Cellular responses elicited by stimulation of neuronal nicotinic acetylcholine receptors

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Cellular Responses Elicited by Stimulation of Neuronal Nicotinic Acetylcholine Receptors

Submitted by

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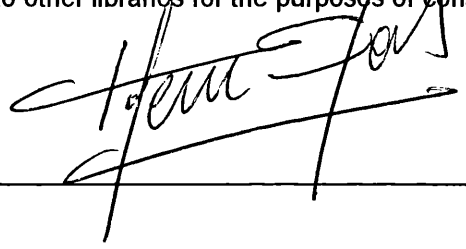

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SUMMARY

The nAChR are ligand gated cation channels formed by the assembly of a variety of receptor subunits into a pentameric transmembrane structure permeable to both Na^+ and Ca^{2+}. Although a considerable amount of information has been gathered about the molecular structure, pharmacology and biophysical properties of nAChR, the role of nAChR in the modulation of various cellular processes of the nervous system has only become apparent in the last decade. The existence of multiple subtypes of neuronal nAChR, the non-uniform distribution in the nervous system and their localisation to both pre- and post-synaptic zones, imply diverse functions for the nAChR. In effect, the ability of nicotine to increase cytoplasmic Ca^{2+} concentrations should make the activation of nAChR an extremely sensitive tool for relying information from activity dependent inputs to the nucleus. The aim of this work was to investigate some of the intracellular signalling processes activated by the stimulation of nAChR in neuronal preparations.

The first part of the project analysed the putative neuroprotective properties of nicotine stimulation using cultures from the hippocampus, a brain region involved in memory and in the progression of Alzheimer's disease. Stimulation with nicotine prevented the NMDA-evoked neuronal loss, an effect that was mediated by the α7 nAChR through a Ca^{2+}-dependent cellular mechanism acting downstream to the Ca^{2+} overload generated by NMDA. Subsequently, the project focused on the analysis of the Ca^{2+} signals activated by the stimulation of nAChR. Using the human SH-SY5Y cell line as a model system it was demonstrated that, similar to hippocampal neurones, nicotine evoked sustained elevations of cytoplasmic Ca^{2+}. The study of these Ca^{2+} signals indicated that besides the direct influx through the nAChR channel and the activation of VOCC, nAChR Ca^{2+} responses involved the activation of store dependent Ca^{2+}-induced and IP_3-mediated Ca^{2+} release.

The recruitment of intracellular Ca^{2+} stores provides further complexity to the Ca^{2+} signals activated by stimulation of nAChR. Eventually, much of the capacity to regulate intracellular mechanisms depends on the activation of specific signalling molecules. In the final part of the present work, it was established that in both SH-SY5Y cells and hippocampal neurones, the activation of α7 nAChR regulates the activity of the ERK cascade using a novel Ca^{2+} and PKA dependent signalling pathway. This mechanism, however, was not mediating the previously observed nicotine neuroprotection.

Although the α7 nAChR constitutes a precise tool for the activation of specific Ca^{2+} signals, we still do not know the exact function of the nAChR-mediated ERK signalling cascade. Probably, the activation of ERK following stimulation of the α7 nAChR is related to the proposed function of nAChR in memory processing. Considering the role that ERK has in synaptic plasticity, and the evidence showing the involvement of nAChR in memory, it is thus suggested that α7 nAChR and the ERK signalling cascade could mediate neuronal plasticity processes in the CNS.

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Ubi dubium ibi libertas

Where there is doubt, there is freedom

Latin proverb

To my parents

and

Virginia.

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Abbreviations

Aβ	β-amyloid peptide
ABP	AMPA receptor binding protein
ACh	acetylcholine
α-Ctx-AulB	α-conotoxin AulB
α-Ctx-IMI	α-conotoxin IMI
α-Ctx-MII	α-conotoxin MII
AD	Alzheimer's disease
ANOVA	Analysis of variance
APP	amyloid precursor protein
α-Bgt	α-bungarotoxin
AL	nucleus of the ansa lenticularis
AnTx-a	anatoxin-a
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
BDNF	brain derived neurotrophic factor
CA	cornu ammonis
CaMK	Ca^{2+}/calmodulin dependent protein kinase
cAMP	cyclic AMP
CaRE	Ca^{2+} response element
CICR	Ca^{2+} induced Ca^{2+} release
CNS	central nervous system
CRE	cAMP regulatory element
CREB	cAMP response element binding protein
DA	dopamine
DAG	diacylglycerol
DAT	dopamine transporter molecule
DHβE	dihydro-β-erythroidine
DMEM	Dulbecco's Modified Eagle's Medium
E18	embryonic day 18
EBS	exposure buffer solution
EC_{50}	agonist concentration which gives half-maximal response
EGTA	ethylenglycol-bis-(β-aminoethylether)-N,N,N',N'-tetracetic acid
EPSC	excitatory post-synaptic current
EPSP	excitatory post-synaptic potential
ER	endoplasmic reticulum
5-HT	5-hydroxy triptophan
GABA	γ-amino-butyric acid

GAP	GTPase activating protein
GEF	guanine nucleotide exchange factors
GKAP	guanylate kinase associated protein
GP	globus pallidus
GRIP	glutamate receptor interacting proteins
HDB	nucleus of the horizontal limb of Broca
IGF-1	insulin-like growth factor 1
IP_3	inositol 1,4,5-trisphosphate
IP_3R	inositol 1,4,5-trisphosphate receptor
IPSP	inhibitory post-synaptic potential
JNK/SAPK	c-Jun amino-terminal kinase/stress-activated protein kinase
LGIC	ligand gated ion channel
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen activated protein kinase
MEK	MAPK/ERK kinase
MEKK	MEK kinase
MLA	methyllycaconitine
MPA	magnocellular preoptic area
MY	basal nucleus of Meynert
nAChR	nicotinic acetylcholine receptor
MPTP	1-methyl-4-phenyl-1,2,3,5-tetrahydopyridine
MTT	3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide
MS	medial septum
NAT	noradrenaline transporter molecule
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NSF	N-ethylmaleimide sensitive fusion protein
PBS	phosphate buffer saline
PD	Parkinson's disease
PICK1	protein interacting with C kinase
PI3-kinase	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PSD	post-synaptic density
RyR	ryanodine receptor
SD	standard deviation of the mean

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SERCA	sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase
SERT	serotonin transporter molecule
SI	substantia innominata
SN	substantia nigra
TSS	Tyrode's salt solution
VDB	nucleus of the vertical limb of Broca
VOCC	voltage operated Ca^{2+} channels
VP	ventral pallidum
VTA	ventral tegmental area

CHAPTER 1

< GENERAL INTRODUCTION >

1.1 The Nicotinic Acetylcholine Receptor (nAChR)

1.1.1 nAChR and the development of Neuroscience

The elucidation of the role and function of nicotinic acetylcholine receptors (nAChR) has played a crucial part in the progress of modern neuroscience. A simple examination of the major contributions to the understanding of the mechanisms of neurotransmission shows how the nAChR figure prominently in many of the cornerstone advances that have occurred in the last 150 years.

It was in the mid nineteenth century that the existence of synaptic transmission and, indirectly, the presence of ligand gated ion channels (LGIC) was first suggested by Claude Bernard (1857) on the basis of the blockage of the neuromuscular junction by curare. In spite of these early studies, research on neuronal nAChR really started in the 1880's with the experiments of Langley on the cat superior cervical ganglion, where nicotine was used as a tool to map the distribution of the sympathetic fibres. More than a decade and a half later, Langley discovered how the application of nicotine to ganglionic preparations generated "effects like those produced by brief stimulation of its preganglionic fibres". Following these studies, experiments on the skeletal muscle allowed him to define the existence of "receptive substances", now more commonly known as receptors, and establish them as specific physiological targets, capable of "receiving the stimulus" and "transmitting it" (Langley, 1914). Although for many years this newly defined "receptive substance" was considered almost as a "mythical" entity, Langley's work allowed the formulation of receptor and signal transduction concepts, setting the foundation for modern neuropharmacology.

In 1914, Sir Henry Dale, one of Langley's students, observed that the action of the esters of choline could be mimicked in some tissues by an alkaloid from *Nicotiana tabacum* (nicotine) and in other tissues by another alkaloid from *Amanita muscaria* (muscarine). In doing so, Dale proved that the same substance (now known to be acetylcholine) could mediate distinctive responses in tissues through different molecules, therefore defining the existence of different receptor subtypes. Ironically, and in spite of all his major contributions to the understanding of neuronal function, Dale never used the term "receptor".

Almost fifty years later, the finding of α-bungarotoxin (α-Bgt) irreversible inhibition of neuromuscular transmission led to the isolation of the embryonic muscle nAChR (Changeux et al., 1970). In spite of this apparent delay in the isolation of the receptor itself, studies by Katz and colleagues in the 1950's and throughout the 1970's relied heavily on the amphibian and mammalian neuromuscular junction to uncover the principles of quantal release of transmitters (Fatt and Katz, 1952; Katz and Miledi, 1965), synaptic delay times and the deconvolution of synaptic noise to single channel events (Katz and Miledi, 1965; 1972), all of which involved the cholinergic synapse and nAChR. In effect, it can certainly be said that by nature's ability to produce precise pharmacological tools targeting the nAChR, as well as due to the advances in experimental techniques applicable to the study of the endplate region of the neuromuscular junction, the nAChR has secured its position as one of the most studied of all receptors in the history of neuroscience.

At present, the research on nAChR has expanded into the study of the neuronal types of nAChR and their role in several processes affecting the function of the brain. The discovery that neuronal nAChR, unlike muscular ones, exist in a variety of subtypes and locations, along with the observation that they can affect several major brain processes like addiction, anxiety, synaptic plasticity, memory processing, neuronal survival, and the progression of neurodegenerative diseases, have been major driving forces in a field in continuous evolution. As part of this progress, a recent and unforeseen discovery emphasised the past and future role of nAChR in the understanding of neuronal transmission. In two consecutive papers, Sixma and colleagues (Smit et al., 2001; Brejc et al., 2001) revealed how in the *Lymnea stagnalis* snail, a glia-derived "releasable substance", similar in sequence and structure to the extracellular domain of the nAChR, can interact with neuronally released acetylcholine, thereby modulating neurotransmission at cholinergic synapses. In fact, the finding of this unique mechanism for controlling synaptic transmission demonstrates that the role of the "nicotinic acetylcholine receptive substance" in the understanding of the novel fundaments of neuronal function is far from over yet.

1.1.2 The superfamily of ligand gated ion channels (LGIC)

Receptors that mediate vertebrate neurotransmission can be divided into two groups: the ionotropic and metabotropic receptors. The ionotropic receptors, which are able to mediate fast transmission, are usually defined as LGIC. Three main families are distinguished among the LGIC: the family of glutamate receptors (kainate/α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, AMPA, and N-methyl-D-aspartate, NMDA), the family of the purinergic receptors and the family of the nAChR. This last one includes the inhibitory γ-

amino-butyric acid ($GABA_A$ and $GABA_C$) receptors, glycine receptors, serotoninergic 5-hydroxy-tryptophan receptors ($5\text{-}HT_3$) and the nAChR (Fig 1.1). Whilst the $GABA_A$ and glycine receptors are selectively permeable to anions and therefore usually inhibitory, the nAChR and $5\text{-}HT_3$ receptors are excitatory by being selective for cations.

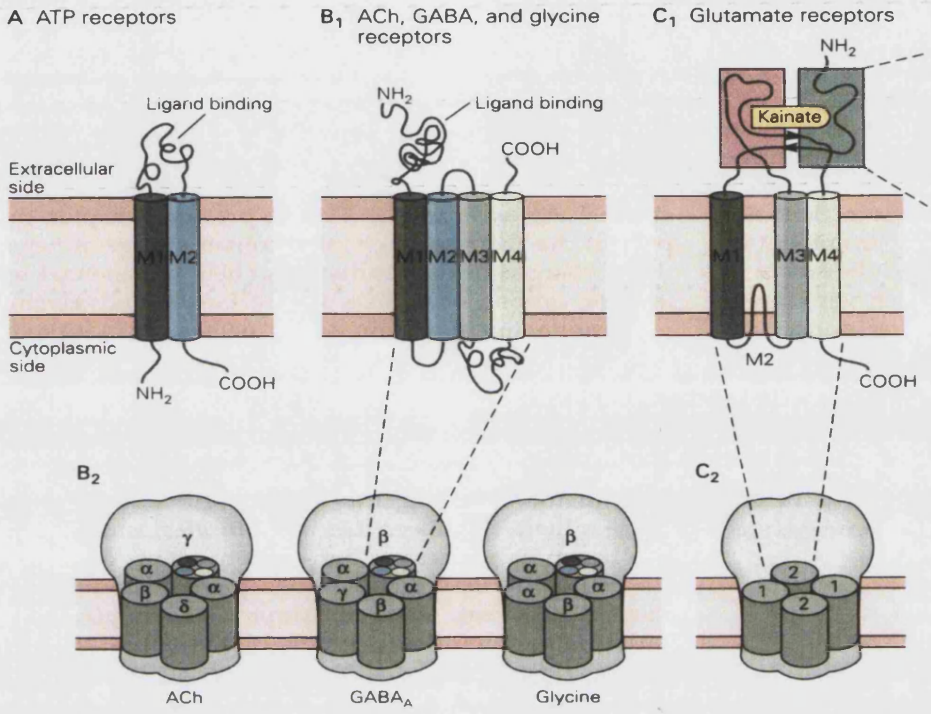

Fig 1.1 Schematic representation of the ligand gated ion channel (LGIC) family members. Three main groups are distinguished: the family of purinergic receptors (A), the family of the nAChR (B_1 and B_2), which also includes the $GABA_A$ and glycine receptors, and the family of glutamate receptors (C_1 and C_2), which include the kainate, AMPA and NMDA receptors. All members of the LGIC superfamily are multiprotein complexes forming an ion selective pathway across the membrane. The upper panels show the transmembrane structure of the typical subunit for each family. The lower panel shows the putative arrangement of the subunits in the formed channel. In the case of the nAChR (B_1) the subunits are arranged in a pentameric structure, with the different subunits conferring distinctive functional and structural properties. Unlike the nAChR, glutamate receptors proved to have only three transmembrane domains, plus a cytoplasm-facing re-entrant membrane loop (M2). Also differing with the nAChR, the ligand binding site in glutamate receptors is formed by the N-terminal and the extracellular M3-M4 loop (Adapted from Kandel, 2000).

When studying a protein-complex as intricate as the LGIC, it is common to subdivide it on the basis of its functional domains into a ligand binding site and a channel domain. Although other sites where allosteric effectors, or any other compounds, may bind must also be considered, these two functional domains, which are thought to have evolved separately, constitute the minimal requirement for the existence of a LGIC. Accordingly, it should be possible to exchange functional domains from one ligand-gated channel with another one. The resulting chimera would display the pharmacological properties conferred by the binding site while the ionic pore selectivity and sensitivity would match those of the corresponding donor. The ensemble of these predictions was verified with the construction of the chimeric

receptor that comprises the N-terminal domain of the α7 neuronal nAChR and the rest of its sequence from the 5-HT_3 receptor (Eisele et al., 1993).

In evolutionary terms, the analysis of DNA sequences also supports the notion that the nAChR share a common ancestor, probably homomeric, with the $GABA_A$, the glycine and the 5-HT_3 receptors (LeNovere and Changeux, 1995; Ortells and Lunt, 1995). However, the most elegant evidence for the structural homologies found in this superfamily is revealed by the ability to change the selectivity of the nAChR ion channel from cation to anion by changing only three channel lining aminoacids to those characteristic of glycine and $GABA_A$ receptors (Galzi et al., 1992).

1.1.3 Structure of the nAChR

All nAChR subtypes are acetylcholine (ACh) -gated cation channels (Cooper et al., 1991; Unwin, 1993; Tierney and Unwin, 2000), organised around a pseudo 5-fold axis that delineates a cation-selective pathway across the membrane when the channel is open, but a robust barrier to the ions when it is closed (Fig 1.2b). As a group, the nAChR are part of a heterogeneous assembly of receptors, which are formed as pentamers from different combinations of various genetically distinct subunits. The differential association of these subunits confer distinctive functional and structural properties to the nAChR types that they form (Fenster et al., 1997). However, and with only few exceptions, the subunit composition, stoichiometry and arrangements of naturally expressed nAChR are not known with absolute certainty. The observed variety of known nAChR subunits is thought to have evolved from the primordial homomeric receptor subunits through repeated gene duplication and spontaneous mutations (LeNovere et al., 1995). To date, 17 receptor subunit genes (α1-α10, β1-β4, δ, ε, γ) have been cloned from vertebrates and initially classified according to the presence or absence of a pair of tandem cysteine residues in the putative N-terminal extracellular region. In the α subunits, these residues are near a site known to engage in agonist binding. None of the other subunits (i.e.: β1-β4, δ, ε, γ) have these tandem cysteines (Karlin, 1993).

In its amino acid sequence, each of the homologus nAChR subunits starts with a signal sequence cleaved during translation (Lindstrom, 1995; Sargent, 1993). The large hydrophilic N-terminal of the mature subunit consists of ~200 amino acids thought to form a large domain on the extracellular surface, which carries the multiple loops that participate in neurotransmitter binding site (Fig 1.2a). This is followed by ~90 aminoacids comprising three closely spaced hydrophobic sequences that form the transmembrane domains M1 to M3.

The hydrophobic segments are thought to stack in the membrane with the amphiphilic M2 helix orientated towards the channel lumen to form an ion permeant pathway.

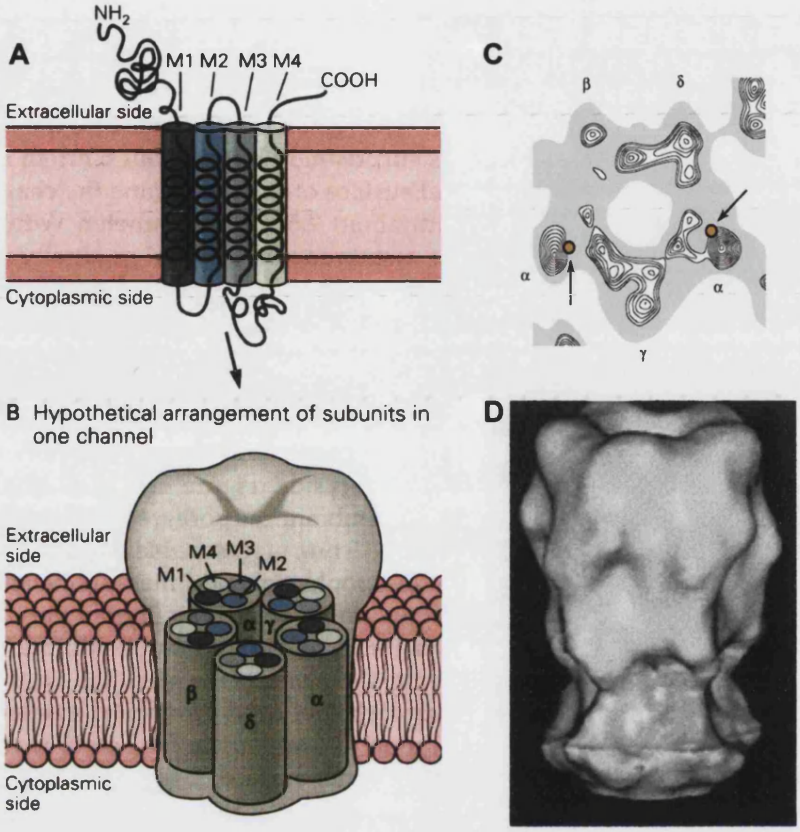

Fig 1.2 Structure of the nAChR

The nAChR are multiprotein pentameric membrane complexes (B, D) that delineate a cation selective pathway across the membrane. The large hydrophilic N-terminal region (200 amino acids) forms a large domain that carries the multiple loops participating in ligand binding (A). Three closely spaced hydrophobic sequences form the M1-M3 transmembrane domains, with the amphiphilic M2 helix orientated towards the channel lumen to form an ion permeant pathway (A, B). Following the M3 transmembrane segment is a large cytoplasmic domain of 100-200 amino acids (A), which constitutes the most variable part of the sequence between subunits, and has several putative phosphorylation sites relevant for signalling processes. An electron-density image of a section of the receptor molecule on the synaptic side of the membrane is shown in C. Arrows indicate the possible site of interaction with the neurotransmitter, and circles indicate the respective positions of the α-Bgt binding sites. In D, an image reconstruction of electron micrographs yielding a structure at 9 Å resolution (Adapted from Kandel et al., 2000 and Unwin, 1993).

The predicted M2 region forms a rather non-selective channel, largely by virtue of hydroxylated serine and threonine residues, which line the internal face and are identical or highly homologous in excitatory (cation channels) and inhibitory (anion channels) receptors (Imoto et al., 1991; Villarroel and Sakmann, 1992; Bertrand, 1993). Ionic selectivity appears to be conferred by rings of fully charged amino acids just outside the bilayer plane (Bertrand et al., 1993). Following the M3 transmembrane segment there is a large cytoplasmic domain of ~100-200 aminoacids, which constitutes the most variable part of the sequence between subunits, and has several putative phosphorylation sites. Next to this large intracellular

domain we find a fourth hydrophobic sequence (M4), finally leading to a small (~ 10-30 aminoacids) extracellular domain at the C-terminus (Fig 1.2a).

Among the structural and functional features that define the LGIC, several structural motifs, which are absent or substantially different in members of the glutamate family in particular, help the characterization of the nAChR family:

- An extracellular N-terminal domain of approximately 200 aminoacids containing the agonist-binding site (Unwin, 1993; Fig 1.1). In glutamate receptors, the agonist-binding site is formed by part of the N-terminal domain and part of the M3-M4 extracellular loop (Dingledine et al., 1999; Fig 1.1).
- A 15 residue cys-loop in the terminal domain (Ortells and Lunt, 1995), which is absent in glutamatergic subunits (Hollman et al., 1994).
- Conserved residues in the second transmembrane segment (M2), proposed to form a primarily α-helical structure lining the ion channel and gating ion-flow (Ortells and Lunt 1995; Unwin et al 1995). Evidence gained using N-glycosylation site tagging (Hollman et al., 1994) and prolactin reporter epitopes (Bennett and Dingledine, 1995) suggests that the "M2" sequence identified in glutamatergic subunits does not traverse the cell's membrane completely, forming instead a re-entrant loop similar to that seen in e.g. K^+ channels. Consequently, the large M3-M4 loop is extracellular and appears to form part of the ligand-binding site, with a C-terminus in the cytoplasmic side (see Fig 1.1c).

At the pentameric structural level, the nAChR are undoubtedly the best characterised of any LGIC. This is largely due to the thorough study of nAChR from skeletal muscle and fish electric organs, whith the latter providing a homogeneous and accessible preparation for electron microscopy studies. In particular, it was the analysis of postsynaptic membranes from the Torpedo electric fish that gave extremely detailed pictures of the pentameric cation channel structure (Fig 1.2d). In these studies, the channel pathway has been reported to narrow across the membrane, but widen into a 20 Å diameter cylinder that extends ~65 Å into the synaptic cleft and ~15 Å into the interior of the cell (Unwin, 1993; Fig 1.2d). The narrow portion of the pore, lined by five α-helical structures from the M2 segment, appears to come together near the middle of the membrane and form the gate of the channel (Unwin, 1993). At the molecular level, it has been proposed that by bending towards the central axis, the helices would allow the leucine-251 large side chains to project inwards and associate in a tight ring, preventing the ions from crossing the membrane (closed channel). In addition, details of the activated receptor suggest that by bending tangentially to the central axis and associating side-to-side, the helices would allow the polar surfaces to become more fully

exposed, while at the same time moving the large side-chains away from the ion path, therefore opening the gate (open channel; Unwin, 1995). The weakening of the "leucine-ring" mode of association of the helices, allowing their side-to-side association around the barrel, must be triggered by localised disturbances at the binding sites in the two α subunits (Fig 1.2c). At the level of the cytoplasmic wall, the receptor was shown to contain openings wide enough for the ions to pass through, made by the spaces between ~30 Å long rods that protrude from each of the subunits towards the cell interior. It was therefore proposed that cations leaving the cell interior are "filtered" through narrow openings in the channel wall. Framing these narrow pores are lines of negatively charged residues, which may help to exclude anions from the vicinity of the transmembrane pore (Miyazawa et al., 1999).

Knowledge about the ligand-binding site comes largely from biochemical studies using high affinity ligands or photo-affinity labelling (reviewed in Galzi and Changeux, 1995) and from functional investigations of receptors engineered by site-directed mutagenesis (reviewed in Bertrand and Changeux, 1999). Most of the evidence obtained up to now indicates that a minimum of two α subunits are present per receptor, forming the ligand binding pockets at the interface with its adjacent subunit (Corringer et al., 1995; Karlin, 1993; Sugiyama et al., 1998; Fig 1.3a). As a result, aminoacid residues from both subunits form the ligand binding structure of the receptor, indicating that the presence of both α and non-α subunits contribute to the physiological and pharmacological properties of nAChR (Couturier et al., 1990; Figl et al., 1992; see review by Bertrand and Changeux, 1995). A particular exception to this rule is given by the α7, α8 or α9 nAChR subunits, which can reconstitute functional homomeric receptors (Schoepfer et al., 1990; Elghoyen et al., 1994; Seguela et al., 1993, Peng et al., 1994). Accordingly, these subunits must possess in their sequences both the principal and complementary components that form the ACh binding pocket, conferring a functional structure probably responsible for the particular characteristics of receptor activation and desensitisation observed with α7, α8 and α9 homomers (see later).

Numerous analyses have identified that the ACh binding pocket results from the contribution of at least six loops. The three loops (A, B, C) of the α subunit are often referenced as the major binding component while the other loops (D, E, F) present on the adjacent subunit are called the complementary component (Bertrand and Changeux, 1995; Galzi and Changeux, 1995; Fig 1.3b). Initial site-directed mutagenesis had highlighted the contribution of aromatic residues in the principal component of the agonist-binding site (Galzi et al., 1991). The participation of these loops has been further documented in several studies where the exchange of short aminoacid sequences has been performed between different subunits (Corringer et al., 1998; Figl et al., 1992). In particular, the introduction of residues between positions 183-191 of the α4 subunit into the α7-5-HT_3 receptor chimera was found to be

sufficient to increase the receptor apparent affinity and equilibrium binding. From these and other microchimera it was concluded that the transfer of residues 151-155 and 183-191 confers typical pharmacological properties of the α4β2 nAChR to the α7-5-HT_3 receptor (Corringer et al., 1998).

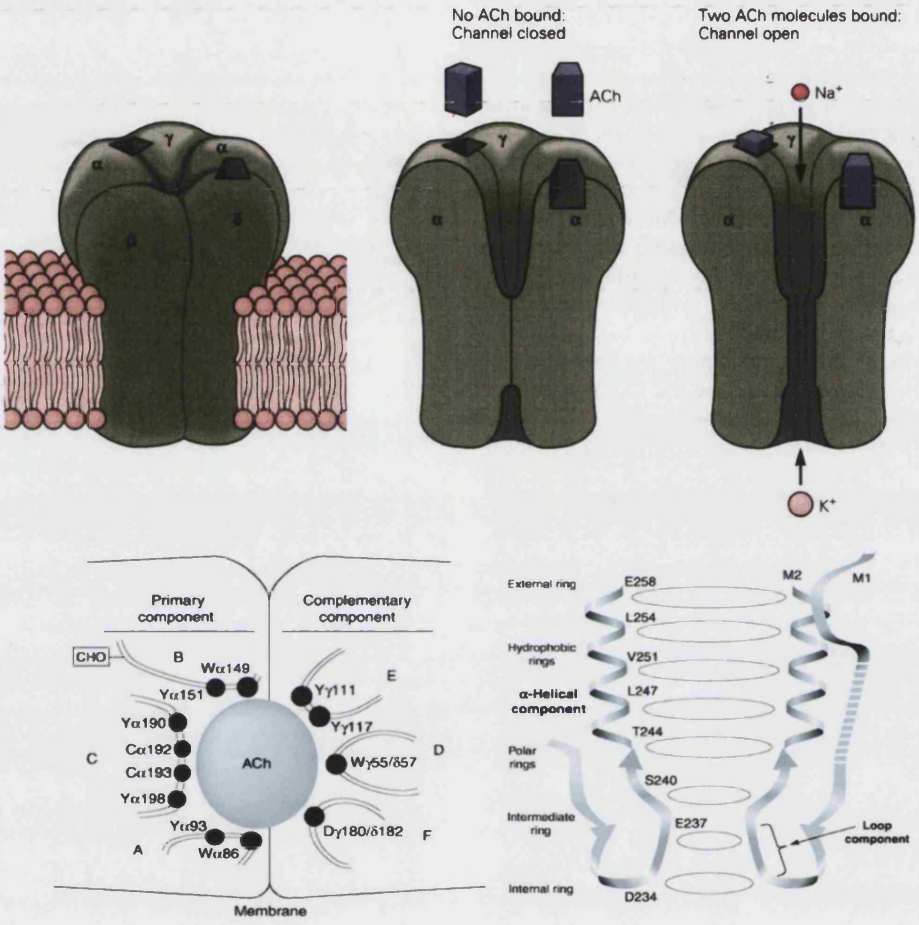

Fig 1.3 nAChR channel and agonist binding site

The upper panel shows the pentameric structure of the nAChR transmembrane channel and the corresponding agonist binding sites in the interfaces between the α and adjacent subunits. In the lower panel, the left picture shows a model of the ACh binding site of nAChR, which summarises the results from photoaffinity labelling experiments. These results revealed that the agonist binding domain is composed of two main components of the N-terminal domain that overlaps the boundary between subunits. The "principal component" is carried by the α subunit and consists of three loops: loop A (Trp-86, Tyr-93), loop B (Trp-149, Tyr 151) and loop C (Tyr-190, Cys-192, Cys-193, Tyr-198), whereas the "complementary component" is carried by the non-α subunits and comprises at least three loops: loop D (Trp-55, Trp-57), loop F (Asp 180, Asp 182) and loop E (Tyr 111, Tyr 117). On the right lower panel, a schematic representation of the transmembrane α7 nAChR channel is shown. The channel pathway has been reported to narrow across the membrane and to widen into a 20 Å diameter cylinder at the synaptic cleft (Adapted from Kandel 2000, and Changeux and Edelstein, 2001).

Additional confirmation for these structural observations of the nAChR came from a totally unexpected source, when the crystal structure of a glia-derived ACh-binding protein was determined with a 2.7 Å resolution (Brejc et al., 2001). This ACh binding molecule is a soluble protein found in the snail *Lymnaea stagnalis*, which is produced and stored in glial

cells, and released in an ACh-dependent manner into the synaptic cleft. The mature ACh-binding protein is 210 aminoacids long and forms a stable homopentamer, although lacking the transmembrane and intracellular domains present in the LGIC superfamily. Of the extracellular portion, nearly all the residues that are conserved within the nAChR family are present in the ACh-binding protein, including those that are relevant for ligand binding. The analysis of this releasable glial protein confirmed many previous assumptions of the nAChR structure. Moreover, it also supported the elaboration of new hypotheses for the subunit assembly and functional structure of the nAChR receptor. The ligand-binding site in this ACh binding protein is found in a cleft or cavity formed by a series of loops from the principal face of one subunit and a series of β-strands from the complementary face of the adjacent subunit. Present at each interface between the subunits and close to the outside of the pentameric ring, the cavity is lined by residues that were biochemically shown to be involved in ligand binding in nAChR (see above). The study of this protein has also shown that the most likely access route to the ligand binding sites are from above or below the double cysteine containing loop that bury the region from the solvent and prevents access from the outside. Although access from the central pore has been previously suggested (Miyazawa et al., 1999) this would require major structural rearrangements at the interface, which are less likely to occur. In spite of the major breakthrough in the understanding of nAChR structure provided by studies of this ACh binding molecule, a cautionary note must arise when studying its binding domain. In fact, it is still unclear whether this ACh binding protein performs the allosteric conformational changes that are important for pentameric LGIC. This apparently minor consideration might, however, have produced a lack of evolutionary restraints necessary for the maintenance of the channel allosteric mechanisms. In the end, this could have diverted the strucutural architecture of the ligand-binding domain in the ACh binding protein, making it different from the ligand-binding site in the nAChR.

1.1.4 Classification of nAChR

In evolutionary terms, muscle nAChR α1, β1, δ, ε, γ subunits are thought to represent one branch of the gene family while neuronal nAChR α2, α3, α4, α5, α6, β2, β3, β4 subunits are thought to form another branch that normally form heteromeric subunit receptors. The third branch consists of neuronal nAChR composed of α7, α8, α9, or α10 subunits, with the first three capable of forming homomeric pentamers. In the case of muscle nAChR, their diversity can be summarised in just two different subtypes, the foetal $(\alpha1)_2\beta\delta\gamma$ and the adult $(\alpha1)_2\beta\delta\varepsilon$. In effect, the ε subunit replaces γ in adult muscle, altering both single channel conductance and channel kinetics (Mishina et al 1986; Camacho et al., 1993). This lack of subtype diversity, together with their specific segregation to neuromuscular synapses, makes muscle

nAChR an easy target for receptor classification. On the contrary, the classification of neuronal nAChR, with their subtype diversity and specific localisation, is a more daunting task. In addition to the evolutionary studies, several approaches have been taken in an attempt to illuminate relationships between nAChR subunits and their genes, in order to allow a rational classification of neuronal nAChR subtypes. However, any protein structure or genomic sequence comparison between different subunits lacks functional significance, as knowledge of the contribution of particular subunits to specific nAChR subtypes is still far from complete. An alternative approximation is given by the use of pharmacological tools for the segregation of particular neuronal nAChR subtypes. Specifically, neuronal nAChR can be divided according to their high affinity binding to the curaremimetic toxin α-bungarotoxin (α-Bgt), which defined a nAChR population independent of the one with high affinity for nicotine (for review see Lindstrom, 2000). Although not totally adequate, the use of pharmacological, functional and anatomical data serves a useful purpose, differentiating the family of nAChR subtypes in three branches:

- The muscle nicotinic acetylcholine receptors (heteromeric).
- The neuronal nAChR with high affinity for nicotine (heteromeric).
- The neuronal nAChR with high affinity for α-Bgt (mainly homomeric).

Thanks to their easier accessibility and straightforward functional role, the synthesis, structure, and function of muscle-type nAChR are known in relatively great detail. On the other hand, the more diverse function, location, structure and roles of neuronal nAChR are much less well characterised. In effect, the functional properties of diverse neuronal nAChR subtypes in vivo, and the actual functional roles in the nervous system of many of the real and potential subtypes of neuronal nAChR remain to be determined. However, it is increasingly apparent that many neuronal nAChR are likely to have functional roles that differ from the clear-cut postsynaptic type of critical link in neurotransmission exemplified by muscle nAChR. Actually, a rapid overview of the suggested functions of neuronal nAChR reveals a molecule involved in such diverse processes as ganglionic neurotransmission in the autonomic nervous system and synaptic plasticity, learning and memory, modulation of transmitter release, addiction, neuroprotection and progression of neurodegenerative diseases in the CNS. The ability to fulfil such a variety of functional roles is, very likely, a result of several interrelated factors. First, the known diversity of neuronal nAChR subtypes can selectively influence their molecular properties (i.e.: desensitisation rates and ligand binding affinity), therefore shaping the actual cellular responses triggered by the activation of particular nAChR subtypes. Secondly, the precise cellular localisation of neuronal nAChR can also determine the activation of different neuronal mechanisms after receptor stimulation. In this regard, the recognised presynaptic location of neuronal nAChR allows the

control of particular neurotransmitter systems, thus affecting a variety of CNS functions (Wonnacott, 1997). Last but not least, the fact that neuronal nAChR are highly permeable to Ca^{2+}, opens the possibility for triggering a variety of intracellular signalling processes with diverse functional outcomes (Role and Berg, 1996).

1.1.4.1 Neuronal nAChR with high affinity for nicotine

A particular population of nAChR can be pharmacologically defined by their high affinity binding to the nAChR specific agonist [^{3}H]-epibatidine, which is weakly displaced, if at all, by α-Bgt (Houghtling et al., 1995). These neuronal nAChR comprise combinations of α2, α3, α4, α5, α6, β2, β3 and β4 subunits, forming heteromeric nAChR with alternate α and β subunits. The functional properties of various subunit combinations expressed in heterologous systems are relatively well known, although the precise nAChR subtypes present in vivo are not specifically defined yet. In *Xenopus oocytes*, functional receptors were obtained with paired combinations of α2, α3, or α4 with β2 or β4 (Papke et al., 1991, 1993). The α6 subunit has been also shown to form functional receptors in combination with β2 or β4 (Gerzanich et al., 1997; Fucile et al., 1998), even though it seems to function more effectively when is combined with another α and β subunit (Fucile et al., 1998). Although the α5 and β3 subunits are incapable of forming a functioning nAChR in pair wise combination with any other subunit and do not contribute to the ACh binding site (Boulter et al., 1990; Groot-Kormelink et al., 1998), they can have an effect on the efficacy and potency of agonists by participating in the overall conformational changes involved in activation and desensitisation, while also contributing to the channel properties by forming one fifth of the channel lining (Groot-Kormelink et al., 1998). This role as a 3rd subunit type is consistent with the suggested α3α5α4 nAChR reported to exist in avian brain (Conroy et al., 1992) and the $\alpha3\alpha4\alpha5(\beta2)_2$ inferred in midbrain dopaminergic nuclei in rats (Klink et al., 2001).

Among the various heteromeric nAChR, most of the attention has focused on the α3* and α4* subtypes. The α3* nAChR, although expressed in some particular brain regions (Sargent, 2000), are better-known as the most abundant subtype in the peripheral autonomic nervous system, where they play a postsynaptic role in ganglionic neurotransmission. However, this is frequently complicated by the presence of perisynaptic α7 nAChR, which can also participate in ganglionic transmission (Zhang et al., 1996; Shoop et al., 1999).

The α4* nAChR are the most prominent subtype with high affinity for nicotine in the CNS. Experiments performed in rodent brain have suggested that more than 90 % of the

mammalian high affinity binding sites for nicotine and the nicotinic ligand cytisine are composed of α4 and β2 subunits alone (Flores et al., 1992; Lindstrom, 2000). Confirming evidence for this observation was given by the loss of nicotine high affinity binding sites reported in β2 null mice (Piccioto et al., 1995). Although when expressed in *Xenopus oocytes* the subunit stoichiometry has been interpreted to be $(\alpha4)_2(\beta2)_3$ (Anand et al., 1991; Cooper et al., 1991), a small fraction of the α4β2 nAChR subtype may have α5 subunits associated with it (Conroy et al., 1992), and the α4-α3/α6-α5-β2 combination has been also proposed to exist in midbrain dopaminergic neurones (Klink et al., 2001).

Unlike their functional role in ganglia and skeletal muscle, where nAChR are the main excitatory LGIC receptor, in the CNS they are outnumbered by several orders of magnitude by the glutamate receptors (Clarke et al., 1985). At present, there is still only limited evidence for heteromeric nAChR with postsynaptic functions in the brain (Clarke 1995; Jones et al., 1999). Nonetheless, numerous reports demonstrate that heteromeric nAChR can function pre-synaptically to modulate the release of various neurotransmitters (Wonnacott, 1997), while they can also fulfil a modulatory role when present on cell bodies and/or dendrites (Blaha and Winn, 1993).

1.1.4.2 Neuronal nAChR with high affinity for α-Bgt

Like muscle nAChR, but unlike the other neuronal nAChR, this set of nAChR can bind with high affinity with the snake venom toxins α-cobratoxin and α-Bgt. Several nAChR subunits are part of this group, including the α7 subunit, and the closely related α8 (Schoepfer et al., 1990), α9 and α10 (Elgoyhen et al., 1994 and 2001), which are mainly associated with sensory organs. Although capable of forming functional homomeric nAChR in heterologous expression systems, it is not certain that the α7 nAChR are always present as homomers, and in avian brain much of α8 (which is not found in mammals) is normally present in heteromeric nAChR with α7 (see Lindstrom, 2000).

In terms of localisation, the highly abundant α7 containing nAChR subtype can be found in many brain regions, being especially concentrated in the hippocampus (Dominguez-Del Toro et al., 1994). In addition, it is also present in peripheral ganglionic neurons along with the α3* nAChR (Conroy and Berg, 1995). The α9 containing nAChR are only expressed in limited neuronal areas, and most notably in the cochlear hair cells (Elgoyhen et al., 1994).

Apart from their ability to function as homomers and bind α-Bgt, the α7, α8 and α9 nAChR exhibit other particular properties, like an exceptionally high Ca^{2+} permeability and very fast

desensitisation kinetics. Rapid desensitisation might intrinsically result from homomeric structure, if the presence of five rather than the typical two ACh binding sites capable of initiating nAChR activation results in faster desensitisation. The exceptionally high Ca^{2+} permeability of α7 (and also α8 and α9/α10) nAChR can have direct functional consequences, as Ca^{2+} entering through these nAChR can facilitate transmitter release when presynaptic α7 nAChR are activated (Gray et al., 1996). In addition, it was also suggested that the α7 nAChR mediated Ca^{2+} influx could initiate intracellular mechanisms leading to the modulation of signalling processes and gene expression (Role and Berg, 1996). In fact, many of the functional properties of α7 nAChR, like fast desensitisation and inward rectification, make them ideal cellular tools for the activation of precise Ca^{2+} signalling pathways.

1.1.5 Ca^{2+} permeability of nAChR

The potential relevance of nAChR in neuronal function has been pressed further by the demonstration of their high relative permeability to Ca^{2+}. As illustrated above, the capacity to modulate intracellular Ca^{2+} concentrations has prompted the suggestion that nAChR regulate many neuronal mechanisms in a Ca^{2+} dependent manner.

Although the report of the nicotine-evoked activation of Ca^{2+} dependent neuronal functions has been a relatively recent development (for review see Role and Berg, 1996), the ability of muscle nAChR to permeate not only monovalent but also divalent cations has been known for some time (Bregestovski et al., 1979). However, at the neuromuscular junction, activation of muscle nAChR yields primarily Na^{+} entry and subsequent depolarisation of the muscle cells. As a result, intracellular Ca^{2+} increases during neuromuscular synaptic activation will be a primary consequence of the activation of VOCC and Ca^{2+} release from the sarcoplasmic reticulum (Martonosi et al., 1984). For some time, this mechanism for the generation of Ca^{2+} signals after nicotine activation was also translated to neuronal nAChR, which were not considered as direct mediators of significant changes in intracellular Ca^{2+} concentrations.

This assumption has dramatically changed in the last decade, when an increasing amount of studies have demonstrated the relative Ca^{2+} permeability of nAChR in neuronal preparations. In freshly dissociated neurones from the medial habenula nucleus, Mulle et al. (1992) unequivocally demonstrated that Ca^{2+} permeates nAChR channels. Nicotine application after extracellular cations were replaced by 100 mM Ca^{2+} resulted in an inward current representing a significant fraction of that recorded in standard medium, suggesting

that the single channel conductance in the pure $CaCl_2$ external solution was comparable to that of NMDA receptors (see below).

Following these initial studies, several reports suggested that the Ca^{2+} permeability of nAChR could be directly related to their subunit composition, therefore proposing different patterns of nicotine evoked Ca^{2+} signals depending on the variable expression of nAChR subtypes. Accordingly, Ragozzino et al. (1998) demonstrated that $\alpha3\beta4$ receptors have a slightly higher fractional Ca^{2+} conductance than $\alpha4$ containing receptors, while Gerzanich et al. (1998) indicated that functional incorporation of the $\alpha5$ subunit in heterologously expressed $\alpha3$ containing human nAChR receptors significantly increased Ca^{2+} permeability.

Although selective Ca^{2+} permeability is a common property of all nAChR, the homomeric $\alpha7$ subtype of nAChR appears to be the most highly permeable for Ca^{2+}. An elegant study by Seguela et al. (1993) examined the relative Ca^{2+} permeability of the $\alpha7$ nAChR by measuring changes in the reversal potential of the nicotine-induced current while changing the external Ca^{2+} concentration from 1 mM to 10 mM. A reversal shift of +3, +7 and +29 mV was observed for muscle nAChR, $\alpha3\beta4$ and $\alpha7$ nAChR respectively. Since the relative permeability of these cation-selective channels to Ca^{2+} versus Na^+ is a function of the magnitude of the shift of the reversal potential, these results indicate that the relative permeability of the $\alpha7$ nAChR to Ca^{2+} is greater than that of the other nAChR (Sands and Barish, 1991; Vernino et al., 1992), and corresponds to a permeability ratio (PCa:PNa) of approximately 20 (i.e. ~ 20 % of the fractional current is carried by Ca^{2+}). Other studies of heterologously expressed $\alpha7$ receptors by Sands et al (1993), in which barium was substituted for Ca^{2+} to minimise any contaminating influence of Ca^{2+}-activated chloride currents, reported a similar PBa:PNa ratio of 17. In addition, studies carried out by Lindstrom and coworkers (1995) demonstrated that 11 % of the $\alpha7$ fractional current was carried by Ca^{2+}, compared to 3 % and 1 % of $\alpha4\beta2$ and muscle nAChR respectively. However, it should also be noted that studies of $\alpha7$ responses in cultured hippocampal neurones indicated that Ca^{2+} carried approximately 6 % of the current (Albuquerque et al., 1997). In spite of these apparent discrepancies in the reported permeability, probably due to differences in experimental preparations, it is widely accepted that $\alpha7$ nAChR are highly permeant to Ca^{2+}, with a relative permeability of Ca^{2+} over Na^+ similar or even higher than that of the NMDA glutamate receptor (Seguela et al 1993; Albuquerque et al 1997). It should be considered however, that the combination of rapid desensitisation and inward rectification in the $\alpha7$ nAChR could lessen the sustained ion flux through these channels. As a result, the overall relative permeability cannot be taken as a direct index of the amplitude of evoked Ca^{2+} responses (further illustrated in Chapters 2 and 3).

