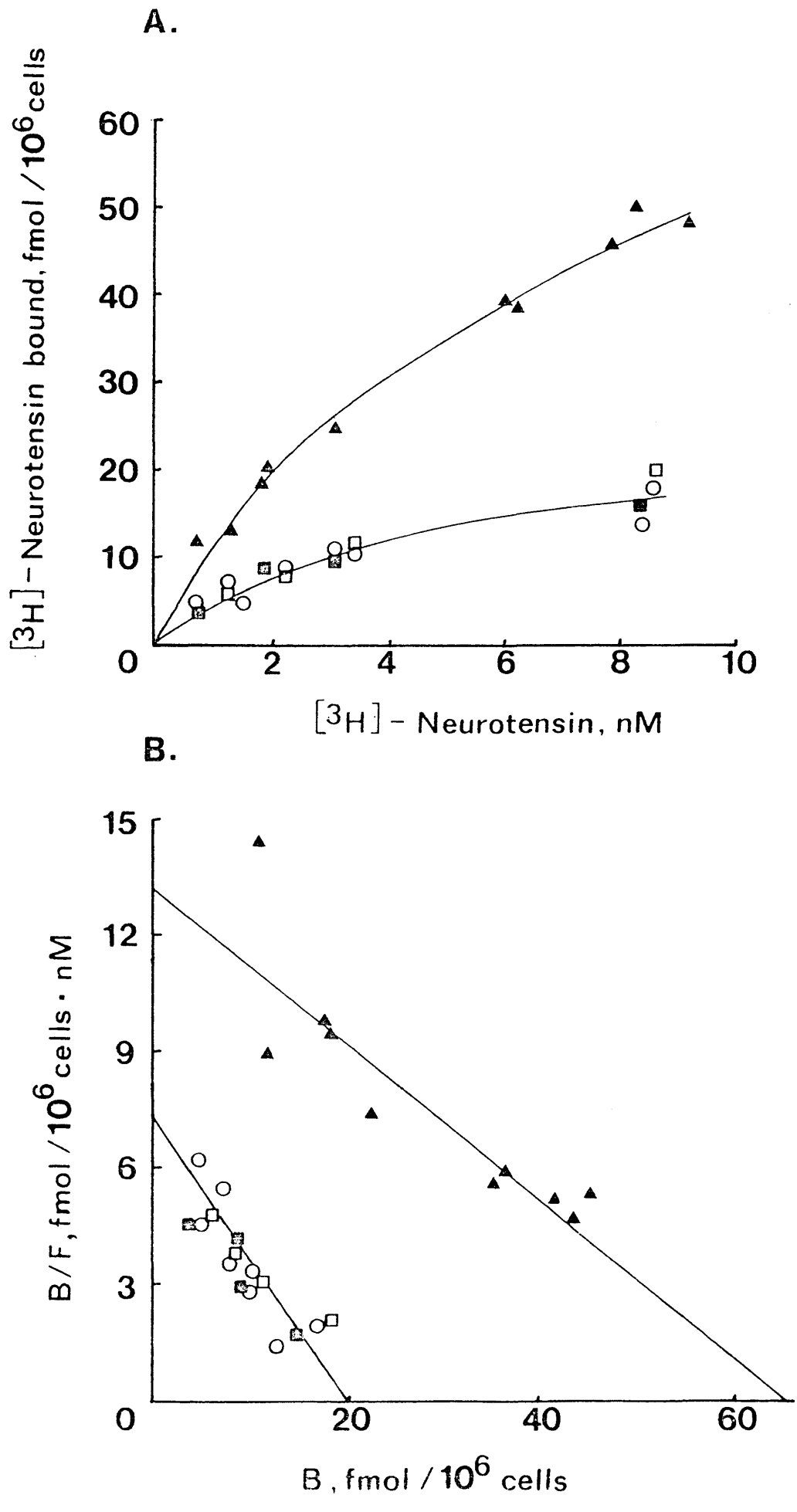


FIG. 19. Saturation (A) and Scatchard (B) plots of specific [^{3}H] NT binding to neuronal and non-neuronal cell lines. Using a solid phase assay (3.2.3.1) the binding of [^{3}H] NT to non-differentiated N1E-115 cells (▲), N1E-115 cells differentiated in the presence of 1.5% (v/v) DMSO (■), C6-Bu1 cells (O) and Swiss 3T3 cells (□) was measured. The results shown are from one of three independent experiments each of which gave similar results.

FIG. 19. Values are the mean of triplicate determinations; SEMs were less than 10%. The variation in the mean values between experiments (n = 3) was 6% or less.


A.
[3H]-Neurotensin bound, fmol / 10^6 cells
60
50
40
30
20
10
0
2
4
6
8
10
[3H] - Neurotensin, nM
B.
B/F, fmol / 10^6 cells • nM
15
12
9
6
3
0
20
40
60
B, fmol / 10^6 cells

The methodology used in the differentiation of cells *in vitro* varies but often involves the use of cAMP; an elevation in the levels of cAMP is regarded as a prerequisite for differentiation and the concomitant rise in the levels of specific enzymes e.g. acetylcholinesterase, tyrosine hydroxylase. In N1E-115 cells Kimhi *et al.* (1976) showed that the addition of DMSO to the culture medium induces process formation and the development of an electrically excitable membrane. The cellular levels of acetylcholinesterase, tyrosine hydroxylase and cAMP however, were unaffected. The conclusion drawn from this by the authors was that differentiation in these cells, as assessed by morphological changes and membrane excitability, occurs independently of enzyme and cAMP changes. However, it is also possible that in these cells DMSO produces an "electrical differentiation" while a "biochemical differentiation" results from cAMP treatment. Both methods of differentiation were investigated with respect to their effect on [^{3}H] NT binding to N1E-115 cells.

The parameters for the binding of [^{3}H] NT to N1E-115 cells differentiated by either of the two methods described and to non neuronal cells were essentially identical (Fig. 19, A, B) with similar K_D (2.5-3 nM), B_{max} (18-25 fmol/10^6 cells) and Hill coefficient ($\tilde{}$1) values. The Scatchard plots for this data were linear (Fig. 19, B) and the K_D obtained, 3.1 $\pm$ 0.3 nM, (n = 9) was significantly lower ($p < 0.005$) than that measured in non-differentiated

N1E-115 cells. The similarity in the [^{3}H] NT binding observed in differentiated N1E-115 cells and both non-neuronal cell lines, together with the results of Poustis et al. (1984) who reported a lack of [^{125}I Trp^{11}] NT binding to rat fibroblasts, suggests that this low capacity binding is physiologically less important than that seen in the non differentiated N1E-115 cells.

The reduction in the number of [^{3}H] NT binding sites after differentiation is clearly at odds with the work of Poustis et al. (1984) who found that NT binding sites appear in the N1E-115 cell line only after differentiation: a contention refuted by other workers (1.6.2.2). To further add to the confusion the maximal binding capacity obtained here for non-differentiated cells is 3-4 fold lower than that seen in a similar study (Gilbert et al., 1986) and is closest to the results from studies involving differentiated cells (Amar et al., 1985, 46 fmol/10^{6} cells; Bozou et al., 1986, 30 fmol/mg protein). The density of [^{3}H] NT binding sites seen in this study is equivalent to ~39,000 sites per non-differentiated N1E-115 cell, an abundance comparable to that for the muscarinic acetylcholine receptor (42,000 sites/cell; Burgermeister et al., 1978) but lower than that of the bradykinin receptor (95,000 sites/cell; Snider and Richelson, 1984) in the same cell line.

Although suitable for the initial assessment of [^{3}H] NT binding to cultured cells the solid phase assay produced results of comparatively high variability. This was as a result of cells detaching from the solid support during

the assay and washing procedure and was particularly pronounced when differentiated cells were used. To alleviate this problem a cell suspension assay was developed and used to further characterize the binding of [^{3}H] NT to the N1E-115 cell line.

3.3.2 CELL SUSPENSION ASSAYS

Neuroblastoma N1E-115 cells were harvested from culture (3.2.3.2) in such a way that cellular disruption was avoided. The binding of [^{3}H] NT was then assayed in a physiological medium (Hanks) to preserve a whole cell structure.

The binding of [^{3}H] NT (2 nM) to intact, non-differentiated N1E-115 cells increased linearly with increasing cell numbers up to approximately 0.7 x 10^6 cells/tube (~0.85 mg protein/tube (500 ul); Fig. 20) and a density of 0.3-0.5 x 10^6 cells/tube was, therefore, used routinely in the assays.

Under the assay conditions used no degradation of [^{3}H] NT was appparent when analyzed as described in the studies with brain membranes (2.2.4).

3.3.2.1 EQUILIBRIUM BINDING STUDIES

Binding of [^{3}H] NT to non-differentiated N1E-115 cells was specific and saturable; non-specific binding was linear and accounted for 40-50% of the total binding at saturation (Fig. 21, A). The Scatchard plot was linear (Fig. 21, B) and gave, in three independent experiments, a

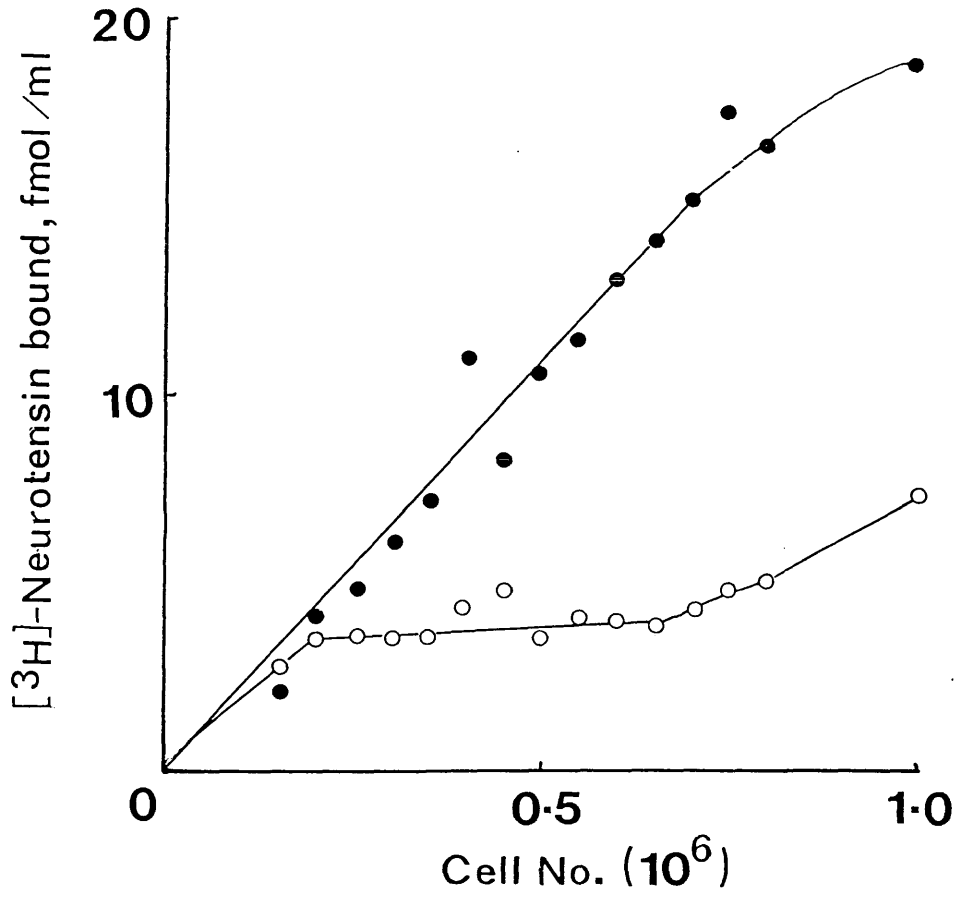
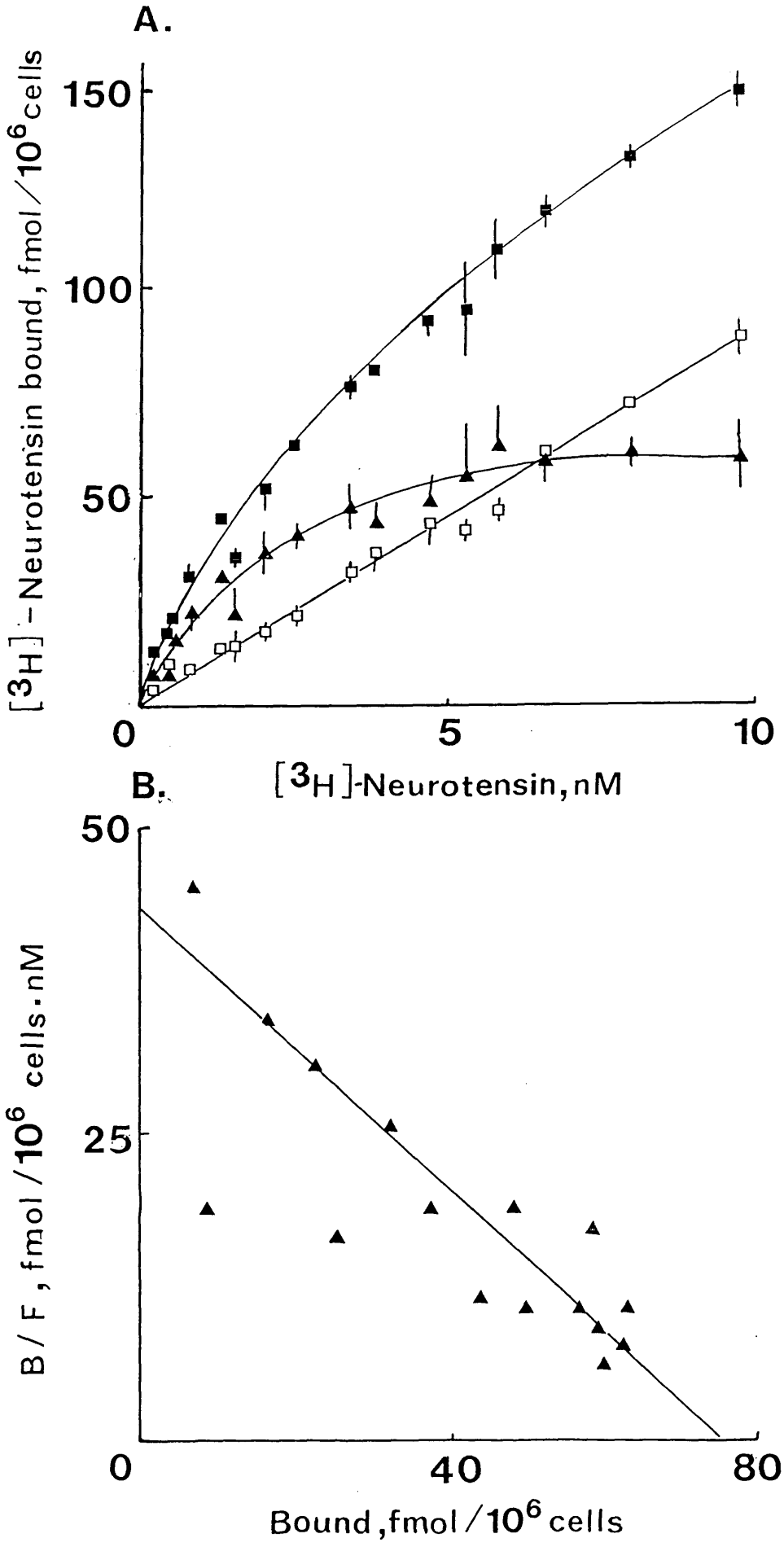


FIG. 20. Binding of [^{3}H] NT as a function of cell number. Using a cell suspension assay (3.2.3.2) the binding of [^{3}H] NT (2 nM) to intact, non-differentiated N1E-115 cells was investigated using increasing numbers of cells per assay tube. Non-specific (O) binding was defined as binding in the presence of 1 uM NT and specific binding (●) obtained by subtracting non-specific from total binding. The data presented are the mean of triplicate determinations and are from one of two independent experiments.

FIG. 21. Binding of [^{3}H] NT to non-differentiated N1E-115 cells as a function of increasing radioligand concentration. The binding of increasing concentrations of [^{3}H] NT to intact, non-differentiated N1E-115 cells was measured using a cell suspension assay (3.2.3.2). Non-specific binding (□) was defined as binding in the presence of 1 uM NT and specific binding (▲) obtained by subtracting the non-specific from the total (■) binding. Results are the mean of triplicate determinations; B. Scatchard plot of the specific binding data. A typical experiment is shown and similar results were obtained in three independent experiments.


A.
150
100
50
0
5
10
[3H]-Neurotensin bound, fmol/10^6 cells
[3H]-Neurotensin, nM
B.
50
25
0
40
80
B/F, fmol/10^6 cells.nM
Bound, fmol/10^6 cells

K_D for NT of 1.9 $\pm$ 0.2 nM and a B_{max} of 75.2 $\pm$ 10.0 fmol/10^6 cells (62.6 $\pm$ 8.3 fmol/mg protein). This value of B_{max} is equivalent to that observed in the solid phase binding studies (Table 9), however, the K_D value is markedly lower (Table 9) suggesting a higher affinity. This discrepancy in the K_D values obtained from the two assay methods probably reflects the significantly faster washing times attainable in the filtration assays i.e. there was minimal loss of specific binding during the washing procedure. In view of this the K_D value obtained from the cell suspension assays is likely to be closer to the `true' value.

Neuroblastoma N1E-115 cells differentiated in the presence of either dibutyryl cAMP or DMSO were found, in five independent assays, to exhibit no specific [^{3}H] NT binding activity. The elongated extensions or processes of differentiated cells (Fig. 18) are often fragile and sensitive to trituration (T. Ashton, personal communication) and consequently may not be transferred during harvesting. Assuming this to be true for differentiated N1E-115 cells the lack of [^{3}H] NT binding seen in the cell suspension assays would imply a localization of the binding sites to the processes. Alternatively, considering the lack of [^{125}I Trp^{11}] NT binding to rat fibroblasts reported by Poustis *et al.* (1984) in a cell homogenate assay, the binding seen here for the differentiated N1E-115 cells using the solid phase assay may represent non-specific binding, possibly to the support. However, BSA was present at a

Table 9. A comparison of two asssay methods for the measurement of [^{3}H] NT binding to N1E-115 cells.

Assay Method	N1E-115 cells Non-differentiated K_D	B_{max}		Differentiated K_D	B_{max}	
Solid Phase	5.09	69.0	(3)	~3	20	(9)
Cell Suspension	1.9	75.2	(3)	NB	NB	(5)

Specific binding of [^{3}H] NT to N1E-115 cells was measured as described (3.2.3) and the K_D and B_{max} values calculated from Scatchard plots of the data. Results are the mean values from several independent experiments (number in parentheses); NB indicates no specific [^{3}H] NT binding detectable.

concentration of 1 mg/ml in the assay buffer and this would tend to occlude such non-specific binding. Similarly, the B_{max} obtained for non-differentiated cells was not dependent on the assay method used (Table 9) and a differential localization of the NT binding sites would, therefore, seem to be a more reasonable explanation.

In a similar study of [^{3}H] NT binding to intact, non-differentiated N1E-115 cells (Gilbert et al., 1986) the K_D value obtained was in the range 9-11 nM although, when determined kinetically the value was lower, 2.8 nM, and closer to that seen here. The EC_{50} values for the NT induced second messenger responses of non-differentiated N1E-115 cells are also in close agreement with the K_D value obtained here i.e. cGMP synthesis 1.5 nM (Gilbert and Richelson, 1986; Gilbert et al., 1986), 1.2 nM (Kanba and Richelson, 1987); Ca^{2+} influx 4 nM (Snider et al., 1986) and phosphoinositol turnover 0.9 nM (Snider et al., 1986).