At present, the concept that nAChR are predominantly permeant to monovalent ions and serve mainly for the control of membrane potential, has been replaced by a more complex hypothesis, in which nAChR channels with significant Ca^{2+} permeability would also be capable of functioning as direct regulators of intracellular signal transduction pathways (see Chapter 4). Consistent with this theory, it has been shown that Ca^{2+} entry through nAChR channels can increase intracellular Ca^{2+} concentration up to the micromolar range, a level sufficient for the activation of Ca^{2+}-dependent regulatory processes (Mulle et al., 1992).

1.1.6 Cellular localisation of nAChR

Functional neuronal nAChR can be located on a cholinergic cell or a cholinergic target (i.e., a cholinoceptive cell). Both types of neuronal nAChR can be present on membranes of the cell body, dendrites, axon or nerve terminals, while functional studies have suggested that neuronal nAChR may also occur in "preterminal" membranes, i.e., on the axon before the spread of nerve terminals (see Wonnacott, 1997). In fact, on the basis of both electrophysiological and transmitter release studies, it has become a common notion that neuronal nAChR are often presynaptic, meaning that they are modulators of the release of various transmitters, including ACh itself (McGehee and Role 1995; Wonnacott 1997). As a result, this terminal or presynaptic location makes possible the modulation of several brain functions, possibly underlying several of the behavioural consequences of neuronal nAChR activation.

In the peripheral nervous system, neuronal nAChR located on terminals, cell bodies and dendrites of autonomic ganglionic neurons are well characterised from a morphological and functional standpoint (Ullian et al., 1997; Zhang et al., 1996). In the CNS, the demonstration of functional neuronal nAChR on cell body/dendrite membranes comes mainly from electrophysiological studies, and although nicotinic responses are less easy to detect than for instance, glutamate responses, much evidence has accumulated for neuronal nAChR on cell bodies/dendrites in a number of CNS regions, including the thalamic nuclei (Lena and Changeux, 1997), cerebral cortex (Roerig et al., 1997; Xiang et al., 1998), ventral tegmental area (Blaha and Winn, 1993; Calabresi et al., 1989), hippocampal pyramidal cells and interneurons (Alkondon and Albuquerque, 1993; Alkondon et al., 1998; Frazier et al., 1998), and locus coeruleus (Egan and North, 1986).

1.1.7 Distribution of nAChR in the nervous system

Several methodologies, including high affinity ligand binding and immunolabelling, have been used to detect and study the distribution of nAChR in the nervous system. However, it was the availability of subunit specific complementary RNA probes that has permitted the analysis of the distribution of nAChR subunit mRNAs in different areas of the nervous system.

In autonomic ganglia neurones, where ACh mediates rapid synaptic transmission, nAChR have been characterised extensively (Conroy and Berg, 1995). Neonatal rat sympathetic ganglia contain mRNAs for α3, α5, α7, β2 and β4, but no mRNA for α2, α4 or β3 (see Sargent, 2000). In the CNS, distribution studies of specific molecular probes have shown that expression of nAChR subunit mRNAs is widespread, with most regions of the brain expressing significant quantities of at least one subunit. The patterns of expression differ, with some subunits being much less common than others. For example, high concentrations of α2 transcripts were only found in the interpeduncular area of the rat, with small amounts detected at limited number of other sites. On the other hand, the α4 mRNA is strongly expressed in a large number of brain regions, although its distribution is less widespread than that of β2 (Wada et al., 1989). Among the β subunits, β2 mRNA expression was found in almost all parts of the brain, including sites with no high affinity [^{3}H]-nicotine binding, where they are presumably assembled with α subunits other than the α4 (Wada et al., 1989). The β4 subunit was found in the medial habenula (Duvoisin et al., 1989), with lower expression at other loci (Dineley-Miller and Patrick, 1992). Finally, α7 mRNA is found in most regions of the brain, but particularly high levels are found in the amygdala, olfactory regions, and distinct layers of the hippocampus and cerebral cortex (Seguela et al., 1993).

Although providing very useful information, in situ hybridisation techniques can only identify cells which express mRNAs for particular subunits, but cannot predict the quantity of that subunit present on the cell surface nor the composition of the receptor and its distribution along the neuron. For that reason, additional mapping studies have been carried out in the CNS using high affinity ligand binding and immunocytochemical detection. The extensive expression of α7, α4 and β2 mRNAs fits well with studies showing the wide distribution of high affinity [^{125}I]-α-Bgt and [^{3}H]-nicotine binding sites. High affinity [^{3}H]-nicotine or [^{3}H]-ACh binding is highest within the interpeduncular nucleus, all thalamic nuclei except the posterior group and intralaminar nucleus, the superior colliculus and the medial habenula. Binding was also noted in the substantia nigra pars compacta, the ventral tegmental area, the molecular layer of the dentate gyrus, the presubiculum and in layers I and III/IV of the cerebral cortex (although not equally in all parts of the cerebrum). When specific antibodies

for the β2 subunit were used, they produced a pattern of binding that is generally similar to that seen with [^{3}H]-nicotine (Swanson et al., 1987; Hill et al., 1993), which is consistent with the fact that virtually all high affinity nicotine binding to rat brain extracts is immunoprecipitated by an antibody against the β2 subunit (Flores et al., 1992; Lindstrom, 2000). The pattern of [^{125}I]-α-Bgt binding is distinct from that seen with [^{3}H]-nicotine, and is highest in the cerebral cortex (especially layers I, IV, V and VI), olfactory bulb, hypothalamus and hippocampus (Clarke et al., 1985).

1.1.8 Brain cholinergic pathways

The study of the neuronal nAChR ditribution constitutes a fundamental question in the process of understanding the role of nAChR in the CNS. In pursuing this objective, the investigation of the cholinergic source responsible for the activation of nAChR is, in many cases, left unattended. In fact, acetylcholine is one of the most ubiquitous neurotransmitters in the mammalian nervous system, and a cholinergic neuron (i.e., a neuron that utilises ACh as a neurotransmitter) has several specific neurochemical features: a releasable pool of ACh; the enzyme necessary for ACh synthesis, i.e., choline acetyltransferase; the uptake site for choline; and the vesicular ACh transporter. The cholinergic innervation of a given brain structure can be intrinsic or extrinsic. The innervation of the striatum, for example, is almost exclusively intrinsic and arises from cholinergic interneurons. In contrast, the cholinergic innervation of limbic structures, neocortex, thalamus and superior colliculus is predominantly extrinsic. The anatomical studies of cholinergic pathways has defined several cholinergic systems, capable of inervating various brain regions:

The *Basal Telencephalic System* is comprised of several large clusters of cholinergic neurons, present in the ventral part of the telencephalon, where, intermingled with different proportions of non-cholinergic neurons, they are detected in a more or less continuous strip. This series of nuclei comprises (from rostral to caudal levels) the medial septum (MS), the nucleus of the vertical (VDB) and horizontal limb (HDB) of the diagonal band of Broca, the magnocellular preoptic area (MPA), the ventral pallidum (VP), the globus pallidus (GP), the substantia innominata (SI), the basal nucleus of Meynert (MY) and the nucleus of the ansa lenticularis (AL). Four principal clusters are distinguished among this basal telencephalic system, which gives origin to four main projection pathways:

- From MS and VDB to the hippocampal formation
- From VDB, HDB, MPA and SI to medial neocortex
- From MPA, SI, MY and AL to lateral neocortex, limbic cortex and amygdala
- From HDB to alfactory bulb

The connectivity of basal telencephalic cholinergic neurons has important functional consequences, as it has been shown that single cholinergic neurons innervate a relatively restricted zone of the cortex. At the level of cell bodies, cholinergic neurons are densely interconnected via dendro-dendritic synapses and functional studies show that even sparse projections to the basal telencephalic system can cause global release of ACh in the cortex (see Butcher, 1992).

In the striatum, a population of cholinergic interneurons that constitute the *striatal system* represent one of the two main populations of interneurons, although only corresponding to a small percentage (1-2 %) of the overall striatal neuronal population. In spite of its size, the population of striatal cholinergic interneurons produces the densest network of cholinergic terminals in the brain (Contant et al., 1996). Other cholinergic systems have been also described, including the *mesopontine tegmental system*, with the highest density of neurones located in the pedunculopontine tegmental, laterodorsal tegmental and parabigeminal nuclei. Their projections are rather widespread, including several thalamic nuclei, the basal ganglia (substantia nigra and ventral tegmental area), basal telencephalic cholinergic nuclei and catecholaminergic brain stem nuclei. Notably, projections of the mesopontine tegmental system are not overlapping with those of the basal telencephalic system. Cholinergic systems have been also described at the brain stem and spinal cord motor nuclei, the medullary tegmentum, the peripheral ganglia and the retina, while it continues to be debated whether or not cholinergic interneurons exist in the cerebral cortex, hippocampus, olfactory bulb and anterior olfactory nucleus of the rat.

In spite of their widespread distribution, the density of cholinergic fibers in neocortex and thalamic nuclei shows major regional variations (Descarries et al., 1997). For example, limbic and paralimbic areas of the cerebral cortex contain a far denser concentration of presynaptic cholinergic markers than immediately adjacent sensory association areas. These limbic and paralimbic areas also seem to be the only parts of the cerebral cortex that have substantial projections back into the basal forebrain cholinergic cell groups. Some of these characteristics may reflect the importance of cholinergic innervation to learning and memory processes. In fact, acetylcholine plays a special role in the cellular events that underlie learning, as demonstrated by the direct role of cholinergic transmission in the establishment of hippocampal long-term potentiation (Matsuyama et al., 2000). Because of this selective concentration, cholinergic agonists may have a relatively greater impact on limbic and paralimbic parts of the brain, areas that are known to play a major role in the organisation of memory and learning.

1.1.9 Neurotransmission in cholinergic synapses

In physiological conditions neuronal nAChR are activated by ACh binding, although the α7 subtype of nAChR may, in addition, be activated by choline (see Chapter 1, part 1.3.2.5). In both cases, it is assumed that activation of neuronal nAChR depends on signals deriving from cholinergic cells at synaptic contacts. However, almost none of the ultrastructural investigations on cholinergic terminals were designed for measuring the frequency with which cholinergic axon terminals, boutons or varicosities are engaged in synaptic contact. Results obtained from more recent ultrastructural studies showed that in several key areas involved in cholinergic transmission, cholinergic terminals presented a low frequency of junctional specialisation (Descarries et al., 1997). These observations imply that rather than a brief pulse of a high concentration (mM) of ACh delivered across the synaptic cleft, ACh may be required to diffuse to adjacent terminals, achieving a lower concentration (μM-nM) but for a longer duration. This hypothesis must assume that sufficient ACh will escape hydrolysis by acetylcholinesterase, while the sensitivity of particular neuronal nAChR subtypes to ACh, and their propensity to desensitise may become important factors in shaping their responsiveness.

Following the above-mentioned observations, the relevance of assessing the spatial relationships between cholinergic neurons and neuronal nAChR is manifestly obvious. Double immunolabelling with choline-acetyltransferase and nAChR subunit antibodies would be the technique of choice for this assessment. Unfortunately, most of the data regarding precise subunit localisation derives from in situ hybridisation, a technique that gives no information on the subcellular distribution of the receptor protein.

1.1.9.1 Wiring versus Volume transmission

In the last decade, the concept that other types of interneuronal, and in general intercellular, communication besides synaptic transmission exist in the CNS is gaining consensus (Agnati et al., 1995; Agnati and Fuxe, 2000). Communication between cells following migration of the transmitter in the cerebral extracellular space has been defined as volume transmission, as opposed to the synaptic communication, which is defined as wiring transmission. Much structural evidence points to the fact that cholinergic transmission in several CNS nuclei is mostly of the volume type. As an example, in both target areas of the basal telencephalic system (neocortex and hippocampus) as well as in the neostriatum of the rat, the percentage of cholinergic varicosities (i.e.: release sites of ACh) that make synaptic contact is only around 10 % (Descarries et al., 1997). This observed asynaptic character and high density of innervation by ACh neurons in many CNS regions has led to the proposal that a

continuous cholinergic tone exists in some brain regions. According to this hypothesis, there would exist, at least in brain regions densely innervated by cholinergic neurons, an ambient level of extracellular ACh to which all tissue elements would be continually exposed (Descarries et al., 1997). This ambient level would be maintained by the spontaneous or evoked release from non-junctional cholinergic varicosities and ACh spill-over beyond the synaptic interfaces of the few junctional ones. In this context, enzymatic degradation by acetylcholinesterase (AChE) would primarily serve to keep the ambient level of ACh within certain limits, rather than totally eliminate ACh from the extracellular space. Supporting the ultrastructural evidence, an increasing amount of microdialysis data seems to indicate the presence of a steady state, resting or basal level of ACh in the extracellular space of different CNS regions. Spontaneous ACh dialysis outputs in the nanomolar range have been repeatedly measured in cerebral cortex, hippocampus or neostriatum of freely moving rats (Damsma et al., 1988; Nilsson et al., 1990; Messamore et al., 1993). In addition, a series of studies performed in rodents have provided strong evidence for the localisation of the CNS predominant molecular form of acetylcholinesterase (G4) in a distinct compartment separate from the junctional area (see Descarries, 1997), leading the belief that this form of the enzyme primarily serves to eliminate ACh molecules which spill over from the synaptic cleft.

Experimental and theoretical models have been proposed to evaluate the diffusion of transmitter molecules in living CNS tissue, which take into account physical factors such as re-uptake, degradation and loss at blood-brain boundaries (Descarries et al., 1997 and references therein). Much of the current work is devoted to dopamine (DA) and nitric oxide and unfortunately little is known about ACh. In the case of neostriatal DA however, it has been calculated that transmitter molecules should be found 10 μm away from release site within 50 msec. In a region as densely innervated as the neostriatum, 10 μm is not a short distance, at least at the scale of axon varicosities. Based on currently available quantitative data on dopamine and ACh innervation density (Doucet et al., 1986; Contant et al., 1996), it may be calculated that a sphere of neostriatal neuropil 10 μM in radius comprises about 400 dopamine and at least as many ACh terminals, five to ten times more unidentified axon terminals and at least several thousand dendritic spines!

The realisation that a significant part of the cholinergic transmission in the CNS may be composed of non-classical synaptic contact fits well with the proposed role of nAChR as modulators of neuronal function. In theory, both terminal and/or somatodendritic nAChR could be regulated by relatively low levels of acetylcholine, able to sustain a basal tone stimulation, or even desensitisation, of nAChR. This may have even further significance in the case of α7 nAChR, which can be activated by the metabolite of acetylcholine, choline. Overall, the integration of classical and non-classical interneuronal contacts may constitute a

key aspect in the modulatory actions of cholinergic transmission and nicotinic stimulation in particular.

1.2 nAChR and Disease

1.2.1 Tobacco smoking and nAChR stimulation: the bad, the ugly, and the good?

In the process of investigating the function of nAChR in the brain, the study of the effects of tobacco smoking has played a significant role. A clear example of this interrelation is given by the examination of the addictive and anxiolitic properties of tobacco consumption, which are closely related with the neuronal consequences of nAChR activation. In general, nicotine is regarded as a toxic and addictive substance, and this negative perception certainly stems from nicotine's presence in tobacco and the many adverse consequences associated with cigarette smoking. In reality, it is generally accepted that the well-known adverse effects of smoking are largely not attributed to nicotine, as cigarette smoke contains many toxic bioactive substances that are sought to be more harmful than nicotine. There is no doubt, however, that nicotine has some major liabilities, which include its addictive properties and adverse effects on the cardiovascular and gastrointestinal systems (for review see Decker and Arneric, 1999).

Although a significant part of human nicotine research has focused on the understanding of nicotine addiction, it has become increasingly accepted in the last 15 years that nicotine can have some beneficial effects on human illness, including neurological disorders such as Alzheimer's (AD) and Parkinson's disease (PD; Donnelly-Roberts and Brioni, 1999). Unexpectedly, some of the initial evidence came from studies of tobacco consumption. In the case of PD, the epidemiological findings suggested that cigarette smoking was negatively correlated with the progression of the disease and, although part of the epidemiological evidence has been controversial, a series of studies by Morens et al. (1994, 1995) demonstrated that tobacco smoking reduces the risk of developing PD and, more important, that this relationship is not due to any obvious confounding factors (Morens et al., 1996). In addition, an inverse correlation between tobacco smoking and the progression of other neurodegenerative diseases of the brain, like AD, has also been reported. Epidemiological studies show that smokers have a lower risk of developing AD than non-smokers, even when other factors (i.e.: age) are controlled (Tyas, 2000). In addition, Van Duijn and Hofman (1991), showed how tobacco smoking might be positively correlated with the delayed onset of AD, while a more recent retrospective case-control autopsy study by Ulrich and colleagues (1997) suggested that an apparent protective action against senile plaque formation could be demonstrated. As a result, the findings reported from epidemiological investigations indicated a potential role for nicotine in the therapy of

neurodegenerative diseases. AD, in particular, has been the foremost therapeutic objective, partly because epidemiological reports have been complemented with studies linking activation of the nAChR with hippocampal function and the process of cognition (this will be further discussed in Chapter 2), but also for the more prosaic reason that substantial economic rewards will surely follow a "successful" therapy for AD.

In addition to AD and PD, nAChR have been also associated with schizophrenia, which constitutes one of the most disturbing disorders of neurological function. Similar to the situation with the neurodegenerative diseases, part of the evidence for the involvement of nAChR came from epidemiological studies of tobacco smokers, which showed how schizophrenic patients are particularly heavy smokers, even when compared with other psychiatric patients (for review see Stitzel et al., 2000). It was therefore suggested that this heavy tobacco use might reflect an attempt at self-medication of an endogenous neuronal deficit. When considering the actual experimental data, much of the support for the role of nAChR in shizophrenia came from studies of paired pulse inhibition, a method developed for the analysis of neuronal mechanisms related to the filtering of sensory inputs to higher brain centers. Clinical evidence suggested that the apparent attention to extraneous stimuli in their surroundings, which is observed in schizophrenics but ignored in normal individuals, is related to the inability to filter sensory stimuli (Waldo et al., 1991). Using an electrically positive evoked potential occurring 50 ms after an auditory stimulus, it was established that both normal humans and rodents showed similar decreased responses to a repeated auditory stimulus (Adler et al., 1986; Freedman et al., 1991). This function was shown to be deficient in schizophrenic patients (Adler et al., 1998). More precise neuronal recordings identified the pyramidal neurones of the hippocampus as a major source of the rat evoked potential in auditory stimulus, and showed that these pyramidal neurones have a decremented response to repeated auditory stimuli that parallels the decrement in the evoked potential (Bickford-Wimer et al., 1990). This observed decrement is lost after transection of the fimbria-fornix, a fibre tract that includes afferents to the hippocampus from cholinergic neurones in the basal forebrain. Moreover, nicotine normalises the inhibition of response (Bickford and Wear, 1995). The inhibition is also selectively blocked by α-Bgt (Luntz-Leybman et al., 1992), which suggests that the α7 nAChR receptor mediates nicotine's effects. Consistent with this apparent involvement of nAChR function in some of the neuronal processes affected in schizophrenia, a report by Freedman et al. (1997) has indicated that the auditory sensory deficit observed in schizophrenic patients is genetically linked to the locus of the α7 nAChR gene on chromosome 15q14.

At present, it is widely recognised that nAChR are involved in a variety of diseases affecting both the nervous system and non-neuronal tissue. These alterations can be divided into

those occurring during development (Tourette's syndrome and schizophrenia), those occurring at any stage of life (epilepsy or depression), and those associated with aging (Alzheimer's and Parkinson's disease, and dementia). The evidence for an involvement of nAChR is supported, in some cases, by direct results showing the nAChR role in the affected brain, while in many others, clinical improvement after nicotine consumption or even tobacco smoking is the sole indicator for a nAChR role. Although it should be considered that the therapeutic benefits might not imply the specific involvement of nAChR in the cause or symptoms of the disease, increasing evidence suggests that abnormalities in nAChR neurotransmission can be associated to various human diseases. Some of the possible mechanisms behind the apparently wide scope of nAChR action in neuronal disease will be further illustrated in Chapter 2.

1.3 The Hippocampus

Although the functional role of nAChR in the CNS is far from being resolved, some of the physiological implications of nAChR stimulation appear to be related to the regulation/modulation of synaptic plasticity and cognitive processes (see below). In addition, the putatively protective properties of nAChR activation can also help in preventing the neuronal loss observed in neurodegenerative disorders, like Alzheimer's, while at the same time, contribute to the cognitive function. Considering that the hippocampus is a key area for memory processing in the brain and one that is affected in the progression of AD, it constitutes a relevant model for the study of nAChR function in the CNS (see Chapters 2, 4 and 5).

1.3.1 The hippocampal region

It was back in the sixteenth century that the anatomist Arantius introduced the term hippocampus, which literally means "horse-caterpillar", to describe a particular area of the brain. In actual fact, the hippocampal region presents, perhaps more than any other part of the brain, many highly formalised and instantly recognisable neuronal assemblages, "so distinctive that cytoarchitectonic boundaries are unmistakable" (Angevine, 1965).

The hippocampal region has been subdivided into the *hippocampal* and *retrohippocampal formation*, with the latter being divided in the pre- and para-subiculum, the parahippocampal cortex and the enthorhinal cortex, which is continuous with the neocortex (Fig 1.4). The *hippocampal formation* itself is probably the one most thoroughly studied, and can be divided into dentate gyrus (area dentate), hippocampus proper (cornu ammonis, CA) and subiculum. Each area is defined by individually distinct cell layers, which are summarised in Fig 1.4.

The hippocampus proper has been divided in CA1, CA2 and CA3 regions, areas that play a crucial part in the understanding of hippocampal neuronal circuitry. These CA fields form a large flap of tightly packed pyramid-shaped neurones that is folded and tucked under the edge of the neocortex. Pyramidal neurons in the hippocampus proper exhibit a large primary apical dendrite with numerous higher order branches and many radially orientated basal dendrites. Their axons arise either from the perikaryon or from a proximal segment of a basal dendrite. Different anatomical layers have been described in the hippocampus proper, which give rise to its lamellar neuroarchitecture. The *stratum moleculare* contains dendritic processes from pyramidal cells projecting from other layers. Next to this layer is the *stratum*

lacunosum with many irregularly spaced cells and a rather large number of fibers projected from other layers. Some fibers end in this layer, while others project into the *stratum moleculare*. Then follows the *stratum radiatum*, populated with few non-pyramidal neurons and containing many fiber systems coursing through it from various points of origin. Also present is a dense network of dendritic arborisations characteristic of the pyramidal neurones from the *stratum pyramidale* situated next to it. The *stratum oriens*, with its densely packed polymorphic cells that project to other parts of the hippocampus, lies adjacent to the *stratum pyramidale* from which receive fibers. Then follows the *alveus*, which for the most part, it is composed of white matter, i.e., axons arising from the *stratum pyramidale*.

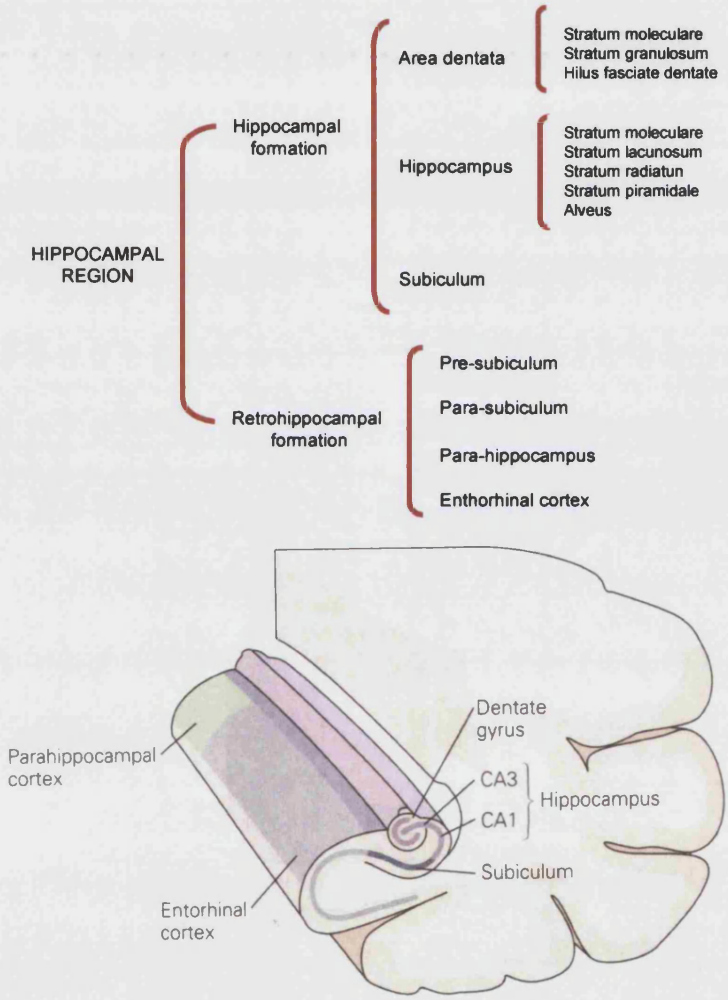

Fig 1.4 The anatomy of the Hippocampus

The description of the defining components of the hippocampus is based on the appearance of the region in horizontal sections. The hippocampal region has been divided into two main formations and various regions and layers. The hippocampal formation itself can be divided into the dentate gyrus (Area dentata), hippocampus proper and subiculum. The general organisation of the hippocampal region in relation to other brain areas is shown in the lower panel. In the case of the hippocampus proper, it has been divided in the CA1, CA2 and CA3 regions, which play a crucial role in the hippocampal neuronal circuitry (Adapted from Angevine, 1975 and Kandel, 2000).

The second sheet of neurones in the *hippocampal formation* is the dentate gyrus, which contains round and also tightly packed neurons called granule cells. These cells extend two or more primary dendrites from one pole of the cell body, while their axons present a dense, mossy appearance, therefore called mossy fibers. The dentate gyrus is separated into three different layers. The stratum moleculare presents many fibers and a few neurones bearing short processes. Next to it is the densely packed granule cell layer (stratum granulosum) followed by a polymorphic zone (hilus fasciate dentate) composed of pyramidal, stellate and bipolar cells. These other morphological cell types (i.e., bipolar, multipolar and aspiny cells) largely correspond to local circuit neurons or interneurons (Milner and Bacon, 1989a, b; Banker and Goslin, 1991). The stratum moleculare of the dentate gyrus blends into the stratum lacunosum-moleculare of the hippocampus proper, while the stratum radiatum of the hippocampus fades out beneath the stratum moleculare-granulosum.

The relatively simple layered structure of the hippocampal region, together with its well-defined connections, made it a model area for the general study of synaptic mechanisms. The axons of pyramidal neurones from many neocortical association areas project into the entorhinal cortex, and information from this area is projected to the dentate gyrus of hippocampal formation via the subicullum, in the so called perforant pathway. This cortical information is processed in the hippocampus through a network of synaptic connections that is not yet completely understood. Three major pathways, called the trisynaptic loop, comprise the thoroughly studied and most important part of the hippocampal synaptic network:

- Perforant pathway, which projects from the enthorhinal cortex and makes synapses onto the granule cells of the dentate gyrus.
- Mossy fiber pathway, which contains the axons of granule cells and runs to the pyramidal cells in the CA3 region of the hippocampus proper. The pyramidal cells of the CA3 field produce branched axons, in which one leaves the hippocampus through the fimbria.
- The Schaeffer collateral pathways consist of the excitatory branches of the pyramidal cells in the CA3 region and ends on the pyramidal cells in the CA1 region of the hippocampus proper.

Axons of the CA1 pyramidal neurones project to dendrites of neurones in the subicullar complex and, to complete the circuit, these neurones send axons back to the entorhinal cortex. However, it has to be noted that apart from the trisynaptic loop described so far, all areas of the hippocampal formation, except the dentate gyrus, contain neurones that project

to various other parts of the brain, mainly through the enthorinal cortex (Lavenex and Amaral, 2000).

1.3.2 nAChR in the hippocampal region

The hippocampus receives rich cholinergic innervation, mainly via the fimbria-fornix, from the medial septum-diagonal band complex (Woolf, 1991). Electron microscopy reveals choline-acetyltransferase-positive terminals synapsing on pyramidal neurones, interneurons, granule cells and their dendrites (Frotscher, 1992). Those cholinergic afferents provide the main source of acetylcholine that activates endogenous nicotinic-cholinergic mechanisms. Indeed, mapping studies indicate that neuronal nAChR are present throughout the hippocampus, and in situ hybridisation experiments show that the strongest labelling by far corresponds to α7 nAChR subunits along with β2, with weaker labelling for α3, α4, α5, and β4 (Seguela et al., 1993; Zarei et al., 1999, and references therein). Immunocytochemical studies by Dominguez-del Toro et al. (1994) demonstrated that the α7 nAChR was the main subunit in the hippocampus. In accordance with this hippocampal high labelling of α7 nAChR subunits, [^{125}I]-α-Bgt binding has also been shown to be high in the hippocampus (Clarke, 1995). Additional evidence for the presence of α7 nAChR in the hippocampus came from the observed loss of [^{125}I]-α-Bgt binding in α7 defficient mice (Orr-Urtreger et al 1997). A more recent study by Fabian-Fine et al. (2001) has reported the ultrastructural distribution of α7 nAChR subunits in the hippocampus. Light microscopic immunolabelling revealed diffuse α7 nAChR immunoreactivity throughout the cell bodies and cell processes of neurones in the dentate gyrus and CA3 and CA1 regions. Immunoreactivity was also visible in the dendritic fields of CA3 and CA1, and ultrastructural data revealed a widespread presynaptic and postsynaptic labelling (Fabian-Fine et al., 2001).

1.3.2.1 Nicotine-evoked ionic currents in hippocampal neurones

Hippocampal neuronal cultures

Much of the existing data on the nAChR function in the hippocampus has been carried out using neuronal hippocampal cultures from rats. This approach allows the easy manipulation of the neuronal environment, permitting also the detailed study of nAChR dependent ionic currents (further analysis of different experimental preparations for the study of hippocampal neurones is given in Chapter 2). Nicotine stimulated currents can be recorded in hippocampal primary cultures from rats, and as Albuquerque and collaborators have demonstrated, hippocampal neurones respond to nicotinic agonists with one of three types of nicotinic whole-cell currents, named type IA, type II and type III (Fig 1.5). These nicotine-

evoked currents are distinguished from one another on the basis of their kinetic and pharmacological properties. Type IA currents are by far the predominant response of hippocampal neurones to nicotinic agonists, and are characterised by fast desensitisation and blockade by α-Bgt, α-cobratoxin and methyllycaconitine (MLA; Alkondon and Albuquerque, 1993; Albuquerque et al., 1997). The fast kinetics of inactivation and the short lived open time of the nAChR channels subserving type IA currents accounts for the unique kinetic properties of these currents (Castro and Albuquerque, 1993). Type II and III currents desensitise more slowly and can only be recorded from a small population of the hippocampal neurones (Albuquerque et al., 1997). Aproximately 10 % of the hippocampal neurones in culture respond to nicotinic agonists with type II currents, whereas no more than 2 % of neurones respond to the agonists with type III currents. Type II and III currents are differentiated from one another on their sensitivity to nicotinic antagonists. The activation of type II currents is inhibited by dihydro-β-erythroidine (DHβE, 10 nM), while type III currents are inhibited by low concentrations of mecamylamine (1 μM), as shown in Fig 1.5.

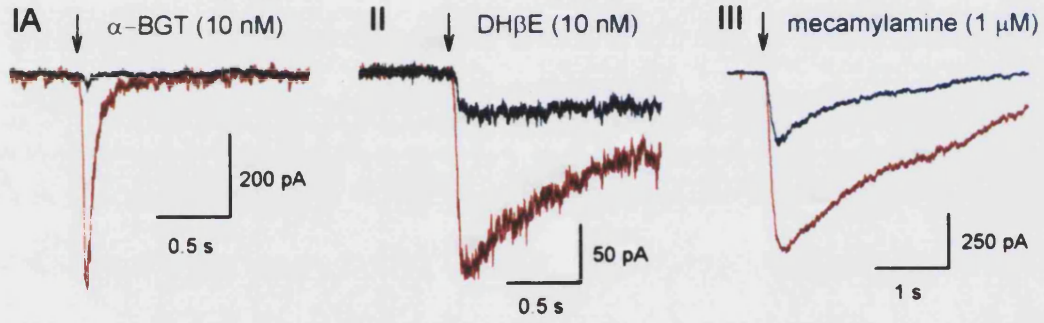

Fig 1.5 Nicotinic currents recorded from the hippocampus
The figure shows the typical family of whole-cell currents evoked by application of ACh to hippocampal neurones, and their sensitivity to blockade by nicotinic antagonists (Albuquerque et al., 1997). Short pulses of ACh (3 mM; 1-2 s) were applied via a U-tube to hippocampal neurones at the time indicated by the arrows. Red traces represent the agonist evoked currents, whereas black traces correspond to currents recorded in the presence of different antagonists (applied by bath superfusion). Type IA currents are by far the predominant response, characterised by fast kinetics and short-lived open time. Type II and III currents desensitise more slowly and can only be recorded from a small population of hippocampal neurones in culture.

A comparison of the kinetic and pharmacological properties of the nicotine-evoked currents in hippocampal neurones to those elicited in oocytes by heterologously expressed nAChR led to the suggestion that an α7 bearing nAChR sub serves type IA currents, while an α4β2 and an α3β4 subserve type II and III currents respectively (Albuquerque et al., 2000). These suggestions are in agreement with mRNA expression studies and with the proportion of cultured hippocampal neurons binding α-Bgt (see above, section 1.3.2). Although it is not completely clear if α7 containing nAChR are functional homomeric receptors in the brain, some recent evidence on the basis of Western blot analysis of neuronal α-Bgt binding

receptors suggests that the rat neuronal α-Bgt binding nAChR is a homo-oligomer of α7 subunits (Chen and Patrick, 1997).

The distribution of nAChR on the surface of hippocampal neurones was addressed by recording whole cell currents evoked by focal application of ACh (Alkondon et al., 1996). In brief, by using an infrared camera and computer-enhanced contrast images of the neuron it was possible to visualise precise areas of the neuronal surface and to estimate distances with a precision of 0.5-1 μm. The focal application of the agonist to well defined areas of the neuronal surface of hippocampal neurones and the exact location of recording electrode allowed the estimation of receptor density distribution (Alkondon et al., 1996). In effect, assuming that the peak amplitude of the current evoked by activation of a single receptor subtype is proportional to the number of individual receptors activated by the agonist, an estimate of the current density at different areas of the neuronal surface can provide important information about the receptor density distribution in such neuronal areas. From these estimations, Albuquerque and collaborators have shown that the density of type IA currents is substantially higher on the apical and basal dendrites of pyramidal neurones and on the dendrites of bipolar neurones than on the soma of these neurones (Albuquerque et al., 1997). The localisation of α7 nAChR in the hippocampus was also detected with the use of specific antibodies, where they were shown to be present along neuronal processes (Zarei et al., 1999). In effect, a correlation of α7 nAChR and the presynaptic marker synaptotagmin provided support for a synaptic location of the receptor (Zarei et al., 1999).

Hippocampal slices

Taking advantage of the knowledge made available by the studies carried out in cultured neurones, electrophysiological studies were also performed in conventional hippocampal slices. Evidence has been provided that in addition to functional α7 nAChR (Alkondon et al., 1997; Frazier et al 1998; Jones and Yakel 1997), functional α4β2 nAChR are also present in CA1 interneurones, and that a single CA1 interneuron can express both α7 and α4β2 nAChR (Alkondon et al., 1999).

Although functional α7 nAChR are also expressed by interneurones of the dentate gyrus (Jones and Yakel 1997), some reports have shown that these receptors are apparently not present in the soma and/or proximal dendrites of the principal (glutamatergic) neurones in the dentate gyrus and in the CA1 field of the hippocampus (Alkondon et al., 1997; Frazier et al., 1998; Jones and Yakel, 1997). It has been also reported that CA1 pyramidal neurones do not respond to ACh with currents that have typical characteristics of responses mediated by α7 nAChR (Alkondon et al., 1997; Frazier et al., 1998; Jones and Yakel, 1997). A more recent study showed that approximately 50 % of the CA1 pyramidal neurones recorded

showed no response to ACh, whereas the other 50 % responded to ACh with slowly decaying currents that were insensitive to blockade by MLA (Alkondon et al., 1999). These results are in apparent discrepancy with the functional α7 nAChR reported to be present in the soma and proximal neurites of hippocamal neurones in culture (see above). However, the existence of nicotine-evoked α7-nAChR mediated post-synaptic currents in CA1 pyramidal neurones after stimulation of the Schaffer collaterals was indeed demonstrated by Hefft et al. (1999) in organotypic hippocampal cultures and slices. In addition, α7 nAChR dependent currents were also detected in most of the CA1 pyramidal neurones after local application of acetylcholine in hippocampal slices (Ji et al., 2001). On the whole, these results are in agreement with the recent study by Fabian-Fine et al. (2001), describing the presence of α7 nAChR immunoreactivity in nearly all synapses in the CA1 stratum radiatum.

1.3.2.2 Functional characteristics of α7 nAChR in the hippocampus

The nAChR present on hippocampal neurones differ from one another not only with respect to their kinetics and pharmacology, but also with respect to single-channel properties, rectification, ion permeability and modulation by Ca^{2+}. The type IA currents generated by α7 nAChR show fast desensitisation in the presence of the agonist, intense rundown and inward rectification dependent on the presence of intracellular Mg^{2+} (Albuquerque, 1997). The α7 nAChR are also distinctive because of their brief open time (~100 μs at –80 mV) and high channel conductance (~73 pS). In addition, most of the important clues about the possible physiological roles of the α7 containing nAChR in the CNS were obtained from studies showing that these receptors have a very high permeability to Ca^{2+} (Seguela et al., 1993; Castro and Albuquerque, 1995). By the analysis of reversal potentials of ACh-induced currents under various ionic conditions it was estimated that, close to the resting potential (~-50 mV) and in the presence of 1 mM extracellular Ca^{2+}, approximately 6 % of the type IA current was carried by Ca^{2+} (Albuquerque et al., 1997). As illustrated previously (section 1.1.5), this estimation is only slightly lower than the one calculated for α7 nAChR expressed in oocytes (Lindstrom, 1995), which was shown to be 11 %. In hippocampal measurements in particular, the Ca^{2+} entry through α7 nAChR appears to be equivalent to approximately 60 % of that through the NMDA receptor. However, considering that the mean opening time and kinetics of inactivation of the NMDA receptor channel are much slower than those of the α7 nAChR in hippocampal neurones (Castro and Albuquerque, 1993; Nelson and Albuquerque, 1994), the Ca^{2+} influx through the NMDA receptor should be longer lasting than that through the α7 nAChR. It is therefore likely that NMDA receptors and native neuronal α7 nAChR mediate qualitatively different Ca^{2+} signals (Teyler et al., 1994). This will be further discussed in Chapter 2 of this thesis.

1.3.2.3 α7 nAChR-mediated synaptic transmission

Direct evidence for the mediation of synaptic transmission by neuronal nAChR in the mammalian CNS is very limited (for review see Albuquerque et al., 2000). The scattered distribution throughout the CNS of cholinergic projections and nAChR-containig targets and the rapid desensitisation of nAChR in general and α7 nAChR in particular have made difficult the isolation of nAChR mediated synaptic transmission in the brain. In fact, it was the development of rapid drug delivery systems and visualised recording techniques in slice preparations that allowed the detection of α7 post-synaptic currents in the hippocampus (Albuquerque et al., 2000). In addition, only the use of precise pharmacological tools made it possible to isolate the α7 nAChR evoked currents from the variety of EPSCs that can be elicited by electrical stimulation.

Two reports, appearing almost simultaneously, described how the stimulation of afferent fibres in the stratum radiatum or stratum oriens of area CA1 of the hippocampus produced α7 nAChR mediated excitatory postsynaptic currents recorded on hippocampal interneurons (Alkondon et al., 1998; Frazier et al., 1998). These postsynaptic currents had their frequency and amplitude reversibly reduced by the selective α7 nAChR antagonists MLA and α-Bgt, and were desensitised by the application of nicotine.

The activation of hippocampal GABAergic interneurons by cholinergic afferents from the medial septum could result in the inhibition of pyramidal neurones. Interestingly, it has been previously shown that EPSPs can be mediated by muscarinic receptors in CA1 pyramidal neurones (Cole and Nicoll, 1983). Altogether these findings may indicate that the transient inhibitory effect mediated by α7 nAChR stimulated GABA neurones, would be opposed by slow cholinergic EPSPs in the pyramidal cells. The combination of a slow excitatory potential (Cole and Nicoll, 1983) with synchronised transient IPSPs might provide a mechanism that could underlie theta rhythm activity. Such patterns of firing have been hypothesised to be an important component of the endogenous patterns of activity that can induce long-term potentiation (LTP) at hippocampal CA1 synapses. In addition, Albuquerque and collaborators proposed a more complex picture of the nAChR role in the hippocampal circuitry. In effect, the finding that treatment of hippocampal slices with nAChR antagonists (MLA and DHβE) causes an increase in the frequency and amplitude of the GABA-mediated post-synaptic currents (Alkondon et al., 1999) led to the suggestion that in the CA1 field of the hippocampus there should be a neuronal network composed of the following circuitry: cholinergic neuron-nAChR bearing interneuron-interneuron-interneuron. This could result in the nAChR-mediated stimulation of the first interneuron and the disinhibition of the activity of the third interneuron. Consequently, nAChR activation in interneurones that synapse directly

onto pyramidal neurones is likely to inhibit the activity of the latter, whereas activation of nAChR in interneurones that innervate other interneurones would cause disinhibition of the pyramidal neuron.

1.3.2.4 Presynaptic α7 nAChR in the hippocampus

The initial difficulty in detecting excitatory nicotinic postsynaptic responses evoked by nAChR stimulation or electrical stimulation of nerve fibres in the brain led to the hypothesis that nAChR may play a role in modulating rather than mediating transmission.

In hippocampal preparations, CA3 pyramidal neurones respond to nicotine with an increase in the rate of spontaneous miniature excitatory postsynaptic currents (mEPSCs) and in the amplitude of evoked EPSCs (Radcliffe and Dani, 1998). Evidence for this effect being mediated by presynaptic nAChR is given by the fact that the nicotine-evoked effect on miniature-EPSCs frequency can be blocked by glutamate receptor antagonists and is absent in the presence of Ca^{2+} free buffer (Gray et al., 1996). Presynaptic α7 nAChR were shown to be responsible for nicotine's effect and α7 nAChR were indeed capable of directly mediating the presynaptic terminal Ca^{2+} influx responsible for the enhanced glutamate release (Radcliffe and Dani, 1998). These findings indicate that properly timed nicotinic activation can alter the output signal of pyramidal neurones excited by glutamate released from presynaptic glutamatergic afferents. When considering the previously illustrated role of nAChR in GABAergic interneurones, it is possible to speculate that the activation of cholinergic fibers synapsing onto GABAergic and glutamatergic neurones can take the full control of timing inhibitory and excitatory signals to hippocampal pyramidal neurones (for review see Albuquerque et al., 2000)

1.3.2.5 Activation of the α7 nAChR by choline

Studies of the efficacy and potency of various agonists in activating different subtypes of neuronal nAChR have led to the discovery that choline, the metabolic product of ACh degradation in vivo, is able to act as a very efficacious agonist at α7 nAChR in hippocampal neurones (Albuquerque et al., 1997). Choline evokes no response in neurones that respond to ACh with type II currents, which indicates that it does not activate the α4β2 subtype of nAChR. Thus, the concept that ACh hydrolysis is the means by which cholinergic activity ends in vivo appears to be true for the cholinergic functions mediated by most nAChR subtypes, with the exception of the α7 nAChR.

There has been no experimental determination of the concentrations of ACh and choline in the synaptic cleft. However, it has been demonstrated that the α7 nAChR is sensitive to activation by a choline concentration as low as 200 μM and it can be desensitised with a concentration of 35 μM (Alkondon et al., 1997). Overall, considering that choline uptake into the presynaptic terminal is a slow process, the concentration of choline in the synaptic cleft during synaptic activity could be sufficient to lead to the activation and/or desensitisation of α7 nAChR. In fact, levels of choline may subserve a tonic basal modulation on both synaptic and non-synaptic sites, which is in agreement with the postulated existence of volume transmission in cholinergic systems.

1.3.3 Memory processing and synaptic plasticity

The hippocampus is well known as a critically important structure for attention and memory processing (for review see Jarrard, 1995). As illustrated above, stimulation of nAChR facilitates hippocampal synaptic activity, and behavioural pharmacology studies of nicotinic agonist and antagonist effects on cognitive performance have shown that nicotine stimulation can improve performance on cognitive tasks, while nicotinic antagonists such as mecamylamine can impair it (Levin and Simon, 1998).

Learning and memory processes are the most important mechanisms by which the environment alters behaviour. Learning being the process of acquiring knowledge about the world, and memory the process by which that knowledge is encoded, stored, and later retrieved. Enduring memories appear to be stored, at least in part, as structural changes at specific synapses within neuronal networks, which encode and connect the sensory impressions associated with each memory. These structural changes are thought to occur in the neocortex. As an example, extensive bilateral lesions of the limbic association areas produces devastating memory deficits characterised by the inability to transfer new short-term memory into long-term memory. Lesion models have also provided insights into the nicotinic involvement in hippocampal cognitive function. In effect, nicotine reverses attention and memory impairments caused by basal forebrain lesions in rats, while ablation of the pathway from the septum to the hippocampus causes significant deficits in memory processes (Levin and Simon, 1998).

The theory that information storage is related to changes in synaptic efficacy has been established for a long time. Although derived from early studies of Cajal and Sherrington, Hebb further developed this concept in the late 1940's, suggesting the existence of activity dependent changes in synaptic efficiency. At present, it is widely recognised that the

strength of the connection between a presynaptic and postsynaptic neuron exhibits a remarkable degree of plasticity. The first of such synapses to be identified in the mamallian brain was the excitatory connection made by perforant path fibres onto granule cells of the hippocampus (Bliss and Lomo, 1973; Bliss and Gardner-Medwin, 1973). Brief trains of high frequency stimulation to monosynaptic excitatory pathways in the hippocampus cause an abrupt and sustained increase in the efficiency of synaptic transmission, an effect called LTP. Since then, changes in the efficacy of synaptic transmission have been associated with short-term adaptation to sensory inputs, changes in behavioural states associated with arousal and short-term memory. More lasting changes have been associated with neuronal development in the immature nervous system and with long-term memory in the mature nervous system. At present, it is possible to say that synaptic transmission can be either enhanced or depressed, and these alterations could span a wide range of time scales, from a transient few milliseconds to enduring modifications that persist for days, weeks and maybe longer (Malenka and Siegelbaum, 2000).

When considering the diverse functions and time scales observed in synaptic plasticity phenomena, it is not surprising that a large number of mechanisms have been described. These include a large set of pre- and post-synaptic mechanisms extensively described in invertebrate organisms (Kandel, 2000). However, perhaps no other form of synaptic plasticity has generated more interest than the LTP in the hippocampal formation. In particular, several aspects of hippocampal LTP have made it a suitable model for memory processing in mammalian brain:

- Evidence from lesion studies in rodents and higher primates, including humans, shows that the hippocampus is a critical component of a neural system involved in the initial storage of certain forms of long-term memory (Zola-Morgan and Squire, 1993).
- Several properties of LTP make it, in theory, an attractive cellular mechanism for information storage (for review see Bliss and Collingridge, 1993). Like memories, LTP can be generated rapidly and be prolonged and strengthened with repetition. It exhibits input specificity (Andersen et al., 1977), such that LTP is elicited primarily at the synapses stimulated by afferent activity, but not at adjacent synapses on the same postsynaptic cell. This may increase dramatically the storage capacity in neuronal circuits.
- LTP is also associative (McNaughton et al., 1978), meaning that temporally pairing activity in a "weak" input (incapable of generating LTP by itself) with activation of a strong input (capable of generating LTP at adjacent synapses on the same

postsynaptic cell) results in the LTP of the "weak" input. This associative property is reminiscent of classical behavioural conditioning.

- LTP is readily triggered in in vitro slice preparations of the hippocampus, making it accessible to rigorous experimental analysis. Indeed much of what is known about the detailed cellular mechanisms of LTP derives from studies on two types of excitatory synapses in hippocampal slices: synapses between the Schaffer collateral-commisural axons and the apical dendrites of CA1 pyramidal cells; and synapses between the mossy fiber terminals of dentate granule cells and the dendrites of CA3 pyramidal cells.
- Finally, LTP has been observed at virtually every excitatory synapse in the mammalian brain that has been studied. This includes excitatory synapses in different regions of the hippocampus; all areas and layers of the cortex, including visual, somatosensory, motor, and prefrontal; the amygdala; the thalamus; the neostriatum and nucleus accumbens, the ventral tegmental area; and the cerebellum. Thus, LTP appears to be a ubiquitous property of mammalian excitatory synapses and may subserve a variety of functions in addition to its postulated role in learning and memory (for review see Malenka and Siegelbaum, 2000).

1.3.3.1 Induction of LTP: role of LGIC and Ca^{2+}

Bliss and his colleagues first described LTP at glutamatergic excitatory synapses in the dentate gyrus in vivo in the early 1970's (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). It is now well established that during low-frequency synaptic transmission the neurotransmitter glutamate can bind to different subtypes of ionotropic receptors that are often, but not always, co-localised at individual dendritic synapses. AMPA receptors (kainate receptors can also be localised at some excitatory synapses) have a channel predominantly permeable to monovalent cations (Na^+ and K^+, although some permeability to Ca^{2+} is also observed), which provides the majority of the inward current responsible for generating synaptic responses when the cell is close to its resting membrane potential. NMDA receptors are blocked at negative potentials by extracellular Mg^{2+} and therefore contribute little to the postsynaptic response during low-frequency synaptic activity. When the cells are depolarised sufficiently for the Mg^{2+} to dissociate from its binding site within the NMDA receptor channel, Ca^{2+}, as well as Na^+, can enter the dendritic spine. The resultant rise in intracellular Ca^{2+} is a necessary step in the triggering of LTP. In support of this model for the initiation of LTP, specific antagonists of the NMDA receptor can block the initiation of LTP, while having minimal effects on basal synaptic transmission (Bliss and Collingridge, 1993). In addition, preventing the rise in Ca^{2+} by loading postsynaptic cells with Ca^{2+} chelators also blocks LTP (Lynch et al., 1983; Malenka et al., 1988). Imaging studies have demonstrated

that NMDA receptor activation causes a large increase in the Ca^{2+} level within dendritic spines (Alford et al., 1993; Yuste and Denk, 1995), whereas direct increases in the level of postsynaptic Ca^{2+} can mimic LTP (Malenka et al., 1988).

NMDA receptor activation and increases in postsynaptic Ca^{2+} that do not reach the threshold for eliciting LTP can generate either short term potentiation (Malenka and Nicoll, 1993) that decays to baseline over the course of 5-20 min or even long term depression (Bear and Linden, 2000). Thus, any experimental manipulation that influences the magnitude or dynamics of the postsynaptic Ca^{2+} signal within dendritic spines may have an effect on the form of the synaptic plasticity caused by a given pattern of synaptic activation.

Another relevant issue that remains not entirely resolved is whether an NMDA receptor-dependent rise in Ca^{2+} alone is sufficient to trigger LTP or if additional factors provided by synaptic activity are required. For example, it is clear that various neurotransmitters found in the hippocampus, such as ACh, can modulate the ability to trigger LTP (Matsuyama et al., 2000; Malenka and Siegelbaum, 2000). It has been indeed reported that nicotine enhanced tetanus-induced LTP in the presence of GABA antagonists in hippocampal slices (Sawada et al., 1994) and that a nicotinic agonist facilitated induction of LTP in hippocampal slices (Hunter et al., 1994). In the ventral tegmental area it was demonstrated that activation of presynaptic nAChR increases glutamatergic transmission and could induce LTP of the excitatory inputs (Mansvelder and McGehee, 2000). A direct role for $\alpha 7$ nAChR in the generation of LTP was also suggested in the hippocampus after the demonstration that nicotinic agonists by themselves can induce LTP in vivo (Matsuyama et al., 2000). However, perhaps due to the different subcellular localisation of Ca^{2+} channels, the LTP due to activation of voltage operated Ca^{2+} channels or nAChR may utilise mechanisms distinct from those involved in NMDA receptor-dependent LTP (Cavus and Teyler, 1996).

A large number of signalling molecules have been suggested to play a role in the Ca^{2+} signal that is required to trigger LTP. In fact, the Ca^{2+} influx through NMDA receptors initiates the persistent enhancement of synaptic transmission by activating at least two Ca^{2+}-dependent serine threonine protein kinases, the Ca^{2+}/calmodulin dependent protein kinase (CaMKII) and protein kinase C (PKC). CaMKII is found in high concentrations in the postsynaptic density and has the interesting biochemical property that, when autophosphorylated on threonine 286, its activity is no longer dependent on Ca^{2+}/calmodulin, thus allowing its activity to outlast the Ca^{2+} signal that originally activated the enzyme (Braun and Schulman, 1995). Inhibiting the activity of CaMKII, by directly loading CA1 pyramidal cells with inhibitors of CaMKII, blocks the ability to generate LTP (Malenka et al., 1989). PKC may play a role analogous to that of CaMKII, as PKC inhibitors have been reported to block LTP and loading

of PKC into CA1 pyramidal neurones can enhance synaptic transmission (Hu et al., 1987; Klann et al., 1991). Several other protein kinases have also been suggested to play important roles in the triggering of LTP. As an example, the initial activation of protein kinase A (PKA), perhaps by activation of calmodulin dependent adenylyl cyclase, has been suggested to boost the activity of CaMKII indirectly by decreasing competing protein phophatase activity (Makhinson et al., 1999). In addition, the mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) has been also addressed as an important mediator of synaptic plasticity events in neuronal preparations (see Chapter 4).

The phenomenon of LTP most probably involves pre- and post-synaptic processes. However, the seemingly simple issue of whether LTP is due primarily to a pre- or post-synaptic modification has generated a lot of controversy over the past decade. In fact, although the postsynaptic model can account for much of the experimental literature, it does not preclude the occurrence of significant presynaptic modifications contributing to LTP. Because of the possibility that NMDA receptor dependent LTP involves presynaptic changes, much effort has been directed towards the putative retrograde messenger that must exist. Pharmacological and genetic experiments have identified nitric oxide, a gas that diffuses readily from cell to cell, as one of the possible candidates. Inhibitors of the enzyme responsible for its synthesis (nitric oxide synthase) block the generation of LTP, while NMDA receptor activation can stimulate the activity of nitric oxide synthase through Ca^{2+}/calmodulin (see Malenka and Siegelbaum, 2000).

1.3.3.2 NMDA receptor independent LTP

Although most of the effort toward understanding long lasting forms of synaptic plasticity has focused on NMDA receptor-dependent LTP, it is clear that at certain synapses a robust form of LTP can be generated independent of NMDA receptor activation. The one most extensively examined occurs at mossy fiber synapses in the hippocampus, which are formed between the axons of dentate gyrus granule cells and the proximal dendrites of CA3 pyramidal cells (Nicoll and Malenka, 1995). The first indication of a different mechanism was that LTP could be triggered in the presence of NMDA receptor antagonists (Harris and Cotman, 1986). This finding suggested either that mossy fiber LTP was triggered presynaptically or that a post synaptic rise in Ca^{2+} was required, but that this was achieved via some other source other than NMDA receptors. However, some authors postulate that mossy fiber LTP can be triggered by postsynaptic Ca^{2+} increases due to activation of either voltage dependent Ca^{2+} channels or metabotropic glutamate receptors that release Ca^{2+} from intracellular stores (Yeckel et al., 1999).

1.3.3.3 Late phase LTP

As with memory storage, LTP has phases. One stimulus train produces an early, short-term phase of LTP (early LTP: 1-3 hours), in a process that does not require new protein synthesis. However, four or more trains induce a more persistent phase of LTP (called late LTP) that lasts at least for 24 hours and requires new RNA and protein synthesis (see Malenka and Siegelbaum, 2000). In addition, it has been demonstrated that the late phase LTP recruits several signalling molecules, including the cyclic-AMP (cAMP)-PKA-MAPK-cAMP response element binding protein (CREB) pathway, and may involve the activation, perhaps the growth, of additional presynaptic machinery for transmitter release and the insertion of new clusters of postsynaptic receptors.

In summary, it is presently accepted that the phenomenon of LTP entail a series of inter and intracellular events that can be modulated by quite a few interacting factors. Among the several signalling molecules that are known to participate in these processes, Ca^{2+} has emerged as the pivotal messenger, able to trigger most of the cellular responses associated with the initiation and maintenance of LTP. The Ca^{2+} permeability of nAChR provides a putative molecular basis for the behavioural data showing a role of nAChR in learning and memory. In effect, the study of the Ca^{2+}-mediated signalling mechanisms activated by stimulation of nAChR constitutes a crucial aspect in the understanding of nAChR neuronal function.

1.4 Neuronal Ca^{2+} Signalling

1.4.1 The ubiquitous messenger

The importance of ion fluxes in fast synaptic transmission and in modulating the biophysical properties of neuronal membranes is well established. However, it has become increasingly clear over the past decade that the presynaptic release of neurotransmitter can have long lasting consequences for the postsynaptic neuron. These long lasting adaptations normally involve the elaboration of complicated intracellular signals, which can modulate a variety of cellular processes, including neuronal differentiation, survival, axon outgrowth and changes in synaptic strength (Ghosh and Greenberg, 1995). Much of the attention upon intracellular messengers in the CNS has been focused on the role played by intracellular Ca^{2+} signals. It has been demonstrated that Ca^{2+} can play a role in the cell survival of developing neurones and in the subsequent mediation of their activity. In the mature nervous system, it is critical for the processing of neuronal responses that could, among others, be a major mechanism of information storage in the brain (Franklin and Johnson, 1992; Bliss and Collingridge, 1993). Interestingly, Ca^{2+} is not only implicated in most of the cellular mechanisms of a living neuron, but it is also invariably involved in their death (Berridge et al., 1998). This incredible versatility is given by its capacity to act in the different contexts of space, time and amplitude. Accordingly, different cell types with particular developmental stages, may integrate Ca^{2+} signals with the precise parameters that fit their physiology.

1.4.2 Extracellular sources of Ca^{2+}

There are several ways of increasing intracellular Ca^{2+} in neurones, which, like other cells, use both extracellular and intracellular sources of Ca^{2+} to mediate cytoplasmic Ca^{2+} signals. The mechanisms responsible for regulating the influx of external Ca^{2+} are well established, and include several Ca^{2+} permeable transmembrane channels. Perhaps the most thoroughly studied way of Ca^{2+} influx to the cells is the activation of the Ca^{2+} permeable glutamate receptor subtypes NMDA and AMPA. However, Ca^{2+} can also enter the cell through other selective channels, including voltage operated Ca^{2+} channels (VOCC) (Catterall, 1995) or the nAChR ligand gated channel.

1.4.2.1 Voltage operated Ca^{2+} channels (VOCC)

VOCC are widely distributed in the animal kingdom and are an essential part of many excitable and non-excitable mammalian cells. The opening of theses channels is primarily regulated by the membrane potential, but is also modulated by a wide variety of hormones, protein kinases, protein phosphatases, toxins and drugs. VOCC have been subdivided into several classes by biophysical and pharmacological criteria. The *low voltage activated channels* are defined by the T-type current, while the *high voltage activated channels* are classified into L (long lasting)-type, P (Purkinje)-type, N (neither L nor T channel)-type, Q-type and R (remaining)-type. The P-, Q-, N-, and R-type currents have been mainly identified in neuronal and endocrine tissues, whereas L-type currents have been found in skeletal, heart and smooth muscles, in fibroblasts, and also in neuronal and endocrine cells (Hofmann et al., 1999). The high voltage activated Ca^{2+} channels are heterooligomeric complexes of five proteins from four genes: the $\alpha 1$ subunit, the intracellularly located β subunit; the $\alpha 2\delta$ subunit, a disulphide-linker dimer, and the transmembrane γ subunit (for details see Catterall, 1995).

The functional roles of VOCC are rather diverse. In skeletal muscle the L-type channels are crucial for excitation-contraction coupling; in heart they are necessary for the generation of electrical impulses and for the initiation of contraction, while in smooth muscle they are involved in tension development (Hoffman et al., 1999). In neurones, L-type channels provide the Ca^{2+} for activation of small conductance Ca^{2+} activated K^+ channels (Marrion and Tavalin, 1998). In addition, the fact that L-type VOCC have a relatively high activation range, a slow inactivation rate and a high single channel conductance, makes them ideal transducers for the passing of large amounts of Ca^{2+} (Catterall, 1995). The N- and P/Q-type channels appear to be linked in many neuronal cells to neurotransmitter secretion (Hofmann et al., 1999).

Most of the prominent features of the Ca^{2+} channel complex can be assigned to the $\alpha 1$ subunit, which contains the ion conducting pore, the selectivity filter of the pore, the voltage sensor and the interaction sites for the β subunits, the $\alpha 2\delta$ subunit, the Ca^{2+} channel blockers and activators. Nine individual genes have been identified for the $\alpha 1$ subunit, which are homologous to each other and encode proteins of predicted molecular masses of 212 to 273 kDa. They belong to the same multigene family as voltage-activated Na^{2+} and K^+ channels and share a common ancestral protein with them. Hydrophobicity analysis of the $\alpha 1$ subunit predicts a transmembrane topology with four homologous repeats, each containing five hydrophobic putative α helices and one amphiphatic segment. An early evolutionary event separated the $\alpha 1$ subunit into the electrophisiolgically distinct low-voltage

activated and high voltage activated Ca^{2+} channels, which share less than 30 % sequence identity (Hofmann et al., 1999).

1.4.2.2 Glutamate receptors

The ionotropic glutamate receptors are LGIC that mediate the vast majority of excitatory neurotransmission in the brain. Three pharmacologically defined classes of these receptors have been described, named according to the selective agonists that identified them: NMDA, AMPA and kainate receptors. The receptor subunits for the different glutamate receptors are encoded by at least six gene families as defined by sequence homology: a single family with four different subunits for AMPA (GluR1-4), two families with 5 subunits for kainate (GluR5-7 and KA1-2) and three families with 6 subunits for NMDA (NR1, NR2A-D, NR3A) (Dingledine et al., 1999). Sequence similarity suggests a common evolutionary origin for all of the ionotropic glutamate receptor genes. With respect to their transmembrane structure, glutamate receptors proved to have only three transmembrane domains (M1, M3 and M4) plus a cytoplasm-facing re-entrant membrane loop (M2). As a result, the N-terminus is located extracellularly and the C-terminus intracellularly (Fig 1.1). The ligand binding site is formed by a conserved amino acid-binding pocket (Sun et al., 1998), proposed to exist in all glutamate receptors, and which would be formed from two globular domains (S1 and S2) drawn from the sequence adjacent to the M1 domain and the M3-M4 loop. It is speculated that the closure of the S1 and S2 lobes places a torque on the receptor that is transmitted to the channel region, and generates the opening.

Early evidence suggested a pentameric structure for glutamate receptors, based on the size of chemically cross-linked NMDA receptor proteins (Brose et al., 1993). However the conclusions from at least six functional evaluations of receptor subunit mixtures are divided between the structure being a tetramer or a pentamer (Dingledine et al., 1999 and references therein). Although the number of subunits composing a receptor is still a matter of dispute, it is generally accepted that a given subunit will only assemble with others within its own family. The precise subunit composition of native glutamate receptors is still not known, but it is apparent that multiple subtypes exist within the AMPA and NMDA receptor families based on subunit composition. In addition to the functional diversity provided by the existence of different genes, ionotropic glutamate receptors are subject to posttranscriptional alterations, which give rise to added structural and functional diversity.

The endogenous transmitter L-glutamate activates both NMDA and non-NMDA receptors, whereas the putative transmitter candidate L-aspartate appears to activate NMDA receptors exclusively (Patneau and Mayer, 1990). In addition, glycine has been shown to be an

essential co-agonist at NMDA receptors (Dingledine et al., 1999). Gating of AMPA and kainate receptors by glutamate is extremely fast, and contrasts with the slow gating of NMDA receptors. In addition, both AMPA and kainate receptors possess a low-affinity binding for the endogenous transmitter glutamate, and are thus thought to deactivate quickly because of the brief mean bound time of the agonist. The rapid activation and brief open time of AMPA facilitates unblock of NMDA receptors by Mg^{2+}, and therefore participation of the more slowly activating and deactivating NMDA receptors in synaptic currents. In effect, one unique feature of the NMDA receptor compared to other LGIC, is the dual dependence of function on agonist binding and membrane potential. This property renders the Ca^{2+} flux through NMDA receptors a coincidence detector for depolarisation and synaptic release of glutamate. The NMDA receptor's voltage dependence follows directly from channel block by submillimolar concentrations of extracellular Mg^{2+}, rather than from the voltage dependence of conformational changes (Nowak et al., 1984). Binding of extracellular Mg^{2+} within the pore is strongly voltage dependent, a property that dominates the physiological role of NMDA receptors. At resting membrane potentials, most subtypes of NMDA receptor undergo rapid channel block by extracellular Mg^{2+}, which reduces the NMDA component of synaptic currents considerably. However, when neurones are depolarised, for example by activation of colocalised AMPA receptors, the voltage dependent block by Mg^{2+} is partially relieved.

All glutamate ionotropic receptors are permeable to cations, and sodium and potassium are thought to be nearly equally permeable. Recombinant NMDA receptors appear to be more permeable to Ca^{2+} than non-NMDA glutamate receptor subtypes. The combination of AMPA/Kainate and NMDA glutamate receptors constitutes a finely tuned association, crucial for glutamate transmission.

As stated in sections 1.1 and 1.3 of this chapter, nAChR can also mediate cytoplasmic changes in Ca^{2+} concentrations due to their high permeability to Ca^{2+}. Interestingly, their specific location and functional properties allows them to activate precise Ca^{2+} signals, not necessarily overlapping with those evoked by other sources of Ca^{2+} influx.

1.4.3 Intracellular Ca^{2+} stores

While much is known about the extracellular Ca^{2+} influx pathways, there is less information on the mechanisms and role of the intracellular supply of Ca^{2+} stored within the endoplasmic reticulum of neurones. The endoplasmic reticulum is a continuous tubular structure composed of distinct heterogeneous subunits with different Ca^{2+} transport characteristics (Golovina and Blaustein, 1997). Perhaps the most remarkable aspect of this endoplasmic

reticulum network is that it appears to be a continuous membrane system (Terasaki et al., 1994), which has a number of regional specialisations of particular significance with regard to Ca^{2+} signalling

The endoplasmic reticulum neuronal network contains inositol 1,4,5-triphosphate receptors (IP_3R) and ryanodine receptors (RyR), both of which are capable of regenerative Ca^{2+} release, enabling the endoplasmic reticulum to play an active role in neuronal Ca^{2+} signalling (Berridge, 1998; Fig 1.6). These two intracellular channels integrate information from the cytoplasm and the lumen, and they are both capable of displaying the phenomenon of Ca^{2+} induced Ca^{2+} release (CICR). It is this regenerative process that is responsible for amplifying Ca^{2+} signals coming from the outside and for setting up Ca^{2+} waves (Berridge, 1998). The Ca^{2+} sensitivity of these receptors can be influenced by a variety of intracellular factors, including IP_3 in the case of IP_3R and cyclic ADP ribose in the case of RyR. In addition, and through a mechanism that has yet to be defined, a buildup of Ca^{2+} within the lumen of the endoplasmic reticulum can exert a positive feedback effect by increasing the Ca^{2+} sensitivity of both the RyR and IP_3R.

1.4.3.1 Ca^{2+} release from IP_{-3} receptors

The phosphoinositide system is particularly well developed in the brain, where a large number of receptors respond by stimulating the hydrolysis of phosphatidylinositol 4,5-biphosphate to form the second messenger's diacylglycerol and IP_3. The latter, can act by modulating Ca^{2+} release from IP_3R, which are widely distributed through the brain. The study of Ca^{2+} release from IP_3R is complicated by their sensitivity to a variety of factors; the most effective activation being when Ca^{2+} and IP_3 are presented together. In addition, IP_3 receptors can be regulated by PKA, PKC and by tyrosine kinases, as well as by the Ca^{2+} dependent phosphatase, calcineurin (for review see Meldolesi, 2001). This may suggest that the IP_3R act as a coincidence detector during integration of synaptic signals (Simpson et al 1995).

1.4.3.2 Ca^{2+} release from ryanodine receptors

RyR resemble the IP_3R in that they are sensitive to Ca^{2+} entering the cytoplasm from outside the neuron or from neighbouring internal stores receptors. Therefore, Ca^{2+} entering from extracellular sources can directly induce Ca^{2+} release from intracellular stores, producing an amplification of the influx signal (Fig 1.6). The extent to which this internal amplification process operates varies considerably between neurones, and internal release can be triggered by either a single action potential (Cohen et al., 1997) or by trains of action

potentials (i.e. hippocampal cells; Jacobs and Meyer, 1997). In addition to the modulation by intracellular Ca^{2+}, a specifc messenger, cyclic ADP ribose, exists for RyR. Although the precise mechanism of receptor regulation by cyclic ADP ribose is not known, it has been proposed as a potentiator, rather than a mediator, of the RyR activation by Ca^{2+}.

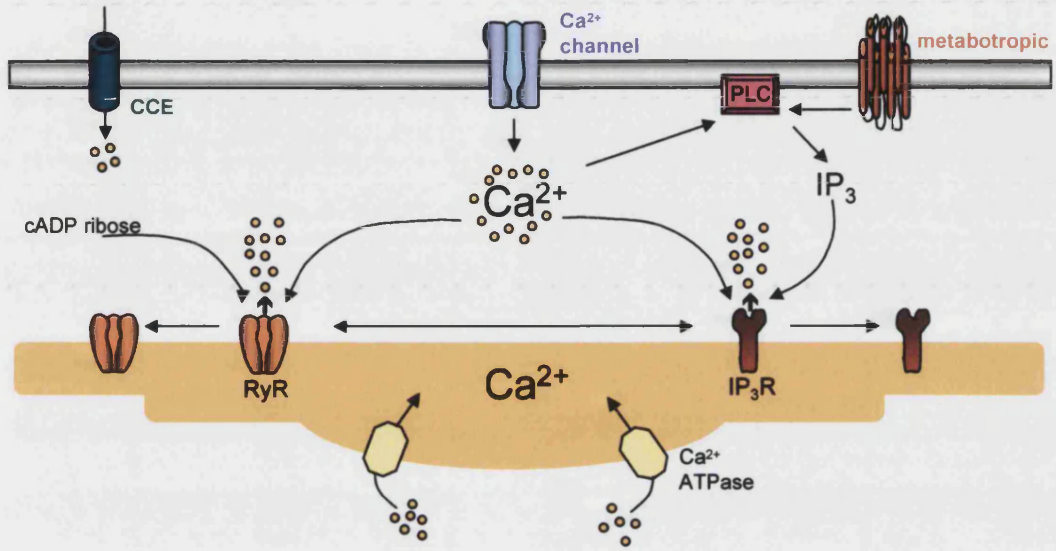

Fig 1.6 Neural Ca^{2+} signalling

The cytoplasmic levels of Ca^{2+} can be regulated by extracellular or intracellular sources, which are operated by various mechanisms. The entry of Ca^{2+} via ion channels can activate release of Ca^{2+} from internal stores via activation of RyR, which can also be modulated by cyclic ADP ribose. In addition, many metabotropic receptors couple to activation of phosphoinositol specific phospholipase-C (PLC) and consequent production of IP_3. In turn, IP_3 can activate the release of Ca^{2+} from IP_3 sentitive internal stores. The influx of Ca^{2+} through plasma membrane ion channels can also increase activation of IP_3R via potentiation of the activity of PLC or via direct sensitisation of IP_3R. Thus, ionotropic receptors or VOCC might induce Ca^{2+} induced Ca^{2+} release via either or both types of intracellular Ca^{2+} channel. This process of Ca^{2+} induced Ca^{2+} release can subsequently initiate the propagation of Ca^{2+} waves. Although the capacitative Ca^{2+} entry (CCE) constitutes another potential source of cytoplasmic Ca^{2+}, its regulation and physiological function is still uncertain.

1.4.3.3 Ca^{2+} spikes and waves

The neuronal endoplasmic reticulum is an excitable organelle capable of regenerative release to produce repetitive Ca^{2+} spikes and waves (Berridge, 1998). These Ca^{2+} waves may function to spread Ca^{2+} signals bidirectionally throughout the cytoplasm (i.e., transferring Ca^{2+} signals from the cell periphery to the nucleus). In addition, it could function much more locally to spread a localised signal between synaptic spines. However, the existence of such Ca^{2+} waves is very dependent on the progressive loading of the endoplasmic reticulum, which thus reflects its dual role as a source and a sink for Ca^{2+}. This faculty gives the potential for integrating not only spatial Ca^{2+} signals, but also temporal ones, as the loading of the endoplasmic reticulum is highly dependent on the previous activity of the neuron (Miller et al., 1996). Thus, the temporal and spatial conjunction of CICR

and IP_3 elicited Ca^{2+} release might play a role in several neuronal processes, like LTP, LTD, transmitter release and neurotoxicity (Simpson et al., 1995). In chapter 4, I will present and discuss evidence regarding the role of intracellular Ca^{2+} stores in the intracellular Ca^{2+} signals activated by nAChR.

1.4.4 Ca^{2+} dependent signalling

The complexity of Ca^{2+}signals is not only associated to the various sources of Ca^{2+} influx to the cell. Once Ca^{2+} reaches the cytoplasmic compartment it can activate numerous signalling mechanisms, capable of modulating almost every aspect of neuronal physiology. Ca^{2+} influx through NMDA receptors and/or VOCC has been extensively studied with regard to the Ca^{2+} dependent neuronal responses. In this chapter I will concentrate on both of them in order to review some of the key components involved on the Ca^{2+} signalling pathways. The role that specific nAChR-evoked Ca^{2+} signals may have on neuronal function will not be discussed here, as it will be extensively examined in Chapters 2, 3 and 4.

Synaptic stimulation that leads to the activation of NMDA receptors and/or VOCC is followed by a series of dramatic Ca^{2+} dependent cellular responses. Stimulation of the NMDA receptor and the activation of VOCC lead to the activation of a variety of distinct protein kinases, including CaMK (I-V), PKC, various components of the Ras/ERK signalling pathway and tyrosine kinases (Greenberg and Ziff, 2000). There is increasing evidence that functions such as LTP and LTD are at least partially mediated by the protein kinase catalysed phosphorylation of specific ion channel subunits, leading to ion channel modulation or changes in the cell surface expression of ion channel subunits (Greenber and Ziff, 2000).

In addition to inducing rapid cellular responses, such as ion channel modulation, the Ca^{2+} evoked signals can have a more long lasting effect that is associated with the activation of down stream signalling effectors, which ultimately lead to induction of new gene expression (Finkbeiner and Greenberg, 1998). However, the way in which changes in gene expression can selectively alter the efficacy of only a few of many synapses is still a matter of discussion. One possibility is the so-called "tagging" of the synapse, which leads to the mutually selective recognition of the new gene products by the "tagged" synapse (Frey and Morris, 1997). On the other hand, another possibility that has gained more recent support is that neurotransmitters modulate gene expression post-transcriptionally by regulating the translation stability of specific mRNA that are localised and translated within the dendritic region of the postsynaptic neuron (Huang, 1999).

The first indication that activation of ionotropic neurotransmitter receptors could lead to the activation of new gene expression came from studies of the c-fos proto-oncogene (Greenberg et al., 1986). It is presently known that its transcription can indeed be rapidly and transiently activated in response to a wide variety of extracellular stimuli, including neurotransmitters and other agents that can lead to membrane depolarisation and the activation of L-type VOCC (Sheng and Greenberg, 1990). The c-fos gene is a member of a family of 50-100 genes termed immediate early genes, which are activated rapidly after stimulation. The best characterised of these genes are the transcription factors, like c-fos and c-jun, that can regulate subsequent programs of late responses. However, some immediate early genes do not represent transcriptional regulators, but encode either soluble factors or receptors that affect neuronal function and enzymes mediating the synthesis of neurotransmitters (Sheng and Greenberg 1990). These immediate early genes, in contrast to the ones that encode transcriptional regulators, are selectively activated in neurons, being capable of modulating synaptic function. One of the best characterised of these neuron specific, Ca^{2+} regulated, immediate early genes is the brain-derived neurotrophic factor (BDNF), which is a member of the neurotrophin family that plays a critical role in regulating the survival and differentiation of selective populations of neurones during development. Activation of VOCC and NMDA receptors could lead to a significant rise in the level of BDNF mRNA and also stimulates the release of BDNF protein, while BDNF has also been shown to be an important mediator in neuronal adaptive responses (Kang and Schuman, 1995).

1.4.4.1 CREB

Promoter deletion analysis in cultured cells revealed the DNA regulatory elements within the c-fos promoter that mediate Ca^{2+} induction of c-fos transcription (Sheng and Greenberg, 1990). One key regulatory sequence described (5'-TGACGTT-3') and designated the Ca^{2+} response element (CaRE), is located 60 nucleotides 5' of the initiation site for c-fos mRNA synthesis. The c-fos CaRE is similar in sequence to the cAMP regulatory element CRE, a DNA regulatory element that mediates the transcriptional responses to elevated levels of cAMP. CRE and CaRE were shown to be functionally indistinguishable, which suggested that the same DNA regulatory element mediates both Ca^{2+} and cAMP responses (Sheng et al., 1991). A member of the basic leucine zipper family of transcription factors, CREB can regulate gene transcription by binding to the CaRE or CRE DNA sequence. When activated by phosphorylation of ser-133, CREB can act as a regulator of immediate early genes that encode transcription factors (i.e., c-fos, c-jun, egr-1) and neuron specific early genes (i.e., BDNF), which made it one of the most studied of the Ca^{2+} regulated transcription factors. At present, CREB has been implicated in several neuronal functions, including long term

memory in flies, long lasting LTP in mice, differentiation and survival (Greenberg and Ziff, 2000).

1.4.4.2 Ca^{2+}-regulated CREB kinases

Considering the clear importance of Ser-133 phosphorylation for CREB activation and function in mature neurones, it is relevant to understand the kinase cascades that may trigger this critical event. In fact, there are multiple Ca^{2+} regulated kinase cascades that catalyse CREB Ser-133 phosphorylation in membrane-depolarised cells (Finkbeiner and Greenberg, 1996). They include CaMK I, II and IV, the ribosomal S6 kinases (pp90Rsk1-3), PKC, PKA, the serine/threonine kinase Akt, and the MAPK-activated protein kinase. These kinases are part of a complex structure of intracellular signalling molecules capable of regulating several aspects of neuronal function, including CREB activation. The Ras/MAPK signalling cascade in particular, has been shown to be activated by Ca^{2+} influx and to trigger the sequential phosphorylation of the kinases MEK/MAPK and Rsk, which can then translocate into the nucleus and phoshporylate transcription factors, including CREB (Greenberg and Ziff, 2000). A more detailed study of the Ras/MAPK cascade and its dependence on nAChR activation will be reviewed on Chapter 4 of this thesis.

1.4.4.3 Importance of Ca^{2+} entry routes

The incredible versatility of Ca^{2+}-activated neuronal responses has suggested that particular neuronal types, or even neuronal compartments, may have precise and unique ways of integrating Ca^{2+} signals. It has been demonstrated that Ca^{2+} influx through VOCC is much more effective at inducing CREB dependent transcription than is Ca^{2+} influx through the NMDA receptors channels (Hardingham and Bading, 1999). These results suggest that the location, duration and amount of intracellular Ca^{2+} concentration may account for the specificity of the Ca^{2+} evoked responses. However, it should be also possible for Ca^{2+} to activate specific signal transduction molecules that are localised in close proximity to its site of entry, therefore conferring specificity towards the influx path. More recent evidence indicates that Ca^{2+} mediated specificity is achieved, in part, by the use of scaffolding and/or anchoring proteins which facilitate the regulation of signal transduction pathways. A more detailed account on the role of Ca^{2+} entry, specificity of Ca^{2+} signalling routes, and scaffolding mechanisms will be reviewed on Chapter 2 and 5.

1.5 Project Aims

Although a considerable amount of information has been gathered about the molecular structure, pharmacology and biophysical properties of nAChR, the role of neuronal nAChR in the modulation of various cellular processes of the nervous system has only become apparent in the last decade. The existence of multiple subtypes of neuronal nAChR, the non-uniform distribution throughout the nervous system and their localisation to both pre- and post-synaptic zones, imply diverse functions of nAChR and indicate that several neuronal mechanisms can be triggered as a result of nAChR stimulation. In spite of the increasing amount of evidence suggesting a functional role for the nAChR, not much is known about the overall cellular mechanisms involved. The aim of this work was to investigate some of the intracellular signalling processes activated by the stimulation of nAChR in neuronal preparations.

The first part of the project was designed to study the putative neuroprotective actions of nicotine stimulation. As a model system we utilised primary cultures from rat hippocampus, a brain region involved in learning and memory processes and one that is affected in the progression of Alzheimer's disease. The specific aims of this part were:

- The analysis of the excitotoxic actions of glutamate and NMDA stimulation in hippocampal primary cultures.
- To demonstrate if nicotine stimulation and nAChR activation, in particular, can prevent the neuronal loss.
- The characterisation of the nAChR subtypes involved and the cellular mechanisms implicated in the observed responses.

The results obtained suggested that the stimulation of neuronal nAChR was capable of protecting hippocampal neurones through the activation of precise and sustained Ca^{2+} signals. Therefore, we decided to centre the attention on the analysis of the Ca^{2+}, and Ca^{2+} dependent, signals evoked after activation of nAChR. As a first approach, experiments were conducted to study the Ca^{2+} sources mediating nicotine evoked Ca^{2+} responses in neurones. In order to do so, we utilised the human neuroblastoma SH-SY5Y cell line as a model neuronal system. In particular, the aims of this part included:

- The replication in SH-SY5Y cells of the sustained Ca^{2+} responses shown to occur in hippocampal primary cultures.
- The analysis of the nAChR subtypes involved in nicotine evoked Ca^{2+} signals.

- The determination of the role of voltage operated Ca^{2+} channels and intracellular Ca^{2+} stores in the nicotine evoked increases in cytoplasmic Ca^{2+}.

Although Ca^{2+} has been suggested as the main candidate for the mediation of nAChR modulated actions, not much is known about the precise cellular mechanisms activated by nicotine evoked Ca^{2+} signals. For that reason, we decided to use both hippocampal primary cultures and SH-SY5Y cells to explore some of the putative signalling pathways activated after nAChR stimulation The particular objectives of this part of the work were:

- The analysis of ERK1/2 activity after nAChR stimulation in both SH-SY5Y cells and hippocampal neurones.
- The study of the nAChR subtypes involved and the Ca^{2+} dependence of nicotine's actions.
- To establish the precise cellular pathways connecting the stimulation of nAChR with the modulation of ERK1/2 activity.
- To determine the relevance of this pathway in the overall processes regulated by nicotine in the hippocampus, and neuroprotection in particular.

CHAPTER 2

< nAChR and Neuroprotection >

2.1 Introduction

2.1.1 Alzheimer's disease and neurodegeneration

Although the cellular bases of neurodegeneration are complex and not completely understood, it is generally assumed that a series of genetic and ambient factors act in combination to trigger the cellular mechanisms leading to neuronal impairment and subsequent loss of brain function. In the case of Alzheimer's disease (AD), the progressive loss of cognitive ability is the result of a neurodegenerative process classically characterised by the extracellular deposition of the β-amyloid (Aβ) peptide in deposits termed senile plaques, and the formation of neurofibrillary tangles in neurones (Kawahara and Kuroda, 2000; Neve et al., 2000). Additional pathological hallmarks of AD include granulovacular degeneration and the overall loss of synaptic contacts and cell density in distinct regions of the brain. AD does not have a simple aetiology. It can occur as a "sporadic" event; it can result from the possession of an extra copy of the chromosome 21 (Down's syndrome); or it can be caused by mutations in the amyloid precursor protein (APP) gene on chromosome 21 or by mutations in the preselinin genes on chromosome 1 and 14 (Neve et al., 2000).

Numerous mechanisms for the neuronal degeneration observed in AD have been proposed, but perhaps the most widely accepted theory is the amyloid hypothesis, in which the deposition of Aβ peptides derived from the APP precursor has been implicated in the pathological cascade that leads to neuronal loss (Kawahara and Kuroda, 2000). This argument is based on in vitro studies showing that Aβ is toxic to neurones, and on the measurement of increased release of Aβ by cells carrying familial AD mutant genes (Neve et al., 2000). The Aβ peptide exists in two major carboxy-terminal variants. $A\beta_{1-40}$ is the major species secreted from cultured cells and found in cerebrospinal fluid, while $A\beta_{1-42}$ is the major component of amyloid deposits (for review see Younkin, 1995). Cells expressing familial AD mutants of APP and the preselinins are reported to secrete increased amounts of $A\beta_{1-42}$, thus suggesting a link of this variant of Aβ to AD pathogenesis (Younkin, 1995). Overall, these mechanisms are linked in some way to the APP, which is the source of Aβ. However, perhaps the most convincing piece of evidence linking AD neurodegeneration to APP and/or its Aβ derivatives is the early finding that the APP gene is located on

chromosome 21, as virtually all individuals trisomic for this chromosome show AD-like neurophatology by the age of 40. In addition, it has been discovered that specific mutations in the APP can be the cause of some forms of familial AD mutants (Neve et al., 2000). These data have raised the possibility that AD may result from an alteration in the normal function of APP (Neve and Robakis, 1998; Nishimoto 1998), and much of the attention has refocused on the delineation of the function that APP subserves in the CNS. It has been shown that in the brain, a percentage of APP is present on the cell surface (Perez et al., 1997) and it is proposed that this cell surface APP mediates the transduction of extracellular signals into the cell via its intracellular C-terminal tail (Perez et al., 1997; Nishimoto, 1998). In addition, APP can also exist in a soluble releasable form that has been associated with promotion of cell survival, neurite outgrowth, synaptogenesis and synaptic plasticity (for reviews see Mattson, 1997). The processing and release of APP can be regulated by a variety of factors, including stimulation of receptors for acetylcholine, serotonin, glutamate and neuropeptides (Nitsch and Growdon, 1994). In particular, it has been shown that the nicotine-evoked increase in soluble APP was inhibited by the α7 nAChR antagonist methyllycaconitine, suggesting that activation of α7 nAChR is sufficient to enhance this secretion (Seo et al., 2001). The α7 nAChR has been also shown to bind selectively and with high affinity with the Aβ peptide in cerebral cortex and hippocampus (Wang et al., 2000). At present, the physiological effect of this interaction is still unclear, as the Aβ peptide has been shown to block nicotine-evoked currents from hippocampal interneurones (Pettit et al., 2001; Liu et al., 2001), but also to activate the ERK cascade via α7 nAChR in an organotipic slice preparation from the hippocampus (Dineley et al., 2001).

The actual mechanisms of Aβ peptide toxicity in vitro might involve the excessive Ca^{2+} influx to the cells (Mattson et al., 1993). It has been reported that antigenic changes of cytoskeletal elements typical of AD are mimicked by excessive Ca^{2+} influx into cultured hippocampal neurones (Mattson, 1990), and when neurones were treated with actin depolymerising agents, known to block receptor-mediated Ca^{2+} entry, Aβ-induced toxicity was prevented (Furukawa and Mattson, 1995). In fact, it may be speculated that the pathology of AD is related to a very protracted chronic form of excitotoxicity (Mattson et al., 1993; see below) caused by a conversion of APP to Aβ. Accordingly, the unprocessed APP protein seems to have a dampening effect on the physiology of cellular Ca^{2+} by hyperpolarizing neurons through the opening of potassium channels (Furukawa et al., 1996). Interestingly, and in agreement with these findings, soluble APP protects neurones from Aβ toxicity (Mattson et al., 1993).

2.1.2 Ca^{2+} and excitotoxicity

As illustrated above, the precise cellular causes of cell death in neuronal degenerative disorders are still being actively investigated. Although it is increasingly accepted that triggering mechanisms might vary according to the particular neurodegenerative disorder, the cellular process of neurodegeneration in the CNS may share some common mechanisms. In particular, the excessive increase in intracellular Ca^{2+} concentrations has been addressed as a fundamental player in the progression of neurodegenerative cell death. One of the most common mechanisms of neuronal death by excessive Ca^{2+} accumulation is initiated by a pathological condition known as excitotoxicity. This is a phenomenon typically encountered in neurones, and follows a stimulation that exceeds the physiologic range with respect to duration or intensity. In general, it describes the supra-physiological activation of glutamate receptors, and in particular the NMDA receptors subtype. A large variety of chronic neurodegenerative diseases seem to have an excitotoxic component (Meldrum and Gathwaite, 1990), while a causal contribution of excitotoxicity to neuronal damage has also been established in stroke or head trauma (Lipton and Rosenberg, 1994) and in acute poisoning phenomena, like 1-methyl-4-phenyl-1,2,3,5-tetrahydopyridine (MPTP; Turski et al., 1991). The excessive increase in intracellular Ca^{2+} observed in excitotoxic processes could then lead to impaired energy metabolism, mitochondrial dysfunction and oxidative stress, resulting in neuronal loss (Meldrum and Garthwaite, 1990; Leist and Nicotera, 1998).

In spite of its recognised role as a contributor mechanism to neuronal death, excitotoxic processes are probably not the initial trigger of the observed neuronal degeneration. In fact, a number of cellular processes could lead to the generation of excitotoxic cell death. For example, an initial insult like the blockade of the mitochondrial respiratory chain, or the perturbation of intracellular Ca^{2+}, could cause a limited disturbance of cellular homeostasis, mitochondrial dysfunction and subsequent energy depletion. This would result in the reduced activity of ion pumps, membrane depolarisation and the opening of VOCC, which consequently leads to hyperexcitability and the increased probability of glutamate release. Subsequent to the increase in glutamate, all subtypes of glutamate receptors can initiate the excitotoxic processes, although is the prolonged activation of the Ca^{2+}-permeable NMDA subtype of glutamate receptor that constitutes the key mediator of excitotoxicity in the CNS (Leist and Nicotera, 1998).

2.1.3 nAChR involvement in cognitive disorders and Alzheimer's disease

ACh is an important participant in the maintenance of cognitive functions, as lesions of forebrain cholinergic nuclei and pathways alter memory and, more specifically, working memory and attentional processes that rely on the integrity of the prefrontal cortex and are deeply affected in patients with AD (Winkler et al., 1995). Although some of these contributions are mediated by muscarinic receptors, nicotine is also able to selectively improve attention, learning and memory through activation of nAChR (Levin and Simon, 1998). These nicotine-induced memory improvements do not diminish with continued treatment and appears to be specific to working memory, with little or no effect on reference memory (Levin et al., 1997; Rezvani and Levin 2001). In addition, studies with transgenic mice lacking functional β2 subunits, demonstrated that the lack of high affinity nAChR leads to an impairment in spatial learning and memory (for review see Changeux et al., 1998).