3.3.2.2 KINETIC STUDIES

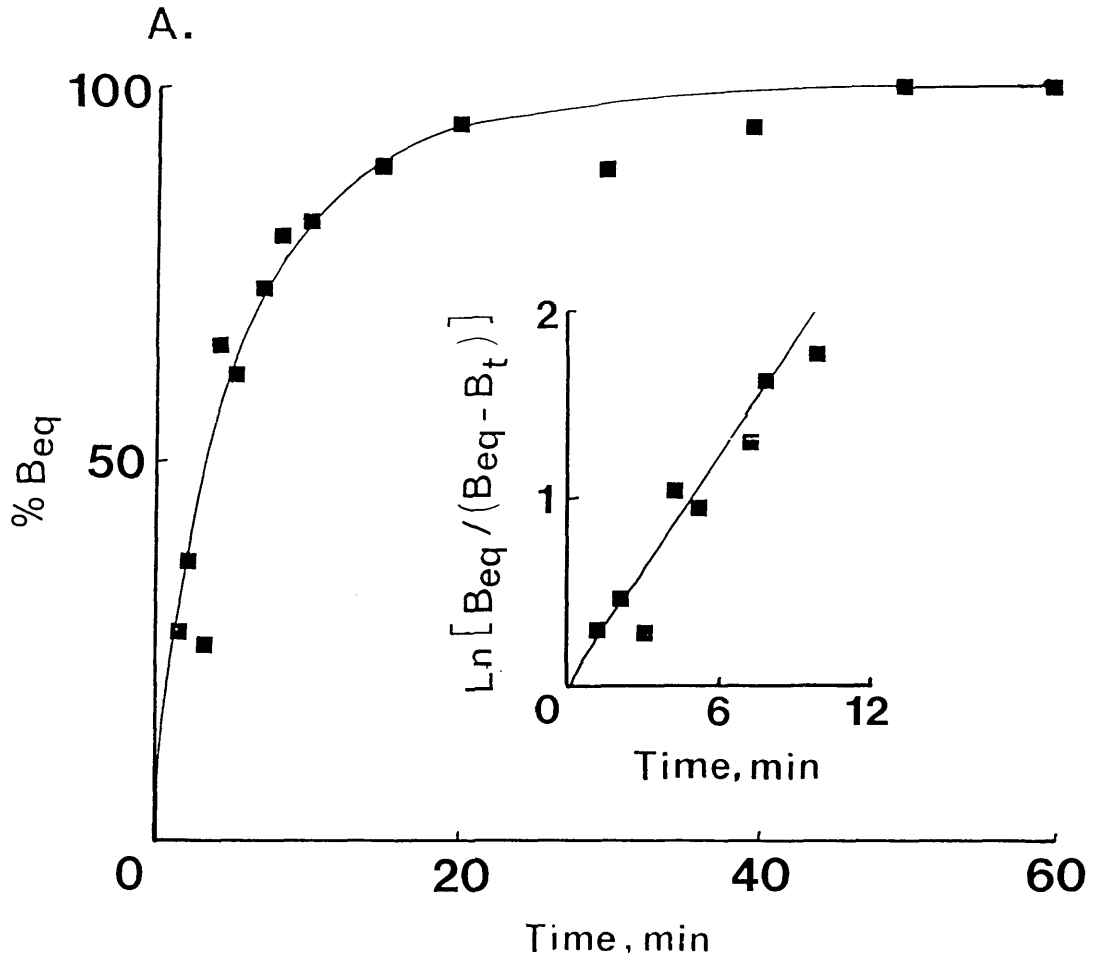
Specific [^{3}H] NT binding was investigated in intact, non-differentiated N1E-115 cells and was found to be time-dependent and reversible.

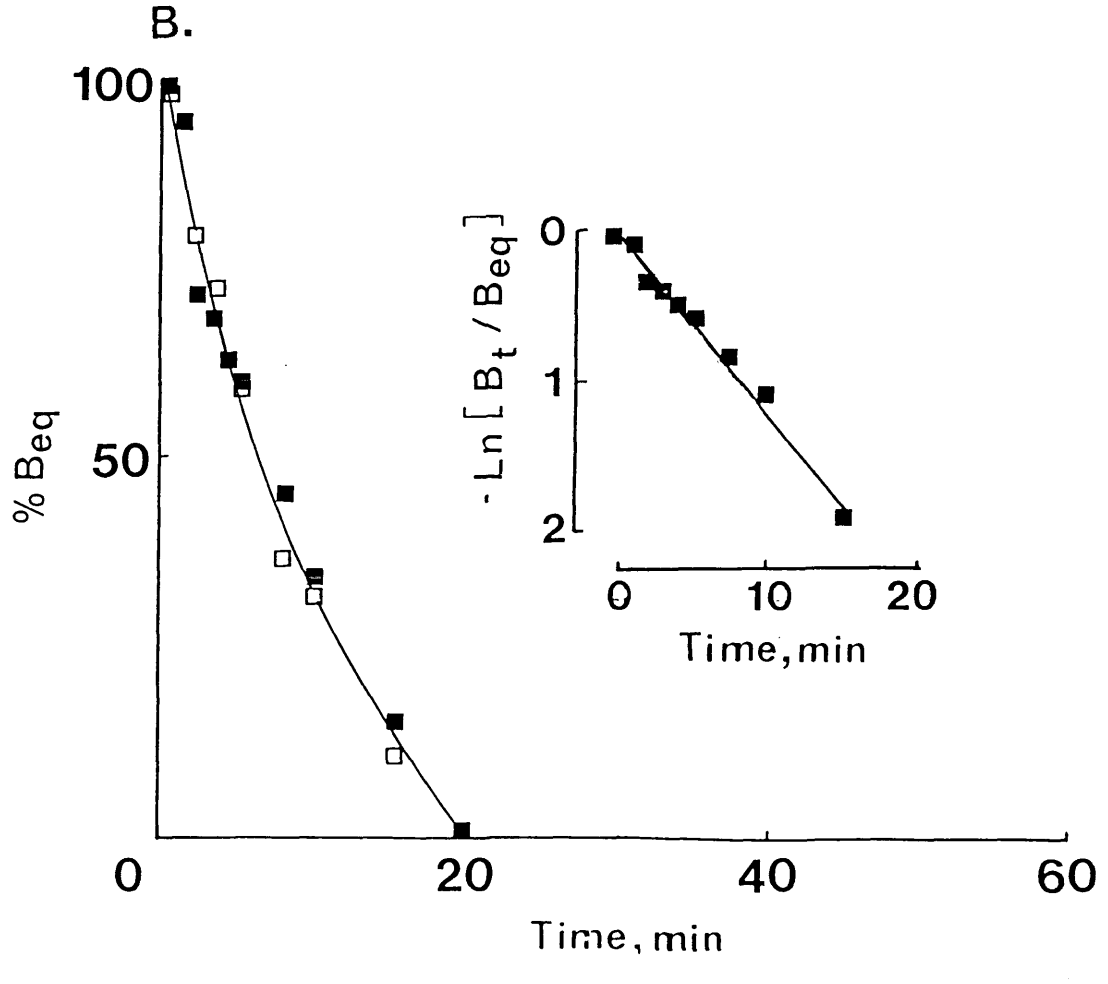
When assayed at a concentration of 2 nM [^{3}H] NT, the specific binding reached a plateau in approximately 20 min (Fig. 22, A). At incubation times of up to 60 min there was no significant decrease in the specific binding observed suggesting that the integrity of the [^{3}H] NT binding sites had been maintained. A semi-logarithmic plot of the data

FIG. 22. Association (A) and dissociation (B) kinetics of [^{3}H] NT binding to non-differentiated N1E-115 cells. Specific [^{3}H] NT binding to intact, non-differentiated N1E-115 cells was measured using a cell suspension assay (3.2.3.2). At each time point the values represent the mean specific binding of triplicate samples as a percentage of B_{eq}, the value at equilibrium. Dissociation was measured by 50-fold dilution of the medium (□) or after the addition of excess (10^{-6} M) unlabelled ligand (■). The results are from one of two independent experiments both of which gave similar results.

Inset: Semi-log plots.

The mean values of the two experiments did not differ by more than 16%.


A.
100
50
% Beq
0
20
40
60
Time, min
2
1
0
6
12
Time, min
Ln [Beq / (Beq - Bt)]


B.
100
50
% Beq
0
20
40
60
Time, min
0
1
2
0
10
20
Time, min
- Ln [Bt / Beq]

(Fig. 22, A, inset) was linear as expected for a pseudo first order reaction.

The reversibility of this binding was demonstrated by dissociation of the bound [^{3}H] NT after the addition of excess unlabelled ligand or a fifty fold dilution (Fig. 22, B). The dissociation rate constant was determined from the slope of a semi-logarithmic plot (Fig. 22, B, inset) and was the same in both cases. The complete dissociation of the ligand indicated that there is no uptake of [^{3}H] NT into the cells i.e. there is no internalization.

From the data shown in Fig. 22, which was typical of three separate experiments, the association and dissociation rate constants were calculated to be 6.7×10^5 M^{-1} s^{-1} and 1.96×10^{-3} s^{-1}, respectively. The equilibrium dissociation constant determined from the kinetic data i.e. k_1/k_{-1} was 2.92 nM which agreed well with the values obtained from equilibrium data, 1.92 nM, and from the kinetic studies of Gilbert et al. (1986; 2.8 nM).

3.2.3 STRUCTURE - ACTIVITY STUDIES

In agreement with the majority of structure-activity studies, the carboxyl terminus of NT, and specifically the 8-13 region, was found to be the biochemically active portion of the peptide for binding to N1E-115 cells (Table 10). Replacement of the Tyr^{11} residue by D-Trp or D-Phe abolished all binding activity: Gilbert et al. (1986) found that these analogues could displace [^{3}H] NT binding to non-differentiated N1E-115 cells although at higher

Table 10. Inhibition of the specific binding of [^{3}H] NT to non-differentiated N1E-115 cells by NT fragments and analogues.

Peptide	K_D^I (nM)	
NT	1.9 ± 0.2	(3)
[Gln^4] NT	1.2 ± 0.3	(3)
NT (8-13)	1.0 ± 0.2	(3)
NT (1-12)	> 10^{-5} M	(2)
[D-Trp^{11}] NT	NC	(2)
[D-Phe^{11}] NT	NC	(2)
NT (1-6)	NC	(2)
NT (1-8)	NC	(2)
NT (1-10)	NC	(2)
Bombesin	NC	(2)
Tuftsin	NC	(2)

Using a cell suspension assay (3.2.3.2) intact, non-differentiated N1E-115 cells were incubated with [^{3}H] NT (2 nM) and varying concentrations of NT or its analogues. Data are mean ± SEM values of several independent experiments (number in parentheses); NC indicates no competition detectable at 10^{-5} M peptide.

concentrations (13-36 uM) than those used here ($\leq$ 10 uM).

As seen in rat and cat brain (1.6.2.2; 2.3.5) and other studies of N1E-115 cells (1.6.2.2) both [Gln^4] NT and NT (8-13) were more potent than NT itself in displacing [^{3}H] NT binding. These two analogues were 1.5-2.0 fold more potent than NT which compares well with their relative potencies (approximately 2.0 fold more active than NT) in rat brain (2.3.5).

In agreement with the results for rat and bovine brain (2.3.5), neither bombesin or tuftsin were able, at a concentration of 10^{-5} M to compete for the [^{3}H] NT binding sites on the N1E-115 cells. In the case of tuftsin it would appear that this tetrapeptide is most likely a competitor for the NT binding sites of non-neuronal origin only.

3.4 SUMMARY

An initial survey was made for the binding of [^{3}H] NT to neuronal and non-neuronal cell lines using a solid phase assay. All of the cell lines investigated exhibited binding activity with the highest density of sites being found on non-differentiated cells of the neuroblastoma N1E-115 cell line. This density was three fold higher than that seen in either the non-neuronal cell lines or the differentiated N1E-115 cells, independent of the differentiation method used.

Using the N1E-115 cells the binding of [^{3}H] NT was further characterized by means of a cell suspension assay which was technically more reliable and simpler in execution than the solid phase assay. Non-differentiated cells bound [^{3}H] NT in a specific, saturable, time dependent and reversible manner whereas, the differentiated cells did not appear to bind [^{3}H] NT specifically. The latter result, which contradicts the findings from the solid phase assays, was attributed to the loss of cell processes during the harvesting procedure.

Both equilibrium and kinetic studies of [^{3}H] NT binding to intact, non-differentiated N1E-115 cells indicated a simple binding behaviour i.e. a single class of non-interacting sites with a K_D of ~2 nM and a B_{max} equivalent to 39,000 binding sites per cell. Additionally, the complete displacement of specifically bound radioligand by excess unlabelled ligand or by dilution discounted the

possibility of any uptake of [^{3}H] NT into the cells.

The ligand specificity of these binding sites correlated well with other structure-activity studies reported in the literature i.e. the carboxyl terminus was required for binding activity. In particular NT (8-13) was more active than NT in competing for the [^{3}H] NT binding sites; this result identifies the N1E-115 [^{3}H] NT binding sites as similar to those reported by other groups using the same cell line and also to those found in rat and cat brain, as previously discussed (1.6.2.2).

Preliminary experiments performed in this laboratory (M. Hanley and T. Jackson, personal communication) have confirmed that the binding of NT to the N1E-115 cells induces phosphoinositol metabolism and Ca^{2+} influx as has been reported by other researchers using the same cell line (Table 5). This, together with the binding assay data, indicates that the [^{3}H] NT binding sites of this cell line represent functional receptor sites. The determination of the binding parameters of these receptors in intact cells enables the use of this cell line as an excellent model for the study of the cellular events occurring upon NT receptor occupancy. The primary objective of this study, however, was to investigate whether any of the cell lines could be used as an enriched source for the solubilization and subsequent purification of the NT receptor; the low density of binding sites (~60 fmol/mg protein) seen in these cells in comparison to rat and bovine brain (~400 fmol/mg protein) makes them unsuitable for such an undertaking.

4. SOLUBILIZATION STUDIES

4.1 INTRODUCTION

To elucidate the molecular mechanisms involved in receptor activity it is desirable to solubilize and purify the receptor in question. Described in this chapter are the detergent solubilization of the NT receptors of rat and bovine brain and the subsequent characterization of the bovine NT binding sites in detergent solution.

4.2 METHODS

4.2.1 SOLUBILIZATION

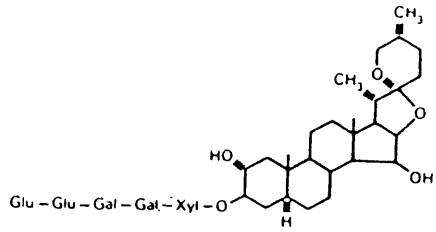
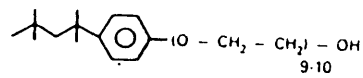
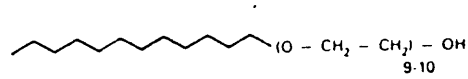
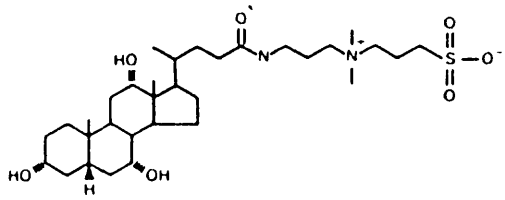
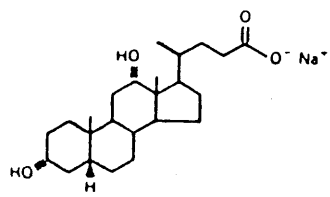
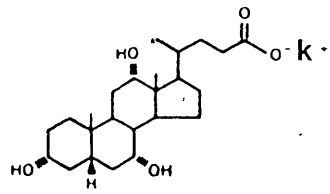
4.2.1.1 PRELIMINARY INVESTIGATIONS

Frozen bovine or rat membranes (2.2.2) were thawed and washed by centrifugation (45,000 x g, 4°C, 30 min) in 20-30 volumes of 10 mM TES KOH, pH 7.5. Using a 25 gauge needle and syringe membranes were resuspended in Buffer C [10 mM TES-KOH, pH 7.5, 1 mM EGTA-K^+, 1mM benzamidine HCl, 0.02% (w/v) bacitracin and 0.002% (w/v) soybean trypsin inhibitor] containing the appropriate detergent (for detergents and concentrations used see Table 11) at a final protein concentration of 4-5 mg/ml. After incubation for 60 min at 4°C (25°C in the case of digitonin) with gentle agitation, non-solubilized membranes were removed by ultracentrifugation (120,000 x g, 4°C, 60 min). The supernatant was immediately assayed for binding activity (4.2.2) and the remainder frozen in liquid nitrogen and stored at -20°C.

4.2.1.2 THE EFFECT OF IONS

The effect of the inclusion of ions in the solubilization procedure was investigated. Frozen membranes were thawed and resuspended at 0.5-1.0 mg protein/ml in Buffer C containing one of the following cations: Mg^{2+} (2 mM); Li^{2+} (4 mM); Ca^{2+} (3 mM) or Na^+ (100 mM). Following incubation for 60 min at 25°C the membranes were pelleted (45,000 x g, 4°C, 30 min) and solubilized as described

Table 11. The six detergents used in solubilization experiments.

Detergent	Concentration	Structure
Digitonin	2% (w/v)	
Triton X-100	1% (w/v)	
Lubrol PX	1% (w/v)	
CHAPS	10 mM	
Na^{+}-deoxycholate	1% (w/v)	
K^{+}-cholate	1% (w/v)	

(4.2.1.1) with the exception that the cation was included in Buffer C throughout.

4.2.1.3 SOLUBILIZATION PROCEDURE

Bovine cortical membranes were preincubated at 0.5-1.0 mg protein/ml in Buffer D [10 mM TES-KOH, pH 7.5, 1 mM EGTA-K^+, 2 mM $MgSO_4$, 1 mM benzamidine-HCl, 0.02% (w/v) bacitracin and 0.002% (w/v) soybean trypsin inhibitor] for 60 min at 25°C. The pelleted membranes (45,000 x g, 4°C, 60 min) were resuspended at 5-6 mg protein/ml in Buffer D containing digitonin at a final concentration of 2% (w/v). Following solubilization for 60 min at 25°C, the supernatant obtained after centrifugation (120,000 x g, 4°C, 60 min) was frozen in liquid nitrogen and stored at -20°C in 1 ml aliquots.

4.2.2 SOLUBLE RECEPTOR BINDING ASSAY

[^{3}H] NT binding to detergent solubilized membranes was measured as described for intact membranes (2.2.3) with the exception that 2mM $MgSO_4$ was included in the assay and BSA was omitted. The final detergent concentration was 0.1% (1 mM for CHAPS) and the protein concentration in the range 0.05-0.1 mg protein/ml assay. All soluble receptor assays were performed in the presence of 2 mM $MgSO_4$ unless otherwise stated.

4.2.3 SOLUBLE PROTEIN ASSAY

Soluble protein samples were precipitated with 1% (w/v) Na^+-deoxycholate/12% (w/v) trichloroacetic acid (TCA) for 15 min at 4°C. Proteins were pelleted by centrifugation (10,000 x g, 4°C, 10 min) the pellet was resuspended in solution 1 (2.2.5) and the protein content measured as described for membrane bound proteins (2.2.5).

4.2.4 FPLC STUDIES

Fast Protein Liquid Chromatography (FPLC) was performed on a Pharmacia FPLC apparatus using a GP 250 Gradient Programmer. Sample injection was via a 3 position, 7 port V7 valve and spectrophotometric monitoring by a single path, single wavelength (214 nm) UV monitor.

All buffers and samples were filtered and degassed prior to use by filtering through a 0.22 um filter.

4.2.4.1 FPLC GEL FILTRATION

A Superose 6 HR 10/30 column (10 mm x 30 cm) containing 24 ml of Superose 6 was used. This matrix has an exclusion limit for globular proteins of 4×10^7 daltons and an operating range of 5×10^3-5×10^6 daltons.

Following equilibration of the column with Running Buffer [10 mM TES-KOH, pH 7.5, 2 mM $MgSO_4$, 1 mM EGTA-K^+, 1 mM benzamidine-HCl, 10 uM 1,10-phenanthroline and 0.05% (w/v) digitonin] standards [250 ul of blue dextran (100 ug), catalase (400 ug), ferritin (50 ug), aldolase (400 ug) and tryptophan (50 ug)] were loaded via a 400 ul

sample loop and eluted with 25 ml Running Buffer at a flow rate of 0.5 ml/min. The absorbance at 214 nm was monitored and a calibration curve plotted of Stokes radius versus average mobility, K_{av} (Fig 23). K_{av} was obtained from the equation:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_t = the total column volume (25 ml)

V_o = the void volume (5 ml)

V_e = the experimentally determined elution volume.

Samples, 200 ul, of undiluted digitonin solubilized receptor (4.2.1.3) together with 50 ul Running Buffer containing blue dextran (100 ug) and tryptophan (50 ug) were loaded and eluted with Running Buffer. Fractions of 0.5 ml were collected and 200 ul aliquots from each assayed for [^{3}H] NT binding activity (4.2.2) in the presence of 0.1% (w/v) digitonin and presence or absence of 10^{-6} M unlabelled NT (single determinations). The experiment was repeated twice.

After use the column was washed with 5 ml of 5 M NaOH, 50 ml H_2O, 50 ml 24% ethanol and stored in ethanol.

4.2.4.2 FPLC CHROMATOFOCUSSING

Chromatofocussing was performed on a mono P HR 5/20 column (5 mm x 20 cm) containing 4 ml anion exchanger. Prior to use the column was washed with 1 ml 5 M NaOH and equilibrated with Start Buffer [25 mM bis-Tris iminodiacetic

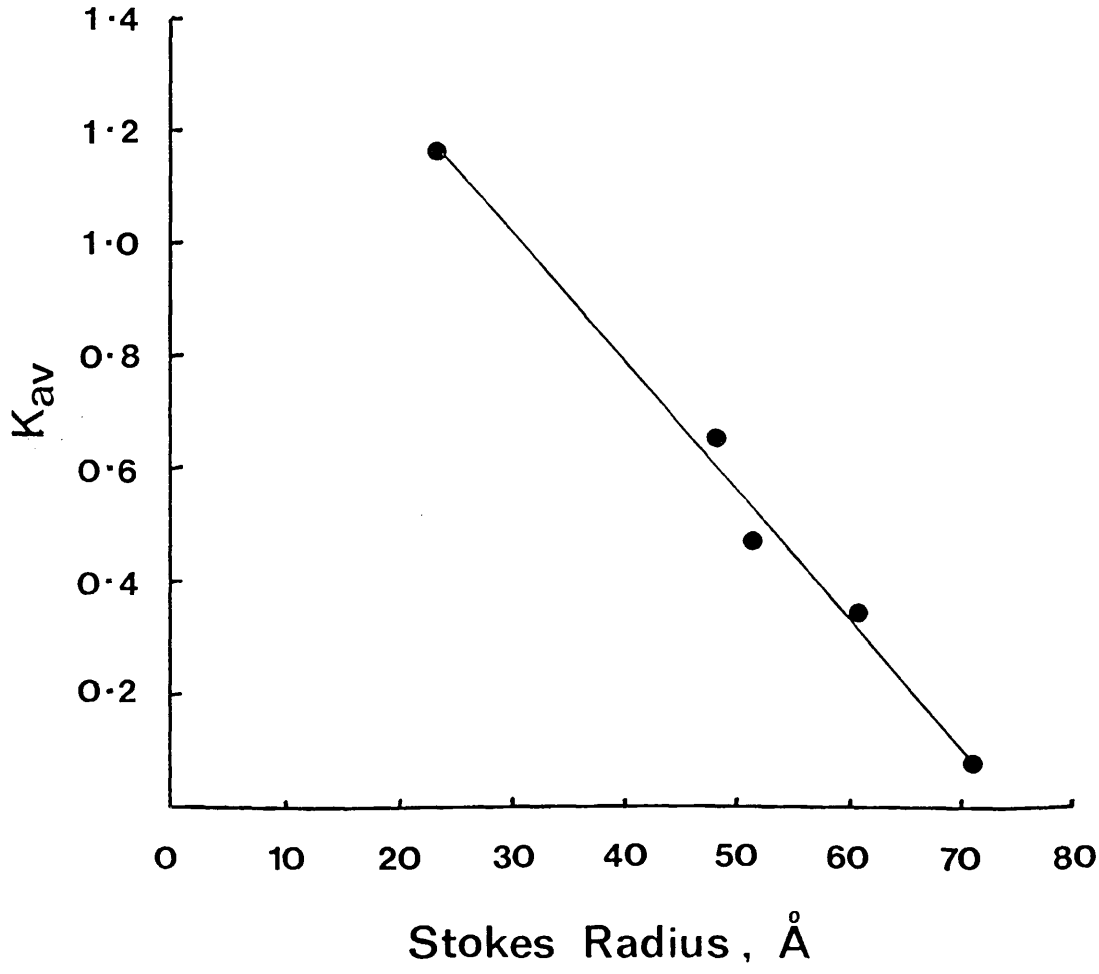


FIG. 23. Calibration curve for gel filtration chromatography. The average mobility, K_{av}, was plotted versus the Stokes' radius of the calibration proteins blue dextran (71 Å), ferritin (61 Å), catalase (52 Å) aldolase (48 Å) and chymotrypsinogen (22 A).

acid, pH 7.1, 0.1% (w/v) digitonin] until the influx and efflux pH were identical (approximately 30 ml). The standards cytochrome C, myoglobin and soybean trypsin inhibitor, all at 0.25 mg/ml in 1 ml of Start Buffer, were loaded through a 1 ml sample loop and eluted with 40 ml of Elution Buffer [10 ml Polybuffer 74 iminodiacetic acid, pH 7.4 in a total volume of 100 ml, 0.1% (w/v) digitonin] at a flow rate of 1 ml/min. Fractions of 1 ml were collected and their pH measured. The column was washed with 1 ml of 2 M Na-acetate and re-equilibrated with Start Buffer. Digitonin solubilized receptor (4.2.1.3), 1 ml, was loaded with a trace quantity of cytochrome C and eluted as above. From each 1 ml fraction collected 0.5 ml was removed and diluted 1:1 with Quench Buffer [100 mM Tris HCl, pH 7.4, 0.1% (w/v) digitonin] the remainder being used for the measurement of pH. Aliquots, 200 ul, from each quenched fraction were tested for [^{3}H] NT binding activity (4.2.2) in the presence of 0.1% (w/v) digitonin and presence or absence of 10^{-6} M unlabelled NT. Determinations were as independent duplicates and the experiment repeated twice.

At the end of each series of experiments the column was washed sequentially with 10 ml 0.5 M Na_2SO_4, 10 ml H_2O, 10 ml 24% ethanol and stored in ethanol.

4.3 RESULTS AND DISCUSSION

4.3.1 DEFINITION AND ASSAY OF SOLUBILIZED NT RECEPTORS

Prior to solubilization experiments two important questions were addressed , namely the definition of solubilization and the means by which soluble [^{3}H] NT binding activity was to be measured.

As a definition of solubilization the most frequently used criterion is the retention of protein or binding activity in the supernatant after centrifugation at 100-200,000 x g for 1 hour. This operational definition, based on the different buoyant densities of the insoluble and soluble fractions, was used in all of the solubilization studies reported here.

Receptor binding in detergent solutions can be measured by a number of different methods; the ones used more frequently are given in Table 12 together with pertinent examples. In a series of control experiments the suitability of each of these assays for the determination of [^{3}H] NT binding to solublized receptor was assessed. Briefly, [^{3}H] NT binding assays, at a single [^{3}H] NT concentration for total and non-specific determinations, were prepared as described for membrane assays (2.2.3.1) but in the absence of membrane protein and BSA and in the presence of 1 mM CHAPS or 0.1% Triton X-100. Separation of bound from free radioligand was then performed according to the assay method being evaluated (for details see El Refai, 1984).

Table 12. Some commonly used soluble receptor assays.

Assay	Receptor	Reference
PEG[a] precipitation	GABA	Sigel *et al.* (1983)
	Opiate	Demoliou-Mason & Barnard (1986).
	Dopamine D_2	Hooper (1986).
Ammonium sulphate precipitation	Benzodiazepine	Gavish *et al.* (1979).
	mAChR	Beld & Ariens (1974).
Charcoal adsorption	Opiate	Cho *et al.* (1986).
	Dopamine D_2	Wheatley & Strange (1983).
	Histamine H_1	Gavish *et al.* (1979).
Gel filtration	Glucagon	Goldstein & Blecher (1976).
	β-adrenergic	Caron & Lefkowitz (1976).
DEAE filters	mAChR	Gorrison *et al.* (1981).
PEI[b] treated filters	Bradykinin	Bruns *et al.* (1983).
	mAChR	"
	α_1, α_2, β adrenergic	"
	Dopamine D_2	"
	Opiate	"

[a]Polyethyleneglycol

[b]Polyethylenimine

Gel filtration or exclusion assays were found to be cumbersome and slow in execution (3-5 min) even after a reduction in the time required to elute bound ligand by the inclusion of a centrifugation step. In any equilibrium binding or time course studies the rapid separation of bound and free ligand is important since the amount of bound ligand depends on the rate of ligand dissociation from the receptor. In numerical terms a separation time of no greater than 0.15 times the $t_{1/2}$ of ligand dissociation must be employed in order to limit the ligand dissociation to less than 10%. In the kinetic studies of [^{3}H] NT binding to membranes of rat and bovine brain (2.3.4), $t_{1/2}$ values of 1-3 minutes were obtained for ligand dissociation suggesting that the optimum separation time must be $<<<$ 1 minute. In view of this, and assuming that the affinity of the NT binding sites will not change when solubilized with detergent, the gel filtration method was discounted as too slow.

The charcoal adsorption assay, in which free radioligand is adsorbed to activated charcoal, also proved to be unsuitable, This method is only effective for small molecules e.g. histamine (see Table 12) and it was found that the proportion of free [^{3}H] NT still remaining in solution was large.

The most convenient method of achieving fast separation times is that of vacuum filtration, where for soluble receptor assays the receptor-ligand complex must

either be specifically precipitated or selectively retained by the filter.

Two agents frequently used in soluble receptor assays for the precipitation of proteins in solution are ammonium sulphate and polyethyleneglycol (PEG); unfortunately, both of these methods had severe disadvantages when used to assay soluble [^{3}H] NT binding activity.

In three independent control experiments, at [^{3}H] NT concentrations of 10-20 nM, the addition of ammonium sulphate to a final concentration of 30% (w/v) precipitated 50-60% of the total radioligand present in the absence of any membrane proteins. This phenomenon was not dependent on the particular detergent used; non-specific precipitation of other small peptides by this method has also been described by El-Refai (1984).

Polyethyleneglycol, at a final concentration of 10% (w/v), also precipitated free [^{3}H] NT at 10 nM radioligand (two independent experiments) but to a considerably lower extent (~2%) than that obtained with ammonium sulphate. However, the PEG precipitation resulted in the specific binding of [^{3}H] NT to the filters: a problem that had been encountered by Goldstein and Blecher (1976) in their analysis of glucagon binding studies. Furthermore, the extent of this non-specific specific binding of [^{3}H] NT did not change by varying either the PEG concentration or the incubation time prior to filtration.

Alternative filtration assay procedures depend on the net anionic charge of the receptor-ligand species at

physiological pH which can be separated from free ligand by virtue of their different interactions with the filter used. Although NT is positively charged at physiological pH, at 15 nM [^{3}H] NT approximately 8% of the total radioligand (two independent experiments) bound to DEAE-cellulose filters as a result of peptide adsorbtion to cellulose (El-Refai, 1984).

The method chosen for the assay of soluble [^{3}H] NT receptor activity was filtration through GF/B filters pre-treated with the cationic polymer polyethylenimine. It has been demonstrated previously in membrane studies that the binding of [^{3}H] NT to these filters is low (2.3.2) and this method has been shown to be suitable for assaying the activity of a large number of solubilized receptors (Bruns et al., 1983; Table 12).

4.3.2 INITIAL SOLUBILIZATION EXPERIMENTS

As is often the case in solubilization studies the initial choice of detergents is made empirically and pragmatically according to availability. Of the six detergents chosen (Table 11) two were ionic (Na^+-deoxycholate, K^+ cholate), three non-ionic (digitonin, Triton X-100, Lubrol PX) and one zwitterionic (CHAPS). All have proven useful in the solubilization of other receptors (Table 13).

Under the solubilization conditions described (4.2.1.1) the percentage of total protein solubilized varied

Table 13. Summary of the detergent solubilization of receptors.

Detergent	Receptor	Reference
Digitonin	Opiate, rat brain	Demoliou-Mason & Barnard (1984).
	β-adrenergic, turkey erythrocyte	Vauquelin et al. (1979).
Triton X-100	Opiate, rat brain	Bidlack et al. (1980).
	β-adrenergic, heart/liver	Strauss et al. (1979).
Lubrol PX	β-adrenergic, heart/liver	Strauss et al. (1979).
CHAPS	Angiotensin II, adrenal gland	Capponi et al. (1983).
	Opiate, NG108 cells	Simonds et al. (1980).
Cholate	Dopamine D_2, bovine striatum	Hooper (1986).
	nicotinic acetylcholine, Torpedo marmorata	Jones et al. (1987).
Deoxycholate	nicotinic acetylcholine, Torpedo marmorata	Briley & Changeux, (1978).
	GABA	Sigel et al. (1983).

according to both the species and the detergent used (Table 14). This result is likely to reflect differences in the lipid composition of brain membranes between species as it is now well documented (Hjelmeland and Chrambach, 1984) that detergents often preferentially solubilize membranes containing particular lipid(s) e.g. digitonin and cholesterol containing membranes (Jones et al., 1987).

Solubilization of rat brain membranes with various detergents resulted in a high yield of solubilized protein (> 40%) but little or no [^{3}H] NT binding activity was retained (Table 14); the highest [^{3}H] NT binding activity was observed in Triton X-100 extracts with a recovery of only ~9% of the activity measured in membranes at the same radioligand concentration (20 nM). The absence of binding activity in Lubrol, K^+ cholate and CHAPS extracts is due to an overall receptor inactivation as suggested by the fact that no activity was measurable in the non-solubilized membrane fraction after ultracentrifugation.

Detergent solubilization of bovine cortical membranes, however, although it gave similar yields for protein content, resulted in the retention of significantly higher levels of [^{3}H] NT binding activity (Table 14). Of the detergents tested the non-ionic detergent digitonin was by far the most effective in solubilizing the receptor in an active state (recovered activity 25%) followed by CHAPS (18%) and Triton X-100 (16%).

The differences in the detergent sensitivity of the NT receptors of rat and bovine brain are not without

Table 14. Comparison of six detergents used for the solubilization of rat and bovine NT receptors.

Detergent	Rat Brain		Bovine Brain	
	Protein (%)	Activity (%)	Protein (%)	Activity (%)
Digitonin (2%)	42 ± 1	5 ± 1 (3)	48 ± 3	25 ± 2 (5)
Triton X-100 (1%)	47	9	41 ± 3	16 ± 4 (3)
Lubrol PX (1%)	46	0	29 ± 2	9 ± 1 (3)
CHAPS (10 mM)	45	0	33 ± 5	18 ± 1 (3)
Na-DOC (1%)	62	6	42 ± 4	14 ± 1 (3)
K^+-cholate (1%)	45	0	44 ± 1	12 ± 2 (3)

Rat or bovine brain membranes were solubilized with each detergent and assayed without delay for specific [^{3}H] NT binding activity (20 nM) as described (4.2.2). The yields of receptor activity and total protein in the soluble fraction are expressed as a percentage of that measured in the membrane fraction. Results are the mean of two independent experiments or mean ± SEM of a number of independent experiments (number in parentheses); all determinations were performed in triplicate.

precedent as a similar phenomenon has been described for the dopamine D_2 receptors. Early attempts to solubilize D_2 receptors of rat brain using digitonin resulted in the loss of high affinity binding (Gorrisen and Laduron, 1978 a, b), whereas it was retained when dog striatum was used as the source tissue (Gorrisen and Laduron, 1979). In view of this and the extremely low soluble NT receptor activity present in detergent extracts of rat brain subsequent studies were devoted to improving the yield of active NT receptor from bovine brain membranes.

In a series of experiments it was found that, irrespective of the detergent used, the [^{3}H] NT binding activity solubilized from bovine brain was unstable with time and there was a complete loss of activity after freezing. It has previously been shown that the inclusion of Mg^{2+} prior to and during membrane solubilization stabilizes opioid receptor activity in detergent solution (Demoliou-Mason and Barnard, 1984); this property was investigated with respect to the stabilization of soluble bovine NT receptor activity.

A significant but not major increase in the percentage yield of solubilized activity (Table 15) and subsequent stability of the preparation was observed when the membranes were treated with Mg^{2+} (2 mM) prior to solubilization and then solubilized with digitonin also in the presence of Mg^{2+}. There was no significant change in the yield of solubilized protein but only moderate yields of

Table 15. Comparison of six detergents used for the solubilization of bovine NT receptors in the presence and absence of magnesium.

Detergent	Activity (%)	
	$-Mg^{2+}$	$+Mg^{2+}$
Digitonin (2%)	25 ± 2 (5)	34 ± 2 (5)
Triton X-100 (1%)	16 ± 4 (3)	16
Lubrol PX (1%)	9 ± 1 (3)	18 ± 3 (3)
CHAPS (10 mM)	18 ± 1 (3)	13
Na-DOC (1%)	14	15
K^+-cholate (1%)	12	13

Bovine brain membranes were solubilized with each detergent in the presence or absence of Mg^{2+} (2 mM) and assayed without delay for specific [^{3}H] NT binding (20 nM) as described (4.2.2). One hundred percent activity (fmol/mg protein) is that obtained in membranes at the same [^{3}H] NT concentration. Results are the mean of two independent experiments or mean ± SEM of a number of independent experiments (number in parentheses); all determinations were performed in triplicate.

binding activity were obtained under these conditions with the other detergents tested (Table 15). The latter detergents were also ineffective in preserving the activity of the solubilized receptor following freezing; it has been reported that some detergents have the ability to chelate divalent cations (Jones et al., 1987) and this may explain their ineffectiveness. The Mg^{2+}/digitonin solubilized bovine NT receptor was stable for at least six months when stored at -20°C and the stabilizing effects of Mg^{2+} could not be mimicked by other cations such as Na^{+} (0.1 M), Ca^{2+} (3 mM) or Li^{2+} (4 mM).

In the solubilization of active and stable dopamine D_2 receptors from bovine striatum a cholate/NaCl procedure is used. The concentration of Na^{+} employed does not significantly increase the yield of solubilized protein nor does it affect the membrane binding properties (Kazmi et al., 1986). It therefore seems likely that the effect of the inclusion of Mg^{2+} in the digitonin solubilization of the bovine NT receptors is analagous to the effect of Na^{+} described above which results in the solubilization of a protein/lipid complex crucial for ligand binding activity as has been suggested (Hooper, 1986; Kazmi et al., 1986).