Additional evidence for the role of nAChR in cognitive processes has been provided by the observed loss of cortical nAChR in AD (Nordberg 1994; Whitehouse and Au, 1986). Studies using neuroimaging techniques also support the involvement of nAChR in AD, as a significant correlation between the temporal cortex labelling of [^{11}C]-nicotine and cognitive function scores in AD patients has been found (Nordberg, 1993). However, epidemiological studies and studies of receptor changes in post-morten or living tissue constitute only indirect evidence that the loss of nAChR is relevant to the cognitive disorder of AD. In particular, they will not demonstrate if the observed nAChR loss is either a cause or a consequence of the neurodegenerative process. On the other hand, studies using nAChR antagonists serve a more direct purpose, by producing a temporary "chemical lesion", which may uncover some of the functional roles of nAChR activation in memory processes. In a series of studies by Newhouse and colleagues (1992; 1994) it was shown that the non-selective nAChR antagonist mecamylamine could produce cognitive impairment, resembling in many cases that seen in AD. Consistent with this proposed role of nAChR in cognitive disorders, symptomatic benefits, such as improvement in attentional functioning, have been reported in patients with AD after nicotine administration (Sahakian et al., 1989; Parks et al., 1996). In addition, the use of cholinesterase inhibitors has been applied as a therapy in the treatment of AD, their positive effects associated not only with an increase of cholinergic transmission, but also with a direct modulation of nAChR function (Maelicke et al., 2001). In fact, galanthamine (Reminyl), a cholinesterase inhibitor with known properties as an allosteric modulator of the nAChR, is administered as a therapeutical treatment for AD (Maelicke et al., 2001).

nAChR have been a recent target for the development of cognitive disorder therapies (Arneric et al., 1995). However, the symptomatic relief produced by cholinergic agents in the treatment of AD are not dramatic, possibly because the treatment is administered at a late stage, when the neuronal degeneration is already advanced. At present, ongoing investigations of the molecular structure and pharmacology of neuronal nAChR are actively used in the development of novel nicotinic agents as potential therapeutic means (Lloyd and Williams 2000). Ideally, the fulfilment of these therapeutic objectives should be intimately associated with the understanding of the cellular mechanisms activated by nAChR in the nervous system. Nonetheless, the true significance of many of the neuronal actions of nicotine stimulation is still far from being resolved.

2.1.4 nAChR mediated cytoprotection

As discussed earlier, epidemiological and clinical data initially suggested the use of nicotinic agonists as therapeutic agents in the treatment of cognitive disorders. In most cases, the rationale behind this clinical conduct was based on the apparent involvement of cholinergic systems in the processing of brain cognitive functions, and was mainly associated with a symptomatic relief rather than neuronal restoration. However, as the results from the clinic prompted the investigation of nicotine actions in neuronal preparations, an initially unexpected observation was unravelled. In effect, in vitro and in vivo work carried out primarily in the last ten years provided direct evidence to support the belief that nicotine stimulation could be acting as an important factor in neuroprotection.

2.1.4.1 In vitro neuroprotection with nAChR ligands

The majority of in vitro models utilised in the study of nicotine neuroprotection try to mimic some of the cellular processes occurring in the onset and/or progression of various neurodegenerative disorders, such as PD, AD and cerebral ischemia (Lipton and Rosenberg 1994; Donnelly-Roberts and Brioni, 1999). In particular, several laboratories have investigated neuronal models associated with the overstimulation of glutamatergic receptors and/or Aβ derived toxicity. These studies have shown that in vitro, nAChR activation can protect neuronal cultures against excitotoxic cell death (Marin et al., 1994; Donnelly-Roberts et al., 1996; Kaneko et al., 1997) and Aβ toxicity (Kihara et al. 1997; Zamani et al., 1997; Wang et al., 2000; Kihara et al 2001), while also preventing the dexamethasone potentiation of kainic acid toxic effects (Semba et al., 1996). In addition, nicotine has been reported to protect PC12 cells against nerve growth factor deprivation (Yamashita and Nakamura, 1996; Li, et al., 1999) and to reduce apoptosis in chick cilliary ganglion neurons (Pugh and

Margiotta, 2000). It is also relevant to mention that the neuroprotective effects observed after stimulation with nicotine in different neuronal preparations are mediated by nAChR, since nAChR antagonists attenuated or completely eliminated the neuroprotection.

In general, nicotine neuroprotection has been demonstrated in various types of neuronal preparations, including cortical and striatal primary cultures (Marin et al., 1994; Kihara et al., 1997; Prendergast et al., 2001). However, previous reports also demonstrated that in certain neuronal models activation of nAChR can produce deleterious effects, such as neurotoxicity in the axons of the medial habenula (Carlson et al., 2001), naturally occurring cell death in motoneurones (Hory-Lee and Frank, 1995) and apoptosis in HC2S2 immortalised hippocampal progenitor cells (Berger et al., 1998). Interestingly, the ability of nicotine to induce apoptosis in undifferentiated HC2S2 cells was associated with the lack of adequate endogenous Ca^{2+} buffering systems. Accordingly, differentiated HC2S2 cells that, unlike their undifferentiated counterparts, have strong expression of the Ca^{2+} binding molecule calbindin, showed no apoptosis after nicotine stimulation (Berger et al., 1998). These results suggest that the capacity of neurones to integrate complex Ca^{2+} signals constitutes a critical factor in the final outcome of nAChR activation. Altogether, the effects of nicotine on cell survival probably depends on a number of factors such as specific gene expression, cell cycle phase, developmental stage and Ca^{2+} buffering capabilities of neuronal populations. As a result, a critical part in the study of nicotine neuroprotection is the understanding of the role of intracellular Ca^{2+} in the mechanisms of neuroprotection and the identification of the nAChR subtypes involved. In fact, although most of the reports have proposed Ca^{2+} as the intracellular mediator of neuroprotection, the actual experimental evidence showing the Ca^{2+} dependence of nicotine protection is scarce (Donnelly-Roberts et al., 1996). Similarly, although most of the studies have not definitively addressed the nAChR subtypes involved, both $\alpha 7$ (Donnelly-Roberts et al., 1996; Kaneko et al., 1997; Carlson et al., 1998; Li et al 1999; Kihara et al 2001) and $\alpha 4\beta 2$ (Kihara et al., 1998) nAChR have been proposed to mediate in vitro neuroprotection. The issues of Ca^{2+} dependence and nAChR subtypes will be further addressed in the Results and Discussion of this Chapter.

2.1.4.2 In vivo neuroprotection with nAChR ligands

In vivo studies showed that nicotine could be protective against MPTP induced neurodegeneration (Maggio et al., 1998), quinolinic acid evoked neuronal death (O'Neill et al., 1998) and systemic kainic acid toxicity (Borlongan, et al., 1995). Nicotine was also shown to reduce the loss of tyrosine hydroxylase immunoreactive neurones after partial transection of the nigrostriatral dopaminergic pathway (Janson et al., 1988) and to delay the ageing process of nigrostriatal neurons (Prasad et al., 1994). However, several studies

assessing nicotine protection in toxically induced parkinsonism reported that chronic continuous nicotine did not prevent the dopamine loss, and even worsened it (Behmand and Harik, 1992; Fung et al 1991a, b). In fact, it seems that the delivery-protocol of nicotine plays a key part in achieving the in vivo neuroprotective actions. This assumption is supported by several studies where acute intermittent nicotine application, in contrast with most of the chronic studies, protected substantia nigra neurones in experimental models of Parkinson's disease (Janson et al., 1992; Maggio et al., 1998; Costa et al., 2000). In vivo, the observed difference between chronic versus intermittent treatments would suggest that nicotine desensitisation of nAChR might be responsible for the lack of protection observed in most chronic studies. Although experimental evidence for the role of nAChR activation in in vivo models of nicotine neuroprotection is largely missing, more recent studies with the nAChR antagonists mecamylamine and chlorisondamine (Maggio et al., 1998; Costa et al., 2000) demonstrate that functional activation of the nAChR is indeed necessary for the achievement of nicotine protection in vivo.

Similar to results obtained in vitro, nicotine effects are also dependent on the neuronal population studied. Consequently, it has been shown that in the septum, nicotine actually has a deleterious effect, producing an increase in the loss of cholinergic neurones (Fuxe et al., 1994). In the hippocampal region however, the reactivity of hippocampal neurones to nicotine's neuroprotective effects has been different compared to the septal counterparts. In an in vivo study of quinolinic induced neurodegeneration, hippocampal pyramidal and granule cells were protected after sub-chronic treatment with nicotine (O'Neill et al., 1997). Quinolinic acid is an endogenous substance that activates the NMDA subtype of glutamate receptor, and it is significantly increased in the cerebrospinal fluid of patients suffering from Huntington's disease and AIDS dementia (Heyes et al., 1993; Lipton and Gendelman, 1995). The finding that in vivo nicotine protects against quinolinic-mediated neurotoxicity supports the notion that the activation of nAChR could be useful to ameliorate the neurodegenerative process associated to NMDA excitotoxicity.

2.1.5 General Aims of this chapter

Taking into account its crucial role in the progression of neurodegenerative processes, the over stimulation of the NMDA receptor is widely used as a model of neurodegenerative-like cell death (Meldrum and Garthwaite, 1990; Stuiver et al., 1996). The high levels of glutamate receptors in the CNS and the vulnerability of the hippocampus to neurodegeneration in Alzheimer's disease have resulted in numerous studies documenting glutamate or NMDA neurotoxicity in hippocampal preparations. As illustrated in the General Introduction, the hippocampus has a particularly high expression of α7 nAChR (Seguela et al., 1993) and

primary hippocampal cultures are predominantly labelled by α-bungarotoxin, indicative of α7 nAChR (Barrantes et al., 1995a). Although the role of nicotine protection has been observed in several preparations, studies on hippocampal neurones are scarce (O'Neill et al 1998; Semba et al 1996; Zamani et al 1997). Even more surprising is the fact that the possible protective role of nicotine activation against NMDA-evoked excitotoxicity was never explored in hippocampal preparations.

Considering its role in the progression of AD, and the high densitiy of both nAChR and NMDA receptors, the hippocampus is an attractive brain region for examining the role of α7 nAChR against NMDA-mediated excitotoxicity. Although both nicotine and NMDA act through ligand gated ion channels with high permeability to Ca^{2+}, their differences in localisation, voltage dependence and magnitude of Ca^{2+} influx may differentiate the intracellular signals activated after receptor stimulation (Rogers and Dani, 1995; Role and Berg, 1996). In fact, both the differences and similarities in the molecular and biochemical properties of NMDA and nAChR may confer interesting insights into the specificity of neuronal Ca^{2+} signalling.

The particular aims of this part of the project were:

- To establish an experimental model for the study of NMDA-evoked excitotoxicity in hippocampal preparations.
- To explore the putative neuroprotective properties of nAChR activation against excitotoxicity in the hippocampus.
- To analyse the Ca^{2+} dependence of both excitotoxic and neuroprotective phenomena.

2.2 Methods

2.2.1 Materials

Tissue culture media were purchased from ICN Pharmaceuticals Ltd. (Basingstoke, Hants, UK). Culture media supplements, 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide (MTT), ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), N-methyl-D-aspartate (NMDA), 7-chlorokynurenic acid and nicotine were obtained from The Sigma Chemical Co. (Poole, Dorset, UK). $^{45}Ca^{2+}$ was from Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK). α-Bgt was obtained from RBI/Sigma (Poole, Dorset, UK). Fura-2 AM was from Molecular Probes (PoortGebouw, Leiden, The Netherlands). Methyllycaconitine and MK-801 were purchased from Tocris Cookson Ltd. (Bristol, UK). All other chemicals used were of analytical grade and obtained from standard commercial sources. Time-mated Wistar rats were obtained from the University of Bath Animal House breeding colony.

2.2.2 Cell Culture

High density hippocampal primary cultures were prepared from the hippocampi of E18 Wistar rat foetuses and maintained in serum free conditions as previously described (Goslin and Banker, 1991; Barrantes et al. 1995). In brief, rats were sacrificed by cervical dislocation and the entire uterus containing the foetuses was removed; each foetus was separated one at a time by cutting the umbilical cord. The foetuses were decapitated, the brains being immediately removed and immersed into fresh culture medium. Microdissection was carried out under 20x magnification, separating the cerebral hemispheres from the diencephalon and brainstem utilising fine spring scissors and style forceps. After the thalamus was removed, the hippocampus could be seen on the posterior half of the hemisphere. At this stage, the meninges were removed in one piece and the hippocampi were dissected by cutting the boundaries between the hippocampus and the adjoining cortex. For further cell dissociation, the dissected hippocampi were placed into a 35 mm Petri dish containing Dulbecco's Modified Eagle's Medium (DMEM), cut into pieces and transferred into a 12 ml test tube. The tissue pieces were washed twice with 10 ml of PBS, centrifuged and the supernatant discarded after each wash. Subsequently, tissue was incubated at 37° in 2 ml of PBS, plus 100 μl of trypsin and D-glucose 20 %. The enzyme reaction was finished after adding 8 ml of serum supplemented defined medium and centrifuging, then tissue was resuspended in the exact volume of serum supplemented media for further plating (1 ml for each well). Cell suspensions were obtained by triturating the tissue with a Pasteur pipette and once a cloudy suspension was obtained cells were ready to be plated in polyethyleneimmine-coated dishes at a plating density of 1.5 x 10^5 cells/cm^2, in serum supplemented (10% fetal calf serum) chemically defined DMEM medium (consisting of

DMEM and Nutrient Mixture F-12 Ham, 3:1), supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin, 100 μM putrescein, 100 μg/ml human transferrin, 5 μg/ml insulin, 30 μM sodium selenite and 20 μM progesterone). After two h to allow cell attachment, the medium was changed to serum-free chemically defined medium (see above). This procedure resulted in viable cultures of neurons with less than 5 % glial contamination. For the long-term survival of neurons, cultures were fed every 3-4 days by replacing half of the medium with fresh medium.

General characteristics of dissociated cell cultures

In the appropriate conditions primary cell cultures develop distinct axons and dendrites, form synapses with one another, and are electrically active. Importantly, neurones in dissociated cultures appear to retain their individual identity, presumably because they are post-mitotic and committed in their differentiation. In general, the morphological and physiological characteristics of the cell populations present in culture correspond closely to the characteristics of the cell populations in the tissues of origin (Goslin and Banker, 1991 and references therein).

Primary dissociated neuronal cultures are particularly useful to study morphological and physiological properties, which can be applied on a cell-by-cell basis. In most cases, they are less well suited to traditional biochemical approaches, mainly because the quantity of material obtainable from these cultures is usually limited and they often contain a heterogeneous population of cells. In effect, developing approaches to deal with the heterogeneity of cell types is integral to the successful use of primary cultures. In the CNS, the presence of single dominant cell types in the hippocampus (pyramidal neurones) and the cerebellar cortex (granule cells) help to overcame this problem, in particular when culture conditions prevent the growth of unwanted glial cells. In the case of the hippocampus, the generation of pyramidal neurones, which begins in the rat at about embryonic day 15 (E15), is essentially complete, while the generation of dentate granule cells, which largely occurs postnatally, has scarcely begun (Bayer, 1980) and the number of glial cells is still relatively modest. The culture protocol previously developed in our laboratory by Barrantes et al. (1995a) avoided the need for glia (a common requirement for neuronal populations) by culturing neurones in a relatively high density. As stated above, this method produces fairly homogeneous hippocampal neuronal cultures, with no significant glial contamination (see Fig 2.1).

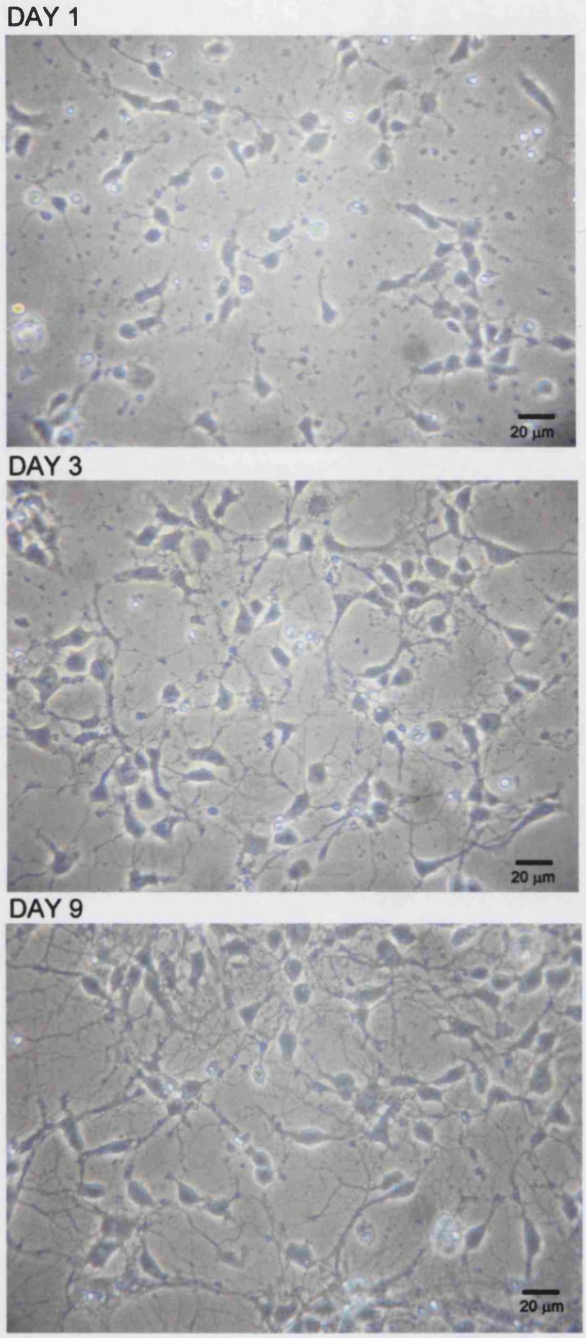

Fig 2.1 Hippocampal neurones in culture.
Hippocampal primary cultures, prepared as described in the methods section, are shown at different stages (1 day, 3 days, or 9 days after dissection and plating). Neuronal processes start to appear on day 2 and extended neurite networks can be seen from day 5-6. In addition to the typical pyramidal cells, stellate, bipolar, and cells with a single dominant process can also be observed. Neurones were cultured for 8 to 10 days before experiments.

In the hippocampal neuronal cultures immunoreactivity to α7 nAChR was observed in the cell body of almost all neurones (95 %) and its localisation paralleled that of α-Bgt binding sites. Using Rhodamine-α-Bgt labelling it was possible to determine that surface α-Bgt sites were on 60 % of neurones, while almost all neurones (95 %) labelled rhodamine-α-Bgt after

permeabilisation (Barrantes et al., 1995a). Therefore, most of hippocampal neurones in culture (10 day old) have the potential to express α7 nAChR, although only 60 % of neurones do so. Studies carried out by Albuquerque and collaborators (1993) with similar, although less dense E-18 primary cultures showed that the cellular density of rhodamine-α-Bgt binding was not uniform, with the highest density occurring at the somato-dendritic region and comparatively less labelling in the processes. A smaller proportion of hippocampal neurones have also revealed immunoreactivity to α4 specific antibodies, although [^{3}H]nicotine/[^{3}H]cytosine binding was not detected (Barrantes et al 1995a). In agreement with these results, electrophysiological studies also reported the predominance of Type IA currents in the hippocampus, which are associated with α7 containing nAChR (see Chapter 1).

Brain slices

Although not ustilised in the present work, brain slices from brain preparations have been widely used for both biochemical and electrophysiological studies, and results obtained with this preparation will be extensively discussed later in this chapter. Unlike tissue culture techniques, brain slices allow the maintenance of a significant amount of the original tissue architecture and connectivity. In particular, perhaps the single biggest advantage of brain slice studies is that it allows patch clamping to study synaptic transmission at synapses that are probably identical to those functioning in vivo. In the particular case of the nAChR, studies in slice preparations allowed the analysis of post-synaptic currents mediated by nAChR at hippocampal interneurones (see Chapter 1). Overall, data from brain slices demonstrated that the hippocampal formation can express functional α7 and α4β2 nAChR, similar to reports in hippocampal primary cultures (Albuquerque et al., 1998).

As illustrated above, the function of nAChR in the hippocampus can be studied using different experimental approaches. The use of the relatively homogeneous hippocampal primary cultures facilitated the biochemical studies developed in the present work. On the whole, hippocampal primary cultures are a suitable model for the study of nAChR and, in particular, they represent a unique culture assay for the functional study of α7 nAChR.

2.2.3 Neurotoxicity and neuroprotection experiments

Drug treatments

After 8-10 days in culture, hippocampal neurons were subjected to the neurotoxic/neuroprotective treatments. The culture medium was removed and kept while the cell cultures were incubated with various drugs at 37°, in an exposure buffer solution (EBS) of the following composition: 2.5 mM $CaCl_2$, 5 mM KCl, 137 mM NaCl, 0.3 mM KH_2PO_4, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM glucose, 0.01 mM glycine, 10 mM HEPES (pH 7.4). NMDA (200 μM and 1 mM) or glutamate (300 μM, 500 μM and 1 mM) were applied for 1 h as the excitotoxic insult. For the assessment of neuroprotection, nicotine was co-applied with NMDA (200 μM), or applied for 1 h immediately after removal of NMDA. Controls consisted of no drug treatment, and were processed in parallel. Where antagonists were used, these were added to the cultures 15 min before the excitotoxic insult and maintained throughout the period of drug treatment. At the end of the incubation with drugs, the EBS was replaced with the medium that had previously been removed. When EGTA was included in the EBS buffer, the estimated concentration of remaining free Ca^{2+} was 130 nM (calculated using the programme WinMAXC v. 1.78; Bers et al., 1994).

Cell Viability assays

MTT method

Cell viability was assayed 20-24 h after the drug treatments using an assay based on the incorporation of the tetrazolium salt 3-[4,5-Dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide, MTT (Carmichael et al., 1987). Dissolved MTT is converted to an insoluble formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes. Active mitochondrial dehydrogenases of living cells will cause this conversion while dead cells will not. After three hours of incubation with the MTT, isopropanol was added and optical density was read at 570 nm.

Fluorescent method

Nicotine protection against NMDA-evoked neuronal loss was also assayed using a Live/Death fluorescent method for culture preparations (Molecular probes LIVE/DEAD viability/cytotoxicity kit, with calcein AM and ethidium homodimer). This assay is based on the ability of the fluorogenic esterase calcein AM to get incorporated into living cells, being then hydrolysed to a green fluorescent product. Therefore, green fluorescence is an indicator of cells that have esterase activity as well as an intact membrane to retain the esterase products. Ethidium homodimer-1 is a high affinity red fluorescent nucleic acid stain that is only able to pass through the compromised membranes of dead cells. The assay was performed according to manufacturer's instructions. In brief, 24 h after drug treatments

fluorescent dyes (320 nM calcein AM and 1.6 μM ethidium homodimer-1) were added to the hippocampal cultures and incubated for 1 h before examination under a fluorescent microscope. At least 15 different fields from two independent experiments were analysed. Data are expressed as a percentage of the total number of cells counted (~ 80 per analysed field) and represents the mean ± SD.

2.2.4 Cytoplasmic Ca^{2+} measurements: Fura-2

Ten-day-old cultures, grown on PEI coated borosilicate glass cover slips (BDH/Merck Ltd., Poole, Dorset, UK) were ester-loaded with 5 μM Fura-2 AM by incubation at 37° for 30 min. The unloaded dye was removed by washing twice with the exposure solution. Fluorescence was measured using a pre-heated (37°) fixed stage microscope (Nikon) equipped with epifluorescence and a photometric analysis system (CAIRN). Excitation of Fura-2 was performed at 340, 360 and 380 nm while emission was measured between 400 and 520 nm. An infrared video camera was used to select and visualize a field containing 8-10 neurons. For UV excitation the equipment used a spinning wheel at 20 rev/s, allowing only brief exposures of the loaded cells to the UV light. The stability of the traces and the reproducibility of responses (Fig 2.7) demonstrated that phototoxicity was not a problem. For more information about Ca^{2+} sensitive dyes and fluorescence measurements see Methods section of Chapter 3 and Appendix 1.

Calculation of "resting" Ca^{2+}: calibration of the dual-wavelength fluorescence data (ratio 340/380) was made using the calcium ionophore ionomycin (2mM), Ca^{2+} free EBS plus 5mM EGTA and $MnCl_2$ (2mM), according to Thomas and Delaville (1991). A Kd of 225 nM for Fura-2 was used in calculations of cytoplasmic Ca^{2+}, made using the background subtraction method described by Thomas and Delaville (1991). The following equation was used: $[Ca^{2+}]_i=K_d*[(R-R_{min})/(R_{max}-R)]*X$, where R_{min} is the fluorescence ratio under nominally "zero" free Ca^{2+} conditions, R_{max} is the fluorescence ratio under saturating conditions, and X = F'_{min380}/F'_{max380} where F' is the fluorescence value corrected with its respective value found after addition of manganese. Responses to NMDA were about 20 % of the R_{max} (R_{NMDA} / R_{max} ratio = 0.202 ± 0.014, n=6), thus they were not close to saturation of the dye.

Ca^{2+} imaging

Nicotine stimulation of hippocampal cultures was also studied at the single cell level using Dynamic video imaging (Concord Hardware, Perkin Elmer, UK) and Merlin data adquisition software (Perkin Elmer). The fluorescent signal was recorded with an intensified Ultrapix low light level camera and a fast wavelengths changing SpectroMaster allowed the illumination of the cells at 340 and 380. Emmited light was collected at 510 nm. Cells were loaded with

Fura-2 as previously described (see above) and experiments were carried out at 37 in the dark.

2.2.5 Neuronal uptake of $^{45}Ca^{2+}$

Hippocampal cells were cultured for 8-10 days before incubation for 1 h, in the presence or absence of stimulus (NMDA, nicotine, KCl), in EBS containing $^{45}Ca^{2+}$ (1 μCi/ml). Cells were then washed three times with EBS, and perchloric acid (0.4 M) was added to lyse the cells. Samples were counted for radioactivity in a Tri-Carb 1600 liquid scintillation counter.

2.2.6 Data analysis and statistics

Statistical significance was determined using paired Student's t test and/or ANOVA plus post hoc Tukey's test as stated in the figure legends, with a statistical significance level of $p < 0.05$. Error bars show the standard deviation of the mean (SD) or standard error of the mean (SEM), as indicated in the legends.

2.3 Results

2.3.1 Glutamate and NMDA excitotoxicity

To determine the most suitable condition producing an excitotoxic insult in hippocampal cultures, the effect of a series of concentrations of glutamate and NMDA were compared. Cell viability was measured using the MTT assay as described in the methods section. Exposure of 8-10 day old cultures to glutamate (300 μM, 500 μM and 1 mM) for 1 h caused a significant and dose dependant reduction in neuronal survival, when compared to cultures submitted to the exposure buffer solution (EBS) alone (Fig 2.2). Similarly, NMDA (200 μM and 1 mM, in Mg^{2+} free conditions) also resulted in decreased cell viability, with slightly higher potency than glutamate (Fig 2.2). As the effect of NMDA can be ascribed to one particular class of glutamate receptor, subsequent experiments used a 1 h incubation with 200 μM NMDA as the excitotoxic stimulus, a condition that decreased cell viability by 25 ± 5 %. The involvement of NMDA receptors in mediating the neurotoxic effects of NMDA was confirmed by the ability of the selective antagonists MK-801 (10 μM) and 7-chlorokynurenic acid (500 μM) to prevent the neuronal loss (Fig 2.2).

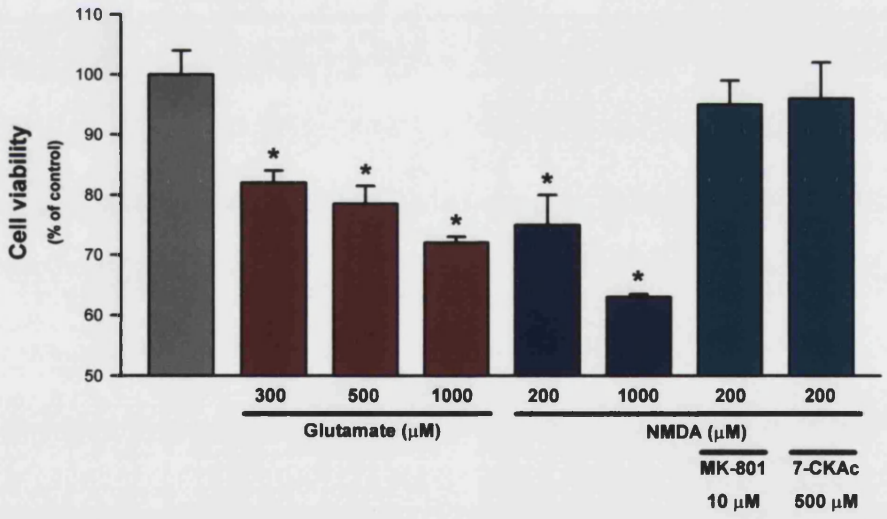

Fig 2.2 Cell viability in hippocampal cultures after excitotoxic treatments.
Hippocampal neurons were cultured for 8-10 days and then incubated with glutamate (300, 500 and 1000 μM) or NMDA (200 and 1000μM) for 1 h. To confirm the involvement of NMDA receptors, NMDA (200 μM) was co-incubated with two NMDA receptor antagonists, MK-801 (10 μM) and 7-chlorokynurenic acid (500 μM). Control cultures, with no added drugs, were processed in parallel. Cell viability was assessed 24 h after drug incubation, using the MTT method. Data are expressed as percentage of controls and bars represents the mean + SD from at least 4 independent experiments. Significantly different from control (*, $p < 0.05$, Student's t test).

2.3.2 Nicotine neuroprotection

Co-application of nicotine (10 μM) with NMDA (200 μM) for 1 h resulted in the complete protection of hippocampal cultures against the excitotoxic effect of NMDA (Fig 2.3), as estimated using the MTT cell viability assay. The nicotine-evoked protection was not observed when lower concentrations of nicotine were used (1 μM and 5 μM; Fig 2.3). Neuroprotection was also analysed using a different method for determining cell viability. Confirming the results obtained with the MTT assay, the live/dead fluorescent method showed that nicotine co-incubation significantly protected hippocampal cultures against NMDA-evoked neuronal loss (from 51 ± 12 % of dead cells after NMDA to 32.5 ± 9 % of dead cells after NMDA plus nicotine; Fig 2.4). Subsequent experiments on neuroprotection used the MTT method for evaluation of cell viability.

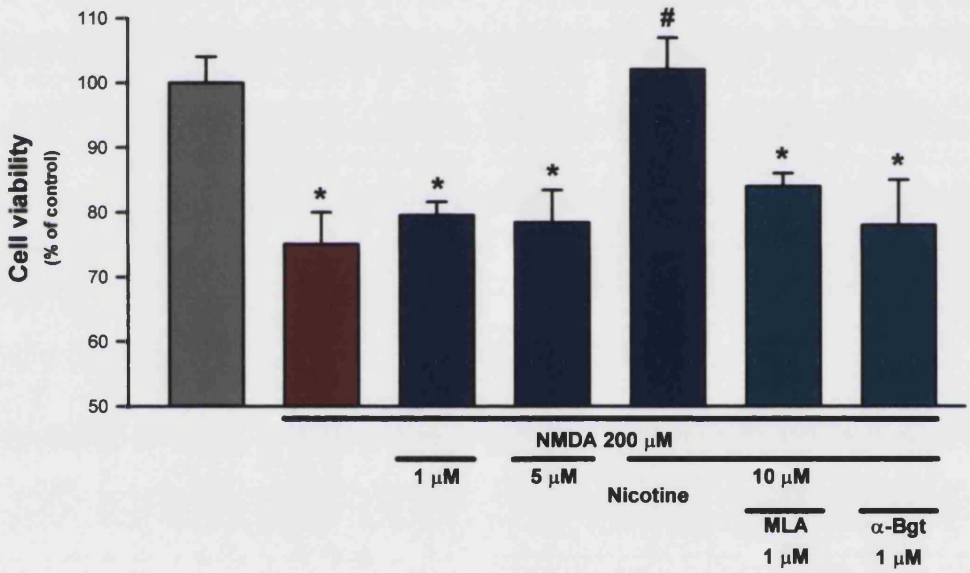

Fig 2.3 Effects of nicotine and nAChR antagonists on NMDA neurotoxicity.
Stimulation with 10 μM nicotine (but not 1 or 5 μM) protected hippocampal cultures when co-incubated with NMDA (200 μM) for 1 h. To assess the involvement of nAChR, two nAChR antagonists, methyllycaconitine (1 μM) and α-bungarotoxin (1μM), were present 15 min before and during the nicotine and NMDA co-incubation. Cell viability was assessed 24 h after drug incubation, using the MTT method. Data are expressed as percentage of controls and bars represents the mean + SD from at least 4 independent experiments. Significantly different from control (*, $p < 0.05$, Student's t test) or from NMDA 200 μM (#, $p < 0.05$, ANOVA and post hoc Tukey's test).

To confirm the presumption that nicotine protection was mediated through nAChR, two antagonists were compared. Both methyllycaconitine (MLA, 1 μM) and α-Bgt (1 μM) completely blocked the neuroprotection afforded by nicotine (Fig 2.3).

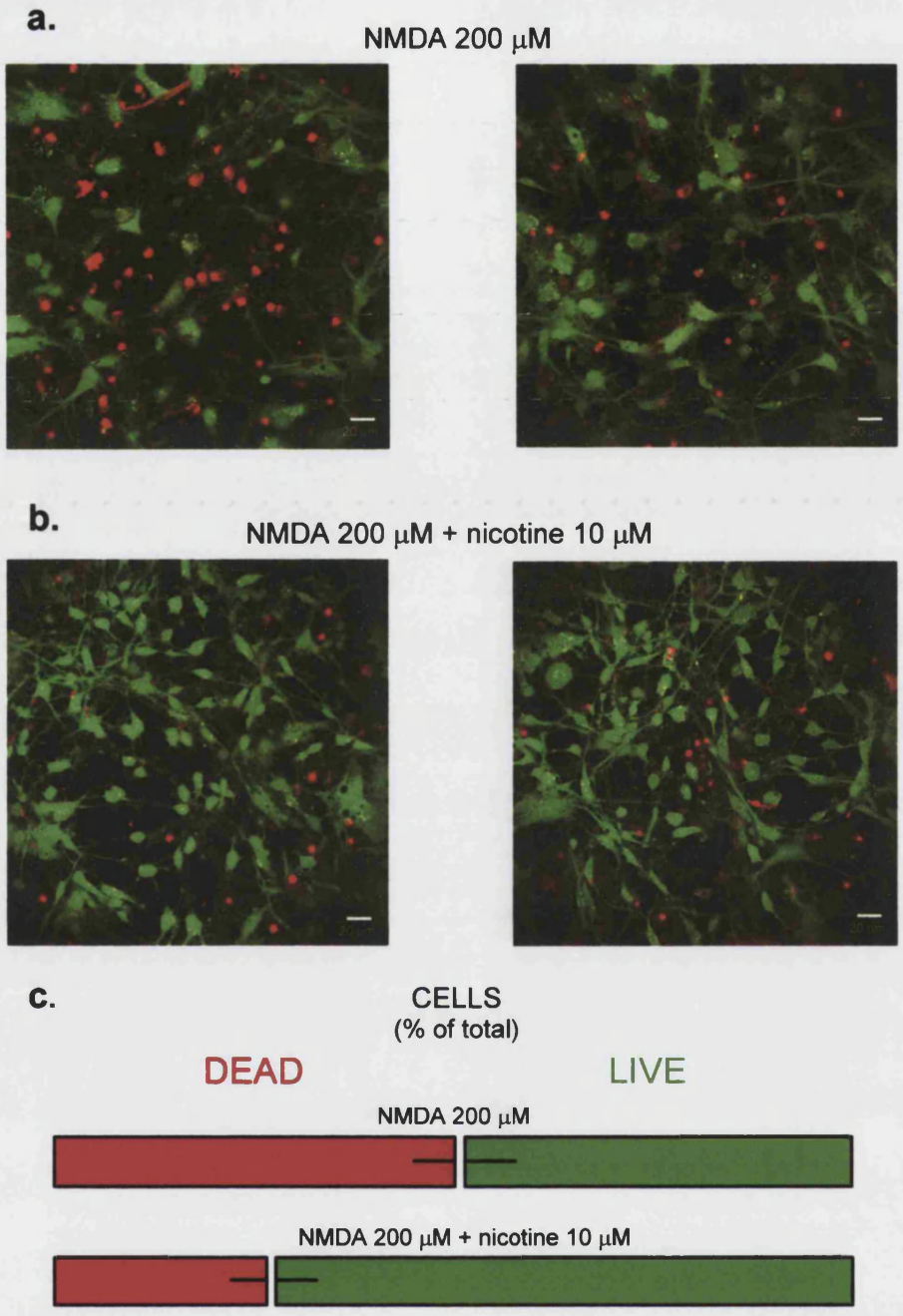

Fig 2.4 Nicotine protection assayed using a Live/Death fluorescent method.
Hippocampal cultures were assayed for neurotoxicity using calcein AM (green fluorescent marker: living cells) and ethidium homodimer (red fluorescent marker: dead cells). Cultures were treated with NMDA (a) or NMDA + nicotine (b) as previously described (see methods). Nicotine (10 μM) protected hippocampal cultures when co-incubated with NMDA (200 μM) for 1 h. The fluorescent assay was performed 24 h after drug treatments, and at least 15 different fields from two independent experiments were analysed. Data are expressed as a percentage of the total number of cells counted (~80 per analysed field) and represents the mean ± SD. The number of dead cells after treatment with nicotine and NMDA was significantly decreased when compared to NMDA alone ($p < 0.05$, Student's t test).

In order to examine the relationship between NMDA and nicotine-mediated effects we devised a post-incubation protocol for nicotine stimulation. In these experiments, nicotine was present for 1 h subsequent to the removal of NMDA from the culture wells, but not together with NMDA. Interestingly, nicotine still prevented the NMDA toxicity when applied after the NMDA stimulation (Fig 2.5). The nicotine protection observed with this post-incubation protocol offered an opportunity to examine the Ca^{2+} dependence of nicotine's effects. Following the NMDA treatment in normal buffer (2.5 mM Ca^{2+}), nicotine was applied for 1 h in buffer containing 5 mM EGTA. Under this "Ca^{2+} free" condition, nicotine failed to prevent the NMDA-mediated decrease in cell viability (Fig 2.5). Paradoxically, the toxic effect of NMDA was also Ca^{2+} dependant, as shown by the lack of significant neurotoxicity when NMDA was administered in the presence of 5 mM EGTA (Fig 2.5). Incubation with EGTA alone produced no significant decrease in cell viability (EGTA: 92 ± 2 % with respect to controls).

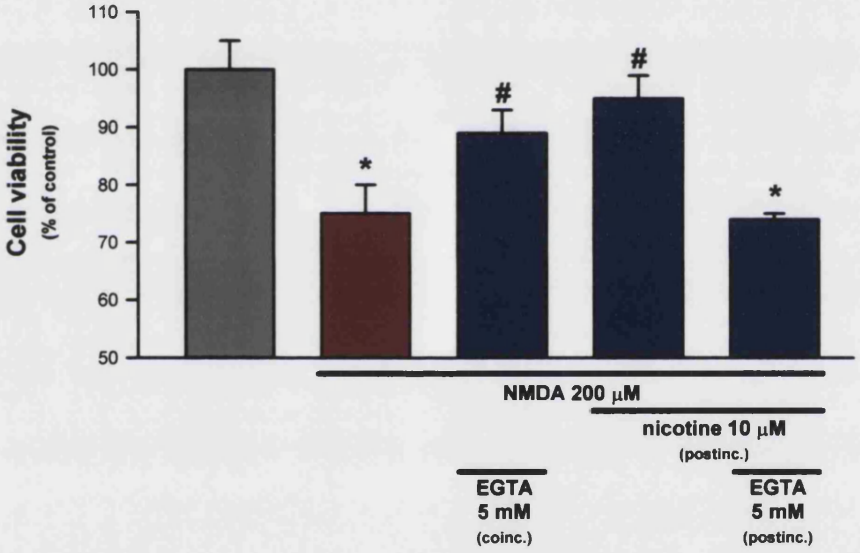

Fig 2.5 Effect of nicotine 1 h post-incubation on neurotoxicity and Ca^{2+} dependence. Hippocampal cultures were incubated for 1 h with NMDA (200 μM) before a subsequent 1 h nicotine (10 μM) incubation was carried out (postincubation). To assess the Ca^{2+} dependence of nicotine protection, EGTA (5 mM) was added to the nicotine post-incubation protocol. In separate experiments, EGTA was also present during NMDA (200 μM) incubations (coincubation). Cell viability was assessed 24 h after drug incubation, using the MTT method. Data are expressed as percentage of controls and bars represents the mean + SD from at least 4 independent experiments. Significantly different from control (*, $p < 0.05$, Student's t test) or from NMDA 200 μM (#, $p < 0.05$), ANOVA and post hoc Tukey's test.

In addition to nAChR or NMDA receptors, Ca^{2+} influx into hippocampal neurones can also occur after activation of VOCC. To examine the effect that Ca^{2+} entry through VOCC might have on the NMDA evoked neurotoxicity of hippocampal neurones, we incubated NMDA treated cells with the general depolarising agent KCl (20 mM). A 1 h co-incubation with 20 mM KCl did not protect hippocampal neurones against the NMDA-evoked excitotoxicity (Fig 2.6). In contrast, preliminary studies with trophic factors showed that incubation with nerve

growth factor (NGF; 50 ng/ml) for 24 hours after the 1 h NMDA insult significantly prevented the neuronal loss (Fig 2.6).

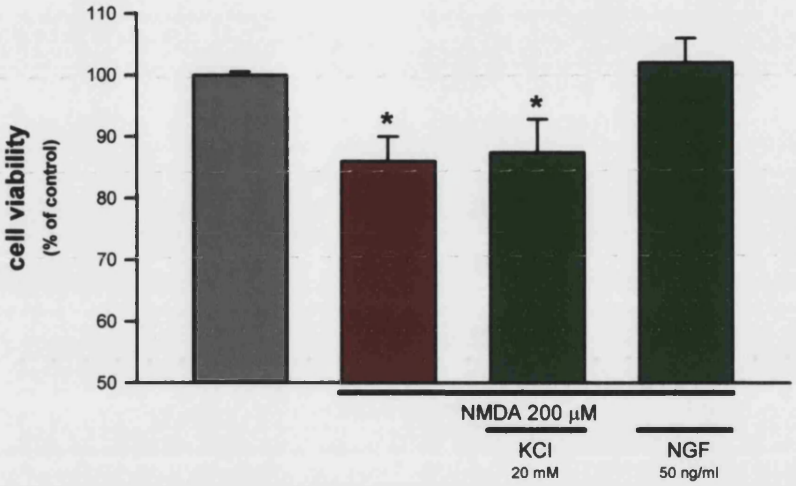

Fig 2.6 Effect of KCL and NGF on NMDA neurotoxicity.
Hippocampal cultures were incubated with NMDA for 1 h and KCl (20 mM, 1 h co-incubation) or NGF (50 ng/ml, 24 h post-incubation) was added to the EBS or culture medium, respectively. Cell viability was assessed 24 h after incubation with NMDA, using the MTT method. Data are expressed as percentage of controls and bars represent the mean + SD from at least 4 independent experiments. Significantly different from control (*, $p < 0.05$, Student's t test).

2.3.3 Nicotine and NMDA mediated increase in cytoplasmic Ca^{2+}

The resting free Ca^{2+} concentration measured in Fura-2 loaded hippocampal neurons was in the physiological range (70.8 ± 5.6 nM, n=9). Acute stimulation with nicotine (10 μM) evoked an increase in the fluorescence ratio (F_{340}/F_{380}) that persisted over the time of drug incubation (5 min, Fig 2.7a). NMDA (200 μM) induced a higher and sustained increase in the fluorescence ratio (5 min, Fig 2.7a), and this was not diminished when added in the presence of nicotine (Fig 2.7b). The nicotine-evoked increase in the fluorescence ratio was completely blocked by 1 μM α-bgt (Fig 2.7c), demonstrating that the α7 subtype of nAChR accounts for this response.

Calculation of the cytoplasmic Ca^{2+} concentration showed that NMDA stimulation (200 μM) increased cytoplasmic Ca^{2+} 4-fold, compared with a 1-fold increase in response to nicotine (Fig 2.7d). This difference in the magnitude of the responses to nicotine and NMDA was also observed when equivalent concentrations of nicotine and NMDA (200 μM) were compared (Fig 2.7d). The absence of interaction between nicotinic and NMDA receptor Ca^{2+} responses was also demonstrated using a lower concentration of NMDA (10 μM, Fig 2.7e). In this

experiment, the increase in fluorescence ratio after NMDA was the same, regardless of whether it was applied before, during or after nicotine stimulation.