Finally, the Mg^{2+}/digitonin procedure for the solubilization of bovine NT receptors was optimized in terms of the most effective detergent:protein ratio. Using a fixed concentration of digitonin, 2% (w/v), the membrane protein concentration was varied to give detergent to protein ratios in the range of ~4:1-34:1 (5-0.6 mg protein/ml). Following

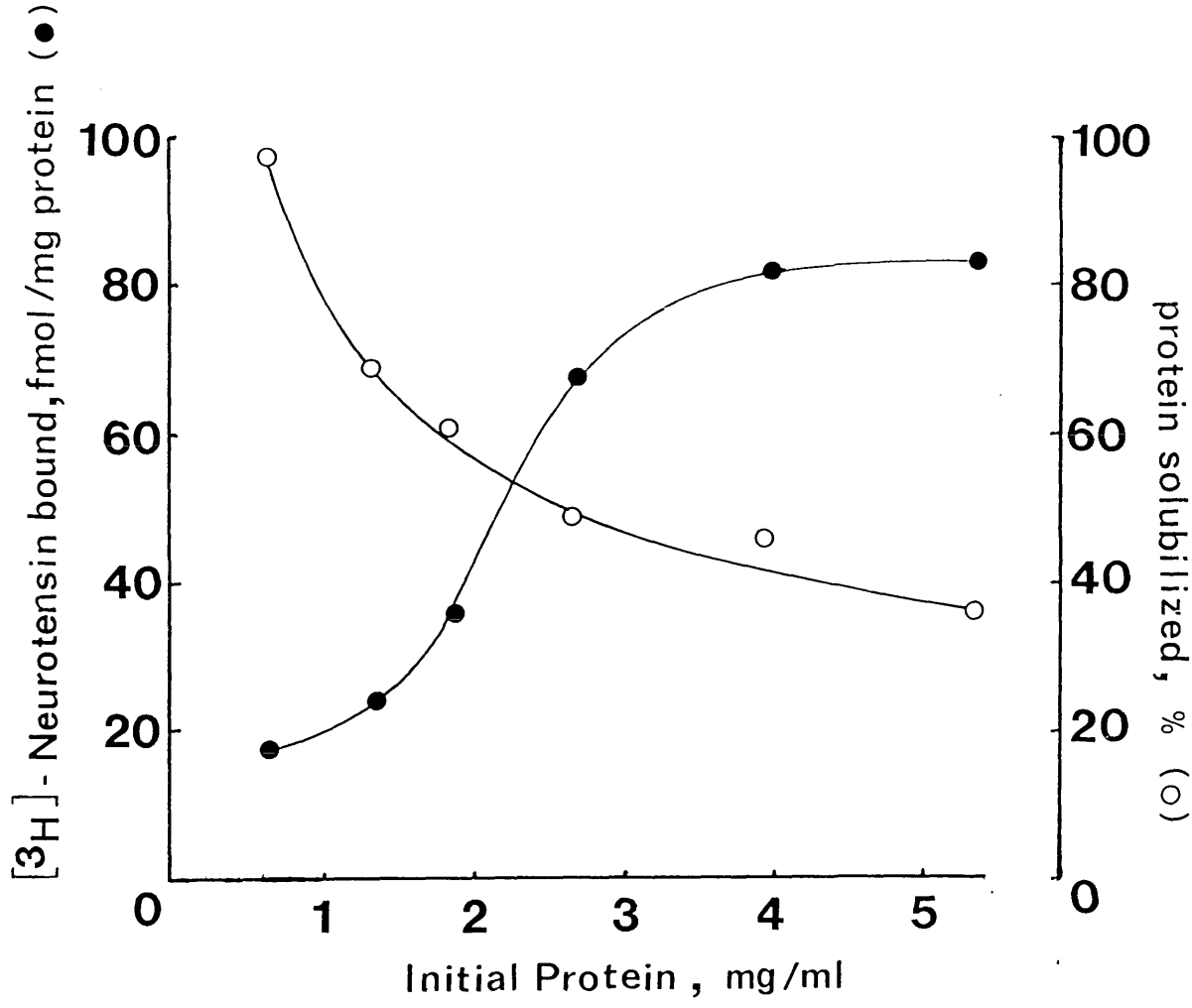


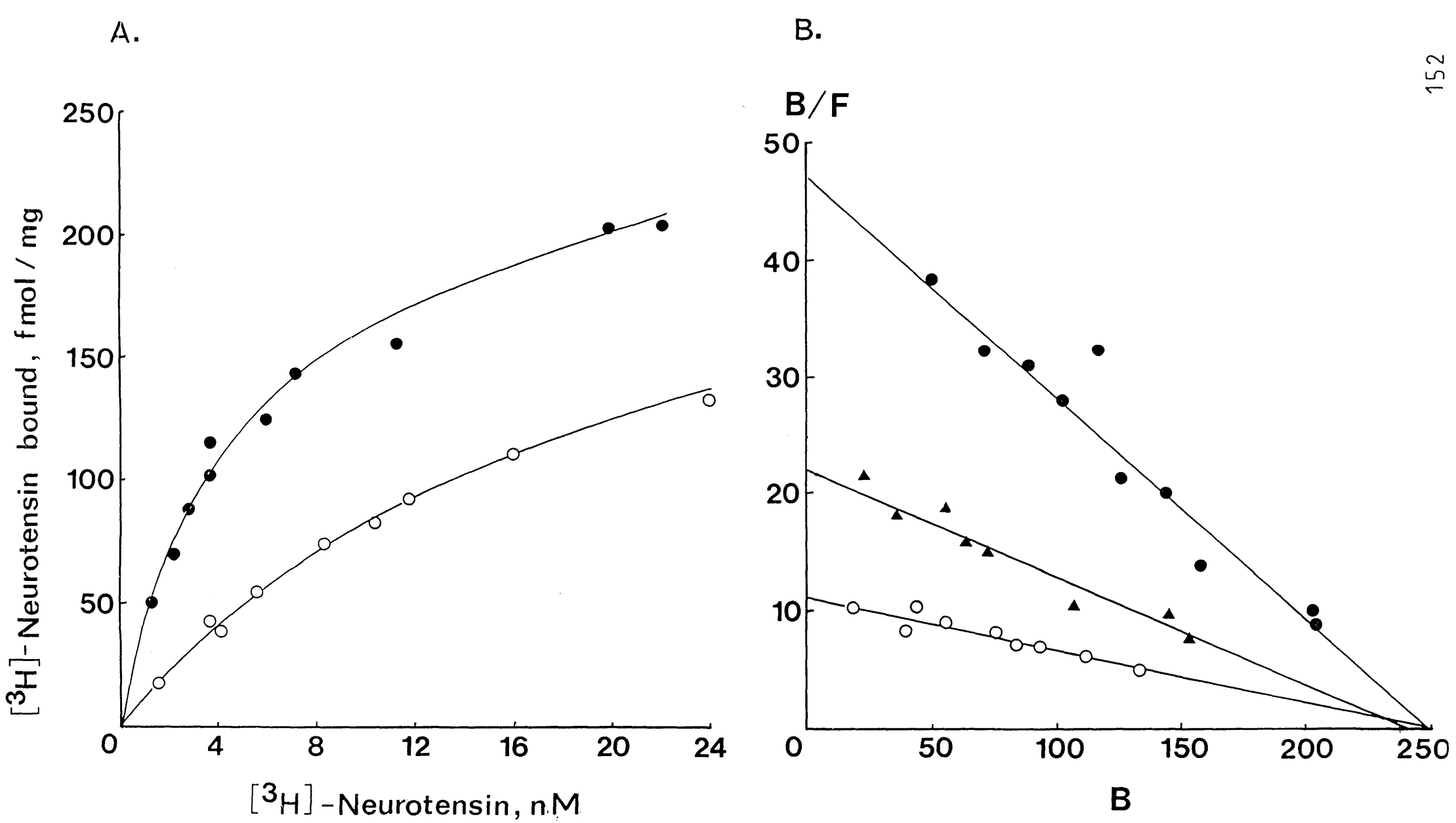
FIG. 24. Solubilization of protein and specific [^{3}H] NT binding sites from bovine cortex by Mg^{2+}/digitonin. Using 2 mM Mg^{2+} and 2% (w/v) digitonin bovine cortical membranes were solubilized at varying inl protein concentrations. Folllowing solubilization (4.2.1.3) soluble protein and specific [^{3}H] NT binding activity were measured as described (4.2.2, 4.2.3). The data are from a single experiment; each point represents the mean of triplicate determinations and SEMs were less than 10%.

solubilization the amount of protein solubilized and the binding of [^{3}H] NT were determined; maximal yields (Fig. 24) were obtained at a protein concentration of ~4 mg/ml with a detergent to protein ratio of ~5:1. There were no significant changes in yields when the initial protein concentration was increased to ~5 mg/ml (detergent:protein ~4:1). Protein concentrations of 4-5 mg/ml were therefore used in all subsequent solubilizations of bovine cortical membranes.

4.3.3 BINDING CHARACTERISTICS OF SOLUBLE [^{3}H] NT BINDING SITES

The specific binding of [^{3}H] NT to Mg^{2+}/digitonin solubilized bovine cortical membranes was concentration dependent and saturable (Fig. 25, A). Non-specific binding increased linearly with increasing concentrations of [^{3}H] ligand and was ~70% of the total binding at the highest radioligand concentrations used routinely (24-25 nM). In five independent experiments the equilibrium binding data (Fig. 25, A) fitted best a theoretical curve in terms of ligand interactions with one class of sites as observed in similar studies of intact membranes (2.3.3). Also, as described for membranes (2.3.3), measurements at [^{3}H] NT concentrations of 40 and 50 nM (one experiment) did not suggest the presence of additional binding sites. The K_D value obtained for soluble [^{3}H] NT binding was 5.5 $\pm$ 0.2 nM

FIG. 25. Saturation (A) and Scatchard (B) plots of [^{3}H] NT binding to Mg^{2+}/digitonin solubilized bovine cortex membranes. Incubations were for 1 h at 20°C with 2 mM Mg^{2+} (●), 2 mM Mg^{2+} and 35 mM Na^+ (▲) or 2 mM Mg^{2+} and 0.1 mM Gpp(NH)p (○). Binding (B) is expressed as fmol/mg protein, free ligand (F) as nanomolar (nM) and B/F as fmol/mg protein.nM. Data are the means of triplicate samples and the SEMs were less than 10%. Similar results were obtained in two to five independent experiments.


A.
B.
B/F
250
200
150
100
50
0
4
8
12
16
20
24
[3H]-Neurotensin bound, fmol / mg
[3H]-Neurotensin, nM
50
40
30
20
10
0
50
100
150
200
250
B

(n = 5) which is close to the value of 3.3 ± 0.2 nM found for [^{3}H] NT binding to membranes. However, the total number of [^{3}H] NT binding sites in the solubilized preparation was only 250 ± 15 fmol/mg protein (n = 5) or 70% of that seen in membranes (2.3.3). The presence of a single class of soluble [^{3}H] NT binding sites was confirmed by the linear Scatchard plot (Fig. 25, B) and a Hill coefficient of 1.0 ± 0.1 (n = 5; Hill plot not shown).

Kinetic studies of [^{3}H] NT binding to solubilized membranes were performed at a radioligand concentration of 5 nM and the relevant rate constants calculated as detailed for membrane binding (2.2.6.3).

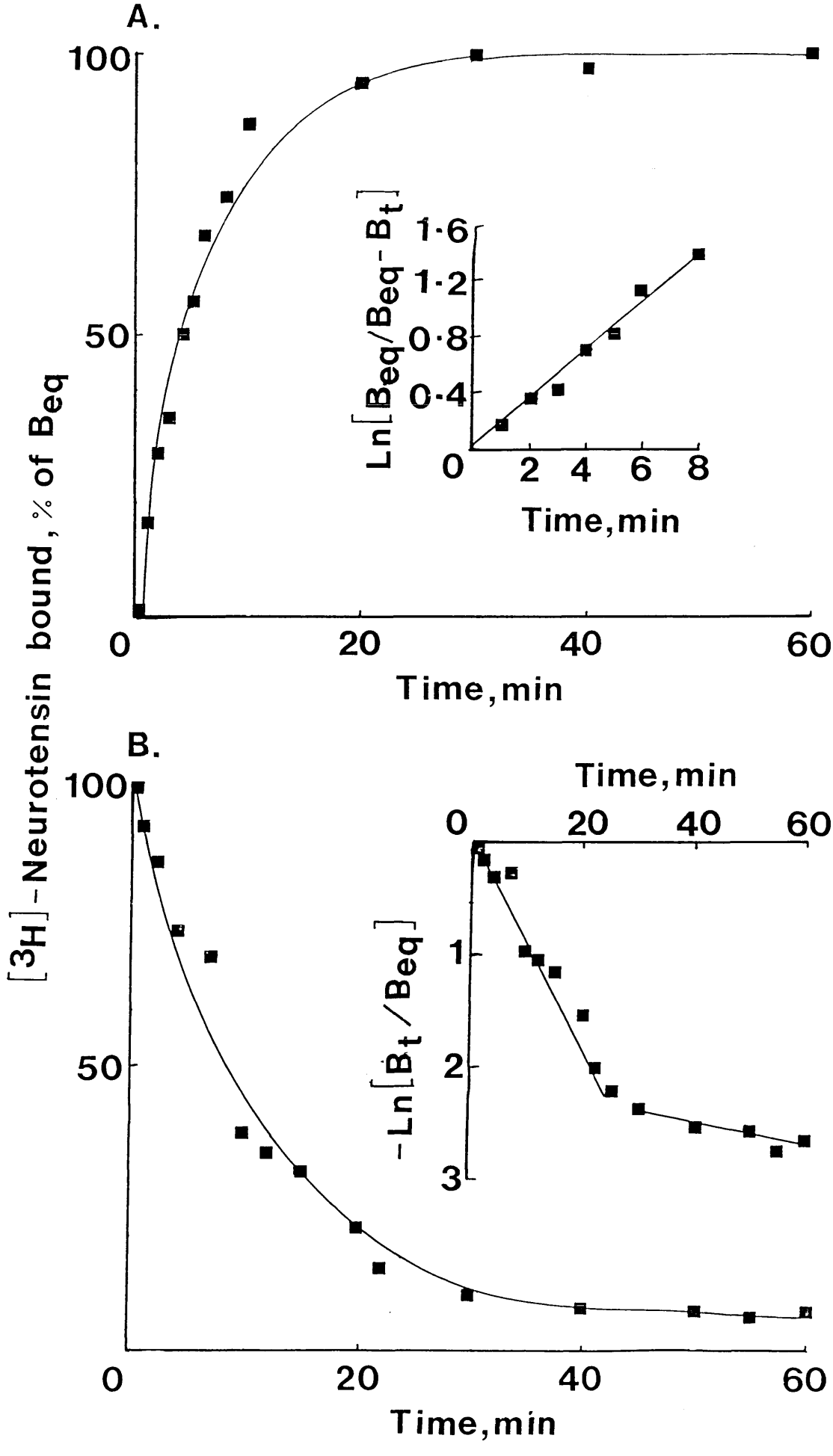
Specific [^{3}H] NT binding to soluble extracts reached a steady state in approximately 30 min (Fig. 26, A) with a $t_{1/2}$ of 3.6 min. The k_{obs} value calculated from the semi-logarithmic plot (Fig. 26, A inset) was 2.9 x $10^{-3}s^{-1}$ which is smaller than the value of 7.2 x $10^{-3}s^{-1}$ obtained with membranes at the same concentration of [^{3}H] NT, suggesting a slower rate of association.

As observed in membranes the dissociation of ligand from the detergent solubilized sites after the addition of excess ligand was biphasic (Fig. 26, B); the `low' and `high' affinity sites represented 88% and 12%, respectively, of the total population of binding sites approximating to the same proportions of sites (82% and 18%) seen in membranes. The dissociation constants calculated from the semi-logarithmic transformation of these data (Fig. 26, B inset) were 1.2 x $10^{-3}s^{-1}$ (`low' affinity sites) and

FIG. 26. Association (A) and dissociation (B) kinetics of [^{3}H] NT binding to Mg^{2+}/digitonin solubilized bovine cortex membranes. At each time point the values represent the mean specific binding of triplicate samples as a percentage of B_{eq}, the value at equilibrium. Dissociation was measured by the addition of excess unlabelled ligand (10^{-6} M). These data are from one of three independent experiments each of which gave similar results.

Inset: Semi-log plots.

The variation in the mean values between experiments (n = 3) was 10% or less.


A.
100
50
0
20
40
60
Time, min
[3H]-Neurotensin bound, % of Beq
Ln[Beq/Beq-Bt]
1·6
1·2
0·8
0·4
0
2
4
6
8
Time, min
B.
100
50
0
20
40
60
Time, min
-Ln[Bt/Beq]
0
1
2
3
Time, min

1.77 x $10^{-4}s^{-1}$ (`high' affinity sites) with corresponding $t_{1/2}$ values of 10 min and ~60 min, respectively. Measurements of the dissociation constants by [^{3}H] ligand dilution was not feasible due to the comparatively low protein concentration of the soluble preparation. However, taking into consideration the equilibrium binding data (vide supra) and the lack of co-operativity in intact membranes (2.3.4) it is unlikely that co-operativity will be detected in the Mg^{2+}/digitonin solubilized membranes.

The equilibrium dissociation constants calculated from the kinetic studies were 3.6 nM for the `low' affinity sites and 0.5 nM for the high affinity sites. A summary of the kinetic and equilibrium binding parameters of membrane bound and soluble [^{3}H] NT binding sites is given in Table 16.

[^{3}H] NT binding to solubilized membranes displayed the same sensitivity to Na^+ and Gpp(NH)p as found in membranes (2.3.6). Na^+ (35 mM) and Gpp(NH)p (0.1 mM) increased the K_D values to 11.0 $\pm$ 0.3 nM (mean of 2 experiments) and 20.0 $\pm$ 0.5 nM (n = 3), respectively, (Fig. 25, A, B) without affecting the total number of binding sites. As observed in membranes, there was no further change in the K_D values when both Na^+ and Gpp(NH)p were included together in the assay medium.

Displacement of specifically bound [^{3}H] NT from the soluble binding sites by competing NT analogues correlated well with that seen in membranes (Table 17). This

Table 16. Kinetic and equilibrium parameters of the membrane bound and Mg^{2+}/digitonin solubilized NT binding sites of bovine brain.

Parameter		Membranes	Solubilized
k_1 ($M^{-1}s^{-1}$)		1.04×10^{6}	3.4×10^{5}
k_{-1} (s^{-1})	Fast	2.01×10^{-3}	1.2×10^{-3}
	Slow	3.32×10^{-4}	1.77×10^{-4}
K_D (nM)	Fast	2.0	3.6
	Slow	0.3	0.5
K_D (nM)	Equilibrium	3.3	5.0

The parameters were calculated as described (2.2.6).

Table 17. Inhibition of the specific binding of [^{3}H] NT to the membrane bound and Mg^{2+}/digitonin solubilized NT receptors of bovine brain.

Peptide	K_D^I (nM)			
	Membrane		Soluble	
NT	3.3 ± 0.2	(7)	5.5 ± 0.2	(5)
[Gln^4] NT	0.13 ± 0.01	(7)	0.23 ± 0.02	(3)
NT (8-13)	0.71 ± 0.03	(3)	1.30 ± 0.05	(3)
NMN	3.7 ± 0.2	(3)	ND	
NT (1-12)	> 10^{-5} M	(2)	NC	(2)
[D-Trp^{11}] NT	NC	(2)	NC	(2)
[D-Phe^{11}] NT	NC	(2)	NC	(2)
NT (1-6)	NC	(2)	NC	(2)
NT (1-8)	NC	(2)	NC	(2)
NT (1-10)	NC	(2)	NC	(2)
Bombesin	NC	(2)	ND	(2)
Tuftsin	NC	(2)	ND	(2)

Membranes or soluble extracts were incubated with [^{3}H] NT (3 nM or 5 nM, respectively) and varying concentrations of NT or its analogues. K_D^I values are mean ± SEM of several independent experiments (number in parentheses). NC indicates no competition detectable at 10^{-5} M peptide; ND not determined.

preservation of the pharmacological specificity suggests that the conformation of the NT binding sites changes very little during solubilization.

4.3.4 FPLC GEL FILTRATION

Prior to application on the FPLC column all samples were passed through a 0.2 um filter; this step caused no significant loss of [^{3}H] NT binding activity indicating that the NT binding sites were truly in solution.

The Mg^{2+}/digitonin/NT binding sites were fractionated on a Superose 6 column in buffer containing 0.05% (w/v) digitonin. This detergent concentration is close to the critical micellar concentration (0.04% (w/v)) of digitonin (Davis, 1984) and therefore minimizes the amount of protein bound detergent whilst avoiding protein precipitation. Under these conditions the NT binding species, as measured with [^{3}H] NT, migrated in two distinct peaks (Fig. 27). These peaks were eluted long after the void volume of the column (Fig. 27) confirming other evidence (vide supra) that the NT binding components present in the Mg^{2+}/digitonin extracts are in true solution. The Stokes radii of the two peaks of soluble binding activity were 52 and 41 A as determined from the standard curve of marker proteins chromatographed under identical conditions (Fig. 23). The dependence of the Stokes radius on the protein bound lipid and detergent and on the solvent composition means that this parameter cannot be used to directly calculate the true molecular weight of the NT binding proteins. The apparent molecular weights, however,

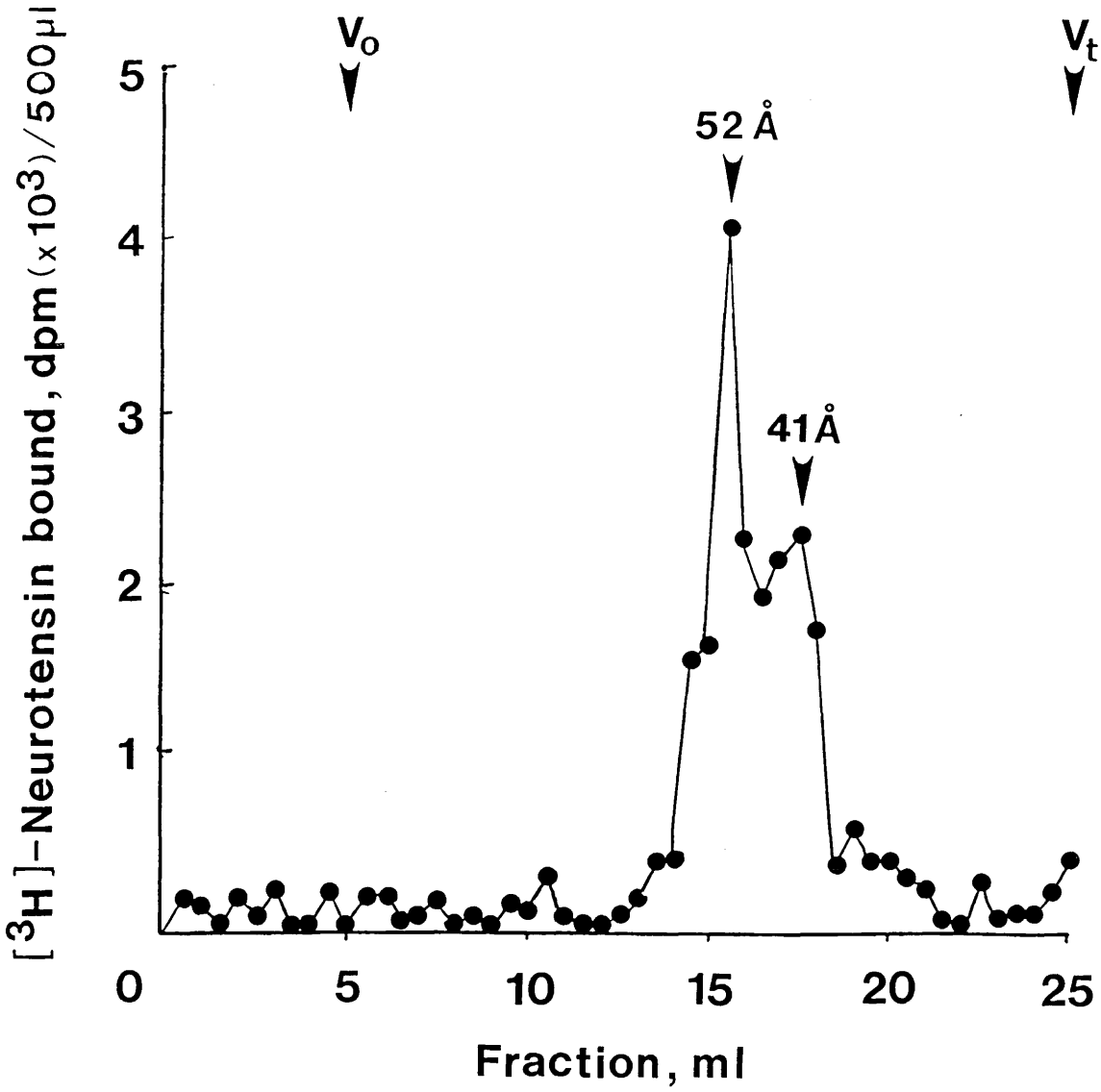


FIG. 27. FPLC gel filtration of Mg^{2+}/digitonin solubilized bovine cortex membranes. Undiluted Mg^{2+}/digitonin solubilized bovine cortex membranes (200 ul) were chromatographed on an FPLC Superose 6 column (4.2.4.1). The specific binding of [^{3}H] NT in each fraction was determined (4.2.2) at a radioligand concentration of 5 nM and the results expressed per 500 ul fraction. The results are from one of three independent experiments each of which gave similar data.

were estimated, using the same marker proteins, and found to be 210 and 140 kdaltons for the 52 and 41 A complexes, respectively.

The presence of two protein species that bind [^{3}H] NT specifically does not correlate with the evidence obtained from equilibrium binding studies which suggest the presence of one class of sites (4.3.3). However, these species may represent different oligomeric forms of the receptor i.e. momomer versus dimer. Alternatively the larger species may represent receptor coupled to effector protein. The difference in the apparent molecular weights of the two species is ~70 kDaltons and it is tempting to speculate that the larger species may be a receptor-guanine nucleotide regulatory protein (molecular weight ~80 kDaltons) complex. Similar results have been obtained with soluble vasoactive intestinal peptide receptors from liver where both receptor and receptor-G protein complexes migrate as distinct peaks after gel filtration chromatography (Couvineau et al., 1986). This, together with the Gpp(NH)p sensitivity of the [^{3}H] NT binding activity observed in membranes and soluble extracts (2.3.6, 4.3.3), makes the putative presence of a G protein an attractive hypothesis. Additional experiments, however, are required before any definite conclusions about the nature of the two [^{3}H] NT binding species can be drawn.

4.3.5 FPLC CHROMATOFOCUSSING

The isoelectric point (pI) of the [^{3}H] NT binding

sites in digitonin solution were determined using an FPLC chromatofocussing column with a pH gradient of pH 7-4. Analysis of this parameter was performed at a Mg^{2+} concentration of 0.05 mM in order to avoid interference with the development of the pH gradient.

To ensure that the column was working in the presence of detergent, samples of cytochrome C, myoglobin and soybean trypsin inhibitor were chromatographed using a pH 7-4 gradient generated in the presence of 0.01% (w/v) digitonin. Cytochrome C, pI 9.2, eluted in the pre-gradient volume of the column whereas myoglobin and soybean trypsin inhibitor were eluted at pI values close to those reported in the literature: myoglobin 6.8, 7.3; soybean trysin inhibitor 4.5 (Malamud and Drysdale, 1978).

Chromatofocussing of the Mg^{2}/digitonin solubilized bovine membranes yielded two peaks of specific [^{3}H] NT binding activity (Fig. 28) as had been observed with gel filtration chromatography (Fig. 27). In two independent experiments the pI values obtained were 6.1/6.2 and 5.5/5.6 for the major and minor peaks of activity, respectively, and are in agreement with the acidic pI values reported for other receptors (for review see Lilly et al., 1984).

The nature of the two populations of [^{3}H] NT binding sites is, at this stage, unclear. The receptor-G protein versus receptor species suggested for the results of gel filtration studies (4.3.4) may also represent the protein species identified here. This would suggest, however, that the receptor is tightly coupled to the G protein. The

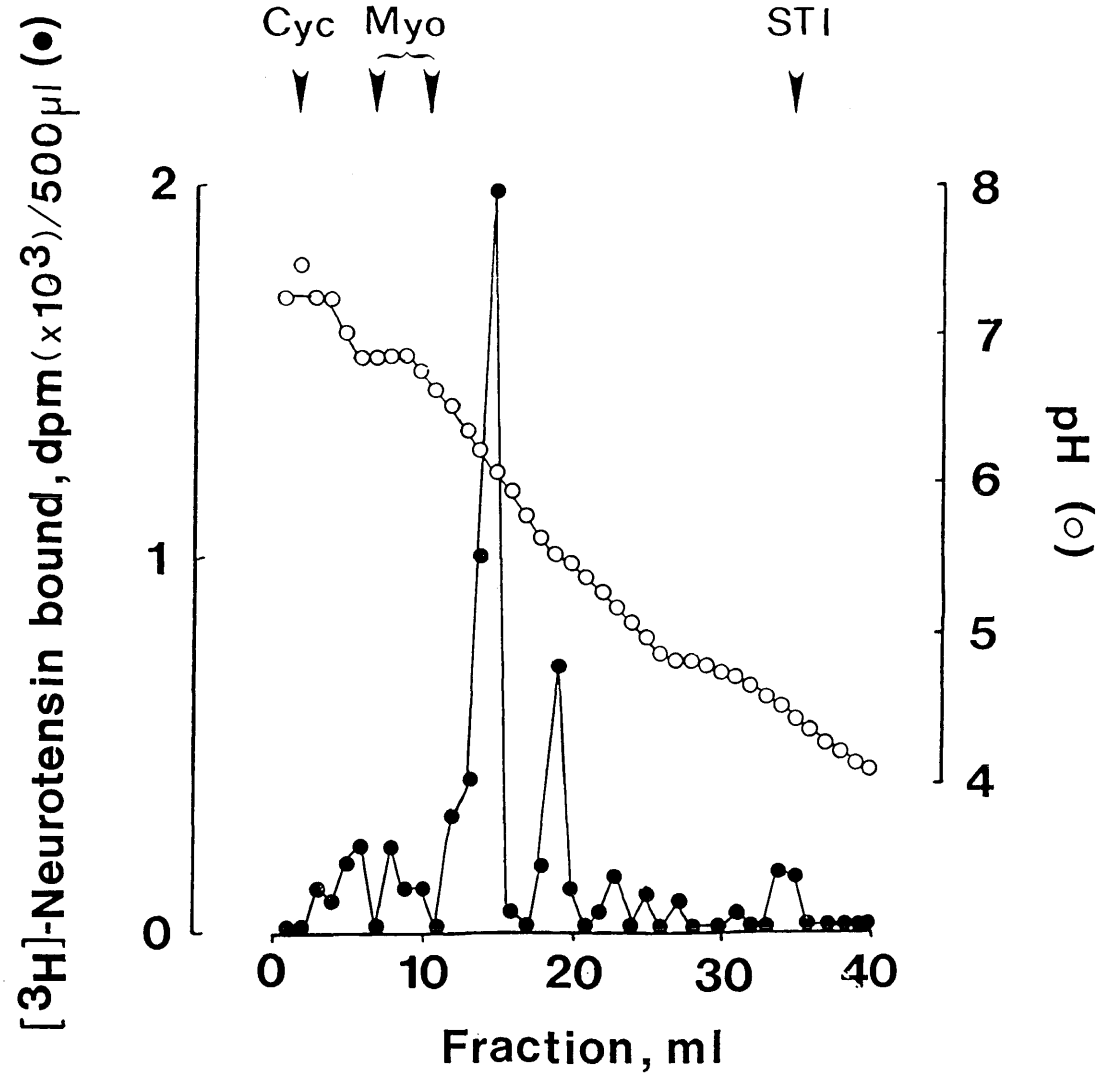


FIG. 28. FPLC chromatofocussing of Mg^{2+}/digitonin solubilized bovine cortex membranes. Mg^{2+}/digitonin solubilized membranes (1 ml, undiluted) were chromatographed on an FPLC mono P column (4.2.4.2). The specific binding of [^{3}H] NT in each fraction was determined (4.2.2) at a radioligand concentration of 5 nM and the results expressed per 500 ul of assay (equivalent to 200 ul of each fraction). The results are from one of three independent experiments each of which gave similar results. The arrows indicate the migration positions of the standard proteins cytochrome C (Cyc), myoglobin (Myo) and soybean trypsin inhibitor (STI).

different pI values may also indicate separate glycosylated forms of the protein or different aggregation states. It has been noted that some proteins have low solubility at their isoelectric point and form precipitates (Lilly et al., 1984). Such precipitates may aggregate and sediment through the column disturbing the formation of the pH gradient. This is, however, unlikely to be the cause of the two peaks of [^{3}H] NT binding activity observed here as the second, minor peak is sharp and well defined and the pH gradient profile is identical to that observed in the absence of membrane proteins.

4.4 SUMMARY

Using vacuum filtration through PEI treated GFB filters as the assay method of choice, various detergents were tested for their ability to solubilize the [^{3}H] NT binding sites of rat and bovine brain.

In a series of experiments it was found that detergent solubilization of rat brain membranes yielded little or no specific [^{3}H] NT binding activity, while detergent treatment of bovine membranes produced significant soluble radioligand binding activity with all of the detergents tested. Subsequent solubilization studies therefore, were performed solely using bovine brain membranes and a preparation with high binding activity was obtained using 2% (w/v) digitonin in the presence of 2 mM Mg^{2+}. These solubilized binding sites were found to retain their activity following freezing and storage at -20°C and the solubilization protocol was optimized with regard to the detergent:protein ratio used.

That the [^{3}H] NT binding sites extracted by Mg^{2+}/digitonin were in a soluble form was suggested by three findings: i) binding activity was present in the supernatant following centrifugation at 100-120,000 x g for 1 hour; ii) the passage of the soluble preparation through a 0.22 um filter without any significant loss of binding activity and iii) the specific [^{3}H] NT binding activity was eluted after the void volume of the Superose 6 gel filtration column.

Analyses of the binding properties of the solubilized [^{3}H] NT binding sites showed that the binding parameters

were almost identical to those obtained with membrane bound receptor sites. Equilibrium binding studies indicated a single class of [^{3}H] NT binding sites with a K_D similar to that observed in membranes but with a 30% decrease in the B_{max} value. A similar situation occurs following solubilization of opioid receptors by digitonin from rat brain membranes (Simon et al., 1986); the authors have attributed the loss of binding sites to a change of conformation during solubilization. The values of the kinetic constants for the rates of [^{3}H] NT association and dissociation suggest slower ligand interactions at the binding sites in detergent solution. As seen in membranes the ligand dissociation profile in digitonin was biphasic and the proportions of the two populations of binding sites were equivelant to those observed in membranes. The [^{3}H] NT binding activity in digitonin retained its sensitivity to both Gpp(NH)p and Na^+ suggesting that the solubilization protocol preserves the interaction of the receptor with its guanine nucleotide binding protein. The relative potencies of NT fragments and analogues in displacing bound [^{3}H] NT were also similar to those determined in membranes suggesting that the specificity of the ligand binding site is maintained in solution.

The results presented here describe for the first time the successful solubilization of NT receptors. The solubilization of the NT binding sites of NG-108 cells has been mentioned (Nakagawa et al., 1984) as well as of rat

brain (S. Amar, personal communication) by the zwitterionic detergent CHAPS but, to date, no data have been presented in the literature to substantiate these claims.

The specific [^{3}H] NT binding sites of Mg^{2+}/digitonin solubilized bovine membranes were resolved into two components by both gel filtration and chromatofocussing analysis. Although, the exact nature of these two populations of binding sites has not yet been determined the large differences seen in the apparent molecular weights after gel filtration means that they are unlikely to correspond to the 49 and 51 kDalton species seen in rat brain (Mazella et al., 1985).

In order to clarify the situation and obtain more information about the NT receptor of bovine brain further purification studies were undertaken.

5. PURIFICATION STUDIES

5.1 INTRODUCTION

Using the detergent solubilization procedure for bovine membranes detailed in the previous chapter a series of purification studies were undertaken. This chapter describes the partial purification of the bovine NT receptor by wheat germ lectin- and hydrophobic chromatography and its purification to homogeneity by NT affinity chromatography. The purified receptor is characterized in terms of [^{3}H] NT binding and subunit composition.

5.2 METHODS

All purification studies were carried out on 2% (w/v) digitonin/2 mM Mg^{2+} solubilized bovine membranes unless otherwise stated.

5.2.1 HYDROPHOBIC CHROMATOGRAPHY

Hydrophobic chromatography was carried out using a 5 ml Phenyl Sepharose column pre-equilibrated with 50 ml Running Buffer [25 mM Tris HCl, pH 7.5, 0.05% (w/v) digitonin] containing 1 M $(NH_4)_2SO_4$. Solubilized membranes (2-10 ml) were diluted with 1 M $(NH_4)_2SO_4$ in 25 mM Tris HCl to 0.1% (w/v) digitonin/0.1 mM Mg^{2+} and loaded onto the column overnight at 30 ml/hour via a circulating system. Proteins bound to the column were eluted in ascending order of hydrophobicity using a stepwise concentration gradient of $(NH_4)_2SO_4$ (0.8, 0.6, 0.4, 0.2 and 0.0 M), followed by digitonin (2% (w/v)) and ethylene glycol (5, 10 and 20% (w/v)) in Running Buffer at a flow rate of 30 ml/hour. The elution volume of each step was 20 ml. Eluates, including the break-through fraction, were stored at 4°C until required; the 2% (w/v) digitonin eluate was collected as a series of 2.5 ml fractions.

Aliquots, 100 ul in triplicate, were assayed for specific [^{3}H] NT binding (2.4.2) in the presence of 0.1% (w/v) digitonin and 2 mM Mg^{2+}. Protein determination was as described for soluble proteins (4.2.3); the experiment was repeated five times.

The column was regenerated by washing sequentially

with 20 ml of each of the following: 6 M urea; water; 25% (v/v) ethanol; 50% (v/v) ethanol; 95% (v/v) ethanol; butan-1-ol; 95% (v/v) ethanol; water and re-equilibrated with Running Buffer/1 M $(NH_4)_2SO_4$ or stored at 4°C in 20% (v/v) ethanol/0.2% (w/v) Na-azide.

5.2.2 FPLC HYDROPHOBIC CHROMATOGRAPHY

See also **4.2**.4.