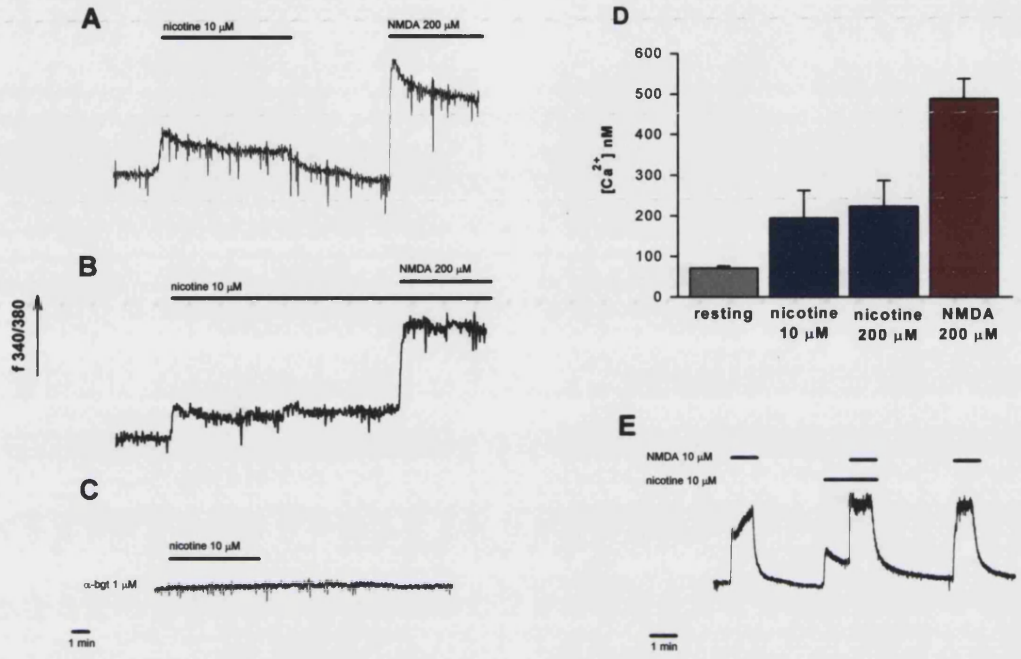

Fig 2.7 Nicotine and NMDA evoked changes in cytoplasmic Ca^{2+}.
Hippocampal neurons were loaded with Fura-2 AM and the fluorescence ratio at 340/380 was measured. A: nicotine (10 μM) or NMDA (200 μM) was applied for 5 min, separated by an intermediate wash. B: NMDA (200 μM) stimulation was performed in the presence of nicotine (10 μM). C: nicotine (10 μM) stimulation was carried out in the presence of α-bungarotoxin (1 μM). D: cytoplasmic Ca^{2+} concentrations were calculated according to Thomas and Delaville (1991) and bars represent the mean + SEM from at least 4 independent experiments. All the treatments were significantly different from resting Ca^{2+} levels ($p < 0.05$, Student's t test). E: NMDA (10 μM) stimulation was performed before, during or after a nicotine (10 μM) stimulus.

2.3.4 Ca^{2+} Imaging

Stimulation of hippocampal cultures with nicotine (100 μM) produced a significant increase in single cell fluorescence as observed with the dynamic video imaging system (Fig 2.8). Interestingly, only a subpopulation of hippocampal neurones was sensitive to the nicotine stimulation. This was not a consequence of poor neuronal viability, as submitting the same cultures to a general depolarisation with KCl produced a significant increase in fluorescence in all neurones analysed (Fig 2.8).

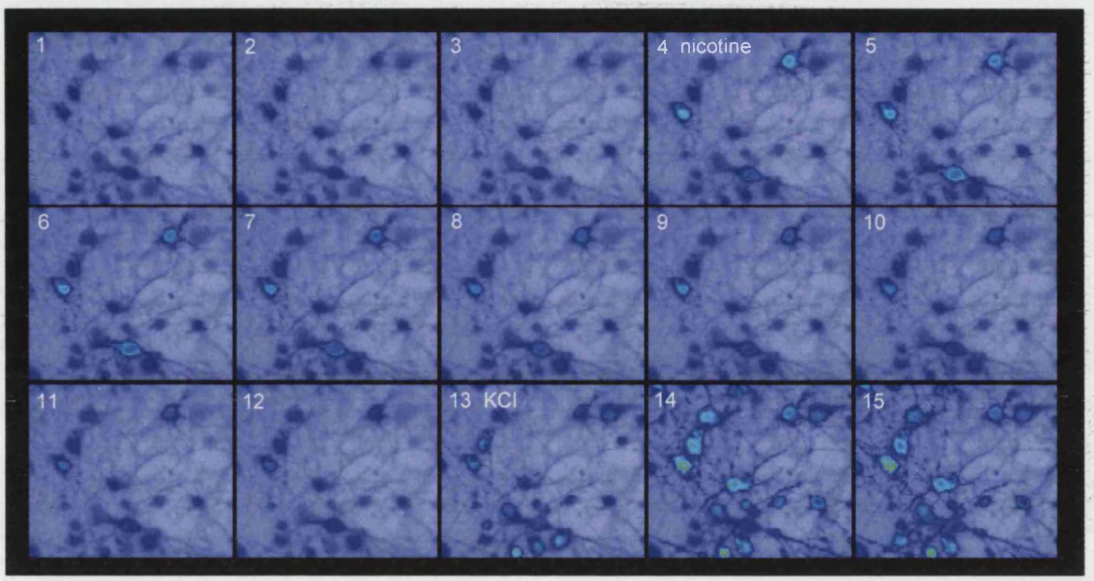

Fig 2.8 Ca^{2+} imaging of hippocampal cultures.
Hippocampal neurones were loaded with fura-2 AM as described in the methods section and changes in cytoplasmic Ca^{2+} levels were analysed using an intensified UltraPix low light level camera, with a fast wavelength changing SpectroMaster. Images shown were acquired before (1-3) and 1s, 2s, 5s, 15s, 30s, 45s, 60s and 120s after nicotine stimulation (100 μM; images 4-11) or 1s, 2s and 15s after stimulation with KCl (20 mM; images 13-15).

2.3.5 Neuronal loading of $^{45}Ca^{2+}$

As the cytotoxic and neuroprotective treatments involved a 1 h incubation, it was important to determine the total Ca^{2+} accumulation (cytoplasmic plus intracellular stores) at the end of this long incubation with the drugs. To measure the extent of Ca^{2+} load into the neurons, $^{45}Ca^{2+}$ was added to the EBS and its intracellular accumulation measured. Notably, the 1 h incubation with NMDA (200 μM) produced a dramatic increase in $^{45}Ca^{2+}$ load, almost 6 fold higher than controls (Fig 2.9). In contrast, incubation with 10 μM nicotine produced no significant increase in $^{45}Ca^{2+}$ accumulation (Fig 2.9). A higher concentration of nicotine (100 μM) also failed to give any increase in $^{45}Ca^{2+}$ loading. The nicotine stimulation did not prevent the NMDA-evoked effect, as co-incubation of NMDA (200 μM) and nicotine (10 μM) increased the $^{45}Ca^{2+}$ load to a level similar to that of NMDA alone (Fig 2.9). For comparison, KCl depolarisation (20 mM; 1 h) was also examined, and found to produce no significant increase in $^{45}Ca^{2+}$ accumulation at the end of the incubation (Fig. 2.9).

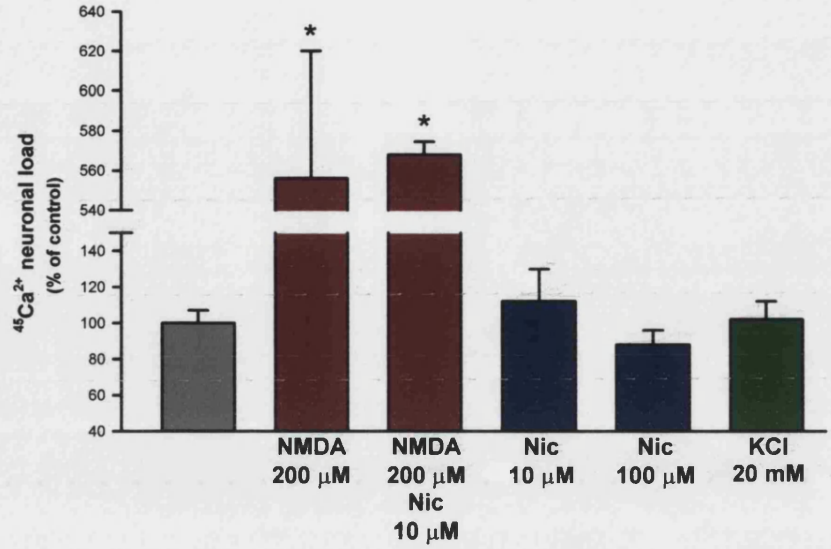

Fig 2.9 Intracellular accumulation of $^{45}Ca^{2+}$ in hippocampal primary cultures.
Hippocampal primary cultures were incubated with nicotine (10 or 100 μM), NMDA (200 μM) or KCl (20 mM) for 1 h, in the presence of 1μCi/ml of $^{45}Ca^{2+}$. When the incubation was over, cells were washed and lysed and $^{45}Ca^{2+}$ accumulation was measured. Data are expressed as percentage of controls and bars represents the mean + SD from at least 4 independent experiments. Significantly different from control (*, $p < 0.05$, Student's t test).

2.4 Discussion

2.4.1 Neurotoxicity and neuroprotection

The results presented in this chapter support the proposition that activation of the α7 subtype of nAChR can protect hippocampal neurons against an excitotoxic insult through a Ca^{2+} dependant mechanism. Both nicotine and NMDA acutely increased intracellular Ca^{2+}, but differed markedly in the magnitude of this response and in the Ca^{2+} load after long incubations.

In recent years, nicotine and other nicotinic agonists have been recognised as neuroprotective agents in several models of neuronal death, both in vivo and in vitro (reviewed by Donnelly-Roberts and Brioni, 1999). However, this is the first time that nicotine has been shown to protect hippocampal cultures against NMDA-induced neurotoxicity. As illustrated in the Introduction of this chapter, the hippocampal region is functionally involved in the progression of AD, while excitotoxic damage due to excess glutamate stimulation has been proposed as a common contributor to neurodegenerative diseases (Meldrum and Garthwaite, 1990; Leist and Nicotera, 1998). In our experimental model, a 1 h exposure of hippocampal primary cultures to glutamate or NMDA produced a significant and concentration-dependent decrease in neuronal viability. The sensitivity of NMDA excitotoxicity to MK-801 and 7-chlorokynurenic acid confirmed that NMDA receptors mediate this effect.

The neuronal loss provoked by over-stimulation of the NMDA receptor has been commonly associated with necrotic-type cell death. In fact, it has been suggested that injury by neurotoxins or glutamate lacked the regulated series of events involved in a death programme. However, more recent evidence suggests that excitotoxic injury can also involve key regulators of apoptosis, like p53, Bax and/or Bcl-2 (Nicotera et al., 1999 and references therein). In fact, the impression that entirely distinct types of cell death take place under a variety of conditions could be misleading. Instead, it is increasingly believed that cell death might be accomplished following a core programme, with a choice of different execution routines that often produce dissimilar morphologies (for review see Nicotera et al., 1999). This concept is directly corroborated in in vitro experiments showing that excitotoxicity can cause both apoptosis and necrosis (Ankarcrona et al., 1995), and with reports demonstrating that caspase inhibitors significantly reduce the extent of excitotoxic damage (Leist et al., 1997). The insult intensity can also contribute to the nature of the neuronal loss. Accordingly, on exposure to mild excitotoxic insults, neurones die exhibiting the typical apoptotic features. With stronger insults, however, the degradative processes

responsible for apoptotic morphology are often prevented and neurones die by necrosis (Bonfoco et al., 1995; Ankarcrona et al., 1995). It has been suggested that this "switch" mechanism could be due to untimely energy dissipation; in fact, recent evidence has revealed that, when energy levels are rapidly compromised, cells triggered to undergo apoptosis are instead forced to die by necrosis (see Nicotera et al., 1999; Roy and Sapolsky, 1999). Taking into account all these variables, it is difficult to predict with certainty the precise mode of neuronal death occurring in our experiments with hippocampal neurones. Although it is probable that both apoptotic and necrotic mechanisms occur, some preliminary experiments using the TUNNEL method for the detection of apoptotic neurones have suggested that, in hippocampal cultures, the stimulation with 200 μM NMDA for 1 h produced a predominantly necrotic form of excitotoxic neuronal loss.

Regardless of the apoptotic or necrotic nature of excitotoxicity, 10 μM nicotine significantly prevented the NMDA-evoked neuronal loss analysed using two different methods, and thus demonstrated the capacity of nicotine to rescue hippocampal neurones from an excitotoxic insult. The α7 subtype of nAChR has been proposed to mediate nicotine neuroprotection in a number of experimental models, mainly because of its high Ca^{2+} permeability (Donnelly-Roberts and Brioni, 1999; Kihara et al., 2001). However, in many cases where protection by nicotinic agonists was shown, there has not been definitive pharmacological evidence to support a role for α7 nAChR. Here, we found that the protection given by nicotine was completely prevented by co-application of 1 μM MLA, a concentration which also blocked the protection by the novel nAChR agonist GTS-21 against NGF and serum deprivation in PC12 cells (Li et al., 1999). Although this MLA antagonism confirms that nicotine is acting via nAChR, the concentration of MLA used would be expected to non-selectively block most nAChR subtypes. Lower concentrations of MLA gave variable results, possibly due to its instability, and were therefore not shown. However, the complete inhibition of nicotine neuroprotection in the presence of the definitive α7 antagonist α-Bgt (Fig 2.3) provides firm evidence that α7 nAChR do mediate nicotine's effect, a result consistent with previous reports showing that α-Bgt blocks nicotine protection against neurotoxicity in cortical cultures (Donnelly-Roberts et al., 1996; Carlson et al., 1997; Kaneko et al., 1997; Kihara et al., 2001). The blockade of nicotine protective effects by nAChR antagonists demonstrates that activation of nAChR is indeed required for the achievement of neuroprotection. At the same time, it argues against the proposition that nAChR desensitisation can act as a functional mechanism in nicotine-evoked neuroprotection (Donnelly-Roberts and Brioni, 1999). Finally, the preponderant role of the α7 subtype of nAChR in the present study is compatible with the high levels of α7 mRNA expression (Seguela et al., 1993) and [^{125}I]α-Bgt and [^{3}H]MLA binding in the hippocampus (Whiteaker et al., 1999), and with the high density of [^{125}I]α-bgt

binding sites (Barrantes et al., 1995a) and type IA currents (Alkondon and Albuquerque, 1993) present in hippocampal primary cultures.

The majority of the previous studies addressing nicotine neuroprotection, used a pre-incubation with nicotine in order to prevent the neuronal loss after a toxic stimulus (e.g.: Donnelly-Roberts et al., 1996; Semba et al., 1996). In our experiments, we found that co-application of nicotine with NMDA was sufficient to ameliorate the toxicity, a result consistent with observations by Marin et al. (1994) and Kihara et al. (1997), in striatal and cortical cultures respectively. An initial explanation for the nicotine protection observed after co-application with NMDA could have been provided by the blockade and/or inhibition of NMDA activation by nicotine stimulation. In fact, it has been previously shown that nAChR agonists, including nicotine, can partially inhibit NMDA evoked responses in rat cortical neurones (Aizenman et al., 1991). In addition, NMDA receptors can be inactivated by Ca^{2+}-dependent mechanisms, including the Ca^{2+}/calmodulin-dependant protein phosphatase calcineurin (Legendre et al., 1993; Lieberman and Mody, 1994; Tong et al., 1995). Nicotine has been shown to elevate intracellular Ca^{2+} and activation of acetylcholine receptors can induce Ca^{2+}/calmodulin-dependant intracellular cascades in PC12 cells (MacNicol and Schulman, 1992). Therefore, a plausible explanation for the protection produced by co-incubation of nicotine with NMDA could have been the direct inactivation of NMDA receptors. However, this hypothesis is not tenable for the reason that nicotine was still neuroprotective when administered after the 1 h NMDA stimulation. In fact, this is the first report to show that nicotine can protect when applied subsequent to the toxic stimulus, an observation that has important consequences for the proposition of functional mechanisms mediating neuroprotection. In this regard, it has been demonstrated that the cellular processes that lead to neurodegeneration occur over relatively long periods, even after the toxic input is over (Choi et al., 1987). In our experimental conditions, the observed neuronal loss is probably a result of a relatively long process of neuronal degeneration occurring over a 24 h period. Therefore, the capacity of nicotine post-stimulation to be protective indicates that nAChR activation can trigger a series of intracellular protective mechanisms that, without blocking the NMDA receptor itself, can prevent the neuronal loss by acting down stream to the initial toxic stimulus. This may well confer a significant advantage in the development of nAChR agonists as therapeutic targets against neurodegeneration.

The ability of nicotine to protect when applied subsequent to the excitotoxic insult not only allowed us to abandon the hypothesis of a NMDA receptor inhibition, but also gave the opportunity to examine some of the mechanisms underlying the nicotine neuroprotection, without interfering with the NMDA receptor activation. Indeed, the removal of extracellular Ca^{2+} from the stimulation buffer during nicotine post-incubation demonstrated that the

nicotine neuroprotection was Ca^{2+} mediated, while parallel experiments showed that the excitotoxic effect of NMDA was, as expected, also dependent on extracellular Ca^{2+} (Fig 2.5). Although this can be viewed as paradoxical, it could be explained by the particular properties of the nAChR and NMDA receptors, which differ not only in cellular localisation but also in Ca^{2+} permeability (Rogers and Dani, 1995; Role and Berg, 1996; more details in Chapter 1). Our studies have addressed some of these questions, by monitoring the changes in intracellular Ca^{2+} after nicotine and/or NMDA stimulation.

2.4.2 Receptor mediated changes in Ca^{2+} levels

The monitoring of intracellular Ca^{2+} levels in hippocampal neurones after nicotine and/or NMDA stimulation produced dissimilar results. Nicotine stimulation increased cytoplasmic concentrations of free Ca^{2+} from 70 to 200 nM, while NMDA generated substantially bigger increases (up to 500 nM). This difference in the magnitude of maximal increases was also observed when equivalent concentrations of nicotine and NMDA were compared (nicotine and NMDA 200 μM), and confirms previous assumptions of the relative Ca^{2+} permeability of nAChR and NMDA receptors. In addition, NMDA and nAChR receptors also produced different results when comparing the overall Ca^{2+} load after the 1 h incubation. In effect, NMDA produced a huge rise in accumulated $^{45}Ca^{2+}$, which was not observed after nicotine stimulation. Although this disparity may be partly explained by the NMDA and nAChR relative permeability to Ca^{2+} (see Chapter 1), it is very likely that the results observed are due to their differences in desensitisation rates (see Dingledine et al., 1999, for NMDA receptors and Role and Berg, 1996, for α7 nAChR), which can dramatically affect the overall changes in intracellular Ca^{2+}. In fact, the sustained increases in cytoplasmic Ca^{2+} concentrations after stimulation with NMDA are in agreement with the relatively slow desensitisation rates of NMDA receptors (Dingledine et al., 1999). When considering the fast desensitisation kinetics of nAChR, the observation that nicotine stimulation also produced sustained Ca^{2+} increases is somewhat more difficult to explain, and may suggests the involvement of various sources of Ca^{2+} influx (see Chapter 3 for related results and further discussion).

The α-Bgt sensitivity of the cytoplasmic Ca^{2+} increase after application of a neuroprotective concentration of nicotine (10 μM, 5 min, Fig 2.7c) confirms that α7 nAChR not only mediates the neuroprotection, but also the observed increase in free Ca^{2+} concentration. In addition, the analysis of cytoplasmic Ca^{2+} levels allowed us to further confirm the notion that NMDA receptor blockade is not necessary to achieve nicotine protection. In effect, we

demonstrated that NMDA-evoked Ca^{2+} signals were not affected when nicotine was applied before or together with the NMDA stimulus (Fig 2.7e).

2.4.3 Ca^{2+} as a life or death intracellular signal

Intracellular Ca^{2+} signals are a fundamental part of neuronal function, and in physiological conditions, they are rapidly buffered after fulfilling their role as mediators of intracellular signalling mechanisms. However, when Ca^{2+} homeostasis is altered and buffering systems are overwhelmed, neuronal Ca^{2+} excess can be linked to injurious processes, like proteolysis, mytochondrial dysfunction and production of toxic reactions (Leist and Nicotera, 1998; Nicholls and Budd, 2000). For that reason, Ca^{2+} has been proposed as a key mediator of neurodegeneration (Tymianski et al., 1993; Berridge, 1998). In general, the cellular influx of Ca^{2+} has been associated with both early neuronal degeneration (Randall and Thayer, 1992) and/or delayed neurotoxicity occurring up to many hours after the initial insult (Choi et al., 1987). Nevertheless, in spite of its clear role as mediator of neuronal degeneration, it has been difficult to demonstrate a relationship between the actual level of intracellular Ca^{2+} load and neuronal death: while early reports showed a correlation between the magnitude of the rise in free intracellular Ca^{2+} and the extent of the neuronal loss (Milani et al., 1991), others could not demonstrate such association (Michaels and Rothman, 1990; Dubinsky and Rothman, 1991). Supporting the link between the amount of intracellular Ca^{2+} load and the level of neuronal toxicity, a "Ca^{2+} set" hypothesis was proposed, in which the trespassing of a set level of intracellular Ca^{2+} concentration should lead to neuronal death (Franklin and Johnson, 1992). As a further development of this theory, it was suggested that moderate levels of Ca^{2+} concentrations should be able to buffer incoming Ca^{2+} insults and thereby promote survival (Franklin and Johnson, 1992). In fact, this latest hypothesis was proposed as a functional mechanism behind the protective effects observed after modest depolarisation (Franklin and Johnson, 1992) and nicotine stimulation (Donnelly-Roberts and Brioni, 1999).

In spite of the initial appeal of the "Ca^{2+} set" level hypothesis of toxicity and protection, several reports have critically challenged this theory, establishing that simple linear relationships were inadequate to demonstrate a significant association between intracellular Ca^{2+} levels and neuronal death (Michaels and Rothman, 1990; Dubinsky and Rothman, 1991; Tymianski et al., 1993). In an elegant report by Tymianski et al. (1993) it was quantitatively demonstrated that the toxicity evoked by NMDA stimulation was not due to the ability to trigger comparatively greater Ca^{2+} increases, but rather to some attribute that, although not defined, must have been specifically associated with the NMDA-mediated Ca^{2+} influx. In support of this proposition, a more recent report by Sattler et al. (1998)

demonstrated that Ca^{2+} loading through VOCC was not toxic, even when maximal Ca^{2+} accumulations were identical to those obtained with a toxic NMDA stimulus. In summary, what these and other studies proposed was that spatially restricted changes in free Ca^{2+} concentration should be more relevant to both signalling and cytotoxicity, than changes in the total amount of cytoplasmic Ca^{2+} (for review see Leist and Nicotera, 1998). In the present study, we report several results that argue in favour of the "Ca^{2+} source" theory rather than the "Ca^{2+} set" hypothesis. In particular, we demonstrate that nicotine did not prevent the NMDA-evoked massive load of intracellular Ca^{2+}. Consequently, this would suggest that a nicotine stimulation that is able to inhibit the NMDA-evoked neuronal loss, is doing so without preventing the overall increase in intracellular Ca^{2+} accumulation observed after NMDA stimulation. Added to this, a one-hour incubation with a general depolarising agent (KCl) did not prevent the NMDA-evoked toxicity, suggesting that only the specific Ca^{2+} influx through the α7 nAChR mediates the protection of hippocampal neurones. It is important to mention that the differences observed in the overall $^{45}Ca^{2+}$ load are not simply a reflection of differences in drug concentration, as higher nicotine concentrations also failed to increase the accumulation of $^{45}Ca^{2+}$ above control levels. When tested for toxicity, even a high nicotine concentration, which differed markedly from NMDA in their Ca^{2+}-evoked effects, did not reduce neuronal viability (107.5 + 6 % of cell viability respect to controls after nicotine 200 μM).

In conclusion, and consistent with the theory that the influx pathway but not Ca^{2+} load determine neuronal vulnerability, we showed that total Ca^{2+} overload, such as that produced by NMDA in the present experiments (Fig 2.9), does not lead inexorably to cell death. In fact, the addition of nicotine was neuroprotective through an autonomous Ca^{2+}-dependent mechanism (Fig 2.10). As well as differences in the magnitude of Ca^{2+} changes, the spatial segregation of nAChR and NMDA receptors, facilitating coupling to distinct Ca^{2+}-dependent signalling pathways, is likely to be crucial in determining the opposing outcomes of nicotine and NMDA application. In this regard, NMDA receptors have been reported to be associated to particular neurotoxic pathways. In a report by Tymianski and collaborators (Sattler et al., 1999), the suppression of PSD-95 protein expression, a member of the postsynaptic density that binds NMDA receptors, produced a specific blockade of NMDA-evoked excitotoxicity in cortical cultures. This deficiency in PSD-95 had no effects on NMDA receptor currents, or in the total Ca^{2+} load after NMDA stimulation. Moreover, suppressing PSD-95 expression affects neither the toxicity nor the Ca^{2+} influx triggered through pathways other than NMDA receptors. Overall, these results provide firm evidence for the existence of specific signalling pathways that link the NMDA receptor with potentially toxic signalling cascades.

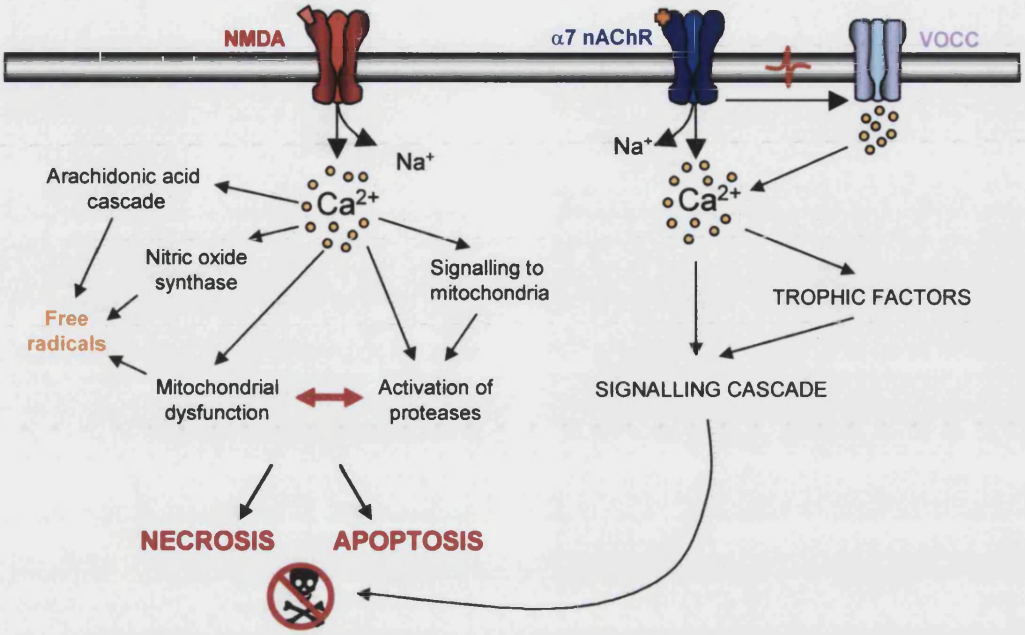

Fig 2.10 Schematic diagram of excitotoxic and protective pathways.
The over-stimulation of NMDA receptors generates several signalling mechanisms and toxic pathways that eventually lead to neuronal death. Activation of α7 nAChR evoked an increase in cytoplasmic Ca^{2+} levels that was neuroprotective through an autonomous Ca^{2+}-dependent mechanism down stream of the NMDA receptor activation. The precise intracellular steps involved in mediating the neuroprotective effects of nAChR activation are still unknown. However, it is likely that a cascade of intracellular events (protein phosphorylation and/or alteration of neurotrophic factor levels) occur. Some of these nicotine evoked signalling processes will be investigated in Chapters 3 and 4.

2.4.4 Possible mechanisms of Ca^{2+}-dependent nicotine protection

The actual intracellular steps involved in mediating the neuroprotective effects of nAChR ligands are still unclear (Fig 2.10). However, it is assumed that a cascade of intracellular events such as protein phosphorylation and/or alteration of the levels of neurotrophic factors occur. In vivo, nicotine was shown to increase the tissue levels of NGF and BDNF (Belluardo et al., 1998; Maggio et al., 1998), and because increased intracellular Ca^{2+} can induce release of neurotransmitters and other substances, it could be suggested that high Ca^{2+} might promote survival by causing release of one or more autocrine factors (i.e. NGF). These factors would then bind to specific neuronal receptors and promote survival. In effect, our results show that incubation with NGF for 24 h after the NMDA insult significantly protected hippocampal preparations, indicating that trophic factors can be neuroprotective in this particular experimental model. Even though the possibility of nicotine protection being mediated by the release of trophic factors is theoretically possible, it seems unlikely, since cell density does not significantly affect the survival-promoting action of depolarisation

(Franklin and Johnson, 1992, unpublished observations) or nicotine stimulation (personal observation). If trophic factors were released by depolarisation, high-density cultures should be more sensitive to protection than low density ones, since a higher amount of trophic factors would be released. Added to this, the stimulation with a depolarising KCl stimulus failed to protect hippocampal neurones against NMDA evoked excitotoxicity. As a result, it seems more likely that increased Ca^{2+} promotes neuronal survival by affecting intracellular signalling mechanisms within the cell.

Although the mechanisms of nicotine protection are not fully resolved, a series of reports that appeared since the results presented in this chapter were obtained indicated some of the possible mechanisms underlying nicotine neuroprotection. According to Garrido et al. (2001) nicotine can protect spinal cord neurones through the inhibition of arachidonic acid induced cellular apoptosis cascades, in a mechanism that does not involve increased expression of Bcl-2. In cortical cultures, nicotine protected against Aβ neurotoxicity through a mechanism that implicated phosphatidylinositol 3-kinase (PI3-kinase) and, unlike spinal cord neurones, upregulation of Bcl-2 (Kihara et al., 2001). Finally, a report by Prendergast et al. (2001) reported that NMDA-evoked toxicity in hippocampal slices could be prevented by chronic pre-exposure to nicotine. Consistent with our results, they show that nicotine does not inhibit NMDA Ca^{2+} signals, and suggest that nicotine protection could be associated with an increase in the expression of the cytosolic Ca^{2+} buffering protein calbindin-D28K.

In summary, the results presented here are consistent with the notion of regulation of cellular function by the specificity of signalling routes. The α7 nAChR has been shown to activate sustained Ca^{2+} signals, which are most probably capable of stimulating specific intracellular mechanisms. Both aspects of cellular function, the integration of various Ca^{2+} signals and the activation of intracellular signalling molecules, must act in conjunction to elaborate the cellular responses after nicotine stimulation. The elucidation of some of these aspects should contribute to the understanding of nAChR function in neuronal systems. Therefore, in the following chapters of this thesis, we analysed some of these particular features that follow the activation of nAChR, including:

- The study of the particular Ca^{2+} sources contributing to the sustained elevations in intracellular Ca^{2+} evoked by nicotine (Chapter 3).
- The analysis of some of the intracellular signalling mechanisms generated after nAChR activation in both hippocampal cultures and human neuroblastoma SH-SY5Ycells (Chapter 4).

CHAPTER 3

< nAChR Mediated Ca^{2+} Signals >

3.1 Introduction

Intracellular Ca^{2+} has a pivotal role in regulating diverse aspects of neuronal function (Berridge, 1998; Rose and Konnerth, 2001), as local changes in Ca^{2+} levels underpin major neuronal processes, such as transmitter release, excitability, synaptic plasticity and gene expression (Ghosh and Greenberg, 1995; Berridge et al., 1998; see Chapter 1). The remarkable versatility and signal specificity of this ubiquitous ion arises from the independent regulation of distinct signalling pathways linked to specific routes of Ca^{2+} influx (Ghosh and Greenberg, 1995). The importance of specific receptor-mediated Ca^{2+} signals was indeed illustrated in Chapter 2, with the activation of Ca^{2+}-dependent protective as well as excitotoxic mechanisms in hippocampal neurones, thus demonstrating the role of Ca^{2+} as a life or death signalling messenger. Overall, the study of neuronal function must be closely associated with the understanding of Ca^{2+} signals and the corresponding Ca^{2+} evoked signalling routes.

In the process of examining intracellular Ca^{2+} signals, there are two aspects of cytoplasmic Ca^{2+} dynamics that are important to consider. First, the cytosol is extensively buffered by Ca^{2+} binding proteins, including mediators of Ca^{2+} action (e.g. calmodulin), and proteins that have no known function other than the binding of the cation itself (e.g. calbindin). Secondly, diffusion of Ca^{2+} throughout the cytosol is very slow, at only one hundredth of the diffusion rate in pure water. As a direct consequence of these properties of cytosolic Ca^{2+} dynamics, any cellular Ca^{2+} influx event would result in the generation of transient high Ca^{2+} microdomains, which would be localised in the proximity of the activated channels (Llinas and Moreno, 1998). However, it is a well established observation that the intracellular consequences of cytoplasmic Ca^{2+} signals are not only limited to changes in Ca^{2+} levels beneath the plasmalemma. In fact, as already discussed in Chapters 1 and 2, neuronal function involves a complex series of intracellular signalling phenomena that are known to depend on Ca^{2+} as a trigger. In the last decade, it has become increasingly clear that most of these processes can be partly sustained by the existence of intracellular Ca^{2+} stores, which are able to rapidly accumulate considerable amounts of Ca^{2+} and to release them at rates in the same order as those of surface influx (for review see Meldolesi, 2001). In effect, the spatial and temporal characteristics of a particular cytoplasmic Ca^{2+} signal are determined by the combination of extracellular and/or intracellular Ca^{2+} sources. Although

voltage- or receptor-operated Ca^{2+} channels are the most common initiators of increases in cytoplasmic Ca^{2+}, it is increasingly accepted that intracellular stores contribute to the generation and regulation of discrete Ca^{2+} signals, while in some cases they can also trigger the initial Ca^{2+} response (Simpson et al., 1995). In the previous chapter, we demonstrated the existence of sustained elevations of cytoplasmic Ca^{2+} after activation of nAChR. It was therefore proposed that Ca^{2+} signals triggered by the stimulation of nAChR most probably involve the integration of various sources of cytoplasmic Ca^{2+} increase.

The combination of extracellular influx and intracellular release of Ca^{2+} has been established as a key premise in the neuronal signalling process. In fact, it has been indicated that Ca^{2+} release from diverse sources is a critical factor in the generation of Ca^{2+} signals with sufficient complexity to regulate the vast number of Ca^{2+} dependent cellular functions (Johnson and Chang, 2000). The spatial and functional heterogeneity of intracellular stores is a crucial element in the generation of such complex Ca^{2+} signals. The identification and characterisation of the cytoplasmic Ca^{2+} signals generated at specific sites by Ca^{2+} release from stores has lead to the proposal of the endoplasmic reticulum (ER) as an intracellular excitable membrane system working co-ordinately with the surface plasmalemma (Berridge, 1998). As illustrated in Chapter 1, intracellular stores not only contribute to segregated intracellular Ca^{2+} elevations, but they also take part in the propagation of intracellular Ca^{2+} signals through the sequential activation of IP_3 and ryanodine receptors. The generation of these intracellular Ca^{2+} waves is particularly relevant within the complex cellular geometry of neurones. In effect, waves generated following the evoked Ca^{2+} influx at the post-synaptic membrane can undergo various developments: they can remain localised in the generating compartment, move to adjacent dendrites, or even invade the whole neuron (Petrozzino et al., 1995; Korkotian and Segal., 1998; Svoboda and Mainen, 1999; Berridge, 1998). These Ca^{2+} waves may not necessarily be confined to single neurones, but can be expanded to their neighbours after store-operated Ca^{2+} influx and stimulation of transmitter release (Taylor and Peers, 2000), or via activation of store operated Ca^{2+} signals in astrocytes (Sharma and Vijayaraghavan, 2001)

3.1.1 Molecular components of the ER membrane system

At variance with other membrane systems, such as the mitochondrial inner membrane, no potential exists across the ER membrane. Instead, the steep Ca^{2+} gradient that provides the driving force for Ca^{2+} release from stores is maintained by a family of ATPase pumps, called the sarcoplamic-endoplasmic reticulum Ca^{2+} ATPases (SERCA), which are molecularly different from the pumps of the plasma membrane. The regulation of the SERCA pump is highly complex, depending on the Ca^{2+} existing at both the cytosolic and luminal surface of

the ER, and at the latter in particular, it has been demonstrated that Ca^{2+} binding proteins can have an inhibitory effect by establishing a direct interaction with the pumps (John et al., 1998). Another particularly important aspect of store function is the low affinity of the major luminal Ca^{2+} binding proteins, about two orders of magnitude lower than corresponding proteins in the cytosol (see Meldolesi, 2001). As a result, diffusion of Ca^{2+} within the lumen is fast, with stores Ca^{2+} concentration expected to be largely the same throughout discrete endomembrane compartments, and to decrease homogeneously after discharge (Park et al., 1999). When considering that ER channels are also sensitive to internal levels of Ca^{2+} in the lumen, the above mentioned properties could play a crucial part in the functional integration of highly localised Ca^{2+} signals.

From the experimental point of view, specific drugs are available to rapidly block the SERCA ATPases either irreversibly (by thapsigargin; Treiman et al., 1998) or reversibly (with cyclopiazonic acid; Plenge-Tellechea et al., 1997). However, in addition to SERCAs, other Ca^{2+} pumps insensitive to thapsigargin and associated to Ca^{2+} pools that are also released after cell stimulation have been described in the stores of a variety of eukaryotic cells (Meldolesi, 2001).

The two major types of store channels, the IP_3R and RyR, are expressed by most, if not all, neurones (Meldolesi, 2001). For both channels, further complexity is provided by the occurrence of different isoforms, which differ in tissue distribution and functional properties (e.g. Ca^{2+} sensitivity and activation mechanisms; Hagar et al., 1998; Sutko and Airey, 1996). This heterogeneous distribution in different brain areas may underlie the occurrence of functionally distinct stores, with independent functions and recruitment mechanisms (Rizzuto, 2001 and references therein). In fact, the intracellular localisation of RyR and IP_3R plays a fundamental role in the appearance and development of the Ca^{2+} release event. In an area with a low density of channel distribution, activation of a single receptor most often results in a local and minor event, whereas in a dense area can trigger activation of adjacent receptors, giving rise to non-decremental processes that may invade the whole cell (Meldolesi and Pozzan, 1998; Berridge, 1998).

The functional regulation of RyR and IP_3 receptors is highly complex (see Chapter 1, section 1.4). In experimental terms, several pharmacological tools have been developed in order to study the internal stores channel function. In the case of IP_3 receptors, generation of the second messenger can be inhibited by phopholipase C blockers such as U-73122, which unfortunately also has some unwanted effects on various other steps of Ca^{2+} homeostasis (Grierson and Meldolesi, 1995). As far as the drugs that act directly at the receptor level, adenophostins, which are Penicillum derivatives, are potent agonists (Takahashi et al.,

1994), while xestospongins are known as blockers (Gafni et al., 1997). However, it has to be mentioned that xestospongin-c has also been reported to inhibit the SERCA pumps (De Smet et al., 1999). Another permeant agent, 2-aminoethoxy-diphenyl borate (2APB), can be better defined as a modulator, since it blocks IP_3R at concentrations of 50 μM and stimulates release at higher concentrations (Maruyama et al., 1997). Blocking of the IP_3R can be also obtained by the application of heparin (Ehrlich et al., 1994), although its inability to cross membranes, resulting in the requirement for microinjection, has limited its use.

The RyR operates primarily as a positive modulator of intracellular Ca^{2+} signals, sustaining their propagation via its regenerative activation along defined pathways. RyR are therefore responsible, more than any other membrane component, for the excitable properties attributed to the endomembrane ER system (Berridge, 1998). RyR receptor regulation depends not only on cytoplasmic Ca^{2+} rises, but also on internal stores Ca^{2+} concentrations. In fact, release responses induced via CIRC, and also by drug agonists, are much larger when the store is full (Meldolesi, 2001). Caffeine, together with a few analogues, is the RyR activator most commonly used (Ehrlich et al., 1994). However, the concentrations needed to induce adequate responses are quite high (up to 25 mM!) and the effect of the drug is far from being specific (Zacchetti et al., 1991). Ryanodine, from which the receptor takes its name, is considered a blocker, although in most cell types it can also be a stimulator at low concentrations (Ehrlich et al., 1994). In recent years, the use of these pharmacological tools has allowed the identification of the role of intracellular Ca^{2+} stores in the modulation of different aspects of neuronal function, and has provided much information in understanding the complexity of cytoplasmic Ca^{2+} signals.

3.1.2 The human neuroblastoma SH-SY5Y cell line as a model system

In the process of studying the function of the nervous system, several experimental models have been developed. Primary cultures from CNS neurones, like the ones described in the previous chapter, constitute a widely used approach, which provides neuronal tissue in an environment that can be easily manipulated. A different approach in the study of neuronal function is given by the use of clonal cell lines derived from tumorigenic tissue. In contrast with primary cultures, clonal cell lines have become immortalised and therefore can be maintained through many passages, giving almost limitless quantities of material. This property has several advantages, but in particular, it allows the increase in the amount of experimental material whenever sensitivity is a problem. An additional benefit of working with neuronal cell lines is the fact that every cell in a clonal line culture is initially isolated from a single cell, thus providing highly homogeneous tissue cultures. In general, this may

also translate into a simplification in the range of macromolecules being expressed and in the interactions occurring between them.

Regardless of the above-mentioned advantages, perhaps the main concern in the use of clonal neuronal cultures is whether the molecules under scrutiny would be regulated in the same way as they are in the analogous, non-neoplastic cells. Although studies on this aspect are scarce, when effects were effectively compared they seem to indicate close similarities in the mechanisms of regulation (for review see Lukas, 1998). In fact, it is increasingly recognised that, in practical terms, it would be very difficult to elucidate supramolecular mechanisms involved in the control of receptors or signalling molecules using brain preparations, intact animals, or many heterologous expression systems. Cell lines are perhaps more useful for such studies, at least in helping to narrow the realm of mechanistic possibilities for regulation of neuronal functional mechanisms, so that other studies can be designed more incisively in particularly relevant models. In this regard, cell lines have been extensively used in the determination of an enormous amount of cellular mechanisms governing neuronal function. These functional studies include ion flux assays, immunologically based assays, intracellular signalling sytems and gene expression studies (Lukas, 1998 and references therein).

In spite of their valuable use, the actual origin of clonal cell lines constitutes one of its major disadvantages in the study of neuronal preparations. This is because dividing CNS neurones are predominantly restricted to periventricular zones in embryonic development and, as a result, the existence of CNS neuron-like tumours, and consequently clonal cell lines, is extremely rare. Various attempts have been made in order to immortalise CNS derived neuronal cultures. These included the fusion of primary CNS neuronal cells with peripheral neuroblastomas or the use of virally transformed or otherwise immortalised neuronal cells (Banker and Goslin, 1991). Although the development of such cell lines holds promise, the task of finding a CNS immortalised cell line expressing functional nAChR has been largely unsuccessful (personal observation).

Clonal cell lines derived from autonomic peripheral ganglia provide the closest alternative for the studies of functional mechanisms in neuronal preparations. The human neuroblastoma clonal cell line was derived through sequential sub-cloning from the SH-SY line and the parental SK-N-SH line, which has been established from a biopsied, metastatic tumour diagnosed as a peripheral neuroblastoma (Biedler et al., 1973; Ross et al., 1983). Cells of the SH-SY5Y clone have neuroblastic morphological character and resemble human foetal sympathetic neurones grown in primary cultures (Ross et al., 1983). The neuronal character of SH-SY5Y cells has been confirmed by a variety of markers, including expression of

neurofilament proteins, glutamic acid decarbolxylase, neuron specific enolase, GABA and noradrenaline uptake and muscarinic acetylcholine receptors (Lukas et al., 1993 and references therein).

3.1.2.1 nAChR in SH-SY5Y cells

A substantial limitation in the use of clonal cell lines is that those established and characterised may not express all of the important nAChR subtypes that mediate nicotine's effect in vivo. In the case of the SH-SY5Y cell line however, several nAChR subunits have been shown to be expressed, including the α3, α5, α7, β2 and β4, which form α7 and α3* subtypes of nAChR (Lukas, 1993; Peng et al., 1994; Wang et al., 1996; Ridley et al., 2001b). This composition of nAChR subunits accurately reflects the expression of corresponding analogues in the autonomic nervous system and supports the view that the SH-SY5Y cell line is a valuable neuronal model for the study of nAChR function.

In SH-SY5Y cells, it has been previously shown that nicotinic receptor activation can mediate the generation of inward currents and the subsequent release of noradrenaline (Gould et al., 1992; Wade et al., 1998), a cellular mechanism most probably mediated by Ca^{2+} influx through the nAChR itself and VOCC. Although a functional implication for the nicotine-evoked Ca^{2+} signals has been suggested, the demonstration of a nicotine-mediated Ca^{2+} elevation is still lacking. In fact, most of the Ca^{2+} studies after cholinergic stimulation have focused on the effect of muscarinic receptors, which have been shown to modulate intracellular Ca^{2+} levels through activation of IP_3-dependent internal stores (Murphy et al., 1991; Forsythe et al., 1992). In more recent studies carried out in our laboratory, we were able to demonstrate that the activation of nAChR could indeed increase the intracellular Ca^{2+} concentrations in SH-SY5Y cells (Ridley et al., 2002).