A Phenyl Superose HR 5/5 column (5 mm x 5 cm) was used with a capacity of ~25 mg protein/ml gel. The column was equilibrated with Running Buffer [25 mM Tris HCl, pH 7.5, 0.05% (w/v) digitonin] containing 1 M $(NH_4)_2SO_4$; the solubilized extract was diluted as described (5.2.1) and loaded onto the column via multiple injections through a 2 ml sample loop at a flow rate of 1 ml/min. Bound proteins were eluted at the same flow rate using a linear, two step gradient of a) 10 ml of 1.0-0.3 M $(NH_4)_2SO_4$ and b) 20 ml of 0.3-0.0 M $(NH_4)_2SO_4$ in Running Buffer. The column was washed with Running Buffer until a steady base line was achieved and the proteins of interest were eluted with 5 ml 2% (w/v) digitonin in 25 mM Tris HCl, pH 7.5. Fractions of 0.5 ml were collected and 100 ul aliquots, in duplicate, were assayed for specific [^{3}H] NT binding (4.2.2) in the presence of 0.1% (w/v) digitonin and 1 mM Mg^{2+}.

The fractions with high activity were pooled and stored at 4°C until required. Protein estimation was as described for soluble proteins (4.2.3); the experiment was

repeated five times.

After each run the column was washed with 10 ml 20% (w/v) ethylene glycol and regenerated as described previously (5.2.1).

5.2.3 LECTIN AFFINITY CHROMATOGRAPHY

Lectin affinity chromatography was performed on a Wheat Germ Lectin Sepharose 6MB column (2 ml) prewashed with 20 ml of each of the following; 0.1 M Tris HCl/0.5 M NaCl [pH 8.5; Base Wash]; water; 0.1 M Na-acetate/0.5 M NaCl [pH 4.5; Acid Wash] and finally 10-20 ml water. The column was equilibrated with 40-50 ml of Running Buffer [10 mM TES KOH, pH 7.5, 1 mM EGTA-K^+, 0.02% (w/v) bacitracin, 1 mM benzamidine HCl, 0.002% (w/v) soybean trypsin inhibitor, 2 mM $MgSO_4$, 0.1% (w/v) digitonin]. Solubilized membranes were diluted to 0.1% (w/v) digitonin with Running Buffer (minus digitonin) and loaded onto the column overnight at 8-10 ml/h using a circulating system. The column was then washed at 20-30 ml/h with 40 ml of Wash Buffer [Running Buffer minus bacitracin and soybean trypsin inhibitor] and specifically bound proteins were eluted with 4 ml of 0.2 M ß-D-N-acetylglucosamine in Wash Buffer.

Aliquots from the eluate (200 ul, in duplicate) were assayed for specific [^{3}H] NT binding; the remaining sample was frozen in liquid nitrogen and stored at -20°C until required. Protein estimation was as described for soluble proteins (4.2.3) after the removal of the sugar by dialysis.

Following each run the column was washed according to

the pre-wash procedure described above and reequilibrated with Running Buffer or stored at 4°C in 0.2% (w/v) Na-azide. The experiment was carried out four times.

5.2.4 NT AFFINITY CHROMATOGRAPHY

NT affinity chromatography was initially performed using 2% (w/v) digitonin/2 mM Mg^{2+} extracts of bovine cortical membranes prepared as described (4.2.1.3). This was later modified by the addition of 0.06% (w/v) asolectin in the solubilization step and these extracts are referred to as 2% (w/v) digitonin/2 mM Mg^{2+}/0.06% asolectin solubilized membranes.

5.2.4.1 PREPARATION OF NT-AFFI-GEL 10

Affi-Gel 10 is an activated affinity support of N-hydroxy-succinimide esters of derivatized and cross linked agarose gel beads. It is highly selective for amino groups and forms stable amide bonds.

Affi-Gel 10 resin (2 ml) was prewashed with three volumes of ice-cold water and gently agitated for 3 h at 4°C in 2 ml of 20 mM HEPES KOH, pH 7.4, containing 12 umoles of NT and a trace amount of [^{3}H] NT. This was followed by a further incubation of 30 min at 20°C at the end of which the unbound ligand was removed and the remaining active sites on the resin blocked by incubation with 1 M ethanolamine HCl, pH 8.0 (1 h, 20°C). The resin was subsequently washed with 20 mM HEPES KOH, pH 7.4, until free of reactants (as detected by absorbance at 280 nm) followed by five volumes

of 10 mM TES KOH, pH 7.5, and stored in the latter buffer containing 0.2% (w/v) Na-azide. The amount of covalently bound NT was estimated from the trace label incorporated to be 4-5 umole/ml resin. The yield of coupling was 30% and did not increase with a longer reaction time.

5.2.4.2 NT AFFINITY CHROMATOGRAPHY

NT-Affi-Gel 10 resin (2 ml) was pre-equilibrated with five volumes of Equilibration Buffer [10 mM TES KOH, pH 7.5, 1 mM EGTA-K^+, 2 mM $MgSO_4$, 0.02% (w/v) bacitracin, 1 mM benzamidine HCl, 10 uM 1,10-phenanthroline, 0.002% (w/v) soybean trypsin inhibitor and 0.01% (w/v) digitonin]; 2% (w/v) digitonin/2 mM Mg^{2+} solubilized membranes were diluted to 0.1% (w/v) digitonin in Equilibration Buffer (minus digitonin) and loaded onto the NT-Affi-Gel 10 column at 20-30 ml/h. The column was subsequently washed at 40-50 ml/h with Wash Buffer [Equilibration Buffer minus bacitracin and soybean trypsin inhibitor] and the affinity bound receptor eluted with 2.5 column volumes of the same buffer containing 10^{-5} M NT, 10^{-5} M [Gln^4] NT or 250 mM NaCl. After dialysis against a total volume of 5 l of Dialysis Buffer [10 mM TES KOH, pH 7.5, 1 mM EGTA-K^+, 2 mM $MgSO_4$ and 1 mM benzamidine HCl] either overnight at 4°C (ligand eluted receptor) or for 2 hours at room temperature (NaCl eluted receptor) the eluates were assayed immediately for [^{3}H] NT binding (4.2.2) or frozen in liquid nitrogen and stored at -20°C.

For 2%(w/v) digitonin/2 mM Mg^{2+}/0.06% asolectin

solubilized membranes the preparation was diluted to 0.1% (w/v) digitonin/0.003% (w/v) asolectin with Equilibration Buffer minus digitonin and loaded onto the column pre-equilibrated with Equilibration Buffer containing 0.003% (w/v) asolectin. All other procedures were as detailed above with the exception that the asolectin concentration in the Wash Buffer was increased to 0.06% (w/v). The dialysed receptor was assayed for specific [^{3}H] NT binding activity in the presence of 0.1% (w/v) digitonin/0.06% (w/v) asolectin.

5.2.4.3 PROTEIN DETERMINATION

The protein content of the purified receptor was estimated by gel densitometry. Samples of purified receptor were run on a 10% SDS-polyacrlamide gel (5.2.5) with standards of 100-500 ng BSA used for the construction of a calibration curve. Following Coomassie Blue staining the gels were analysed on a Joyce Loebel Chromoscan Gel Densitometer at a wavelength of 595 nm.

5.2.5 POLYACRYLAMIDE GEL ELECTROPHORESIS

Gel electrophoresis was carried out on cross-linked 10% polyacrylamide gels prepared according to the method of Laemmli (1970) as modified by Douglas and Butow (1976).

5.2.5.1 SAMPLE PREPARATION

Protein samples were precipitated by the chloroform/methanol method of Wessel and Flugge (1984). To

each 150 ul protein sample was added, in sequence, 600 ul methanol, 150 ul chloroform and 450 ul water; the samples were vigorously vortexed and centrifuged for 9 s at 10,000 x g after each addition. After the addition of water the top (aqueous) layer was removed, 450 ul methanol added and the sample mixed by gentle agitation. Following centrifugation for 4 min at 10,000 x g the supernatant was removed by aspiration to within 1-2 mm above the pellet and the rest evaporated under vacuum. Precipitated proteins and molecular weight markers were dissolved in 50 ul of Sample Buffer which contained 8 M urea, 6% (w/v) SDS, 10% (v/v) glycerol, 100 mM DTT and 0.15 mg/ml bromophenol blue in 0.063 M Tris HCl, pH 6.8. For studies of protein mobility under non-reducing conditions DTT was omitted from the Sample Buffer. The samples were boiled for 3 min and applied to the gel using a Hamilton syringe. The molecular weight markers used for the construction of a calibration curve (Fig. 29) were phosphorylase b (M_r 92,500); BSA (M_r 66,200); ovalbumin (M_r 45,0000; carbonic anhydrase (M_r 31,000); soybean trypsin inhibitor (M_r 21,000) and lysozyme (M_r 14,400).

5.2.5.2 PREPARATION OF SLAB GEL

The separating gel (0.7 mm x 12 cm x 12 cm) contained 10% (w/v) acrylamide, 0.27% (w/v) N,N'-methylene-bisacrylamide, 0.1% (w/v) SDS, 0.025% (v/v) tetra-methylenediamine (TEMED), 0.1% (v/v) glycerol, 0.075% (w/v) ammonium persulphate in 0.38 M Tris HCl, pH 8.8. The gel was

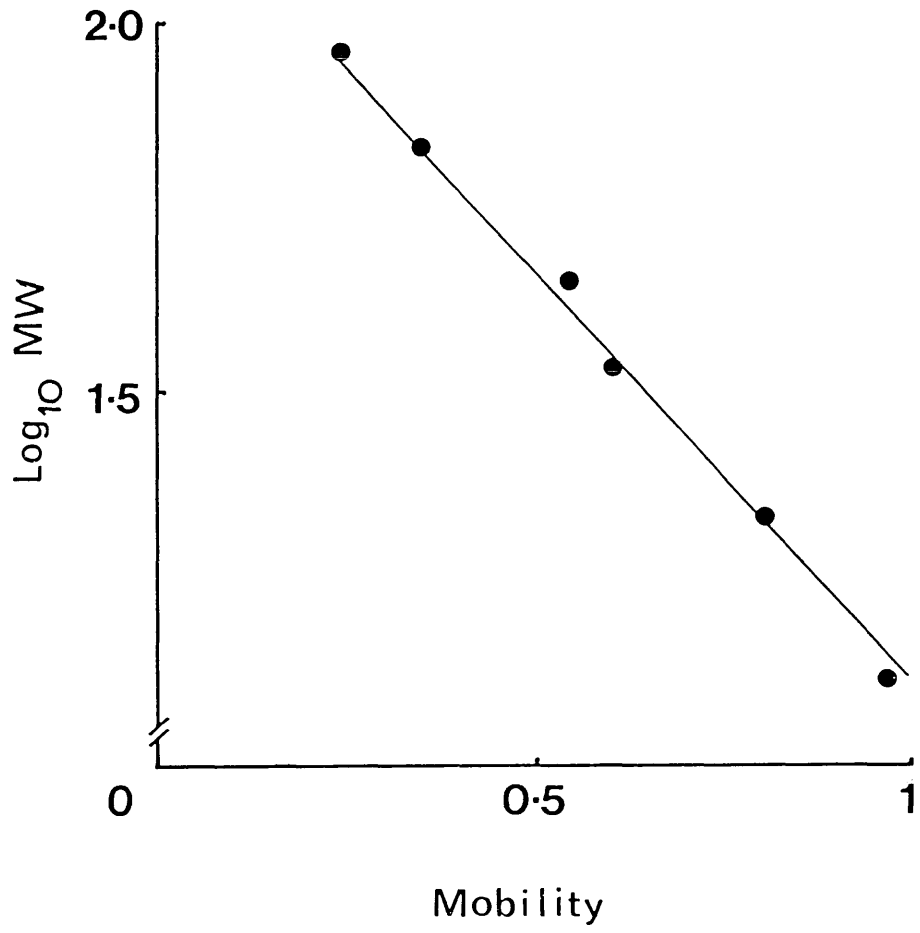


FIG. 29. Molecular weight calibration curve for 10% SDS-PAGE. The standard proteins are, from left to right, phosphorylase b (91 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14 kDa).

left to polymerize for 1-2 h at room temperature. The stacking gel contained 5% (w/v) acrylamide, 0.15% (w/v) N,N'-methylenebisacrylamide, 0.1% (w/v) SDS, 0.05% (v/v) glycerol, 0.075% (v/v) TEMED and 0.06% (w/v) ammonium persulphate in 0.125 M Tris HCl, pH 6.8; the gel was left to polymerize for 0.5-1 h at room temperature before use. The electrode buffer was 25 mM Tris glycine, pH 8.8, containing 0.1% (w/v) SDS; the samples were electrophoresed at 20 mA (constant current) through the stacking gel and at 200 V (constant voltage) through the separating gel.

5.2.5.3 COOMASSIE BLUE STAINING

The gel was fixed and stained overnight with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) ethanol, 10% (v/v) acetic acid and destained with frequent changes of 25% (v/v) ethanol, 10% (v/v) acetic acid.

5.2.5.4 SILVER STAINING

Silver staining was performed by a modification of the method of Morrisey (1981). Briefly, after electrophoresis the proteins were fixed in 50% (v/v) methanol, 10% (v/v) acetic acid for 30 min. This was followed by a series of washes (100 ml each): 5% (v/v) methanol; 7% (v/v) acetic acid; water and 5 ug/ml DTT, the gel being heated at each stage for 1-2 min in a microwave oven set at its highest setting. The gel was then incubated with 0.1% (w/v) silver nitrate for 30 min. At the end of this time the gel was rapidly rinsed with water followed by two rapid rinses with a small volume of developing solution

which contained 3% (w/v) Na_2CO_3, 0.02% (v/v) formaldehyde. The gel was developed in ~100 ml of developing solution and the reaction stopped by the addition of 7.5 ml 2.3 M citric acid. Before drying under vacuum on a gel drier the gel was washed for 30 min in 0.03% (w/v) Na_2CO_3 to prevent bleaching.

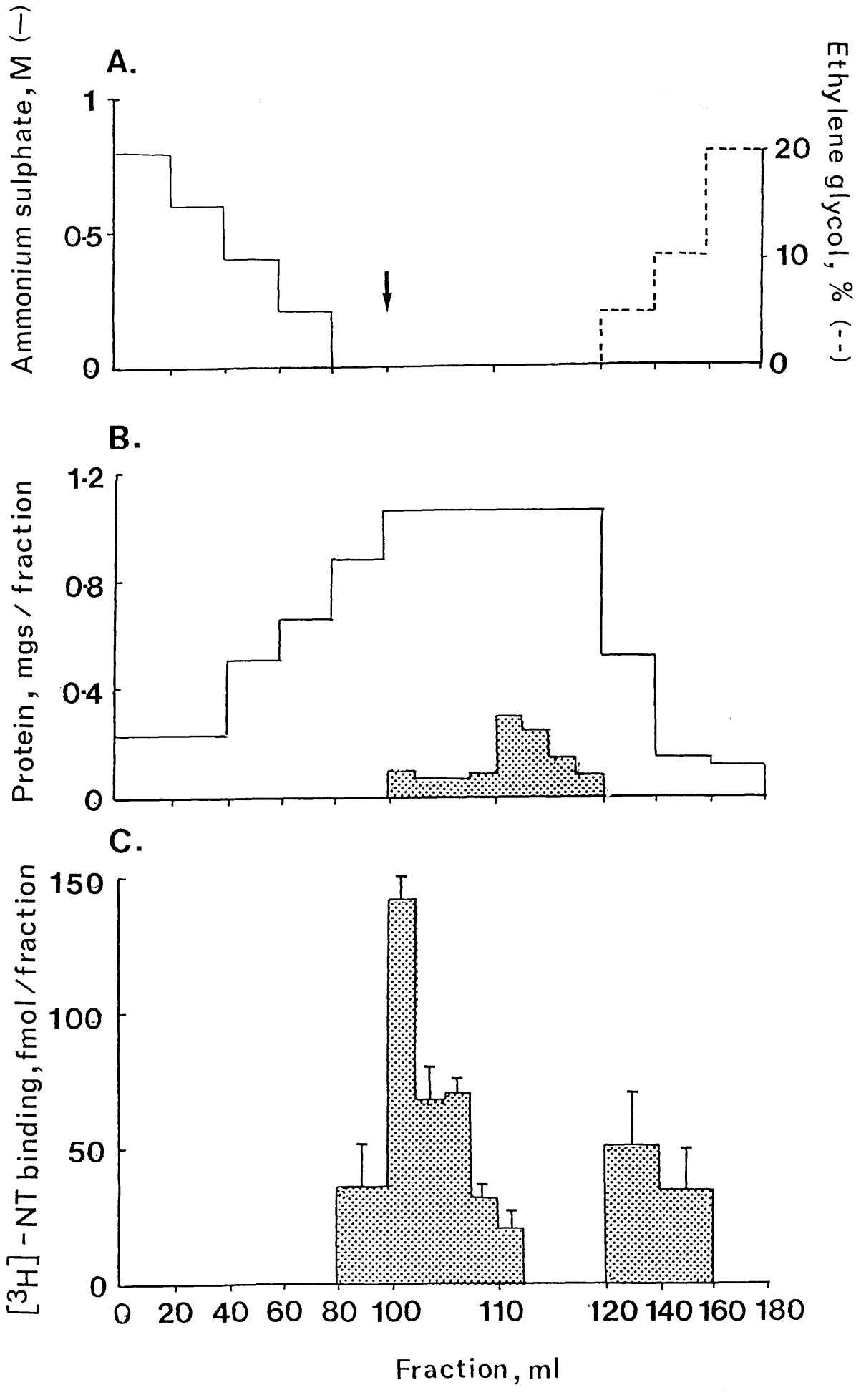
5.3 RESULTS AND DISCUSSION

5.3.1 HYDROPHOBIC CHROMATOGRAPHY

Separation of proteins by hydrophobic chromatography is achieved on the basis of their differential hydrophobic interactions with an uncharged bed material possessing hydrophobic groups. In this study the matrix used was Sepharose beads to which phenyl groups are attached to the monosaccharide units of the agarose support via uncharged ether linkages.

Bovine brain membranes solubilized with Mg^{2+}/digitonin were applied to a Phenyl Sepharose column in buffer containing 1 M ammonium sulphate, which promotes hydrophobic interactions. Under these conditions 76.6 $\pm$ 3.8% (n = 6) of the applied protein and 100% of the specific [^{3}H] NT binding activity were adsorbed onto the column. After washing the column extensively with decreasing concentrations of ammonium sulphate, tightly bound proteins were eluted with 2% (w/v) digitonin followed by increasing concentrations of ethylene glycol (Fig. 30, A). Each fraction , 20 ml (salt elution) or 2.5 ml (digitonin elution), was assayed for protein content (Fig. 30, B) and specific [^{3}H] NT binding (Fig. 30, C) at a radioligand concentration of 5 nM. Compiled data of six independent experiments showed the recovery of total protein and [^{3}H] NT binding activity to be 100% and 83 $\pm$ 12%, repectively, of that initially adsorbed. Of the retained [^{3}H] NT binding activity 69 $\pm$ 13% (n = 6) was eluted by the addition of 2%

FIG. 30. Hydrophobic chromatography of Mg^{2+}/digitonin solubilized bovine cortex membranes. Soluble extracts were bound to a phenyl sepharose column (5 ml) in 1 M ammonium sulphate and eluted in ascending order of hydrophobicity using stepwise gradients of ammonium sulphate and ethylene glycol (A). Elution of the NT binding activity was by the addition of 2% (w/v) digitonin (arrow). Fractions (20 ml) were analysed for protein content (B; the shaded bars represent the protein content per 2.5 ml fraction) and specific [^{3}H] NT (5 nM) binding activity (C) as described (4.2.2, 4.2.3). Binding data are expressed as the mean of triplicate determinations $\pm$ SEM values. These results are from one of six independent experiments each of which gave similar data.

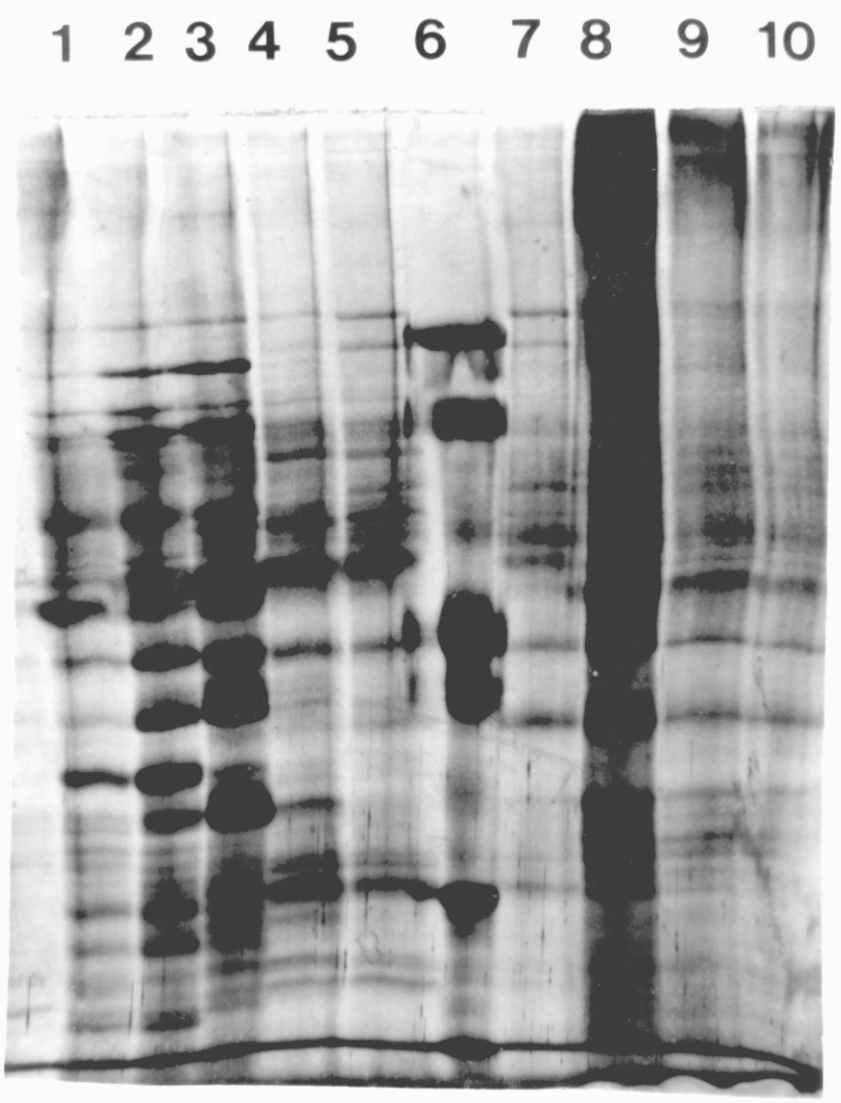

A.
Ammonium sulphate, M (—)
Ethylene glycol, % (--)
1
0·5
0
20
10
0
B.
Protein, mgs / fraction
1·2
0·8
0·4
0
C.
[3H] - NT binding, fmol / fraction
150
100
50
0
0 20 40 60 80 100 110 120 140 160 180
Fraction, ml

(w/v) digitonin, the remaining activity was eluted by ethylene glycol (Fig. 30, C). The variation in the NT binding activity eluted with digitonin in the various experiments is probably due to variations in the ambient temperature; all experiments were performed at room temperature and any increase in temperature would result in stronger hydrophobic interactions thus limiting the amount of protein eluted with digitonin. The strong interactions of the NT receptors with the Phenyl Sepharose column suggests a high content of hydrophobic amino acids as has also been observed for the opioid receptors of frog (Borsodi et al., 1986) and rat (Y.H. Wong, personal communication) brain.

The maximum purification of the NT receptor activity achieved by this chromatographic procedure was 14-15 fold in the fractions eluted with digitonin (Fig. 30, C) and in a series of six experiments this value ranged from 10-20 fold. This is considerably lower than the 50 fold purification of the opioid receptor from frog brain reported by Borsodi et al. (1986) using the same technique. In their case, however, the opioid receptor activity was eluted at low ammonium sulphate concentrations (0.6-0.0 M) without having to increase the detergent concentration.

The SDS-PAGE analyses of 10-20 ug protein samples taken from each Phenyl Sepharose fraction are shown in Fig. 31. Lane 7 which contains proteins from the fraction with the highest specific [^{3}H] NT binding activity showed no apparent enhancement of any specific protein bands when

FIG. 31. Silver staining of hydrophobic chromatography eluates after SDS-PAGE. Eluates from the phenyl sepharose column (10-20 ug) were electrophoresed as described (5.2.5) and stained with silver reagent (5.2.5.4). Lanes 1-5, proteins eluted with decreasing ammonium sulphate 0.8-0.0 M, respectively; lane 6 molecular weight markers (91, 66, 45, 31, 21 and 14 kDa, respectively); lane 7 proteins eluted with 2% (w/v) digitonin; lanes 8-10 proteins eluted with increasing ethylene glycol 5, 10 and 20% (w/v), respectively.



compared with the protein profile of the other fractions. However, a number of the heavily stained proteins present in lanes 1-5 and 8-10 were noticeably absent.

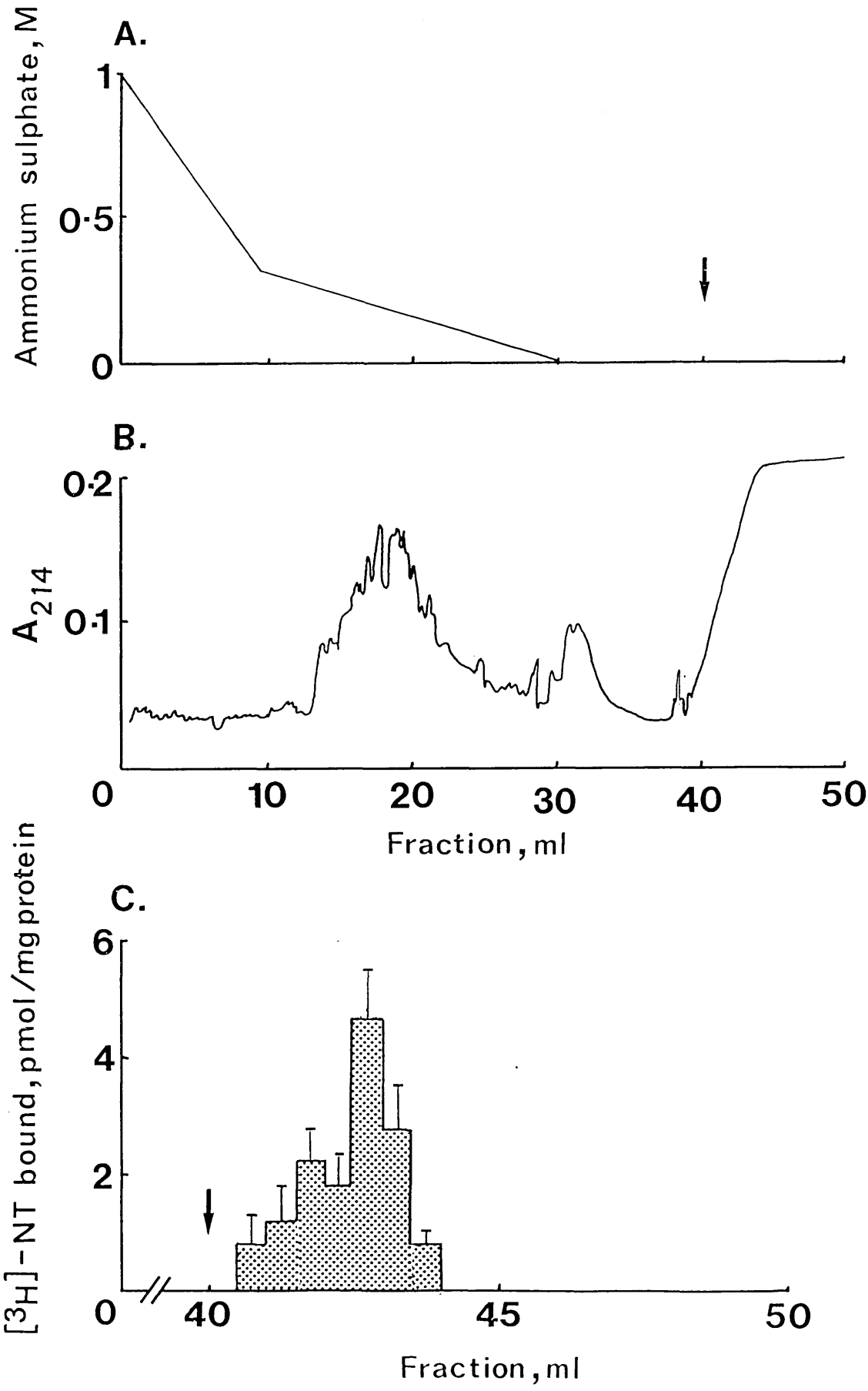
To ascertain whether the yield and purification of the NT binding sites could be improved using this technique a number of modifications were investigated using an FPLC Phenyl Superose column.

5.3.2 FPLC HYDROPHOBIC CHROMATOGRAPHY

Solubilized bovine membranes were chromatographed on an FPLC Phenyl Superose column using two linear concentration gradients of ammonium sulphate (Fig. 32, A). The proteins eluted were monitored by measuring the absorbance at 214 nm (Fig. 32, B). As previously observed (5.3.1), the majority of the bound proteins eluted at ammonium sulphate concentrations of < 0.4 M. Elution with 2% (w/v) digitonin (Fig. 32, arrow) resulted in a large increase in absorbance mainly due to the detergent itself and was therefore not indicative of the protein concentration which was determined separately (4.2.3).

In the representative experiment shown in Fig. 32 the [^{3}H] NT binding activity in the major peak eluted with digitonin (Fig. 32, C) gave a 45 fold purification as compared to the activity present in the crude soluble extracts. This value ranged from 35 to 50 fold in a series of six experiments; all of the retained [^{3}H] NT binding activity (90 $\pm$ 2%) was eluted with 2% (w/v) digitonin. The higher fold purification achieved by the use of FPLC

FIG. 32. FPLC hydrophobic chromatography of Mg^{2+}/digitonin solubilized bovine cortex membranes. Proteins were bound to an FPLC phenyl superose column in 1 M ammonium sulphate and eluted with linearly decreasing concentration gradients of ammonium sulphate (A). The protein efflux was monitored at 214 nm (B). The NT binding activity was eluted with 2% (w/v) digitonin (arrow). Aliquots of the digitonin eluted fractions were assayed for specific [^{3}H] NT (5 nM) binding activity (C). The values represent the mean of triplicate determinations $\pm$ SEM values. These results are from one of six independent experiments each of which gave similar data.


A.
Ammonium sulphate, M
1
0·5
0
B.
A_{214}
0·2
0·1
0
10
20
30
40
50
Fraction, ml
C.
$[^3H]$-NT bound, pmol/mg protein
6
4
2
0
40
45
50
Fraction, ml

hydrophobic chromatography can be attributed to the improvement in the separation of bound proteins by the use of linear ammonium sulphate gradients. Additionally, the faster elution of the bound proteins (5.2.2) minimizes the receptor inactivation due to proteolytic degradation and protein oxidation.

Irrespective of variations in the method used the hydrophobic chromatography technique resulted in a partial purification of the NT binding sites which were inactivated after storage at -20°C; this inactivation could not be prevented by the addition of either Mg^{2+} or glycerol to the digitonin eluates. The [^{3}H] NT binding activity, however, remained stable for 18-24 hours at 4°C and therefore this technique can be used as a preliminary purification step.

5.3.3 WHEAT GERM LECTIN CHROMATOGRAPHY

The glycoprotein nature of the NT receptor of bovine brain was investigated by wheat germ lectin chromatography.

In four separate experiments all of the applied specific [^{3}H] NT binding activity was retained by the lectin and 80 $\pm$ 7% of this was eluted by the addition of N-acetyl-β-D-glucosamine; a representative profile of this activity is shown in Fig. 33. This procedure gave a purification of only 15-20 fold (range of 4 experiments) as compared to the activity present in the Mg^{2+}/digitonin crude extracts although the protein composition of the sugar eluates, as evidenced by SDS-PAGE (Fig. 34), was qualitatively

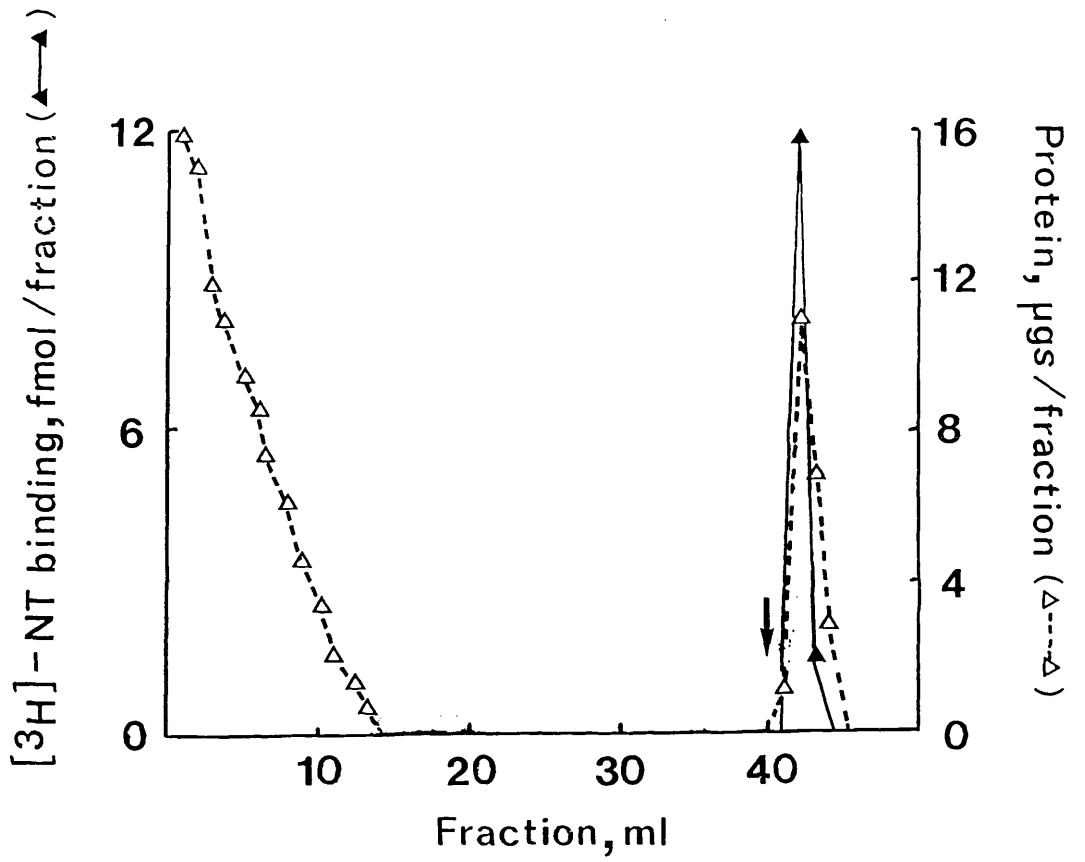
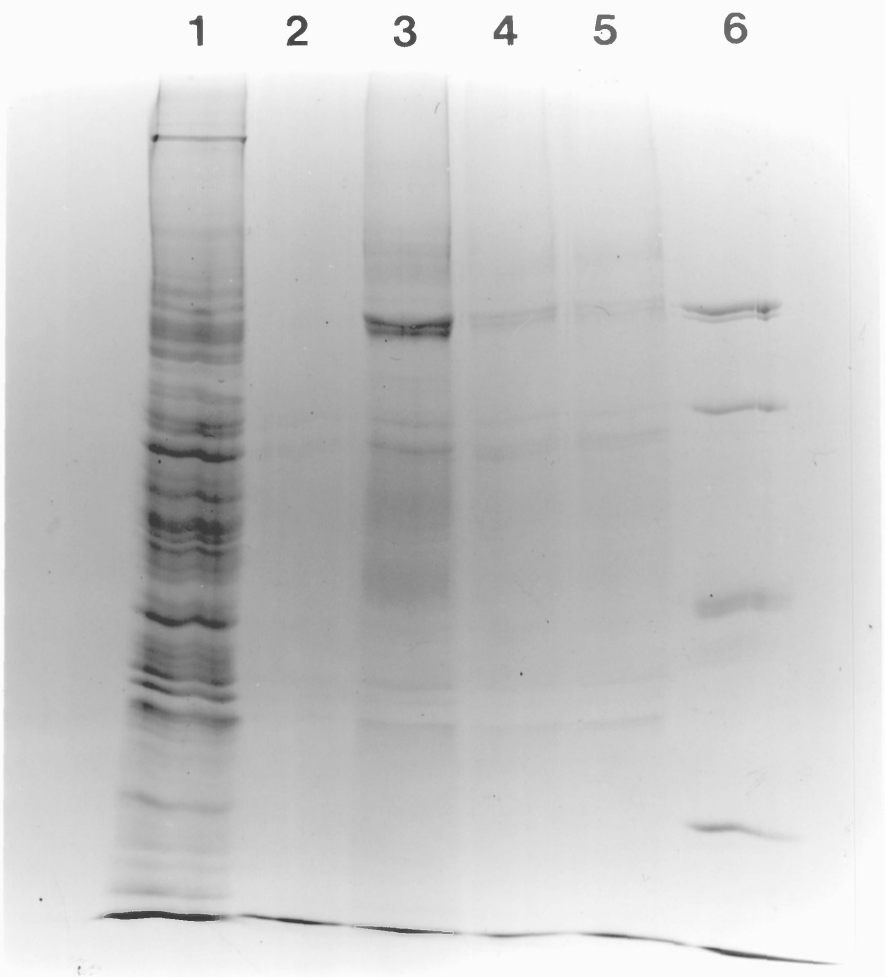


FIG. 33. Wheat germ lectin chromatography of Mg^{2+}/digitonin solubilized bovine cortex membranes. Solubilized membranes were chromatographed on a wheat germ lectin-sepharose 6MB column as described (5.2.3). The elution of bound glycoproteins was carried out by the addition of 0.2 M N-acetylglucosamine (arrow). Fractions (1 ml) were assayed for specific [^{3}H] NT (5 nM) binding activity and protein content (4.2.2, 4.2.3).

FIG. 34. Coomassie blue staining of wheat germ lectin chromatography eluates after SDS-PAGE. Proteins specifically bound to the wheat germ lectin column were eluted with 0.2 M N-acetylglucosamine (4 x 1 ml). Aliquots of 0.5 ml from each fraction were precipitated, electrophoresed on a 10% polyacrylamide-SDS gel and stained with Coomassie blue. Lane 1, crude soluble extract; lanes 2-5, N-acetylglucosamine eluates; lane 6, molecular weight markers (91, 66, 45 and 21 kDa, respectively).



different.

The use of FPLC hydrophobic chromatography and wheat germ lectin chromatography in tandem (2 experiments) did not give a higher fold purification than that obtained by hydrophobic chromatography alone.