3.1.2.2 Ca^{2+} studies in SH-SY5Y cells

SH-SY5Y cells have been extensively used in the study of intracellular Ca^{2+} signals and their modulation after diverse stimuli. They have been shown to express both L- and N-type Ca^{2+} channels (Morton et al., 1992), while the existence of whole cell Ca^{2+} channel currents was verified as well (Reeve et al., 1994). The induced depolarisation of SH-SY5Y cells promoted the cytoplasmic influx of Ca^{2+} through the activation of VOCC, indicating the functional coupling of Ca^{2+} channels (Lambert et al., 1990). In addition, the existence of internal stores channel proteins was also suggested, when the expression of IP_3 and ryanodine receptors was demonstrated using isoform specific antibodies (Mackrill et al., 1997).

The activation of intracellular Ca^{2+} signals in SH-SY5Y cells has been shown after stimulation with platelet-derived growth factor (Wheldon et al., 2001), neuropeptide Y and somatostatin (Connor et al., 1997), acetylcholine and muscarinic receptor selective agonists (Lambert et al., 1990; Murphy et al., 1991; Forsythe et al., 1992) and potassium chloride (Lambert et al., 1990; Morton et al., 1992). Added to this, studies with muscarinic agonists suggested the involvement of an acetylcholine-induced production of IP_3, with the subsequent modulation of internal stores Ca^{2+} release (Lambert et al., 1990; Murphy et al., 1991). The Ca^{2+} mobilisation from IP_3-dependent stores was suggested to contribute to the bradykinin-evoked noradrenaline release (Purkiss et al., 1995), and is consistent with a functional role of internal stores in SH-SY5Y physiology.

3.1.3 General Aims of this chapter

All nAChR, and particularly the $\alpha 7$ subtype, exhibit high relative permeability to Ca^{2+} (see Chapter 1 and 2). The opening of the nAChR channel can lead to a direct increase in cytoplasmic Ca^{2+} concentrations, whilst Na^+ influx can cause neuronal depolarisation, activation of VOCC and subsequent amplification of Ca^{2+} transients (Vijaraghavan et al., 1992; Rathouz and Berg, 1994). The Ca^{2+} entry after activation of nAChR has been reported to regulate a diverse array of cellular processes, including neurotransmitter release (Soliakov and Wonnacott, 1996; Gray et al., 1997; Kulak et al., 2001), neuroprotection (Donnelly-Roberts et al., 1996; Dajas-Bailador et al., 2000) and synaptic plasticity (Matsuyama et al., 2000; Mansvelder and McGehee, 2000). nAChR-evoked Ca^{2+} entry can also result in the activation of key signalling molecules, such as PKC (Messing et al., 1989), CaMK II (Tsutsui et al., 1994), ERK1/2 (Dineley et al., 2001; see Chapter 4) and CREB (Dineley et al., 2001).

The evident versatility of nAChR-mediated cellular functions suggests the involvement of elaborated Ca^{2+} signals, which probably result from the integration of different sources of intracellular and/or extracellular Ca^{2+}. In fact, the contribution of internal Ca^{2+} stores to the sustained Ca^{2+} signals activated by nAChR stimulation has been reported in chick cilliary ganglion cells (Shoop et al., 2001) and substantia nigra brain slices (Tsuneki et al., 2000), while nicotinic activation of store-operated Ca^{2+}-induced Ca^{2+} release has been recently demonstrated in hippocampal synaptic neuronal boutons and astrocytes, and at single terminal varicosities of postganglionic sympathetic nerve terminals (Emptage et al., 2001; Sharma and Vijayaraghavan, 2001; Brain et al., 2001). However, the relationship between different Ca^{2+} sources in the elaboration of nicotine-evoked Ca^{2+} signals should be further complicated by the contribution of different nAChR subtypes. The understanding of how

diverse nAChR subtypes may differentially integrate a variety of extracellular and intracellular Ca^{2+} signals is required.

The aim of the present study was to explore the relative contributions of particular nAChR subtypes and different Ca^{2+} sources to the nicotine-evoked modulation of intracellular Ca^{2+} signals. In particular, we wanted to use the human neuroblastoma SH-SY5Y cell line in order to characterise the generation of sustained elevations in cytoplasmic Ca^{2+} observed after nicotine stimulation, modelling similar responses observed in hippocampal cultures (Fig 2.7). The particular objectives addressed in the present chapter were:

- The replication in SH-SY5Y cells of the sustained Ca^{2+} signals shown to occur in hippocampal primary cultures.
- The characterisation of the nAChR subtypes involved in the generation of Ca^{2+} responses.
- The analysis of the different Ca^{2+} sources contributing to the nicotine-evoked Ca^{2+} signals, including VOCC and internal stores.
- The study of possible differences in the Ca^{2+} signals evoked by diverse subtypes of nAChR, with particular emphasis on the $\alpha 7$ subtype.

** Some of the results presented in this Chapter were obtained in collaboration with Adrian Mogg.*

3.2 Methods

3.2.1 Materials

Tissue culture media, serum and plasticware were obtained from Gibco BRL (Paisley, Renfrewshire, Scotland). Media supplements, α-Bgt, cadmium chloride, (-)-nicotine hydrogen tartrate, nifedipine, ryanodine and mecamylamine were purchased from The Sigma Chemical Co. (Poole, Dorset, U.K.). Xestospongin-c and α-conotoxin Iml (α-Ctx-Iml) were from Calbiochem (Nottingham, UK). α-Conotoxin MII (α-Ctx-MII) was synthesised as previously described (Cartier et al., 1996; Kaiser et al., 1998). Fluo-3 AM and pluronic F127 were purchased from Molecular Probes, (Oregon, USA). The concentrations of nAChR antagonists, VOCC blockers and ryanodine and IP_3 receptor inhibitors were selected in order to give maximum blockade of their targets, based on previous studies (Kaiser et al., 1998, for α-Ctx-MII; Johnson et al., 1995, for α-Ctx-Iml; Pereira et al., 1999, for α-Bgt; Sharma and Vijayaraghavan, 2001, for $CdCl_2$; Taylor and Peers, 1999, for nifedipine; Gafni et al., 1997, and Gueorguiev et al., 2000, for xestospongin-c; Shoop et al., 2000, for ryanodine).

3.2.2 Cell culture

Human neuroblastoma SH-SY5Y cells (ECACC, Salisbury, UK; passages 14-20) were cultured as previously described (Sharples et al., 2000; Ridley et al., 2001). In brief, cultures were maintained in 175 cm^2 tissue culture flasks in DMEM:F12, supplemented with 15 % foetal bovine serum, 2 mM L-glutamine, 1 % non-essential amino acids and 190 U/ml of penicillin and 0.2 mg/ml of streptomycin, until confluent (see Fig 3.1). Cells were plated (1:5 dilution from flask) into 96 well Primaria plates (Falcon, New Jersey, USA) and experiments were performed 72 h later with confluent cultures.

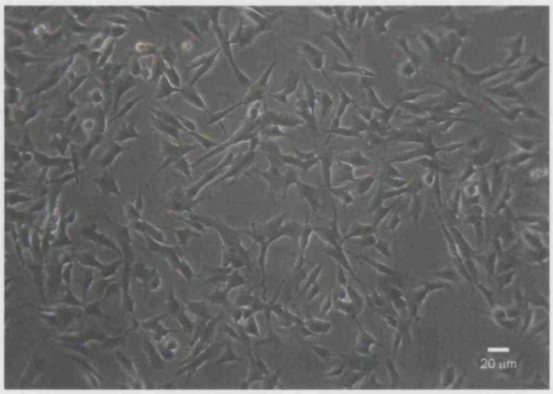

Fig 3.1 SH-SY5Y cells in culture.
Human neuroblastoma SH-SY5Y cells (passages 14-20) were cultured in 75 cm^2 tissue culture flasks. A 75 % confluent 3-day old culture is shown in the figure. For experiments, cells were cultured until they reach 100 % confluency.

3.2.3 Cytoplasmic Ca^{2+} measurements: Fluo-3

After removal of the medium from the confluent cultures, SH-SY5Y cells were washed twice with Tyrode's Salt Solution (TSS: 137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.2 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.5 mM glucose; pH 7.4) and incubated with the membrane permeable Ca^{2+}-sensitive dye fluo-3-AM (10 μM) and 0.02 % pluronic for 1h at room temperature in the dark. Cells were then washed twice with TSS, before adding 80 μl buffer, with or without added drugs, per well. After 15 min pre-incubation (or 30 min in the case of α-Bgt or thapsigargin) at room temperature in the dark, changes in fluorescence (excitation 485 nm, emission 538 nm) were measured using a Fluoroskan Ascent fluorescent plate reader (Labsystems, Helsinki, Finland).

Fluorescence was monitored in short-term or long-term protocols as follows. Basal levels of fluorescence were monitored for 5 s (short term protocol) or 20 s (long term protocol) before addition of nicotine (20 μl) using an automatic dispenser. After stimulation, changes in fluorescence were monitored for 20 s (initial increase; short term protocol) or 10 min (sustained response; long term protocol). For normalising fluo-3 signals, responses from each well were calibrated by determination of the maximum and minimum fluorescence values. At the end of each experiment, addition of 0.2% Triton (F *max*) was followed by 40 mM $MnCl_2$ (F *min*). The mean F *min* / F *max* ratio was 0.24 ± 0.03 (mean ± SEM, n = 32) and 0.26 ± 0.04 (mean ± SEM, n = 30) for short- and long-term measurements respectively; these values were consistent with those reported for this dye in other systems (Minta et al., 1989; Sharma and Vijayaraghavan 2001). According to previous studies, the manganese-saturated fluorescence corresponds to an intracellular Ca^{2+} concentration of 100 nM (Minta et al., 1989). The average baseline fluorescence normalised to F *min* was 1.15 ± 0.02 (n = 25). Data were calculated as a percentage of F *max* - F *min*. Values were expressed as a percentage of the response to 30 μM nicotine (included in all experiments), unless otherwise stated. Maximal responses to nicotine were approximately 20 % of the F *max* – F *min*, thus not close to saturation of the dye. No significant effects on basal levels of fluorescence were observed after incubation with any of the antagonists or blockers used, with the exception of $CdCl_2$. Incubation with $CdCl_2$ produced a rather slow but sustained increase in basal fluorescence, which precluded its use in long-term experiments.

3.2.4 Statistical analysis

Data represent the mean ± SEM of 3 or more independent experiments (each with 4 replicates). Statistical significance was determined using Student's t test or one-way ANOVA

plus post hoc Tukey's test, as stated in the figure legends. Values of $p < 0.05$ were taken to be statistically significant.

3.3 Results

3.3.1 Nicotine-evoked Ca^{2+} signals

In SH-SY5Y cells loaded with the Ca^{2+}-sensitive dye fluo-3, nicotine (30 μM or 100 μM) produced rapid and significant increases in fluorescence. The time course of the response to 30 μM nicotine indicated that maximal fluorescence levels were reached within 100 s, and were sustained for the duration of the recording in the continued presence of nicotine (10 min; Fig 3.2a). In comparison, stimulation with 100 μM nicotine evoked a more rapid increase in fluorescence values (Fig 3.2) with a moderately higher maximum level (Fig 3.2b), which was, however, not significantly different from the maximum increase in response to nicotine 30 μM (Fig 3.2).). In contrast to the sustained response to 30 μM nicotine, elevated fluorescence evoked by 100 μM nicotine slowly decreased towards a lower plateau after approximately 30 s (Fig 3.2a, b). Subsequent experiments concentrated on the analysis of the factors contributing to the initial and sustained responses to 30 μM nicotine.

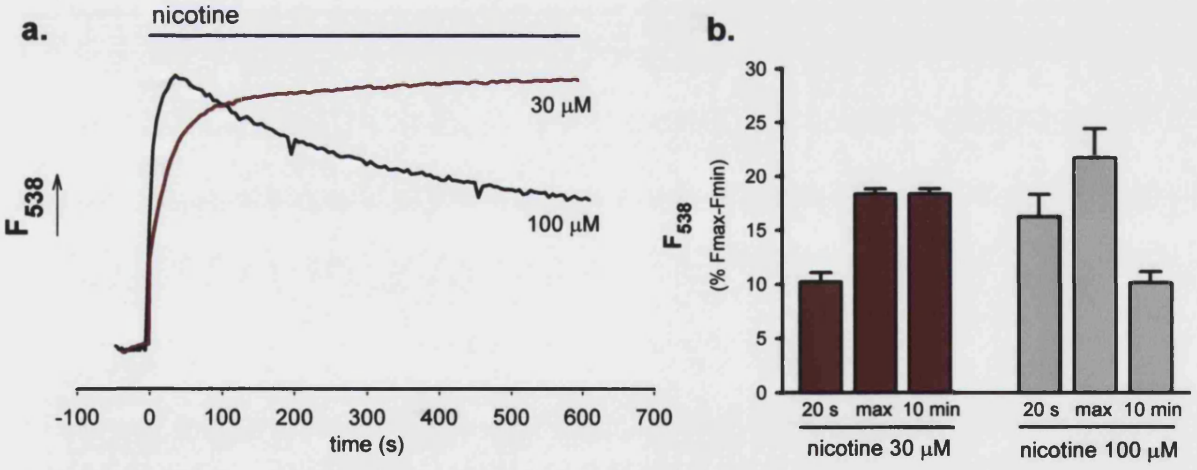

Fig 3.2 Elevation of intracellular Ca^{2+} levels after nicotine stimulation.
Representative traces from SH-SY5Y cells loaded with fluo-3 AM and stimulated with 30 μM or 100 μM nicotine are shown in (a). Changes in fluorescence were monitored for 10 min. In (b), the increase in fluorescence in response to 30 μM and 100 μM nicotine stimulation was determined at 20 s and 10 min, as well as the respective maximum values (max). Data are expressed as the percentage of *Fmax* - *Fmin* (mean ± SEM), as described in the methods, from at least four independent experiments.

Pre-incubation with the general nAChR antagonist mecamylamine (10 μM) completely prevented the nicotine evoked increase in fluorescence (see Fig 3.4), confirming that nicotine exerted its effect through nAChR. The contribution of nAChR activation to the

sustained elevation in fluorescence after incubation with 30 μM nicotine was examined by applying mecamylamine (10 μM) during the nicotine stimulus (Fig 3.3). The addition of mecamylamine 150 s after nicotine stimulation (when the maximum response has been reached) produced an almost immediate and complete inhibition of the nicotine-evoked increase in fluorescence (Fig 3.3a, c). When the same application protocol was performed with buffer alone, only a small decrease (15 %) in fluorescence was observed, which can be attributed to dilution and/or mechanical perturbation of the sample (Fig 3.3b, c). The rapid decrease in the Ca^{2+} signal observed in response to the application of mecamylamine indicates that the dye was not rate limiting in determining the duration of the response. In fact, the relatively low affinity of fluo-3 for Ca^{2+}, which will give comparatively faster dissociation rates (Minta et al., 1989), should allow a good temporal resolution of Ca^{2+} signals, as the bound dye would not artificially extend the time course of intracellular Ca^{2+} elevations. Therefore, the inhibition seen with mecamylamine indicates that nAChR activation is required throughout the response in order to sustain the long-term increase in fluorescence values.

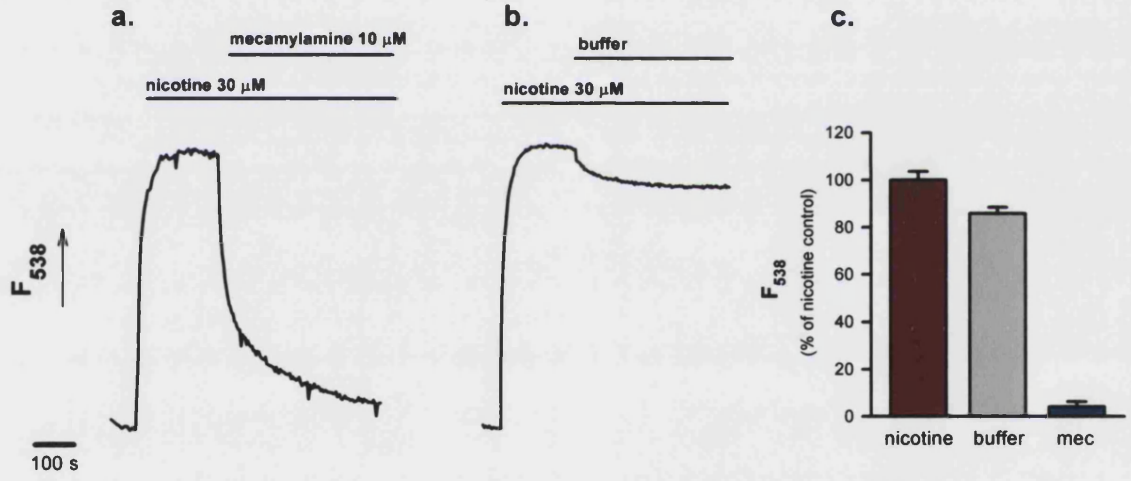

Fig 3.3 Effect of mecamylamine on the sustained elevation of intracellular Ca^{2+} after nicotine. SH-SY5Y cells were stimulated with 30 μM nicotine and fluorescence was monitored for 5 min. Then (a) mecamylamine or (b) buffer was added and fluorescence was monitored for further 10 min. Representative traces are shown. (c) Percentage inhibition of the nicotine-evoked fluorescence signal after mecamylamine (mec) or buffer application, measured at the end of the 15 min recording period. Data represent the mean ± SEM of three independent experiments, each carried out in quadruplicate.

3.3.2 nAChR subtypes involved

The relative contribution of nAChR subtypes and different Ca^{2+} sources to the initial (20 s) rise in fluorescence evoked by 30 μM nicotine stimulation was examined. In order to define the nAChR subtypes involved, maximally effective concentrations (see methods) of the α7 nAChR selective antagonists α-Bgt (40 nM) and α-Ctx-Iml (1 μM), and the α3β2*-selective

antagonist α-Ctx-MII (200 nM) were used. In the presence of each of these antagonists nicotine-evoked responses were significantly inhibited by about 20 % (Fig 3.4). Co-application of α-Ctx-MII plus α-Bgt or α-Ctx-Iml produced an additive effect, inhibiting about 40 % of the response to 30 μM nicotine (Fig 3.4). The α3β4 selective antagonist α-Ctx-AuIB was also used in some experiments, inhibiting only 11 % of the nicotine evoked response (Fig 3.4). Although attempts were also made to determine the nAChR subtypes involved in the long term Ca^{2+} response, these were largely unsuccessful. Application of α-Bgt (40 nM) and α-Ctx-MII (1μM) after nicotine-evoked Ca^{2+} levels have reached maximal values, produced only a small decrease of fluorescence, not distinguishable from the reduction after buffer addition (data not shown). Interestingly, when stimulating with 100 μM nicotine (Fig 3.5) α-Bgt did not block any significant component of the initial nicotine-evoked response, a result that probably indicates the rapid desensitisation of α7 nAChR receptors when using higher nicotine concentrations. This is also indicating that the progressive decrease of the long-term fluorescence elevation after 100 μM nicotine might be due to desensitisation of α7 nAChR. When lower concentrations of nicotine were used (10 and 30 μM), α-Bgt significantly blocked nicotine-evoked responses (Fig 3.5).

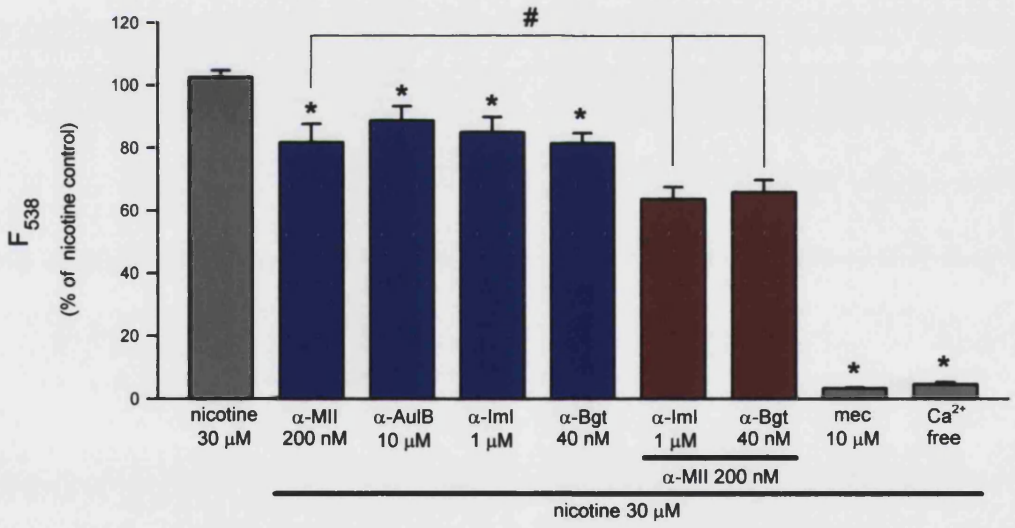

Fig 3.4 Contribution of nAChR subtypes to the nicotine-evoked increase in intracellular Ca^{2+}. SH-SY5Y cells loaded with fluo-3 AM were stimulated with 30 μM nicotine in the presence or absence of mecamylamine (mec; 10 μM), α-Ctx-MII (α-MII; 200 nM), α-Ctx-AuIB (α-AuIB; 10 μM), α-Ctx-Iml (α-Iml; 1 μM) or α-Bgt (40 nM). Nicotine stimulation was also carried out after co-incubation with α-Ctx-MII plus α-Ctx-Iml or α-Bgt. Antagonists were incubated with the cells for 15 min, or 30 min in the case of α-Bgt, before addition of nicotine. Fluorescence was monitored for 20 s after nicotine stimulation. Values at the 20 s timepoint are expressed as a percentage of the nicotine control response, measured in parallel. Data represent the mean ± SEM of at least 4 independent experiments, each carried out in quadruplicate. Significantly different from nicotine stimulation, * $p < 0.05$, Student's t test; or from nicotine stimulation in the presence of α-Ctx-MII, # $p < 0.05$, ANOVA and post hoc Tukey's test.

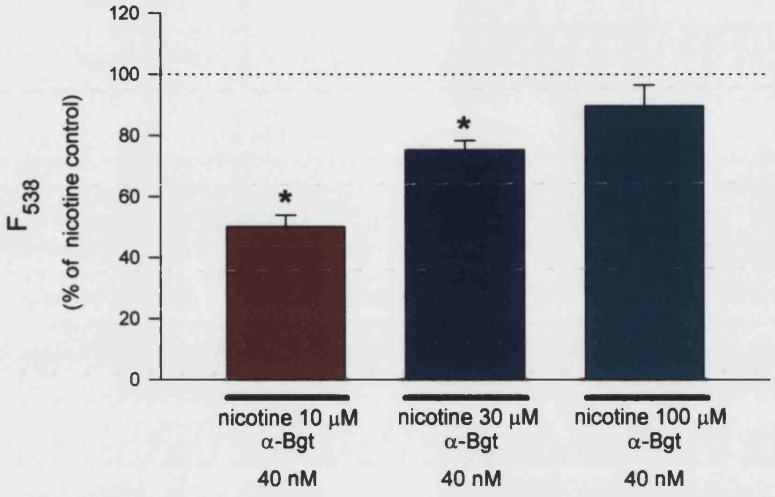

Fig 3.5 Contribution of α7 nAChR to the nicotine-evoked increase in intracellular Ca^{2+}. SH-SY5Y cells loaded with fluo-3 AM were stimulated with 10, 30 and 100 μM nicotine in the presence or absence of α-Bgt (40 nM, 30 min pre-incubation). Fluorescence was monitored for 20 s after nicotine stimulation. Values at the 20 s timepoint are expressed as a percentage of their respective nicotine control responses, measured in parallel and represented with a dotted line. Data represent the mean ± SEM of at least 4 independent experiments, each carried out in quadruplicate. Significantly different from nicotine stimulation, * $p < 0.05$, Student's t test.

3.3.3 Role of VOCC

The nicotine-evoked increase in fluo-3 fluorescence was totally dependent on extracellular Ca^{2+}, as no response was observed when nicotine stimulation was carried out in the absence of extracellular Ca^{2+} (Ca^{2+} free buffer plus 2 mM EGTA; Fig 3.4). In standard conditions (1.8 mM extracellular Ca^{2+}) $CdCl_2$ (100 μM) blocked more than 80 % of the nicotine-evoked increase in fluorescence (Fig 3.6), indicating that VOCC mediate most of the response. The residual nicotine-evoked increase in fluorescence in the presence of $CdCl_2$ was significantly decreased, by a further 9 %, by α-Bgt (Fig 3.6), whereas α-Ctx MII had no additive effect (Fig 3.6). This suggests that α7 nAChR contribute to the VOCC-independent increase in intracellular Ca^{2+}. The L-type VOCC blocker nifedipine (5 μM) inhibited 67 % of the nicotine-evoked increase in fluorescence. In the presence of nifedipine, both α-Bgt and α-Ctx-MII produced an additional, statistically significant, inhibition of 9 % and 5 % respectively (Fig 3.6).

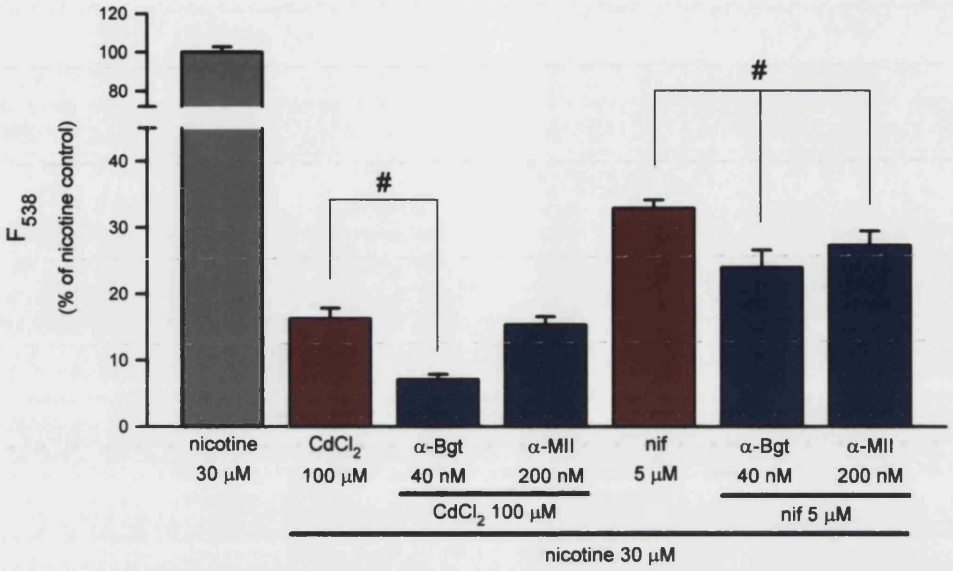

Fig 3.6 VOCC-dependent and -independent Ca^{2+} influx activation of nAChR.
The contribution of VOCC-dependent and -independent Ca^{2+} influx to the nicotine-evoked elevation of intracellular Ca^{2+} was examined. SH-SY5Y cells loaded with fluo-3 AM were stimulated with 30 μM nicotine after incubation with $CdCl_2$ (100 μM) or nifedipine (nif; 5μM), and after co-incubation with $CdCl_2$ plus α-Bgt (40 nM) or α-Ctx-MII (α-MII; 200 nM), and nifedipine plus α-Bgt or α-Ctx-MII. Fluorescence was monitored for 20 s after nicotine stimulation. Values at the 20 s timepoint are expressed as a percentage of the nicotine control response, measured in parallel. Data represent the mean ± SEM of at least 5 independent experiments, each carried out in quadruplicate. All drug treatments were significantly different from nicotine stimulation, $p < 0.05$, Student's t test. Significantly different from $CdCl_2$ or nifedipine are indicated, # $p < 0.05$, ANOVA and post hoc Tukey's test.

3.3.4 Intracellular Ca^{2+} stores and the nicotine-evoked Ca^{2+} signals

To establish if intracellular Ca^{2+} stores also contributed to the overall increase in cytoplasmic Ca^{2+}, we used selective inhibitors of IP_3- and ryanodine-sensitive stores. Pre-incubation with ryanodine (30 μM), a selective blocker of Ca^{2+}-induced Ca^{2+} release from ryanodine receptors, produced a significant decrease (39 %) in the nicotine-evoked rise in fluorescence measured at 20 s (Fig 3.7). Consistent with this observation, stimulation of SH-SY5Y cells with caffeine (30 mM), an agonist of ryanodyne sensitive Ca^{2+} stores, produced an increase in fluorescence equivalent to ~ 30 % of the nicotine-evoked response (Fig 3.7). No further inhibition was observed when α-Bgt or α-Ctx-MII was co-applied with ryanodine (Fig 3.7). Xestospongin-c (10 μM), a membrane permeant, non-competitive inhibitor of IP_3R (Gafni et al., 1997), produced greater inhibition than ryanodine (60 %, Fig 3.7), and when applied together, the two agents showed a partially additive effect, reducing the nicotine-evoked response by 77.5 % (Fig 3.7).

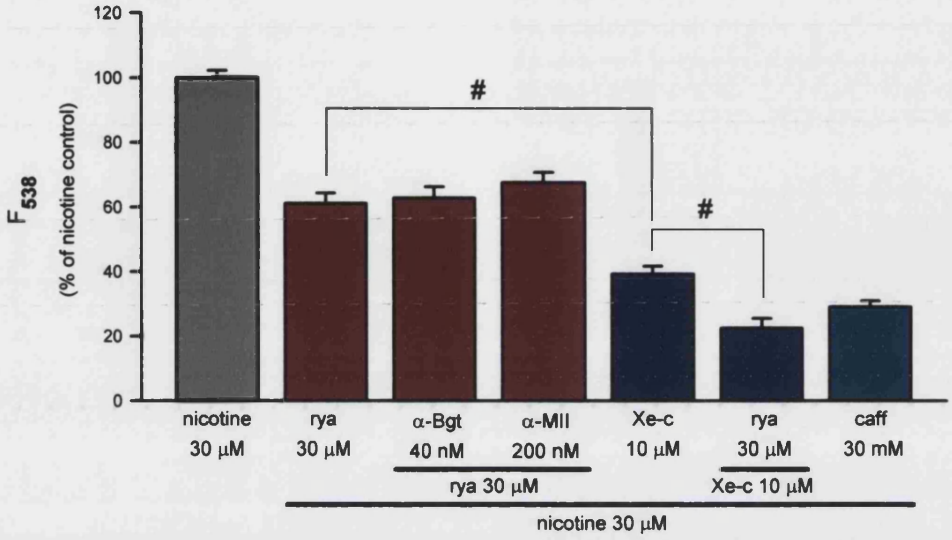

Fig 3.7 Contribution of intracellular stores to the nicotine-evoked increase in intracellular Ca^{2+}. SH-SY5Y cells loaded with fluo-3 AM were stimulated with 30 μM nicotine after incubation with ryanodine (rya; 30 μM), xestospongin-c (Xe-c; 10 μM), or after co-incubation with ryanodine plus α-Bgt (40 nM) or α-Ctx-MII (α-MII; 200 μM) and ryanodine plus xestospongin-c. In a separate group of experiments, SH-SY5Y cells were also stimulated with 30 mM caffeine. Fluorescence was measured for 20 s after nicotine stimulation. Values at the 20 s timepoint are expressed as a percentage of the nicotine control response measured in parallel. Data represent the mean ± SEM of at least 4 independent experiments, each carried out in quadruplicate. All drug treatments were significantly different from nicotine stimulation, $p < 0.05$, Student's t test. Significantly different from ryanodine or xestsopongin-c is indicated, # $p < 0.05$, ANOVA and post hoc Tukey's test.

The contribution of intracellular Ca^{2+} stores has been thus far described for the initial phase of the Ca^{2+} response to nicotine (20 s time point; Fig 3.2b). We also analysed the involvement of internal stores in the long-term responses, by examining the role of Ca^{2+} release through ryanodine and/or IP_3 receptors in the sustained increase in fluorescence evoked by 30 μM nicotine. Consistent with the involvement of Ca^{2+} stores, inhibition of the SERCA ATPase pump after a 30 min preincubation with thapsigargin, produced a 69 ± 5 % reduction of the nicotine-evoked increase (data not shown in figure). Pre-incubation with ryanodine (30 μM) produced a significant depression of the nicotine response throughout the time course monitored (Fig 3.8a, b). However, comparison of the effect of ryanodine at the initial (20 s) versus the later phase of the fluorescence response (10 min) showed significantly greater inhibition at the early stage, than at the end of the 10 min incubation (Fig 3.8b). In contrast to ryanodine, the response to nicotine in the presence of xestospongin-c showed a quite different profile (Fig 3.8a). The initial inhibition produced by xestospongin-c at 20 s (60 %) was followed by a progressive decrease in the nicotine-evoked fluorescence, resulting in the complete abolition of the response by the end of the experiment (Fig 3.8a, b).

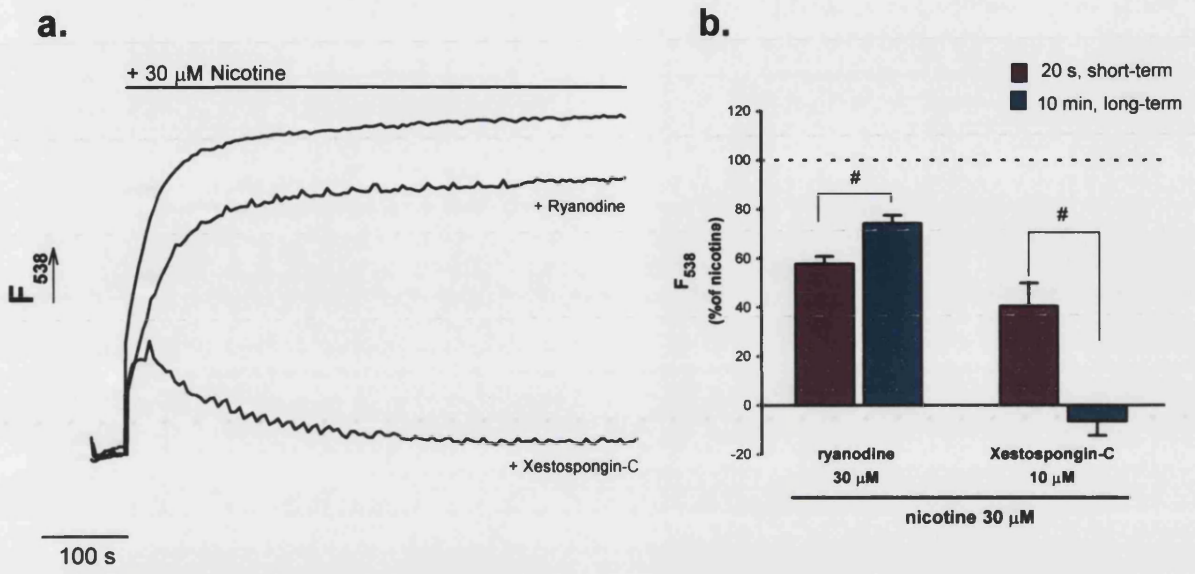

Fig 3.8 Involvement of Ca^{2+} stores in the sustained elevation of intracellular Ca^{2+}.
In (a), representative traces of long-term fluorescence measurements (10 min) of SH-SY5Y cells loaded with fluo-3 AM are shown. Cells were stimulated with 30 μM nicotine in the presence or absence of ryanodine (30 μM) or xestospongin-c (10 μM). (b) Percentage inhibition of nicotine's response in the presence of ryanodine or xestospongin-c, measured at 20 s or 10 min after stimulation. Data are expressed as a percentage of the corresponding nicotine control at that time point (dotted line) and bars represent the mean ± SEM of at least 3 independent experiments, each carried out in quadruplicate. All drug treatments were significantly different from nicotine stimulation, $p < 0.05$, Student's t test. Significantly different from ryanodine (20 s) or xestsopongin-c (20 s) is indicated, # $p < 0.05$, ANOVA and post hoc Tukey's test.

3.4 Discussion

In this chapter, experiments designed to investigate the intracellular Ca^{2+} signals generated by the activation of nAChR in SH-SY5Y cells loaded with the Ca^{2+}-sensitive dye fluo-3 are described. The results demonstrate that stimulation of nAChR produces rapid and sustained elevations in cytoplasmic Ca^{2+} levels, which are dependent on the activation of different Ca^{2+} sources, including extracellular Ca^{2+} influx through nAChR and VOCC channels and intracellular Ca^{2+} from internal stores.

3.4.1 nAChR subtypes, VOCC and the nicotine-evoked Ca^{2+} signals

The rapid and sustained elevations of intracellular Ca^{2+} levels in response to nicotine stimulation were prevented by the general nAChR antagonist mecamylamine (Fig 3.3; 3.4) or when carried out in the absence of extracellular Ca^{2+}, demonstrating that activation of nAChR and extracellular Ca^{2+} influx mediate nicotine-evoked Ca^{2+} signals in SH-SY5Y cells. The use of selective nAChR antagonists disclosed the contribution of both $\alpha7$ and $\alpha3\beta2^*$ nAChR in the initial phase (20 s) of the Ca^{2+} increase after stimulation with 30 μM nicotine. The α-Ctx-MII is a relatively novel antagonist of nAChR, which blocks the response to acetylcholine in oocytes expressing $\alpha3\beta2$ nAChR, being 2-4 orders of magnitude less potent on other nAChR subunit combinations (Cartier et al., 1996). Although the co-application of α-Ctx-MII with $\alpha7$ selective antagonists was additive, it only inhibited nicotine's response by 40 %, indicating that other nAChR subtypes must be also involved. SH-SY5Y cells express $\alpha3$, $\alpha5$, $\alpha7$, $\beta2$ and $\beta4$ nAChR subunits (Lukas et al., 1993, Peng et al 1994), which can form a variety of nAChR subtypes (Wang et al., 1996). Immunoprecipitation studies suggest that in SH-SY5Y cells, 50 % of the $\alpha3^*$ nAChR, also contain the $\beta2$ subunit (Wang et al., 1996); the presence of an $\alpha3\beta2$ interface should make such nAChR susceptible to α-Ctx-MII (Cartier et al., 1996), and previous experiments carried out in this lab show that 200 nM is enough to maximally inhibit $\alpha3\beta2^*$ responses (Kaiser et al. 1998; Ridley et al., 2002). This study suggests that 40 % of the Ca^{2+} response is attributable to $\alpha3\beta2^*$ and $\alpha7$ nAChR, whereas 60 % is mediated by a non-$\alpha7$, non-$\alpha3\beta2^*$ nAChR subtype, with the $\alpha3\beta4^*$ nAChR being the most likely candidate. In fact, the relatively high Ca^{2+} permeability (Ragozzino et al., 1998) and slow rate of desensitisation (Cachelin and Jaggi, 1991; Vibat et al., 1995;) of heterologously expressed $\alpha3\beta4$ nAChR may be relevant to the sustained Ca^{2+} changes observed in the present study. Although there is no commercially available antagonist of the $\alpha3\beta4^*$ subtype of nAChR, preliminary experiments with the newly described α-Ctx-AuIB

(donated by M. McIntosh) indeed demonstrated that a significant, although relatively modest, component of the nicotine evoked Ca^{2+} increase is mediated through α3β4 nAChR. The small amount of toxin available precluded its use in co-incubation experiments with other toxins.

The sustained increase in Ca^{2+} levels throughout the duration of the nicotine stimulation is consistent with the results obtained in hippocampal cultures (see Chapter 2; Dajas-Bailador et al., 2000), where long-term Ca^{2+} responses were also detected. At first, this characteristic of the nicotine-evoked response seems to be in conflict with the reported rapid desensitisation of nAChR (McGehee and Role, 1995). However, consistent with the results shown in hippocampal neurones and SH-SY5Y cells, sustained elevations of intracellular Ca^{2+} after nicotine stimulation have been also observed in chick cilliary ganglia neurones (Shoop et al., 2001) and PC12 cells (Gueorguiev et al., 2000). As a first approach towards the study of these long-term Ca^{2+} responses, it was important to analyse if the activation of nAChR was a constant requirement for sustaining the prolonged Ca^{2+} signals. The ability of mecamylamine, applied after Ca^{2+} levels had reached a plateau, to provoke the rapid return of intracellular Ca^{2+} to basal values, suggests that activation of nAChR is required throughout the nicotine-response in order to maintain the sustained elevation in the Ca^{2+} signal (Fig 3.3a). This is likely to reflect the inability of 30 μM nicotine to fully desensitise the entire nAChR population concurrently. As a result, there is the probability that at any given time during the nicotine stimulation, a proportion of the nAChR population would be responsive to activation by the agonist. This interpretation is consistent with the failure to sustain maximum Ca^{2+} increases at higher nicotine concentrations (100 μM; Fig 3.2), which would desensitise a greater proportion of nAChR.

Attempts to define the relative contribution of nAChR subtypes to the nicotine-evoked long-term increase (5-10 min) in Ca^{2+} signals were largely unsuccessful. Unlike mecamylamine, addition of α-Bgt or α-Ctx-MII after the nicotine-evoked Ca^{2+} increase had reached maximal values, failed to give any significant blockade. In the case of α-Bgt this may be partly explained by the slow kinetics of interaction with the receptor, which would prevent the rapid blockade of the nicotine response (Lukasiewicz and Bennett, 1978). However, it is also possible that the rapidly desensitising α7 nAChR do not contribute significantly to the long-lasting elevation in cytoplasmic Ca^{2+}. In fact, long-term responses after stimulation with a higher nicotine concentration (100 μM) produced a progressive decrease after reaching maximal values, an indication that a particular population of nAChR is more susceptible to functional desensitisation. In addition, pre-incubation with α-Bgt failed to produce any significant block of the initial Ca^{2+} rise after stimulation with 100 μM nicotine. Most likely, this would indicate that the α7 nAChR are primarily active in the initial phases of the 10-30 μM

nicotine-evoked Ca^{2+} signal, while long-term agonist exposure or high agonist concentration might induce a shift of the population of α7 nAChR towards desensitised states. The failure of α-Ctx-MII to produce a significant block when applied halfway during nicotine stimulation is more difficult to explain, although it may be possible that the α7 and α3β2 nAChR (both with relatively fast desensitisation rates; see Role and Berg, 1996) are not mediating the sustained elevations of Ca^{2+}. Instead, this could be a functional role for the α3β4, a nAChR subtype with a comparatively slow rate of desensitisation (Cachelin and Jaggi, 1991; Vibat et al., 1995; Role and Berg, 1996), and therefore a more likely mediator of sustained Ca^{2+} responses. However, it is important to note that the rapid decrease observed after addition of mecamylamine might be a direct consequence of its channel blocker properties, which allows the rapid prevention of nicotine responses, even in the presence of the agonist. Unlike mecamylamine, α-Bgt and α-Ctx-MII do not prevent nicotine response by blocking the ion channel, a difference that may also explain the results obtained.

Despite the high relative Ca^{2+} permeability of neuronal nAChR, the vast majority of the nicotine-evoked increase in intracellular Ca^{2+} in SH-SY5Y cells is dependent on the activation of VOCC, as incubation with $CdCl_2$ blocked more than 80 % of the response (Fig 3.6). This is consistent with previous studies in other preparations, including hippocampal neurones (Barrantes et al., 1995b) and chick cilliary ganglia (Rathouz and Berg, 1994; Shoop et al., 2001), where it was also demonstrated that $CdCl_2$ does not block nAChR directly (Rathouz and Berg, 1994). The $CdCl_2$ resistant increase in intracellular Ca^{2+} was not further blocked by α-Ctx-MII (Fig 3.6), suggesting that α3β2* nAChR do not account directly for any measurable part of the Ca^{2+} increase. This is compatible with the elegant study by Shoop et al (2001), which concluded that the role of α3* nAChR in generating Ca^{2+} responses in chick cilliary ganglion somatic spines could be primarily that of a catalyst for the mediation of membrane depolarisation and VOCC activation. In contrast to these observations, co-application of α-Bgt and $CdCl_2$ produced an additive effect, which suggests that, unlike α3β2* nAChR, the α7 nAChR subtype may be responsible for a component of the Ca^{2+} increase that is independent of VOCC activation. This is consistent with previous results found in chick ciliary ganglia (Rathouz and Berg, 1994) and hippocampal slices (Gray et al., 1996).

SH-SY5Y cells have been shown to express both L- and N-type VOCC (Morton et al., 1992). In effect, L-type VOCC appears to be responsible for part of the $CdCl_2$-sensitive response, as incubation with nifedipine significantly decreased the nicotine-evoked increase in Ca^{2+} levels (Fig 3.6). However, the ability of dihydropyridines to block the neuronal nAChR, especially α3* subtypes (Lopez et al., 1993; Herrero et al., 1999 and references therein; Shoop et al., 2001), compromises the estimation of the extent of the L-type VOCC

contribution. Nevertheless, co-application of nifedipine and α-Ctx-MII resulted in significantly greater inhibition (Fig 3.6) possibly implying that a subpopulation of α3β2* nAChR are preferentially associated with non-L-type VOCC. The suggestion of a specific coupling is in agreement with the previous finding of Kulak et al. (2001), interpreted in favour of a functional association between α3β2* nAChR and N-type VOCC in rat brain synaptosomes. Although indicating the specific segregation of nAChR associated Ca^{2+} signalling pathways, the functional consequences of these forms of association are still unknown.