Despite the lower purification achieved by wheat germ lectin chromatography this technique may, however, be preferable to hydrophobic chromatography since the specific [^{3}H] NT binding activity is eluted in 0.1% digitonin. The partially purified NT receptor activity can therefore be applied directly onto an affinity column without having to dilute the detergent prior to application. Both the lectin and the hydrophobic chromatography (FPLC) purified preparations were used in the subsequent purification of the NT receptors by ligand affinity chromatography.

5.3.4 NT AFFINITY CHROMATOGRAPHY

For the affinity gel used in the NT affinity chromatography studies reported here NT was immobilized on Affi-Gel 10 beads (5.2.4.1). Since the N-terminus of NT is blocked by a pyroglutamyl residue the N-hydroxysuccinimide reactive group on the spacer arm can be expected to react only via the side chain groups of Lys^6 or Asn^5 of NT. This would leave free the 8-13 segment of NT which is essential for receptor binding (1.6.2.2; 2.3.5).

In a series of preliminary experiments bovine NT receptor partially purified by either FPLC Phenyl Superose or wheat germ lectin chromatography was applied to the NT

affinity gel. Although 50-70% (range of 4 experiments) of the specific [^{3}H] NT binding activity was retained on the column less than 2% of this was eluted by the addition of 10^{-5} M NT. A slight improvement in the yield of recovered binding activity (4-6%; range of 3 experiments) was obtained when Mg^{2+}/digitonin solubilized membranes were chromatographed without any prior purification.

To allow for the possibility of delipidation occurring during the ligand affinity chromatography bovine membranes were solubilized as described (4.2.1.3) but in the presence of 0.06% (w/v) asolectin which was also present at the same concentration in the chromatography buffers. Using this Mg^{2+}/digitonin/asolectin solubilized preparation the binding of [^{3}H] NT to the solubilized binding sites was found to be identical to that obtained in the absence of added phospholipid; binding to the NT affinity column was 40-70% of that applied (range of 3 experiments) and 8-10% of the retained binding activity could be recovered by NT elution.

The low recovery of binding activity from the column after NT elution was not significantly improved by elution with 10^{-5} M [Gln^4] NT and it was subsequently found that, in both cases, NT receptor activity was present in the Na^+-cholate wash of the column after each run. Since the SDS-PAGE profiles of the NT or [Gln^4] NT eluted and Na^+-cholate eluted protein were found to be the same, recovery of the tightly bound receptor was accomplished by

elution with 250 mM NaCl. It had been shown previously that Na^+ decreases the affinity of the bovine NT receptor for its ligand (2.3.6) and complete recovery of receptor bound to the column was possible using this method.

5.3.5 PROPERTIES OF THE PURIFIED NT RECEPTOR

Following the removal of NaCl by dialysis, the binding of [^{3}H] NT to the purified receptor was specific and appeared to be saturable within the same concentration of [^{3}H] NT seen for the crude soluble receptor (Fig. 35): non-specific binding was low and even at the highest [^{3}H] NT concentration used it was less than 30% of the total binding. A Scatchard plot of the data (Fig. 35, inset) identified a single set of sites with an equilibrium dissociation constant similar to that observed in crude Mg^{2+}/digitonin/asolectin extracts (Table 18).

On the basis of the estimated protein content of the purified receptor (5.2.4.3) the fold purification achieved in three experiments was in the range of 18,000-36,000 (Tables 18, 19) when compared with the NT binding activity present in intact bovine cortex membranes. Comparison with the activity in crude soluble extracts gave a higher fold purification, 30,000-51,000 (Tables 18, 19), since there was some loss of binding activity during the detergent extraction of the membranes (4.3.3).

SDS-PAGE of the purified receptor protein, when denatured in the presence of 100 mM DTT, showed a single band of M_r 72,000 after either Coomassie Blue staining or

Table 18. The binding parameters of the bovine brain NT receptor.

Sample	K_D (nM)	B_{max} (fmol/mg protein)	n
Membranes	3.3 ± 0.2	350 ± 10	5
Crude soluble	5.5 ± 0.2	250 ± 15	5
Purified	5.5 ± 0.1	12.7 x 10^6–7.4 x 10^6	3

The parameters were determined from Scatchard plots of the equilibrium specific binding data. Values are the mean ± SEM of a number of independent experiments (n), except that for the purified samples, due to the variation in the estimated (5.2.4.3) values of the protein content, the B_{max} values are the highest and lowest obtained.

Table 19. Purification of the bovine brain NT receptor.

Sample	Total protein (mg)	Specific binding (fmol/mg protein)[a]	Purification factor	Recovery (%)
Membranes	54	260	1	100
Soluble	19.3	154	0.6[b]	21.2[b]
Purified[c]	4.4×10^{-4}	4.7×10^{6}	18,000;36,000[d]	14.8[b]

[a]The activity was determined at 12 nM [^{3}H] NT in the assay conditions described (2.2.3.1; 4.2.2).

[b]Not corrected for the higher K_D value in solution (see Table 18) and therefore an underestimate.

[c]Values for a single preparation (except where noted). For the range of binding activities found see Table 18.

[d]The maximum purification found in the series of preparations and using the B_{max} values was 36,000-fold (see Table 18) relative to the membrane specific activity.

FIG. 35. Specific binding of [^{3}H] NT to the purified bovine NT receptor. Bovine NT receptor purified on NT-Affi-Gel 10 was assayed for specific [^{3}H] NT binding activity as described (4.2.2) in the presence of 0.1% (w/v) digitonin/0.06% (w/v) asolectin. All points denote the mean of triplicate determinations and SEMs were less than 10% of the mean. Similar results were obtained in three independent experiments.

Inset: Scatchard plot.

The variation in the mean values between experiments (n = 3) was 5% or less.

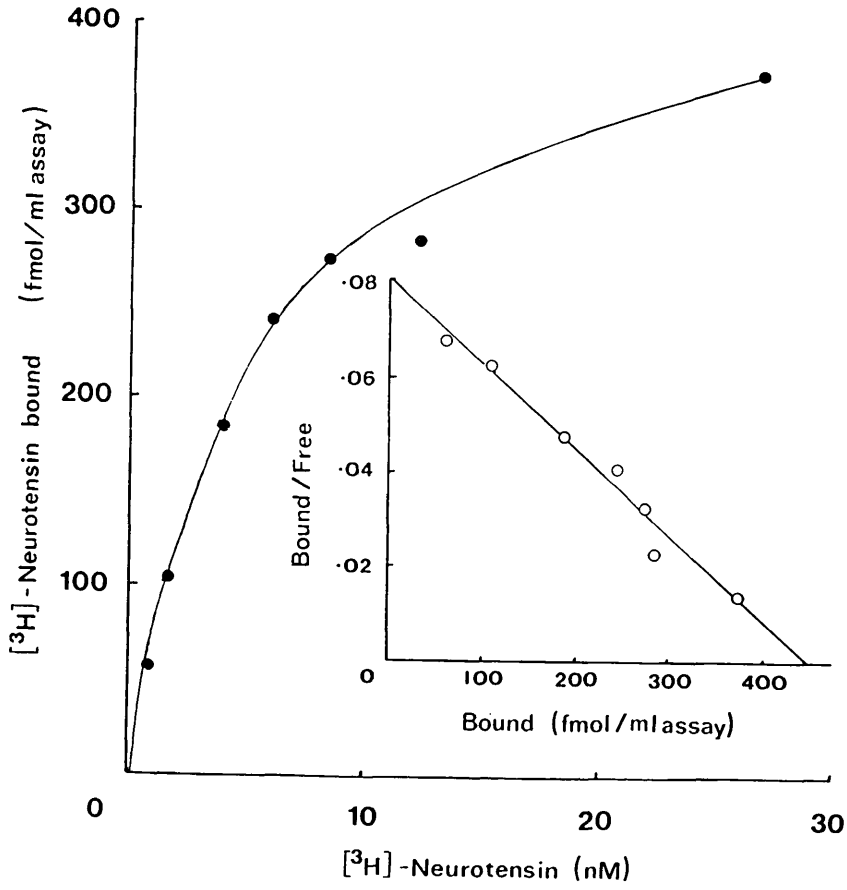
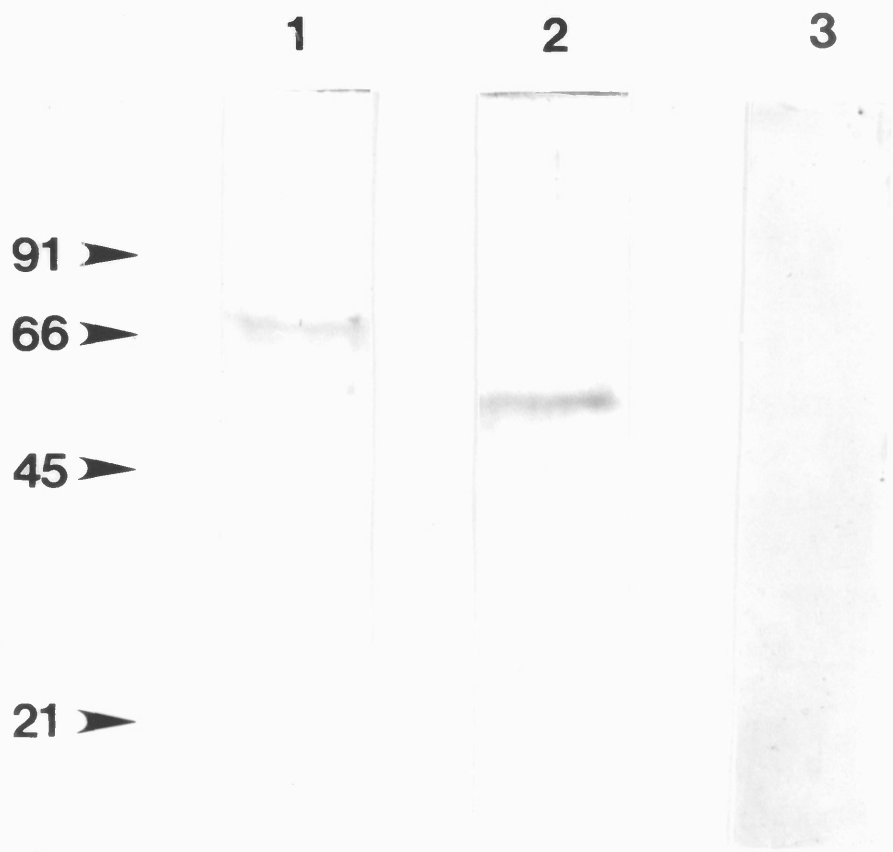


FIG. 36. SDS-PAGE of the purified bovine NT receptor. The protein eluted from the NT-Affi-Gel 10 affinity column was analyzed on a 10% polyacrylamide gel, with silver staining. The receptor, $\tilde{}$ 350 ng, was first dentured with SDS at 100°C either with 100 mM DTT (lanes 1 and 3) or without DTT (lane 2). Lane 3, an equivalent volume of sample from a control preparation, in which crude extract was preincubated with 10^{-6} M NT (1 h, 20°C) prior to its application to the affinity gel, was similarly analyzed; all stages were as for the unblocked preparation of lane 1, but the gel was overstained for the detection of any minor bands. The migration positions (kDa) of standard proteins (phosphorylase b, BSA, ovalbumin and soybean trypsin inhibitor) are shown on the left. Coomassie blue staining of similar gels showed only the one band seen here.


1
2
3
91
66
45
21

silver staining (Fig. 36, lane 1). Under non-reducing conditions (Fig. 36, lane 2) a single band of M_r 50,000 was instead visible, suggesting the presence of intra-molecular disulphide bonds. A larger amount of aggregated protein was observed on the top of the gel under non-reducing conditions (Fig. 36, lane 2) indicating that there was some aggregation of the protein during precipitation for SDS-PAGE. Increases in apparent M_r after strong DTT reduction have been found, and interpreted similarly, for several other homopolymeric receptors including the β_2-adrenergic receptor (Fraser and Venter, 1980), the interleukin-3 receptor (Sorenson et al., 1986), a gonadotropin receptor (Wimalasena et al., 1986) and the u opioid receptor (Gioannini et al., 1985; Newman et al., 1986).

In a control experiment, in which an excess of free NT was incubated (1 h, 20°C) with the sample prior to the stage of binding to the NT-Affi-Gel 10 column (with all other steps as before), no protein bands were detected after analysis by SDS-PAGE even after overstaining the gel (Fig. 36, lane 3); this pre-blocking effect confirmed that the M_r 72,000 polypeptide (M_r 50,000 non-reduced), which was also seen after NT or [Gln^4] NT elution, was specific to the NT receptor.

On the basis of the apparent size (M_r 72,000) of the single protein identified by SDS-PAGE, the theoretical binding activity (assuming one binding site per receptor molecule) would be 13.9 umol/g protein; this is reasonably

close to the B_{max} (7.4-12.7 umol/g protein) obtained from the saturation binding measurements on the purified receptor. Taking into account the difficulty in estimating the low amounts of protein present, these results suggest that the NT receptor has been purified to homogeneity.

5.4 SUMMARY

It is true of many receptors, including the u opioid receptor (Gioannini et al., 1985; Cho et al., 1986), the atrial natriuretic factor receptor (Takayanagi et al., 1987) and the muscarinic acetylcholine receptor (Haga and Haga, 1985), that several chromatographic steps are required in sequence for the purification of the protein to homogeneity. Considering this, the techniques of wheat germ lectin- and hydrophobic chromatography were investigated with respect to the partial purification of the bovine NT receptor prior to NT affinity chromatography. Both of these procedures gave only moderate improvements in the purity of the preparation but did show the NT receptor to be a highly hydrophobic glycoprotein. Subsequently, it was found that the complete purification of the NT receptor was possible in a single step using NT affinity chromatography.

A single protein band of M_r 72,000 was evident following SDS-PAGE analysis and, assuming one [^{3}H] NT binding site per receptor molecule of M_r 72,000, the theoretical specific binding activity calculated was approximate to the B_{max} value obtained from the equilibrium binding studies with the purified receptor. These two observations indicate that the NT receptor has been purified to homogeneity.

Apparent molecular weights, as measured under similar conditions, in the region of 70,000-80,000 daltons have been reported for other single chain receptor species associated with G-proteins e.g. the β-adrenergic receptor (M_r 65,000-

70,000; Fraser and Venter, 1980), the muscarinic acetylcholine receptor (M_r 80,000) of a number of species (Venter and Fraser, 1984; Haga and Haga, 1985; Kerlavage et al., 1987) and the rat u opioid receptor (M_r 66,000-72,000; Gioannini et al., 1985; Barnard et al., 1986; Newman et al., 1986). The sizes of the two protein subunits of the NT receptor (M_r 49,000 and 51,000) previously identified in rat brain membranes by photolabelling and cross-linking studies (Mazella et al., 1985) are in disagreement with the single, larger size observed here. This may be the result of proteolytic degradation and/or deglycosylation or may indicate the existence of receptor subtypes as exemplified by the β_1 and β_2 adrenergic receptors which are monomeric and dimeric, respectively (Venter and Fraser, 1984).

6. SUMMARY AND FUTURE PROSPECTS

6. SUMMARY AND FUTURE PROSPECTS

In this thesis [^{3}H] NT binding to membranes of rat and bovine brain and to whole cells of the murine neuroblastoma cell line N1E-115 were investigated in an attempt to characterize the system and resolve some of the controversy surrounding the binding properties of the mammalian NT receptors. A single class of [^{3}H] NT binding sites was observed in all cases from equilibrium binding studies although, in bovine membranes kinetic studies identified two populations of sites the significance of which is unclear. The ability of the various NT fragments and analogues to displace specifically bound [^{3}H] NT was similar in rat brain membranes and N1E-115 cells and correlated well with the results reported in the literature. However, the rank order of potencies of these compounds in bovine brain differed from those observed in the other two tissues examined providing evidence for the existence of species related NT receptor subtypes. The binding properties of all of the [^{3}H] NT binding sites examined in this study fulfilled most of the criteria necessary for their classification as receptors and were therefore identified as putative NT receptors.

Solubilization of the [^{3}H] NT binding sites of bovine brain in an active form and with binding properties similar to those observed in intact membranes was achieved using 2% (w/v) digitonin in the presence of 2mM Mg^{2+}. The specific [^{3}H] NT binding components of this crude soluble extract were resolved into two species by gel filtration

chromatography or chromatofocussing. Lectin affinity- and hydrophobic chromatography showed that the NT receptors are highly hydrophobic glycoproteins.

Purification of the bovine NT receptor to homogeneity was possible in one step using NT affinity chromatography. SDS-PAGE of the purified receptor under reducing conditions revealed a single protein band of M_r 72,000. Under non-reducing conditions a single band of M_r 50,000 was identified by SDS-PAGE suggesting that the receptor contains intra-molecular disulphide bonds. Since larger molecular weight species were not identified under these conditions it would appear that the receptor does not form disulphide bond linked oligomers; this does not exclude the possibility that an oligomeric form of the receptor may be stabilized by ionic or hydrophobic interactions. In view of this, and the Gpp(NH)p sensitivity of the solubilized receptor, it is possible that the higher molecular weight species observed in gel filtration studies may represent an oligomeric form of the receptor or alternatively is a receptor-guanine nucleotide regulatory protein complex. Further investigations, however, are required in order to confirm this as well as to elucidate the nature of the two populations identified in chromatofocussing studies.

The method described here for the purification of the bovine NT receptor utilizes the native ligand and is thus applicable to all NT receptors that can be successfully solubilized. Furthermore, the purification procedure is

both simple and rapid and, when scaled up, could allow the purification of large amounts of receptor protein which may be used for:

1. Reconstitution studies,

2. The production of polyclonal and/or monoclonal antibodies, and

3. The determination of protein sequence which could be used to begin the cDNA cloning of the receptor. Oligonucleotide probes specific for the NT receptor can be constructed once the protein sequence is known and used to screen a cDNA library. The RNAs produced from any positive cDNA clones can then be tested for the correct expression of the receptor by injection into Xenopus oocytes, a system which has been shown to express functional NT receptors following injection of brain specific mRNAs (Parker et al, 1986, Hirono et al., 1987).

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APPENDIX 1: MATERIALS

[3,11-tyrosyl-3,5,-^{3}H] Neurotensin (40-70 Ci/mmol) was purchased from New England Nuclear and neurotensin and its analogues from Cambridge Research Biochemicals. 1,10-Phenanthroline, polyethylenimine, dithiothreitol, bovine serum albumin (fraction V and Radioimmunoassay grade), soybean trypsin inhibitor, bacitracin, benzamidine, Triton X-100, Lubrol PX and the buffers TES, Tris and HEPES were from Sigma. Sodium deoxycholate was from Koch Light, CHAPS from Serva, digitonin from BDH and Gpp(NH)p from Boehringer. All other materials were of the highest purity available commercially.

APPENDIX 2: PUBLICATIONS

Mills, A., Demoliou-Mason, C.D. and Barnard, E.A. Characterization of neurotensin binding sites in intact and detergent solubilized bovine brain membranes. J. Neurochem (in press).

Mills, A., Demoliou-Mason, C.D. and Barnard, E.A. Purification of the neurotensin receptor from bovine brain. J. Biol. Chem. (in press).

APPENDIX 3: ADDITIONAL DATA

Table 20. Inhibition of the binding of [^{3}H] NT to purified bovine neurotensin receptor.

Peptide	K_D^I (nM)
NT	5.4
[Gln^4] NT	0.2
NT (8-13)	1.4
NT (1-12)	NC
NT (1-8)	NC

Purified bovine neurotensin receptor was incubated with 5 nM [^{3}H] NT and varying concentrations of NT or it's analogues. K_D^I values are the mean of two independent experiments. NC indicates no competition detected at 10^{-5} M peptide.