3.4.2 nAChR and intracellular Ca^{2+} stores

It is widely recognised that neuronal Ca^{2+} signals initiated by voltage- or receptor-operated Ca^{2+} permeable channels can induce Ca^{2+} mobilisation from intracellular Ca^{2+} stores, which can play a crucial role in defining complex Ca^{2+} responses (for review, Simpson et al., 1995; Berridge, 1998; Rose and Konerth, 2001). SH-SY5Y cells have both IP_3 and ryanodine receptors (Mackrill et al., 1997), and we have shown that both contribute to the nicotine-evoked Ca^{2+} signals. Indeed, pre-incubation with ryanodine prevented a significant part of the nicotine-evoked elevation in cytoplasmic Ca^{2+}, suggesting that Ca^{2+} influx through the nAChR receptor itself, or through VOCC, provokes Ca^{2+} induced Ca^{2+} release from ryanodine dependent storage sites (Fig 3.7). The increase in Ca^{2+} observed after incubation with caffeine, an agonist of the ryanodine receptor, further confirms the functional presence of ryanodine-dependent Ca^{2+} stores in SH-SY5Y cells (Fig 3.7). Although Ca^{2+} release from ryanodine receptors contributes to both the initial and sustained Ca^{2+} elevation in response to nicotine (Fig 3.7; 3.8), the contribution of Ca^{2+} mobilisation from this source seems to decrease with time, as ryanodine has a larger effect during the initial phase of the response (Fig 3.8a, b). In contrast, Ca^{2+} released via IP_3 receptors, disclosed by inhibition with xestospongin-c, plays a fundamental role in the maintenance of sustained Ca^{2+} responses evoked by nicotine (Fig 3.8). IP_3 receptors are sensitive to both Ca^{2+} and the intracellular messenger IP_3. In PC12 cells, it has been shown that nicotine stimulation can lead to the production of IP_3 (Gueorguiev et al., 1999), while consistent with our findings, IP_3 dependent storage sites are involved in the long-term elevations in intracellular Ca^{2+} concentrations after activation of nAChR (Gueorguiev et al., 2000). It is important to mention that taking into account the reported ability of xestospongin-c to partially block the ER pump, the precise extent of the IP_3 dependent Ca^{2+} elevation is difficult to estimate. In fact, we have shown that a 30 min pre-incubation with thapsigargin, a SERCA ATPase inhibitor, produced a drastic inhibition of the nicotine's responses (69 %). However, the IP_3 involvement should not be invalidated, when considering that in experiments with xestospongin-c, the inhibitor was only preincubated for 10 min before nicotine stimulation. In thapsigargin experiments, a more

prolonged incubation (30 min) in Ca^{2+} free buffer was required in order to obtain the significant reduction of the nicotine response. When thapsigargin was applied in similar conditions to xestospongin-c, the inhibition observed after thapsigargin was relatively modest (22 ± 4 % inhibition with respect to controls, data not shown in figure).

The functional interaction of ryanodine- and IP_3-dependent Ca^{2+} stores has been described as a key signalling mechanism in neurones (Berridge, 1998), where they are considered to be separate but interacting processes (for review see Simpson et al., 1995). Interestingly, the inhibition of the response to nicotine after the simultaneous addition of ryanodine and xestospongin-c was only partially additive (Fig 3.7), suggesting that part of the store dependent Ca^{2+} release is occurring through sequential activation of ryanodine and IP_3 receptor dependent Ca^{2+} stores.

It is increasingly assumed that the versatility of specific Ca^{2+} signals is accomplished by the selective segregation of precise Ca^{2+} sources with particular signalling mechanisms. As discussed above, we found that after stimulation with nicotine, a fraction of the Ca^{2+} influx seemed to be directly mediated by the α7 nAChR (Fig 3.6). As α-Bgt produced no further inhibition in the presence of ryanodine, the increase in Ca^{2+} levels mediated by α7 nAChR could be specifically associated with ryanodine-sensitive Ca^{2+} stores. This would indicate that Ca^{2+} influx via α7 nAChR might activate ryanodine receptors directly, without the contribution of VOCC. The existence of this functional coupling between Ca^{2+} influx through α7 nAChR and Ca^{2+} induced Ca^{2+} release from ryanodine receptors may be pertinent to the particular activation of specific Ca^{2+}-dependent signalling mechanisms by α7 nAChR.

Overall, the functional coupling between nAChR and intracellular store channels provides a further spatial and temporal dimension to the Ca^{2+} signals evoked by nicotine stimulation. Indeed, recent evidence has highlighted the importance of the intimate cross-talk between the Ca^{2+} entry and Ca^{2+} release mechanisms, which should play a fundamental role in defining the specificity of nicotine-evoked Ca^{2+} signals. At present, several mechanisms are known to cooperate in the definition of the spatio-temporal patterns of cytoplasmic Ca^{2+} signals. These include the molecular repertoire of internal stores Ca^{2+} channels expressed and their intracellular distribution. In effect, the Ca^{2+} release channels show a complex and finely regulated distribution in the various neuronal subtypes, and throughout development (Rizzuto, 2001). The expression patterns of Ca^{2+} release channels are also modified by neural activity (Zhao et al 2000) and in pathological conditions (Lin et al., 2000). Interactions between organelles and/or the cell membrane can provide local signalling domains that control the diffusion of the cytoplasmic Ca^{2+} rise and the activation of effector systems. The wide extension of the ER network allows the propagation of Ca^{2+} induced Ca^{2+} signals

throughout the neuron (Berridge, 1998). This already intricate scenario should be further extended when considering the huge variety of cell membrane Ca^{2+} channels, including the nAChR, which could interact with these Ca^{2+} sources. The mechanisms underlying this seemingly complex system of channels, receptors and membranes, bring to light an incredible variety of specific Ca^{2+} signals that are able to transduce a specific stimuli into particular patterns of cellular activity.

3.4.3 nAChR and Ca^{2+} stores: functional implications

The complex repertoire of intracellular Ca^{2+} sources and sinks, as well as the molecular diversity of the machinery for Ca^{2+} accumulation, release and storage, translates functionally in the regulation of neuronal development, activity and eventually death (for review see Rizzuto, 2000). These signals follow a well-defined spatio-temporal pattern, occurring as waves that usually diffuse from specific pacemaker regions (Rizzuto, 2000; Meldolesi, 2001). The diffusion of the Ca^{2+} signal to the cell nucleus, by acting on transcription factors (Bito et al., 1997; Hu and Chrivia et al., 1999) or repressor proteins (Carrion et al., 1999), changes the gene expression patterns and is thus responsible for long term changes in neuronal morphology and activity.

The results presented here indicate that different nAChR and various Ca^{2+} sources, namely VOCC and ryanodine and IP_3 dependent Ca^{2+} stores, mediate the elevation of intracellular Ca^{2+} after nicotine stimulation of neuroblastoma cells (see Fig 3.9). Using combinations of antagonists, we deduce that whereas $\alpha3\beta2^*$ nAChR couple exclusively to VOCC, a portion of $\alpha7$ nAChR-mediated Ca^{2+} influx may directly activate Ca^{2+} induced Ca^{2+} release from ryanodine receptors. Furthermore, analysis of the sustained elevations of Ca^{2+} indicate that continuous activation of nAChR is required and that IP_3-sensitive stores play a crucial role in maintaining the sustained Ca^{2+} responses after nicotine stimulation. These data provides insights into the complexity of Ca^{2+} signals emanating from the activation of nAChR, a functional characteristic that probably underlies the remarkable versatility of nAChR-dependent neuronal functions.

Ultimately, changes in cytoplasmic Ca^{2+} constitute the conventional key that opens the Pandora's box of downstream signalling. The intricate regulation of the spatial and temporal patterns of Ca^{2+} signalling leads to the alteration in the activity of specific signalling molecules, and most probably, the modification of gene expression. The demonstration of precise Ca^{2+} signals mediated by the $\alpha7$ subtype of nAChR, both in the stimulation of Ca^{2+} induced Ca^{2+} release and in the activation of neuroprotective mechanisms, certainly suggest

that specific signalling mechanisms might be involved in the aftermath of α7 nAChR activation. In this regard, results presented in the next Chapter will explore the specific activation of signalling kinases following nicotine stimulation of SH-SY5Y cells and hippocampal neurones.

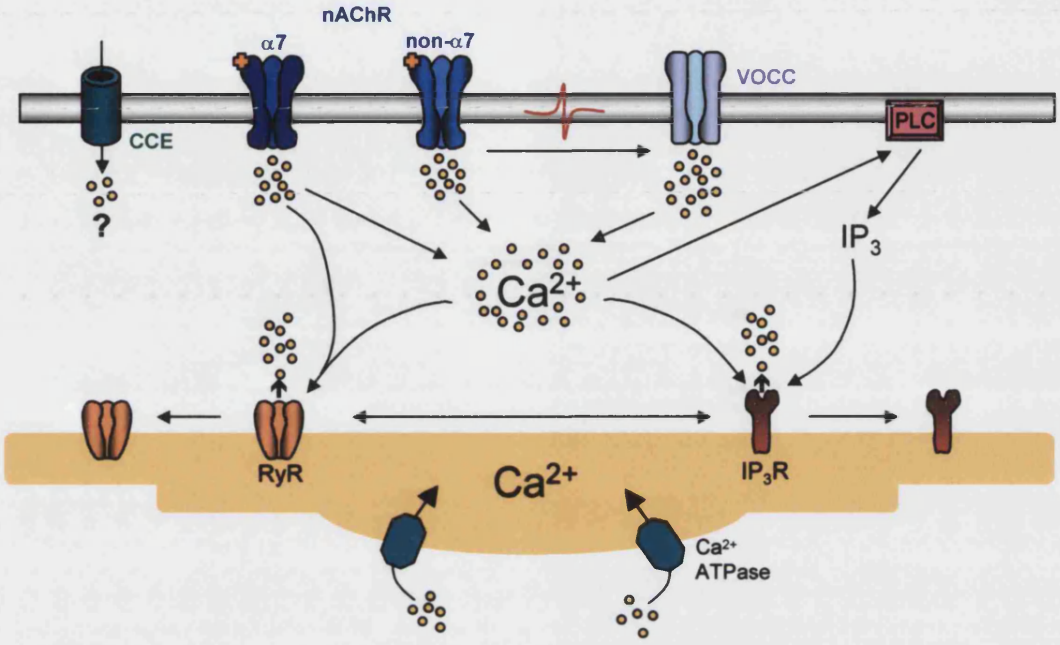

Fig 3.9 Generation of complex Ca^{2+} signals after stimulation of nAChR in SH-SY5Y cells. The results presented indicate that different nAChR and various Ca^{2+} sources, namely VOCC and ryanodine and IP_3 dependent Ca^{2+} stores, mediate the elevation of intracellular Ca^{2+} after nicotine stimulation. Using combinations of antagonists we deduce that a portion of the α7 nAChR-mediated Ca^{2+} influx may directly activate Ca^{2+} induced Ca^{2+} release from ryanodine receptors. In adition, we demonstrated that continuous activation of nAChR is required, and that IP_3 sensitive stores play a fundamental role in maintaining the sustained Ca^{2+} responses after nAChR stimulation. Although capacitative Ca^{2+} entry (CCE) might be anticipated to participate in the observed process, its role in nicotinic evoked signalling remains unanswered. The data depicted in the schematic diagram demonstrates the complexity of Ca^{2+} signals activated by nicotine, and suggests the involvement of downstream signalling processes.

CHAPTER 4

< nAChR Mediated ERK Signalling >

4.1 Introduction

4.1.1 Intracellular Ca^{2+} signalling

Neuronal systems must be able to integrate activity-dependent changes in synaptic inputs into concerted responses and long-term cellular processes, which are normally dependent on intracellular messengers and protein synthesis (Bailey et al., 1996, and see Chapter 1). The integration of voltage signals and generation of action potentials is one of the key mechanisms for information processing in neurones. The other main integrator of synaptic inputs is the nucleus, which elaborates the long-term responses of cellular activity. In fact, both the axon initiation zone and the nucleus can be considered as functional integrators of synaptic activity. However, while the axon hillock takes one type of input (membrane voltage) and generates one type of output (the action potential), the nucleus has many different types of inputs (multiple signalling cascades) and generates many different types of outputs (gene expression at different levels). Although the generation of slow and persistent signal inputs (i.e. hormones) is an important mechanism in neuronal processing, it is also fundamental to consider the fast changes in synaptic input, which relay important and crucial information as well.

As a widespread strategy for rapidly transducing fast synaptic information to the nucleus, excitable cells developed the fast conversion of electrical to biochemical signals through an intense and local influx of Ca^{2+} (Bito et al., 1997). LGIC and VOCC are able to respond quickly to millisecond-scale electrical events and generate quite complex cytoplasmic Ca^{2+} signals (see Chapters 1 and 3). The decoding of this Ca^{2+} signals by the appropriate Ca^{2+}-sensitive proteins provides the functional basis for the Ca^{2+} mediation of synapse-to-nucleus signalling. In effect, the use of Ca^{2+}-dependent molecules with different Ca^{2+} sensitivity and/or Ca^{2+} targets that are activated and inactivated over time provides discrimination between synaptic signals of varying amplitude and duration. Added to this, the source of the highly regulated Ca^{2+} signal offers a localised and precise source of specificity for the signalling-to-nucleus process (see Chapters 2 and 5).

The intracellular processes activated by Ca^{2+} influx are part of an extremely intricate and complex scenario, involving an enormous amount of signalling molecules that are dependent on Ca^{2+} for activation. As a result, the study of the nAChR activated Ca^{2+} signals can be an extremely complicated task. In the next sections of the Introduction to this chapter I will try to summarise the rationale behind our investigation of nicotine dependent signalling processes.

4.1.2 nAChR and Ca^{2+} signalling

The capacity of nAChR to increase cytoplasmic Ca^{2+} levels has been extensively illustrated and discussed in the preceding chapters of this thesis. In fact, the capacity of nicotine to increase cytoplasmic Ca^{2+} concentrations through permeation of the nAChR channel per se and by recruiting VOCC might grant the activation of nAChR with the ability to relay information from activity dependent inputs to the nucleus. The α7 nAChR, in particular, has been implicated in several cellular processes, including long-term potentiation (Matsuyama et al., 2000; Mansvelder and McGehee, 2000), neuroprotection (Donnelly-Roberts et al., 1996; Dajas-Bailador et al., 2000; Kihara et al., 2001) and learning and memory (Levin et al., 1999). However, although the potential of nAChR signalling has been apparent, the great majority of studies on the general mechanisms of Ca^{2+} signalling have relied, by far, on the investigation of Ca^{2+} signals following glutamate receptors and/or VOCC activation. In part, this is probably a result of the comparatively lower Ca^{2+} responses obtained after nAChR stimulation, which have made the NMDA receptor or VOCC a much more attractive model for the study of Ca^{2+} mediated signalling processes. As a result, the role of nAChR in the activation of Ca^{2+} dependent signalling cascades has remained, in comparison, largely unexplored. However, in spite of the relative lack of information, nicotine has been shown to activate various kinases, including PKC (TerBush and Holz, 1986; Messing et al., 1989; Tuominen et al., 1992; Cox and Parsons, 1997) and CaMK II; Tsutsui et al., 1994). In PC12 cells, human lung cancer cells and hippocampal neurones, nicotine has been reported to activate MAPK/ERK (Cattaneo et al., 1997; Heusch and Maneckjee, 1998; Tang et al., 1998; Dineley et al., 2001) through PKC dependent (Tang et al., 1998) or PKC independent pathways (Heusch and Maneckjee, 1998). Overall, these results clearly indicate that nAChR are relevant targets for the activation of specific signalling pathways in neuronal systems.

Given the various signalling molecules that are dependent on Ca^{2+} for activation, it was important to narrow the realm of possible targets for the nicotine-evoked Ca^{2+} activated signalling mechanisms. The reasons for studying the MAPK/ERK are illustrated in the following sections of the introduction. On the whole, the role of MAPK/ERK in diverse neuronal processes in the hippocampus (summarised below) and its "location" down-stream

of many signalling molecules, made it a suitable and relevant target for the study of nAChR dependent signalling processes in neuronal models.

4.1.3 Mitogen activated protein kinase (MAPK)

The MAPK cascade is one of the various pathways used to convey extracellular signals into intracellular biochemical responses. Being a member of the serine/threonine protein kinase family, it has been classically associated with the regulation of a diverse array of cellular processes, such as cell growth and proliferation, differentiation and apoptosis (Fukunaga and Miyamoto, 1998; Grewal et al., 1999).

The MAPK cascades are found in all eukariotic organisms and basically consist of a three-kinase module that includes a MAPK activated by a MAPK kinase (MEK), which is in turn activated by a MEK kinase (MEKK). To date, 12 member proteins of the MAPK family, 5 of the MEK and 14 of the MEKKs have been identified in mammalian cells, and it is increasingly believed that the specificity of MAPK responses is achieved by the activation of different modules in response to different stimuli (Garrington and Johnson, 1999). Although the combination of different members of the module may seem extraordinarily complex, certain themes start to emerge on a more detailed inspection. First, the MEKs have the fewest number of members in the MAPK module, which considering their high specificity for their MAPK substrates, allows minimal variation of the MEK-MAPK section. In contrast, the fourteen defined MEKKs are more diverse in structure, with particular regulatory motifs that are not found in the other members of the module. As a result, MEKKs can be differentially activated by a variety of upstream inputs, thus producing the selective regulation of the MEK/MAPK pathway (Garrington and Johnson, 1999).

MAPKs are grouped into subfamilies on the basis of sequence similarity, mechanisms of upstream regulation, and sensitivity to activation by different MEKs (Widmann et al., 1999). Most family members require phosphorylation on one tyrosine and one threonine residue for activation (Cobb and Goldsmith, 1995). At present, three subfamilies have been described, including the ERK (sometimes also referred as MAPK), c-Jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 (Robinson and Cobb, 1997). In addition, several different subtypes have been described for each of the three subfamilies, including five MEK family members (MEKs 1-5; Robinson and Cobb 1997). With respect to subtype specificity, the MEK1/2 act on ERK1/2, MEK3 acts on p38, MEK4 acts on JNK/SAPK and perhaps also p38, while MEK5 has no proven substrates (Robinson and Cobb, 1997). Among the MEKKs, the first described members were the Raf proteins, which were originally studied as oncogene products (Robinson and Cobb, 1997). Unlike the MEK family, MEKKs

are difficult to categorise by primary sequence similarity. The diversity of regulatory domains in different MEKKs gives the family of MAPK the flexibility to respond to a wide range of cellular stimuli. The variety of motifs include proline rich sequences for binding SH (Src homology) 3 domains, binding sites for GTP-binding proteins, leucine-zipper dimerisation sequences, and phosphorylation sites for tyrosine and serine/threonine kinases (Garrington and Johnson, 1999).

4.1.4 Extracellular signal-regulated kinase (ERK)

The genes of ERK are highly expressed in the CNS (Thomas and Hunt, 1993), and proteins of both ERK1 and ERK2 are widely but differentially distributed in various regions of the rat brain (Ortiz et al., 1995). In situ hybridisation studies revealed that the signal for ERK2 mRNA was highly intense in many regions, including the cerebral cortex, olfactory bulb, hippocampus, amygdala, basal ganglia (except the globus pallidus), brain stem nuclei and cerebellum, among others. In contrast, hybridisation signals for ERK1 were relatively weak and were observed in restricted regions in comparison to ERK2 (Thomas and Hunt, 1995). These included the olfactory bulb, cortex, cerebellum and some hypothalamic and brain stem nuclei. In the hippocampus, ERK1 was highly expressed in the dentate gyrus and was practically absent in the CA1 region, which contrasts to expression of ERK2 in all regions of the hippocampus (Thomas and Hunt, 1995). Expression of the upstream kinases of ERK1/2 has been also noted in the brain. The direct upstream kinases MEK1 and 2 are both expressed in the brain, as indicated by northern blot analysis (Brott et al., 1993). In addition, a number of MEK activating kinases, MEKKs, have been reported in the CNS, most notably c-Raf-1 and B-Raf, with the latter being the most abundant (Storm et al., 1990; Barnier et al., 1995).

4.1.5 Activation of ERK by neurotrophic factors

In the last decade, the role of ERKs in the regulation of neuronal function has received increasing attention, and although various routes to ERK activation have been recently described (see later), much of the characterisation of ERK signalling cascades was initially focused on the actions of neurotrophic factors (Segal and Greenberg, 1996). It is well established that multiple neurotrophins regulate the development of the nervous system. Accordingly, extensive studies have been centred on intracellular signal transduction of neurotrophins and in the elucidation of the role of ERK activation in neurones. In effect, the examination of the actions of NGF has provided important information about neurotrophin-mediated signalling pathways. It has been established that at least three proteins, namely,

the adaptor protein Shc and its neural specific isoform N-Shc, the phospholipase PLC-γ1, and the phosphotyrosine phosphatase SHP directly associate with NGF-activated tyrosine kinase receptor, Trk (for review see Kaplan and Miller, 1997), thus coupling Trk to several intracellular signalling pathways. For example, Shc association with Trk, and its tyrosine phosphorylation by it, results in the rapid activation of phosphatidylinisitol 3-kinase, Ras, and the serine-threonine kinases B-Raf and ERK (Kaplan and Miller, 1997; Grewal et al., 1999, and references therein).

Recent studies have also identified a novel mechanism for NGF-induced stimulation of ERKs (Yao et al., 1998). This pathway involves the PKA-dependent activation of the Ras-related small G-protein Rap1 and the subsequent activation of B-Raf, a Raf isoform. In fact, given the high localisation of B-Raf in neurones, it has been suggested that the ability of PKA and Rap1 to couple to ERKs activation may have significant implications for neuronal signalling, including the regulation of neuronal survival and synaptic plasticity (Grewal et al., 1999).

4.1.6 Ca^{2+} dependent activation of ERK

As discussed in chapters 1 and 2, Ca^{2+} influx can modulate various cellular events, including neuronal survival, excitotoxicity and also synaptic remodelling and adaptive responses. Many of the Ca^{2+} functions in neuronal activity are associated with changes in gene expression, which may then lead to structural and functional changes, underlying long-term adaptive responses. For many years, the majority of studies examining Ca^{2+} dependent signal transduction in neuronal models have focused on the PKC and the CaMK family. Many PKC isoforms as well as the CaMK require Ca^{2+} for activity in vitro, and contain conserved domains responsible for direct binding of Ca^{2+} or Ca^{2+}/calmodulin complexes (Hanson and Schulman, 1992). More recent studies have demonstrated that Ca^{2+} influx in neurones might also lead, by indirect mechanisms, to the stimulation of other intracellular signals, including ERK (Rosen et al., 1994). In effect, ERK does not require Ca^{2+} for activity in vitro, but is instead regulated by phosphorylation on conserved threonine and tyrosine residues (Seger and Krebs, 1995). As a result, activation of ERK in response to Ca^{2+} influx implicates the mediation of one ore more additional second messengers.

Rosen et al., (1994) first demonstrated that Ca^{2+} influx through VOCC activates the ERK cascade, via activation of the small GTP-binding protein Ras, which is also associated with the NGF dependent ERK activation. In recent years it has become evident that Ras, and other Ras related G-proteins like Rap-1, are able to function as molecular switches that transmit receptor signals to downstream MEK/ERK cascades (Finkbeiner and Greenberg,

1998; Grewal et al., 1999). Prior to stimulation, Ras resides at the inner face of the plasma membrane in an inactive state, bound to GDP. The activity of Ras and Rap1 is tightly regulated by specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Grewal et al., 1999). In effect, Ras has been implicated as a major target for Ca^{2+} signalling to ERKs, particularly through the stimulation of a specific GEF, RasGRF, which is activated by binding to calmodulin (Farnsworth et al., 1995), or via tyrosine kinases such as Src or Pyk2 after recruitment into multiprotein complexes (see Finkbeiner and Greenberg, 1996 for review). More recent studies have also identified other GEFs that can be regulated by a variety of intracellular second messengers. For example, CalDAG-GEFI and CalDAG-GEFII can bind directly to and be activated by both Ca^{2+} and DAG (Ebinu et al., 1998; Kawasaki et al., 1998). Likewise, cAMP-GEFI and cAMP-GEFII are activated directly by cAMP, independently of PKA, while C3G is regulated by cAMP in a PKA dependent manner (Grewal et al., 1999 and references therein). Furthermore, nitric oxide has been reported to be an intermediate between NMDA receptor activation and Ras stimulation (Yun et al., 1998). The use of multiple signalling pathways probably allows neurones to regulate the kinetics of ERK activation, and such mechanisms may be important for translating specific patterns of neuronal activity into qualitatively different intracellular signals (see Fig 4.1 for a schematic diagram of some of the ERK pathways).

4.1.7 Functional role of the Ras-ERK cascade in neurones

Taking into account the many signalling paths to ERK, it is important to establish the biological consequence of ERK activation in the nervous system, and the significance of the various possible pathways of Ca^{2+} dependent ERK signalling. ERK phosphorylate a wide range of substrates, which include various downstream Ser/Thr effector kinases. For example, the transcription factor CREB can be phosphorylated and activated in an ERK dependent manner via stimulation of the CREB kinase RSK2 (Xing et al., 1996). This action is a major target of both neurotrophin and neuronal Ca^{2+}, while PKA has been shown to use an ERK-dependent pathway to CREB (Roberson et al., 1999). More recent studies have also suggested that ERKs have multiple roles in the stimulation of transcription, and more importantly, that these include the translocation of ERKs from the cytoplasm to the nucleus, where they can directly phosphorylate and thereby stimulate transcription factors (Thomson et al., 1999). The mechanisms leading to ERK nuclear localisation are still unclear, but they appear to require both ERK phosphorylation and dimerisation (Khokhlatchev et al., 1998).

The present understanding of the functional effects of ERK activation are still far from complete, however, several lines of evidence suggest that ERKs, and in particular the Ras-

ERK pathway, may contribute to the regulation of differentiation, survival and synaptic plasticity (Finkbeiner and Greenberg, 1996):

4.1.7.1 ERK and neuronal survival

It has been demonstrated that Ras is an important factor for neurotrophin induced neuronal survival, and the introduction of Ras into embryonic neurones mimics growth factor treatment (Borasio et al., 1993). In addition, an anti-apoptotic role for ERKs has been reported in PC12 cells after growth factor withdrawal (Xia et al., 1995), and it has been also demonstrated that ERKs can protect certain populations of neurones against specific toxic stimuli (Maher et al., 2001; Punn et al., 2000; Anderson and Tolkovsky, 1999). In spite of these reports, other studies indicate that ERK might not be required in NGF-mediated survival (Creedon et al., 1996; Virdee and Tolkovsky, 1996). Overall, although under certain circumstances ERK activity may not be sufficient to mediate survival, it appears as an important factor for survival under some physiological or pathological conditions (Anderson and Tolkovsky, 1999).

4.1.7.2 ERK and synaptic plasticity

A relatively recent set of studies suggests that the ERK cascade plays a fundamental role in vertebrate and invertebrate memory consolidation, in particular, through the regulation of synaptic activity after increases in intracellular Ca^{2+} (for review see Grewal et al., 1999). In fact, ERK signalling components are highly enriched in the adult CNS, while expression of many ERK regulators, including many GEFs and Rafs, is largely restricted to the CNS. In addition, expression of ERK signalling components is especially high in association areas implicated in learning and memory, such as the hippocampus, neocortex and cerebellum (for review see Impey et al., 1999).

Several aspects of the ERK signalling cascade make it an attractive candidate for the mediation of some of the Ca^{2+}-dependent intracellular mechanisms observed in synaptic plasticity. First, the sequential nature of the ERK cascade provides multiple points at which the response can be regulated by phosphorylation and desphosphorylation, allowing for tremendous amplification of extracellular signals. Secondly, because Ca^{2+} signals are highly regulated in the contexts of space and time, the Ca^{2+} dependent activation of ERK can be spatially and temporally graded according to the specific synaptic input.

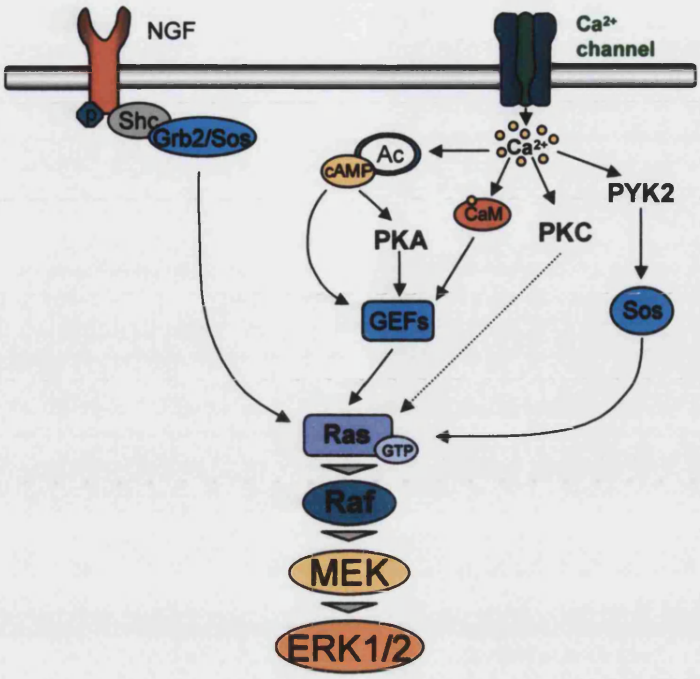

Fig 4.1 Schematic model for the activation of ERK1/2 in neurones
Activation of the ERK1/2 signalling pathway can occur through a variety of intracellular routes. In the context of growth factor signalling, various adaptor molecules (Shc, Grb2) are capable of recruiting specific guanine nucleotide exchange factors (GEFs), such as Sos, into multiprotein complexes that signal to the downstream Ras pathway. Ca^{2+} influx can also activate the ERK1/2 cascade through various mechanisms, of which only some are depicted in the present model. The essential link is provided by the GEF proteins, which connect various Ca^{2+} dependent processes into an increase of Ras activity. Recent studies have identified specific GEFs that can be regulated directly by intracellular second messengers, such as cAMP, PKA, Ca^{2+}/calmodulin (CaM) and PYK2, among others. The complexity and, at the same time, specificity of these signalling pathways may explain the ample role of ERKs in the regulation of activity dependent neuronal events, such as synaptic plasticity and cell survival.

As illustrated in Chapter 1, LTP is an activity-dependent strengthening of synaptic efficacy, proposed as a cellular model for vertebrate memory formation. Relatively recent reports indicate that ERK signalling plays an important role in the induction of LTP. For example, stimuli that induce LTP in area CA1 of the hippocampus also potently activate ERK (English and Sweatt 1996, Sweatt, 2001), while pharmacological inhibition of MEK partially inhibits LTP formation in area CA1 of hippocampal slices (English and Sweatt, 1997). In addition, the inhibition of MEK prevents the gene expression-dependent long-lasting LTP (Impey et al., 1999). Therefore, ERK signalling not only regulates short-term synaptic function, but may also promote the transcription and translation of new proteins required for long-lasting LTP.

Several transgenic mouse mutants have also implicated the ERK pathway in vertebrate long-term memory formation. Mice deficient for RasGRF, an activator of Ras (see above), show a marked deficiency in LTP in the basolateral amygdala (Brambilla et al., 1997), a

structure believed to help encode certain forms of associative fear conditioning. Consequently, Brambilla et al. (1997) examined the cued fear conditioning, in which mice learn to fear a normally innocuous auditory tone by paired aversive stimuli, in Ras-GRF deficient mice. In fact, the long-term memory formation for cued fear conditioning was markedly compromised in Ras-GRF mutant mice. Considering that Ras-GRF is a potent activator of the ERK cascade, these results suggest a role for Ras-ERK in long-term memory formation.

Complementary studies in animal models of long-term memory have been also carried out utilising pharmacological inhibitors of MEK. This approach avoids the problems associated with gene disruption studies, in which compensatory changes can affect the outcome synaptic functioning. Atkins et al. (1998) demonstrated that systemic injection of the MEK inhibitor SL327 blocks cued fear conditioning in rats, suggesting that ERK activity is required for memory formation. In addition, the behavioural model of contextual fear conditioning utilised also induces a marked activation of ERK in the rat hippocampus, as demonstrated by western blotting experiments. Interestingly, SL327 also blocks hippocampal LTP (Atkins et al., 1998), a results that when added to the evidence showing the necessity of ERK activity for LTP, suggests that ERK signalling is essential for hippocampus dependent long term memory, and supports the notion that LTP and memory consolidation share similar mechanisms.

In summary, these findings indicate that the ERK cascade should join the CREB/CRE and PKA pathways as evolutionarily conserved regulators of memory formation. Of particular interest is the fact that the activity dependent Ca^{2+} influx in CNS neurones can lead to the increase in cAMP, and through particular GEFs couple to activation of ERK (Vossler et al., 1997; Grewal et al., 1999). Consistent with this, activation of adenylyl cyclase markedly increases ERK activity in hippocampal neurones (Impey et al., 1998). Thus, in addition to being activated by Ca^{2+}, ERK may also be a major cAMP-stimulated kinase in the CNS. In fact, these studies raise the possibility that some of the effects of cAMP, which have been attributed to PKA, may be the result of ERK activation (Impey et al., 1999).

4.1.8 General Aims of this chapter

The stimulation of the ERK1/2 constitutes one of the possible candidates for the mediation of some of the neuronal actions of nAChR activation. In fact, nAChR modulate many cellular processes in which ERKs are implicated, including cell growth, differentiation and synaptic plasticity (Role and Berg, 1996; Fukunaga and Miyamoto, 1998; Sweatt, 2001).

As illustrated earlier in this chapter, the cellular signalling pathways that can lead to activation of ERK1/2 are complex and may proceed through a variety of intracellular routes (Grewal et al., 1999). In general, the common downstream pathway is comprised by the activation of the Raf MEKKs, which leads to the activation of the ERK1/2, through phosphorylation by activated MEK (Sweatt, 2001). Several second messenger systems converge on the MEK-ERK1/2 cascade, including signalling routes that involve the integration of Ca^{2+} signals. In addition, the generation of cAMP can also result in the activation of the ERK1/2 cascade (Vossler et al., 1997), while PKA has been more recently described as a novel link between Ca^{2+} entry through VOCC and subsequent activation of the ERK1/2 signalling pathway (Grewal et al., 2000a,b).

In view of the above-mentioned role of the ERK signalling cascade in neuronal processes of survival and synaptic plasticity, nicotine-evoked ERK activation constituted an attractive candidate for the mediation of nAChR intracellular actions in primary hippocampal cultures. In order to analyse the involvement of ERK in nicotine-evoked signalling processes, and to define the precise mechanisms involved, the first part of the study was carried out using the SH-SY5Y cell line as a model system, which allowed a more detailed definition of the particular signalling steps occurring. Subsequent studies defined the involvement of ERK signalling after nicotine stimulation in the hippocampus. In particular, the aims of this chapter were:

- To determine if nicotine could increase ERK1/2 activity in SH-SY5Y cells and hippocampal primary cultures.
- To establish the nAChR subtype involved and the intervening signalling steps leading to activation of ERK1/2.
- To determine if the ERK1/2 cascade has a role in the previously observed nicotine-evoked protection in hippocampal cultures.

** Some of the results presented in this Chapter were obtained in collaboration with Dr. Lev Soliakov.*

4.2 METHODS

4.2.1 Materials

The following antibodies were used in this study: mouse E10 monoclonal anti-phospho-p44/42 ERK1/2 (Thr 202/Tyr 204) and rabbit polyclonal anti-p44/42 ERK1/2, anti-phospho-MEK1/2 (Ser 217/221) and anti-MEK1/2 antibodies were purchased from New England Biolabs Ltd. (Hertfordshire, UK). Rabbit polyclonal anti-Raf-B (C-19) and E10 mouse monoclonal anti-Raf-1 antibodies were obtained from Santa Cruz Biotechnology Inc. (Autogen Bioclear UK Ltd, Mile Elm Calne, Wiltshire). Recombinant inactive mouse p42 MAP kinase 2/ERK 2 (62 kDa fusion protein) and recombinant inactive mouse MEK 1 (71 kDa fusion protein) were provided by Upstate Biotechnology (TCS Biologicals, Buckingamshire, UK). Anti-mouse IgG-horse radish peroxidase (HRP), myelin basic protein (MBP), (-)-nicotine, insulin-like growth factor 1 (IGF-1), L-glutamine, gentamicin, non-essential aminoacids, penicillin and streptomycin were from Sigma-Aldrich (Dorset, UK). Anti-rabbit IgG-HRP conjugates were from Vector Laboratories (Burlingame, USA). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham International (Little Chalfont, UK). Mecamylamine and α-bungarotoxin were provided by Tocris Cookson (Bristol, UK). PD98059, KT5720, H-89, forskolin, Ro 31-8220, KN-62, LY294002 were from Calbiochem (Nottingham, UK). COMPLETE^{TM}, a protease inhibitor cocktail, was purchased from Boehringer (Lewes, UK). DMEM, Neurobasal medium and B-27 supplement were from Gibco BRL (Paisley, UK). All other chemicals and reagents were obtained from the usual commercial sources.

4.2.2 Cell culture

Human neuroblastoma SH-SY5Y cells, (ECACC, Salisbury, UK; passages 14-20) were cultured in 75 cm^2 tissue culture flasks and maintained in DMEM, supplemented with 15 % foetal bovine serum, 2 mM L-glutamine, 1 % non-essential amino acids and 190 U/ml of penicillin and 0.2 mg/ml of streptomycin, until confluent. In experiments on SH-SY5Y cells, culture medium was replaced by serum-free medium (0.1 % albumin instead of foetal bovine serum) 24 h prior to drug treatments.

High-density hippocampal cultures were prepared from the hippocampi of E18 Wistar rat foetuses and maintained in serum free conditions as described in the methods section of Chapter 2. Kinase inhibitors or nAChR antagonists were incubated with cells for 30 min prior to nicotine treatment. In "Ca^{2+}-free" experiments, EGTA (3mM) was co-applied with nicotine. Forskolin treatment was carried out for 10 min before nicotine. In each case stimulation was terminated by aspiration of the medium, followed by two washes with 15 ml of ice-cold PBS (120 mM NaCl, 19 mM Na_2HPO_4, 6 mM KH_2PO_4). Cells were then lysed by adding 1 ml of

lysis buffer (137 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 10% glycerol, 1% NP-40, 20 mM Tris, pH 8.0, supplemented with protease inhibitor cocktail, 1 mM PMSF, 1 mM orthovanadate and 1 mM dithiothreitol), followed by shaking on ice for 10 min. The lysed cells were passed through 25 G needles and centrifuged at 13,000 rpm for 10 min, the supernatant fraction was collected and pre-cleared by rotation with protein-A-agarose beads at 4°C. The protein concentration was estimated by the Bradford assay and equal amounts of protein were used for immunoprecipitation or Western blot analysis.

4.2.3 Western blotting

Samples (cell lysates or immune complexes) were heated for 7-10 min at 95° C, resolved using 7.5% SDS-PAGE and blotted onto PVDF (Millipore) membranes. Western blotting was performed with phospho-specific (anti-phospho-p44/42 ERK1/2; diluted 1:1000) or phosphorylation state independent (anti-p44/42 ERK1/2, anti-Raf-B and anti-Raf-1; diluted 1:1000) primary antibodies. HRP-conjugated secondary antibodies, anti-mouse IgG (diluted 1:3000) or anti-rabbit IgG (diluted 1:4000) were used accordingly. Electro Chemi-Luminescence (ECL) western blotting reagents (Amersham International) were utilised for the detection of bands. For data analysis see corresponding section below.

4.2.4 Immunoprecipitation

Immune complexes were prepared by incubating SH-SY5Y cell lysates (250 μg of protein) with either phospho-specific p44/42 ERK1/2, Raf-1 or Raf-B antibodies for 1.5-2.0 h at 4°C, followed by mixing the suspensions with protein-A-agarose beads at 4°C overnight. Immune complexes were then washed twice with each of the following solutions: lysis buffer (see above), PBS and kinase buffer (20mM β-glycerolphosphate, 0.1 mM pyrophosphate, 0.1 mM orthovanadate, 2 mM dithiothreitol, 20 mM Tris, pH 7.5); kinase activity associated with immune complexes was measured as described below.

4.2.5 ERK1/2 kinase activity assay

ERK1/2 activity was assayed by incubating the immune complexes in kinase buffer (as above) with 20 μM ATP, 5μCi [γ-^{32}P] ATP and 3-6 μg of myelin basic protein (MBP) substrate in a final volume of 30 μl. Following incubation for 20 min at 30° C, phosphorylation was stopped by addition of 15μl of 3x concentrated Laemmli sample buffer, followed by heating at 95° C for 10 min. Samples were resolved using 12% SDS-PAGE and gels were subsequently dried and exposed to BioMax film (Kodak) for 18 hours at -70° C.

4.2.6 Raf-1/B-Raf kinase assays

This assay was performed in accordance with the manufacturer's instructions (Upstate Biotechnology), with minor modifications. Immune complexes containing Raf-1 or B-Raf were incubated with inactive GST-MEK-1 (0.4μg) and inactive GST-ERK2 (1.4 μg) in kinase assay medium (125μM ATP, 20mM β–glycerolphosphate, 0.1 mM pyrophosphate, 0.1 mM orthovanadate, 2 mM dithiothreitol, 20 mM Tris, pH 7.5, in a total volume of 40μl). After 30 min at 30° C, an aliquot (10 μl) of the medium was taken and kept on ice for subsequent kinase assay (see below), and the residual amount was mixed with 3x Laemmli sample buffer, heated for 10 min at 95° C and resolved using 7.5% SDS-PAGE. Gels were blotted onto PVDF (Millipore) membranes and bands corresponding to phosphorylated GST-MEK1 were identified using phospho-MEK1/2 specific primary antibodies (dilution 1:1000) and secondary anti-rabbit IgG-HRP conjugates (1:3000-1:4000 dilution). ECL western blotting reagents were used for detection.

An additional method for determining Raf-1/B-Raf activity was used, in which the reserved aliquots were mixed with 150μM ATP, 10μM [γ-^{32}P] ATP and 20μg MBP in 30μl of kinase assay buffer. After 10 min at 30° C, the reaction was stopped by spotting 25 μl aliquots of the samples on to P81 filter paper (Whatman, 2x2 cm each). Filters were washed three times in 0.75% phosphoric acid (5 min per wash) and once with acetone, before being placed in scintillation vials and counted for radioactivity in a liquid scintillation counter (Packard 6000).

4.2.7 Cell Viability

Neuronal survival of hippocampal primary cultures after different treatments was estimated using the MTT method for cell viability, according to the procedures described in the Methods section of Chapter 2.

4.2.8 Data analysis and statistics

Autoradiographs were quantified by densitometry using OneDscan v 1.0 (Scanalytics Inc., Fairfax, USA). Results are expressed as a percentage of control responses (stimulated with buffer alone) and data represent the mean ± SEM of 3 or more independent experiments. Statistical significance was determined using Student's t test and one-way ANOVA plus post hoc Tukey's test, as stated in the figure legends. Values of $p < 0.05$ were taken to be statistically significant.

4.3 RESULTS

4.3.1 nAChR-mediated activation of ERK1/2 in SH-SY5Y cells

In initial experiments, SH-SY5Y cells were stimulated with a range of nicotine concentrations (100 nM - 100 μM) for 40 s, and ERK1/2 phosphorylation and activity were analysed by western blotting and kinase assay respectively. The highest concentration of nicotine tested (100 μM) consistently induced a significant enhancement of ERK1/2 phosphorylation (Fig 4.2a) and activity (Fig 4.2b), approximately 50% above basal levels. This response was relatively modest compared with ERK1/2 phosphorylation after IGF-1 (Fig 4.2a).

The time course of activation of ERK1/2 by 100 μM nicotine in SH-SY5Y cells showed that ERK1/2 phosphorylation was increased at 10 s and persisted over 40 s of nicotine stimulation (Fig 4.2a). Activity of ERK1/2, estimated using MBP as substrate, was also increased at 40 s and 10 min (Fig 4.2b) but then declined with prolonged stimulation (up to 20 min, Fig 4.2b). A 40 s treatment with 100 μM nicotine was used for further experiments on SH-SY5Y cell cultures.

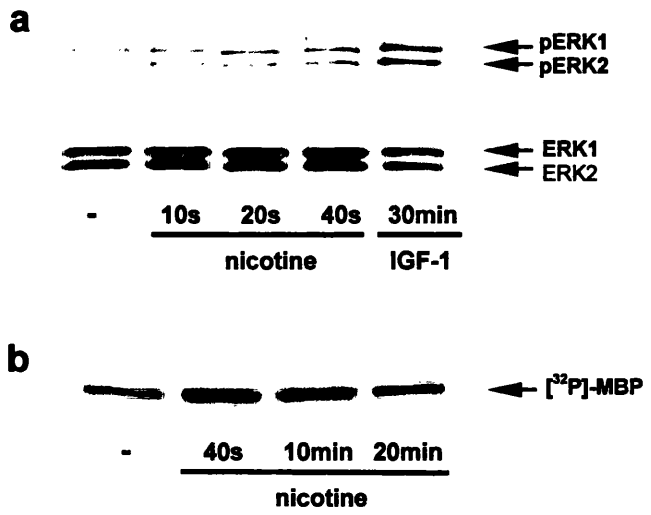

Fig 4.2 Time-course of nicotine-evoked activation of ERK1/2.
SH-SY5Y cells were stimulated with nicotine or IGF-1 for the indicated times and then lysed for further analysis. Activation of ERK1/2 was determined directly by Western blotting of cell lysates or by in vitro kinase assay using immune complexes. (a) Time-dependent phosphorylation of ERK1/2 after nicotine, identified in cell lysates by Western blotting using anti-phospho-p44/42 ERK monoclonal antibodies (top panel). The total amount of ERK1/2 in each sample was estimated with an anti-p44/42 ERK polyclonal antibody (bottom panel). (b) Time-dependent increase in ERK1/2 activity after nicotine stimulation. Immune complexes containing activated ERK1/2 (phospho-ERK1/2) were incubated with MBP as a substrate in the presence of [γ-^{32}P] ATP and subjectd to SDS-PAGE. Incorporation of $^{32}P_i$ into MBP was identified by autoradiography as described in the Methods section.

In the presence of a selective inhibitor of the ERK1/2 kinase, MEK (PD98059; 50μM, 30 min), the activation of ERK1/2 by nicotine was totally prevented (nicotine: 146.0 ± 17.9 %; nicotine + PD98059: 103.3 ± 14.8 % of control values; n=3, $p<0.05$, Fig 4.3a), thus demonstrating that the nicotine-induced activation of ERK1/2 required MEK activity.

To ascertain if nicotine was acting through a nAChR to increase ERK1/2 activity, SH-SY5Y cells were pre-incubated with either the general nAChR antagonist mecamylamine (10μM, 30 min) or the α7-specific nAChR antagonist α-Bgt (10nM, 30 min), before stimulation with 100 μM nicotine (40 s). Incubation with either antagonist completely abolished the nicotine-dependent activation of ERK1/2, from 160.7 ± 19.0 % to 105.7 ± 11.8 % of controls for mecamylamine (n=6, $p<0.05$, Fig 4.3b) and from 151.7 ± 15.9 % to 100.7 ± 11.5 % of controls for α-Bgt (n=7, $p<0.05$, Fig 4.3c), thus implicating the α7 subtype of nAChR.

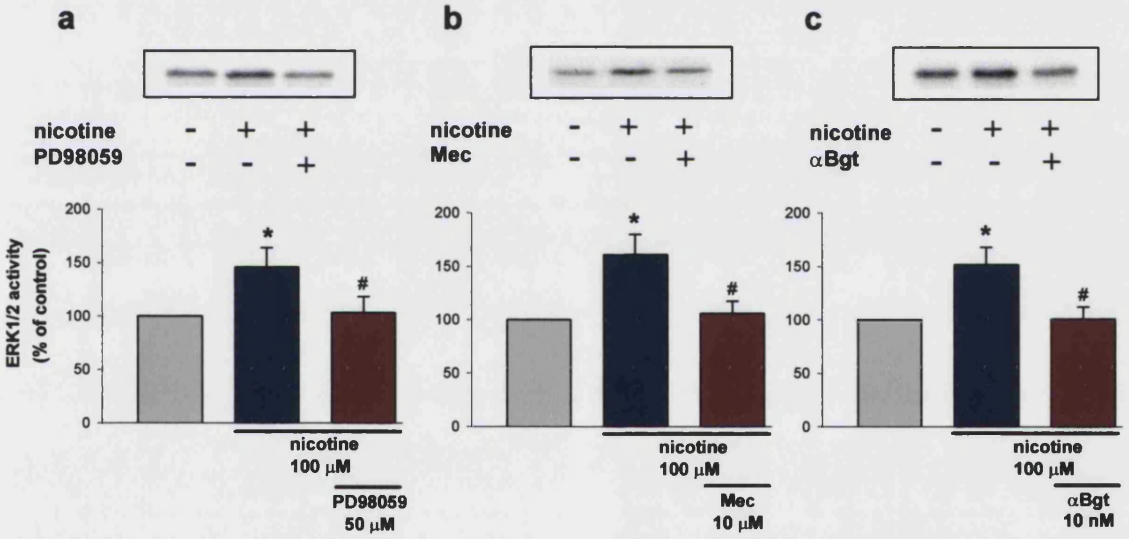

Fig 4.3 Nicotine-induced activation of ERK1/2 via α7 nAChR and the upstream kinase MEK. SH-SY5Y cells were treated with (a) the MEK inhibitor PD98059 (50μM), (b) mecamylamine (Mec, 10μM) or (c) α-bungarotoxin (α-Bgt, 10nM) for 30 min, followed by nicotine stimulation (100μM, 40 s). Controls were treated with buffer. Cell lysates were immunoprecipitated with phospho-p44/42 ERK monoclonal antibodies and the in vitro kinase assay was carried out using MBP as a substrate, as described in the Methods section. Autoradiographs were analysed by densitometry and values are expressed as a percentage of the buffer control run in parallel; representative autoradiographs are shown in the upper panels. Data are expressed as the mean ± SEM of 3 to 7 experiments. Significantly different from control, * $p<0.05$, Student's *t*-test. Significantly different from ERK1/2 activity in the presence of nicotine, # $p<0.05$, ANOVA and post hoc Tukey's test.

The high Ca^{2+} permeability of α7 nAChR suggests that this receptor may activate second messenger cascades by increasing cytoplasmic Ca^{2+} concentrations. To examine the Ca^{2+}-dependence of the nicotine-evoked increase in ERK1/2 activity, SH-SY5Y cells were stimulated with nicotine in nominally Ca^{2+}-free medium, by co-incubation with EGTA (3 mM). This "Ca^{2+}-free" condition significantly reduced the nicotine-evoked activation of ERK1/2, from 149 ± 15.5 % to 107.6 ± 8.5 % of controls (n=5, p<0.05, Fig 4.4). EGTA itself did not alter the basal levels of kinase activity in SH-SY5Y cells (Fig 4.4).

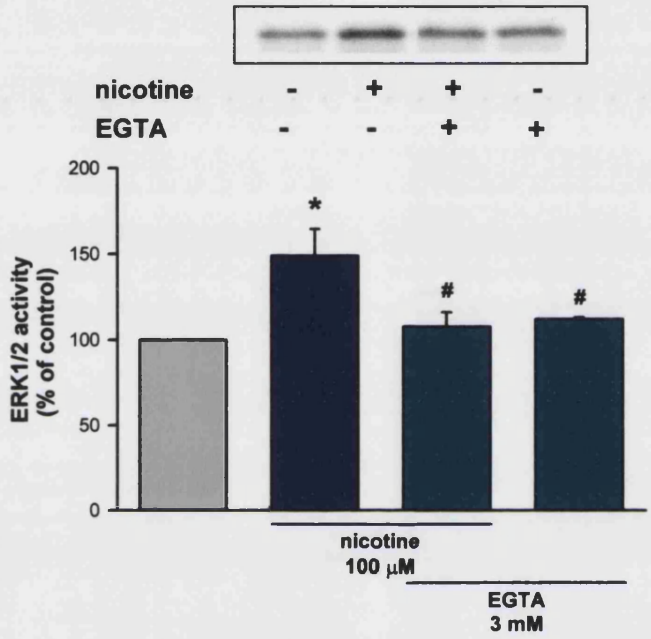

Fig 4.4 Nicotine-induced activation of ERK1/2 is dependent on extracellular Ca^{2+}.
SH-SY5Y cells were treated with nicotine (100μM, 40 s) in normal medium or in the presence of EGTA (3 mM), followed by lysis and immunoprecipitation with phospho-p44/42 ERK monoclonal antibodies. The in vitro kinase assay with MBP as a substrate was performed as described in the Methods section. Autoradiographs were analysed by densitometry and values are expressed as a percentage of the buffer control run in parallel; representative autoradiographs are shown in the upper panel. Values are the mean ± SEM of 5 experiments. Significantly different from controls, * p<0.05, Student's t-test. Significantly different from ERK1/2 activity in the presence of nicotine, # p<0.05, ANOVA and post hoc Tukey's test.

4.3.2 Kinase pathways and the nAChR-induced activation of ERK1/2

A variety of intracellular kinases can be activated by increases in cytoplasmic Ca^{2+}. To elucidate the signalling pathway that mediates the Ca^{2+}-dependent nicotine stimulation of ERK1/2, SH-SY5Y cells were incubated for 30 min with inhibitors of several Ca^{2+}-dependent kinases reported to operate upstream of ERK1/2, prior to the addition of nicotine (100μM, 40 s). Ro 31-8220 (1μM), KN-62 (5μM) or LY294002 (50μM) did not significantly modify the

nicotine-evoked activation of ERK1/2 (Fig 4.5a), indicating the lack of a major involvement of PKC, CaMK II or PI3 kinase, respectively. In contrast, two selective and structurally different inhibitors of PKA, KT5720 (0.5μM, 30 min) and H-89 (5μM, 30 min) reduced the nicotine-evoked increase in ERK1/2 activity from 151 ± 15.4 % to 98.6 ± 8.7 % (n=10, $p<0.05$) and 106.7 ± 11.8 % (n=8, $p<0.05$) of controls respectively (Fig. 4.5b).

The capacity of PKA to lead to the activation of ERK1/2 in SH-SY5Y cells was supported by the ability of forskolin (10μM, 10 min) to elevate the activity of ERK1/2 (136.0 ± 15.9% of controls, n=4, $p<0.05$) to an extent comparable with the effect of 100 μM nicotine (Fig 4.5c). Application of nicotine (100 μM, 40 s) in the presence of forskolin (10 μM, 10 min) showed no additive effect (146 ± 26.6% of controls, n=3, Fig 4.5c) compared with either drug alone. The effect of forskolin plus nicotine was completely abolished by pretreatment with KT5720 (0.5μM, 30 min, 97.7 ± 6.1% of controls, n=3; Fig. 4.5c). Forskolin stimulation produced no significant increase in cytoplasmic Ca^{2+} concentrations (forskolin: 12 ± 8 % compared to control nicotine stimulation) and neither forskolin nor the PKA inhibitor H-89 significantly affected the increase in cytoplasmic Ca^{2+} evoked by nicotine (nicotine + H-89: 95 ± 3 % of control nicotine stimulation), as determined by fluorescence measurements using the Ca^{2+} sensitive dye Fluo-3.

The intermediate steps connecting the nAChR-mediated activation of PKA to the activation of MEK and ERK1/2 in SH-SY5Y cells were investigated by examining the activation of the MEK kinases Raf-1 and B-Raf. The activities of Raf-1 and B-Raf were estimated in immunoprecipitates, either by direct measurement of MEK1 phosphorylation by Western blotting, or by indirect measurement of $^{32}P_i$ incorporation into MBP. Both methods showed that nicotine differentially affected Raf-1 and B-Raf kinase activities in SH-SY5Y cells. The ability of Raf-1 to phosphorylate MEK-1 was slightly but significantly increased by nicotine treatment, compared to controls (116.8 ± 5.4 %, n=7, $p<0.05$, Fig 4.6a), whereas B-Raf phosphorylation of MEK-1 was significantly reduced (85.5 ± 4.8%, n=7, $p<0.05$, Fig 4.6b). Changes in Raf-1 and B-Raf activity after nicotine stimulation were completely prevented by the PKA inhibitor H-89 (5μM, 30 min; 103.2 ± 2.8 %, n=10 and 100.8 ± 5.5 %, n=10 respectively, Fig 4.6a, b). The indirect determination of Raf-1 activity by analysis of the MBP phosphorylation in an in vitro kinase assay indicated a tendency for Raf-1 activity to increase after nicotine stimulation, but this did not reach statistical significance (Fig 4.7). On the other hand, the in vitro kinase assay for B-Raf showed a significant decrease in B-Raf activity after nicotine (79.7 ± 6.0% of control, $p<0.05$, n=5; Fig 4.7), consistent with the Western blot results. This effect was also prevented by H-89 pretreatment (96.5 ± 4.7% of control, n=5, Fig 4.7).

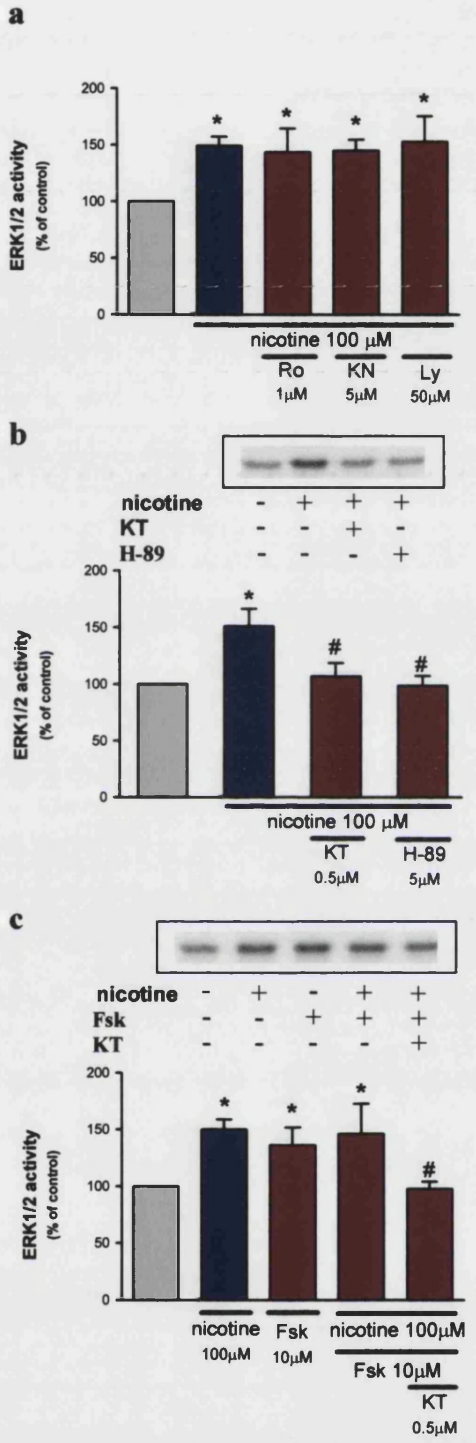

Fig 4.5 Nicotine-induced activation of ERK1/2 occurs via a PKA-dependent pathway. SH-SY5Y cells were treated for 30 min with various kinase inhibitors. (a) PKC inhibitor Ro 31-8220 (Ro, 1μM), CaM kinase II inhibitor KN-62 (KN, 5μM) or PI3 kinase inhibitor LY294002 (LY, 50μM); (b) PKA inhibitors KT5720 (KT, 0.5μM) or H-89 (5μM). In (c) cells were treated with forskolin (Fsk, 10μM) for 10 min prior to incubation with nicotine (100μM, 40 s) or buffer. Cell lysates were immunoprecipitated with anti-phospho-p44/42 ERK monoclonal antibodies and assayed for kinase activity as described in the Methods section. Representative autoradiographs are shown (top panels). Data are expressed as the mean ± SEM of 3 to 8 experiments. Significantly different from control, * $p<0.05$, Student's *t*-test. Significantly different from ERK1/2 activity evoked by nicotine stimulation, # $p<0.05$, ANOVA and post hoc Tukey's test.

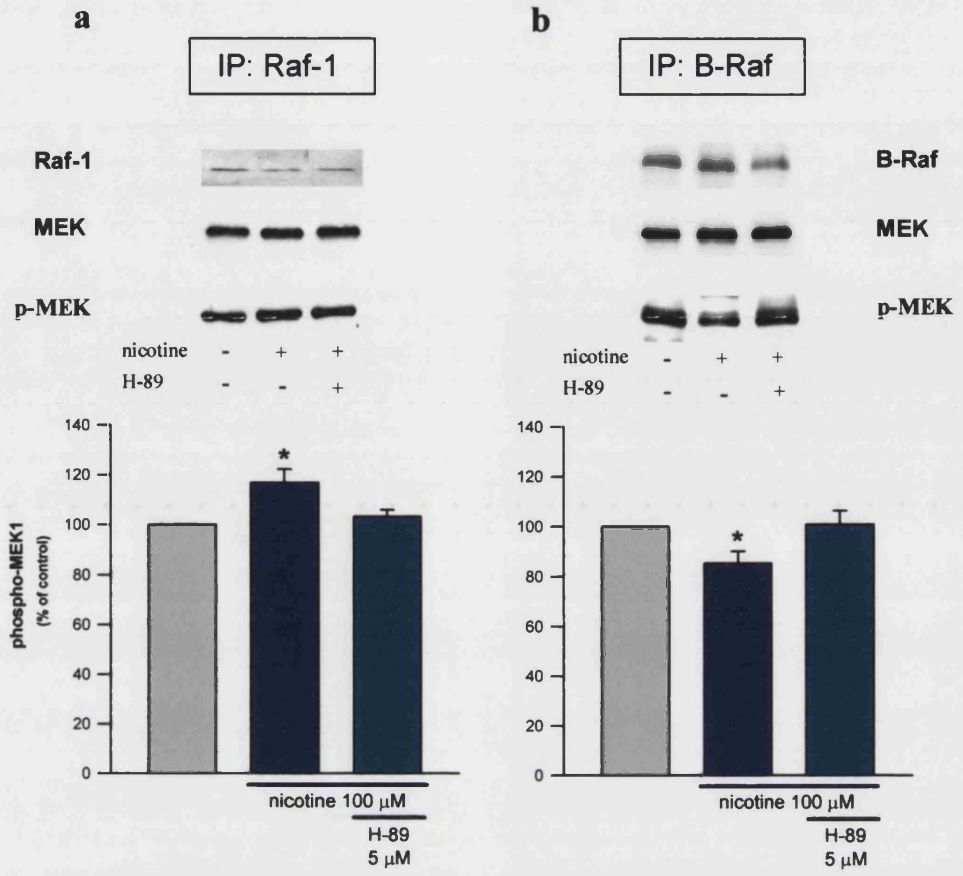

Fig 4.6 Effects of nicotine treatment on phosphorylation of MEK by Raf-1 and B-Raf. Raf-1 (panel a) and B-Raf (panel b) activities in SH-SY5Y cells were estimated from MEK-1 phosphorylation as determined by Western blots with phospho-MEK-1 specific antibodies, see Methods section for details. SH-SY5Y cells were pretreated with H-89 (5μM, 30 min) or buffer prior to stimulation with nicotine (100μM, 40 s). Cell lysates were immunoprecipitated with antibodies against Raf-1 or B-Raf and immune complexes were incubated with equal amounts of GST-MEK1. The upper panel of blots demonstrates the presence of the respective Raf isoforms in the corresponding immunoprecipitates. The levels of total MEK present were assayed in parallel with MEK specific antibodies and shown in the middle panel. Phosphorylation of MEK1 was identified using phospho-MEK1/2 specific antibodies and the lower panel shows representative blots for phospho-MEK1. The summary of data for phospho-MEK1 obtained from 5 independent experiments is represented in the histograms (values are the mean ± SEM). Significantly different from control, *$p<0.05$, Student's *t*-test.

4.3.3 nAChR-mediated activation of ERK1/2 in hippocampal neurones

The activation of ERK1/2 after nicotine stimulation was also analysed in hippocampal neurones by Western blotting with phospho-specific ERK1/2 antibodies. ERK 2 showed higher levels of phosphorylation and the results for this isoform only were quantitated by densitometry. Incubation of hippocampal primary cultures with nicotine (100 μM, 5 min) produced a significant increase in ERK 2 phosphorylation (134.6 ± 3 % of controls, $p<0.05$, n=6, Fig 4.8a). Similar to SH-SY5Y cells, the increase in ERK activation after stimulation with nicotine was substantially reduced by 30 min incubation with 10 nM α-Bgt (from 135.0 ±

6 to 112.0 ± 5 % of controls, $p<0.05$, n=3, Fig 4.8b) and was dependent on extracellular Ca^{2+}, as shown by the decrease in phosphorylation in the presence of EGTA (from 136.9 ± 9 after nicotine to 63.5 ± 3.5 % of controls with nicotine + EGTA, $p<0.05$, n=3, Fig 4.8c). Interestingly, the "Ca^{2+}-free" condition obtained with EGTA, significantly reduced the basal phosphorylation of ERK 2 to 74 ± 10.5 % of controls ($p<0.05$, n=3). A significant decrease in the basal phosphorylation of ERK 2 was also observed after inhibition of MEK by PD98059 (50 μM, 30 min; 68.0 ± 10 % of controls, $p<0.05$, n=4). When incubated before nicotine, PD98059 prevented the stimulation of ERK 2 activity, and reduced levels of phospho-ERK1/2 to those observed in the presence of PD98059 alone (nicotine + PD98059: 66.6 ± 8.6 % of controls, n=3). The total amount of ERK2 was analysed in parallel and no significant differences were observed between experimental conditions.

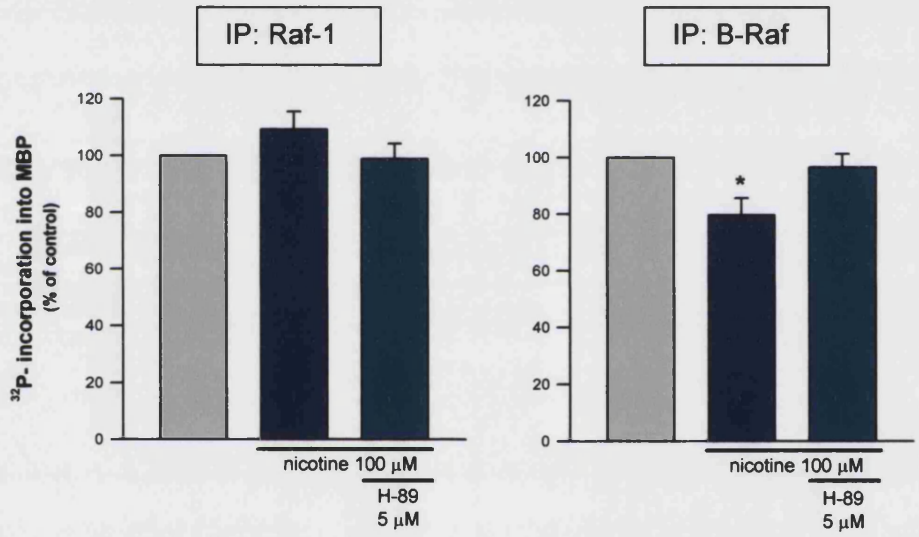

Fig 4.7 Effects of nicotine on $^{32}P_i$ incorporation into MBP using Raf-1 and B-Raf immunoprecipitates.

Raf-1 and B-Raf activities in SH-SY5Y cells were estimated after sequential phosphorylation of downstream kinases MEK1, ERK2 and its substrate MBP, see Methods section for details. SH-SY5Y cells were pretreated with H-89 (5μM, 30 min) or buffer prior to stimulation with nicotine (100μM, 40 s). Cell lysates were immunoprecipitated with antibodies against Raf-1 or Raf-B and immune complexes were incubated with equal amounts of GST-MEK1, GST-ERK2 and MBP. $^{32}P_i$ incorporation into MBP was measured by scintillation counting. Values are the mean ± SEM of at least 5 experiments. Significantly different from control, *p<0.05, Student's *t*-test.

As we demonstrated in SH-SY5Y cells (Fig 4.5b), the PKA inhibitor H-89 completely prevented the nicotine-evoked activation of ERK 2 in hippocampal neurones, reducing values from 137.3 ± 4 % to 75.5 ± 12 % of controls, ($p<0.05$, n=4, Fig 4.8d). Basal levels of ERK 2 phosphorylation were slightly reduced after H-89 alone (76 ± 21 % of controls), but this was not significantly different from control values. Although ERK 1 showed generally low levels of basal phosphorylation in hippocampal neurons compared with ERK 2, nicotine

stimulation increased amounts of phospho-ERK 1, and this was also dependent on α7 nAChR activation and PKA (see blots in Fig 4.8).

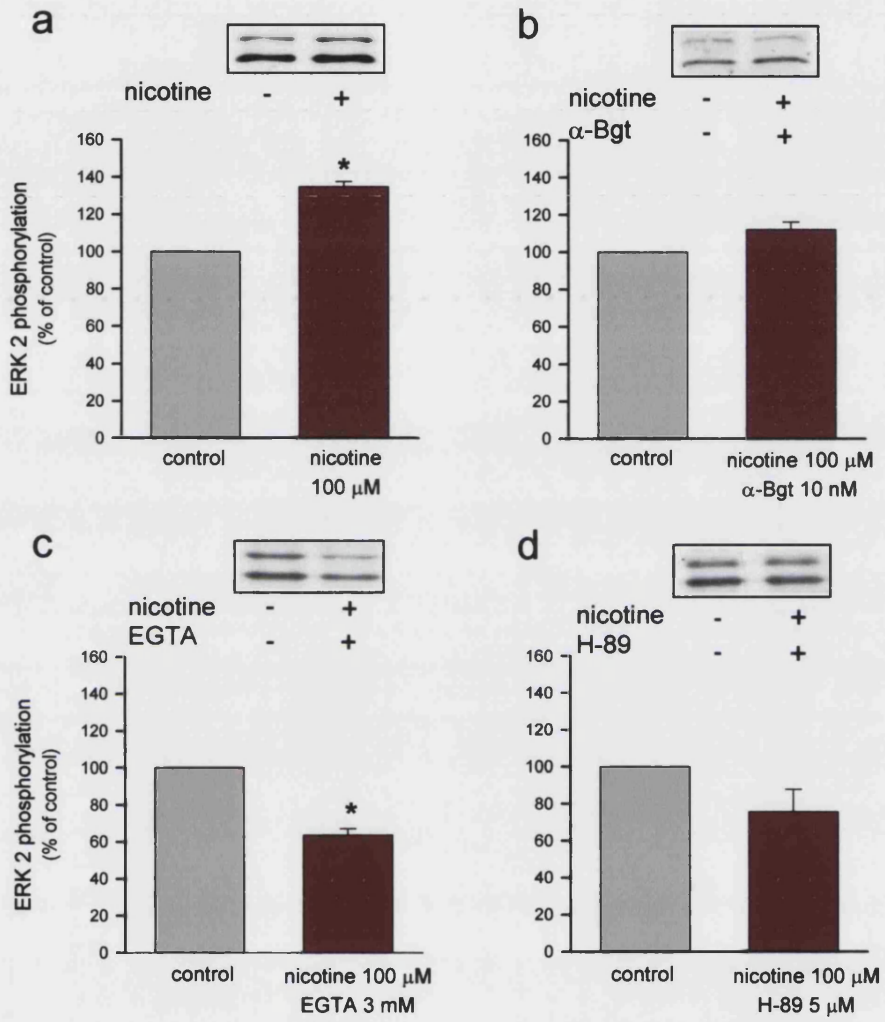

Fig 4.8 Nicotine-induced activation of ERK 2 in hippocampal neurones.
Hippocampal primary cultures were stimulated with (a) nicotine alone (100 μM, 5 min), or nicotine in the presence of (b) α-Bgt (10 nM, 30 min), (c) EGTA (3mM) and (d) H-89 (5 μM, 30 min). ERK1/2 phosphorylation was determined by Western blotting using hippocampal culture cell lysates. Representative autoradiographs are shown in the upper panels. Autoradiographs were analysed by densitometry and values of ERK 2 are expressed as a percentage of the buffer control run in parallel. Values are the mean ± SEM of at least 3 experiments. Significantly different from controls, * $p<0.05$, Student's *t*-test.

4.3.4 ERK1/2 and the nAChR mediated neuroprotection in hippocampal neurones

Taking into account the observed activation of ERK1/2 activity after nicotine stimulation, it was relevant to establish if the ERK1/2 signalling cascade was mediating the previously observed nicotine protection in hippocampal primary cultures. In order to do so, we submitted hippocampal cultures to the previously characterised NMDA (200 μM) insult (see Chapter 2), while nicotine's ability to prevent NMDA excitotoxicity was tested in the presence (30 min pre-incubation and 1 h co-incubation) of the MEK inhibitor PD98059 (50 μM). Nicotine was still able to protect hippocampal cultures after inhibition of MEK by PD98059 (Fig 4.9), thus not supporting the involvement of the ERK1/2 cascade in nicotine protection. It is important to mention that PD98059 was not able to protect hippocampal neurones per se, therefore suggesting that NMDA excitotoxicity was not mediated by ERK1/2 activity (Fig 4.9).

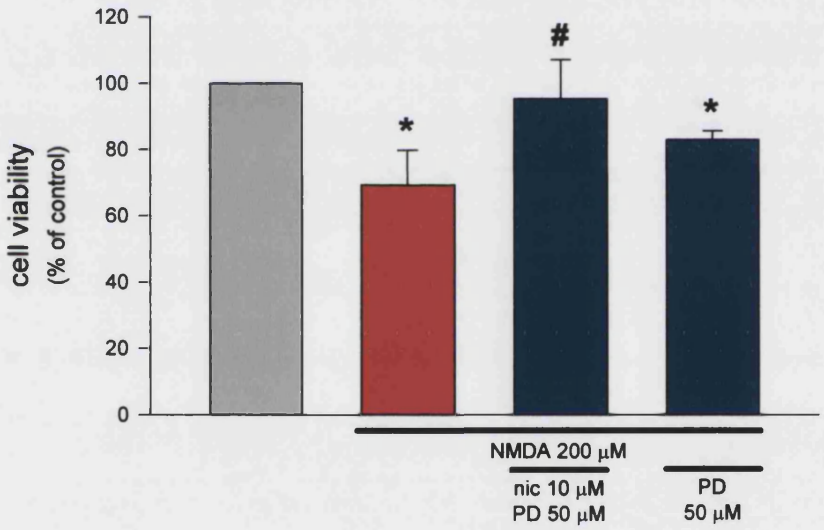

Fig 4.9 Nicotine neuroprotection in hippocampal cultures is not mediated by ERK1/2. Hippocampal primary cultures were submitted to the previously described excitotoxic insult (NMDA 200 μM, see Chapter 2), and nicotine's ability to protect hippocampal neurones was assayed in the presence of the MEK inhibitor PD98059 (PD, 50 μM, 30 min pre-incubation and 1 h co-incubation). Control cultures, with no added drugs, were processed in parallel. Cell viability was assessed 24 h after drug incubation, using the MTT method. Data are expressed as percentage of controls and bars represents the mean + SD from 3 independent experiments. Significantly different from control (*, $p < 0.05$, Student's t test) and from NMDA 200 μM (#, $p < 0.05$, ANOVA and post hoc Tukey's test).

4.4 DISCUSSION

The results presented in this chapter indicate that nicotine can activate the MEK-ERK1/2 signalling cascade in the human neuroblastoma SH-SY5Y cell line and hippocampal neurones, via stimulation of the α7 subtype of nAChR, through a mechanism that is dependent on extracellular Ca^{2+} and requires PKA as an upstream mediator (Fig 4.10). Although the nicotine-evoked increase in ERK1/2 activity is relatively modest when compared with more robust stimulators of the ERK1/2 cascade, such as IGF-1 (Fig 4.2), these observations define a precise and novel cell signalling route through which α7 nAChR may exert a modulatory influence on cellular function.

4.4.1 α7 nAChR and the Ca^{2+}-dependence of nicotine-evoked ERK1/2 activation

In SH-SY5Y cells, the sensitivity of the nicotine-induced increase in ERK1/2 activity to mecamylamine and α-Bgt indicates that this effect is mediated by a nAChR of the α7 subtype. Although SH-SY5Y cells express α7 subunits (Lukas et al., 1993) and surface [^{125}I]-α-Bgt binding sites have been detected (Ridley et al., 2001), studies have failed to demonstrate α7-mediated currents (Gould et al., 1992; Puchacz et al., 1994) or an α7-mediated component of nicotine stimulated Rb^+ efflux (Lukas et al., 1993). However, results presented in Chapter 3 demonstrated the existence of a significant α-Bgt-sensitive component in the nicotine-evoked elevation of intracellular Ca^{2+} in SH-SY5Y cells. This situation is reminiscent of chick ciliary ganglion cells, in which α3* nAChR dominate the agonist-induced currents but α7 nAChR mediate a detectable increase in cytoplasmic Ca^{2+} (Vijayaraghavan et al., 1992). The results presented here provide further evidence for functional α7 nAChR on SH-SY5Y cells, which are capable of activating the ERK1/2 signalling cascade. Interestingly, although other nAChR subtypes also contribute to Ca^{2+} responses in SH-SY5Y cells (see Chapter 3), the complete blockade of ERK activation after α-Bgt, suggests that only the α7 nAChR is associated with this particular signalling pathway. In addition, it should also be mentioned that stimulation of the α7 nAChR mediated the activation of ERK at a concentration (100 μM) that failed to produce a detectable α7 component of the Ca^{2+} increase in SH-SY5Y cells. As demonstrated in Chapter 3, stimulation with 100 μM nicotine produced a rapid and significant increase of cytoplasmic Ca^{2+}, which was, however, not significantly blocked by α-Bgt, probably indicating the rapid desensitisation of α7 nAChR. Nevertheless, the ability of α7 nAChR receptors to still mediate the activation of ERK when stimulated with 100 μM nicotine would suggest that a

brief and localised Ca^{2+} signal is sufficient to activate the ERK signalling cascade. Accordingly, these results might indicate the existence of a specific and efficient association between α7 nAChR and the ERK signalling pathway in SH-SY5Y cells.

Hippocampal neurones also express α7 nAChR subunits (Seguela et al., 1993) and surface [^{125}I]-α-Bgt binding sites (Barrantes et al., 1995a; Ridley et al., 2001a), but in contrast to SH-SY5Y cells, activation of α7 nAChR in hippocampal neurones gives rise to robust, rapidly desensitising currents (Alkondon et al., 1992). We have also shown that in hippocampal primary cultures the nicotine-evoked elevation in cytoplasmic Ca^{2+} levels was primarily mediated by the α7 nAChR (see Chapter 2). In the present study, we demonstrate that 100 μM nicotine, acting through the α7 subtype of nAChR, can also increase the activity of ERK 2 in hippocampal primary cultures (Fig 4.8). This concentration of nicotine gives almost maximal activation of α7 currents in hippocampal neurones (EC_{50} 27 μM; Alkondon and Albuquerque, 1993), and our findings are in agreement with the recent report of Dineley et al. (2001) that used somewhat higher nicotine concentrations (500 μM).

In both SH-SY5Y cells and hippocampal neurones, the nicotine-induced ERK1/2 activation was dependent on extracellular Ca^{2+} (Fig 4.4, Fig 4.8c), consistent with the ability of neuronal nAChR to elevate intracellular Ca^{2+} concentrations, and with the reported Ca^{2+} dependence of ERK1/2 activation in PC12 cells (Rosen et al., 1994; Rusanescu et al., 1995; Grewal et al., 2000a) and CNS neurones in vitro (Kurino et al., 1995, Dineley et al., 2001). As illustrated in Chapter 3, results from various systems have shown that neuronal nAChR, including the α7 subtype, can increase intracellular Ca^{2+} by different routes, including the direct permeation of the nicotinic channel and the activation of VOCC (Rathouz et al., 1994; Barrantes et al., 1995b; Soliakov and Wonnacott, 1996). However, we have not distinguished between a direct and/or indirect route of Ca^{2+} entry with respect to nicotine-induced activation of ERK1/2: attempts to block VOCC with 200 μM Cd^{2+} resulted in a further substantial enhancement of ERK1/2 activity. This observation is in agreement with the reported Cd^{2+}-evoked ERK1/2 activation in rat mesangial cells (Ding and Templeton, 2000), and precluded the use of Cd^{2+} to dissect the contribution of VOCC in the present study. Interestingly, in hippocampal primary cultures (but not in SH-SY5Y cells) EGTA, and also the inhibition of MEK by PD98059, significantly reduced ERK1/2 activity below basal levels. This most probably reflects the occurrence of endogenous signals in hippocampal cultures that can activate ERK1/2 through Ca^{2+} and MEK dependent pathways.

4.4.2 Signalling pathway linking α7 nAChR stimulation to ERK1/2 activation

As ERK1/2 activity is exclusively regulated by the upstream, dual specificity kinase MEK (Sweatt, 2001), the inhibition by the MEK inhibitor PD 98059 of nicotine's effect on ERK1/2 in SH-SY5Y cells and hippocampal neurones is persuasive evidence that nicotine elicits the phosphorylation (and hence activation) of ERK1/2 in both cell types. The intermediate steps coupling α7 nAChR activation and Ca^{2+} entry to the activation of MEK are likely to depend on stimulus-specific factors. The Ca^{2+}-dependent activation of the ERK1/2 cascade could indeed proceed through a variety of upstream Ca^{2+}-dependent kinases, including PI3-kinase, CaMK II, PKC and PKA (Sweatt, 2001). In PC12 cells in particular, nicotine-evoked activation of ERK1/2 was shown to occur through PKC dependent pathways (Tang et al., 1998). Although classical or Ca^{2+}- and diacylglycerol-dependent PKC isoforms are expressed in SH-SY5Y cells (Kramer and Simon, 1999), the failure of Ro 31-8220 to significantly reduce nicotine-stimulated activation of ERK1/2 (Fig. 4.5a) indicates the lack of involvement of PKC. In the investigation of PC12 cells (Tang et al., 1998), a higher concentration of nicotine (1 mM) and much longer exposure time to nicotine (30 min - 12 h) was employed, which may have favoured activation of another nAChR subtype and / or a different route to MEK-ERK1/2. In cultured striatal neurones the Ca^{2+}-dependent stimulation of ERK1/2 by AMPA (50 μM; 5 min) was not affected by Ro 31-8220, but proceeded through a PI3-kinase dependent cascade (Perkinton et al., 1999). Moreover, Kihara et al. (2001) have recently demonstrated the nicotine-dependent activation of PI3-kinase in cortical primary cultures. However, the failure of a maximally effective concentration of the PI3-kinase inhibitor LY294002 to prevent the nicotine-evoked increase in the ERK1/2 activity in SH-SY5Y cells demonstrates that PI3-kinase is not an essential intermediate between nAChR stimulation and ERK1/2 activation in these cells. Similarly, although nAChR activation can lead to the stimulation of CaMK II in adrenal medullary cells (Tsutsui et al., 1994), the lack of effect of the CaMK II inhibitor, KN 62, on the nicotine-induced activation of ERK1/2 suggests that CaMK II does not mediate this response.

In contrast, the complete abolition of nicotine-evoked ERK1/2 activation in SH-SY5Y cells and hippocampal neurones by PKA inhibitors implicates PKA as the predominant mediator of this response. This is compatible with recent evidence for the Ca^{2+}- and PKA-dependent activation of ERK1/2 in PC12 cells (Grewal et al., 2000a) and with the observation that forskolin can increase ERK1/2 activity, independently of nicotine stimulation (Ambrosini et al., 2000). Previous reports have suggested that nAChR may control some of its cellular functions through cAMP and PKA activation. In particular, nicotine has been shown to increase cAMP levels in PC12 cells (Gueorguiev et al., 1999), possibly through

Ca^{2+}/calmodulin-sensitive adenylyl cyclases (Xia and Storm, 1997). In addition, the nAChR-mediated expression of tyrosine hydroxylase was prevented in PKA deficient cells or after inhibition of adenylyl cyclase (Hiremagalur et al., 1993; Gueorguiev et al., 1999). The regulation by PKA of the MEK-ERK1/2 pathway after stimulation of the α7 nAChR constitutes a novel signalling cascade in both SH-SY5Y cells and hippocampal neurones.

Potential targets for PKA in the ERK1/2 pathway are the serine/threonine kinases Raf-1 and B-Raf, which have typical PKA phosphorylation consensus sites and whose cAMP-dependent modulation is well documented (Cook and McCormick, 1993; Peraldi et al., 1995; Vossler et al., 1997). It was recently demonstrated in both PC12 cells and hippocampal neurones that depolarisation-mediated Ca^{2+} influx induces the stimulation of the Ras-related small G-protein Rap1 and its association with B-Raf, thus leading to the formation of a Rap1/B-Raf signalling complex; this was dependent on PKA activity (Grewal et al., 2000b). However, the present study found that, at least in SH-SY5Y cells, the activity of B-Raf, estimated by two approaches, was significantly reduced by nicotine stimulation, and this was dependent on PKA as it was prevented by the PKA inhibitor H-89 (Fig 4.6b; 4.7). Peraldi et al. (1995) reported the cAMP-dependent inhibition of B-Raf kinase activity in PC12 cells, and although no alternative for MEK activation was proposed, it was demonstrated that in cAMP treated cells the association between Ras and B-Raf was virtually abolished. Interestingly, dissociation of the Ras/B-Raf complex could favour the association of GTP Ras with the other Raf isoform, Raf-1. In accordance with this hypothesis, our data show that the phosphorylation state of MEK-1 following incubation with Raf-1 immunocomplexes was significantly increased by about 15% after nicotine treatment, suggestive of an increase in Raf-1 activity, which was dependent upon the activity of PKA (Fig 4.6a). Raf-1 activity, measured by in vitro kinase assay with Raf-1 immunocomplexes and MBP as a substrate, showed a tendency to increase, but this did not reach statistical significance (Fig 4.7). In neuronal models, the modulation of Raf-1 activity by PKA has been generally found to be inhibitory (Grewal et al., 1999; Sweatt, 2001). Nevertheless, a Ca^{2+}- and PKA-dependent increase in Raf-1 and ERK1/2 activity has been reported in neonatal rat cardiomyocytes (Yamazaki et al., 1997), and a positive link between PKA and Raf-1 may also exist in neurones.

4.4.3 ERK1/2 and the nicotine protection against NMDA excitotoxicity

ERK1/2 is a plausible mediator of the nicotine stimulation in neuronal survival (Chapter 2), and this hypothesis was tested in our hippocampal primary cultures. The results obtained indicate that ERK1/2 are not the key mediators of nicotine-induced neuroprotection in

hippocampal cultures, as inhibition of MEK did not prevent the nicotine-evoked protection. In fact, although the prominent role of the Ras-ERK pathway in many NGF-mediated differentiation events is widely accepted (Kaplan and Miller, 1997), more recent evidence has not substantiated the hypothesis of the sufficiency of sustained Ras, MEK or ERK activity for many aspects of neuronal differentiation and survival (see below). Neither sustained ERK activity nor expression of activated MEK was sufficient to induce neuritogenesis in immortalised rat hippocampal cells (Kuo et al., 1996). Moreover, sustained activation of Ras and ERK was insufficient for survival responses of hippocampal pyramidal neurones (Marsh and Palfrey, 1996), while inhibition of MEK failed to block NGF-dependent survival of rat sympathetic neurones (Virdee and Tolkovsky, 1996). Overall, it is becoming apparent that other signalling pathways must act in concert with the Ras-ERK signalling cascade to induce survival and differentiation responses in neurones (Kaplan and Miller, 1997). The PI3-kinase constitutes one of these possible candidates, as a number of studies have implicated PI3-kinase and its downstream substrate, the serine/threonine kinase Akt, in neuronal differentiation and survival (Kimura et al., 1994; Yao and Cooper, 1995). During the final stages of the present work, a report from Kihara et al. (2001) further substantiated the role of PI3-kinase in nicotine-evoked neuronal survival. In effect, it was demonstrated that nicotine activation protected cortical primary cultures against Aβ neurotoxicity through the activation of PI3-kinase. Although this may suggest a role for PI3-kinase in the nicotine protection of hippocampal cultures, the experimental evidence is still lacking, and it is possible that various signalling pathways act in conjunction to achieve the observed effect.

4.4.4 α7 nAChR activation of ERK1/2: functional implications

The preservation of nicotine protection after inhibition of MEK most probably indicates that nicotine-evoked activation of ERK activity is not related to the protective properties of nicotine stimulation (see Chapter 2). In fact, several results suggest that the ERK stimulation in response to Ca^{2+} influx is distinct from the pathways activated by NGF. It was demonstrated by Rosen et al., (1994) that concentrations of KCl and NGF that were individually saturating for ERK activation, together produced an additive response at each time-point. In addition, the same authors reported that Ca^{2+} activation of ERK presented a relatively transient kinetic of activation, which peaked 3 min after stimulation and decreased to only 20 % of peak within 1 h. This was significantly different when compared with the more prolonged activation after NGF, which reached a maximum at 20 min and remained elevated for at least 1 h. In summary, the activation of ERK by stimulation of α7 nAChR is probably mediating an intracellular effect other than neuroprotection.

The increased recognition that both the α7 nAChR and ERK1/2 are important players in the cellular mechanisms of learning and memory makes the signalling pathways elaborated in this study (Fig 4.10) of particular relevance for the hippocampus. In the case of the α7 nAChR, its role in memory processes is supported not only by behavioural studies (Levin and Simon, 1998), but also by the demonstration at the cellular level that α7 nAChR may contribute to the induction of LTP in the intact mouse dentate gyrus (Matsuyama et al., 2000, and see Chapter 1 for more details). As illustrated in the Introduction of the present chapter, activation of ERK1/2 is considered critical for LTP and various forms of hippocampus-dependent memory formation (Sweatt et al., 2001). It is therefore possible that activation of the ERK pathway constitutes one of the key cellular mechanisms that mediate the functional role of nAChR in the modulation of memory processes in the CNS.

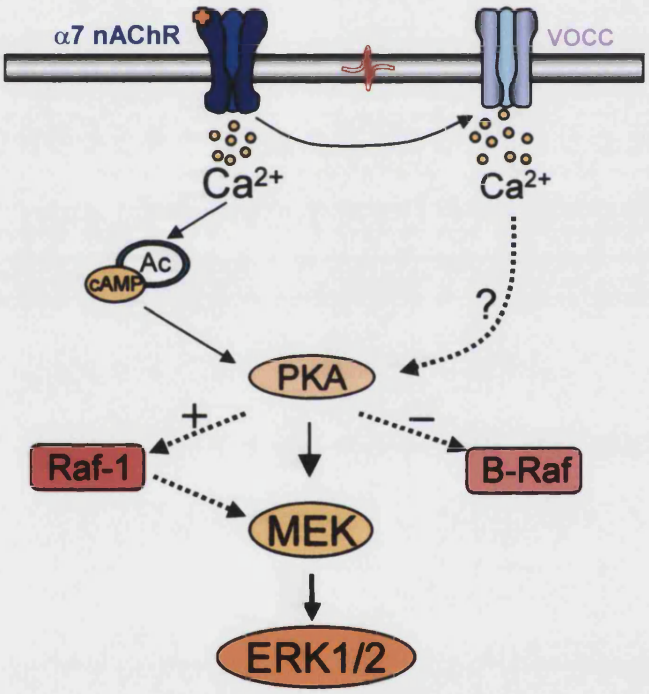

Fig 4.10 Model for the nAChR-evoked activation of ERK1/2
Stimulation of the α7 nAChR by nicotine will increase intracellular Ca^{2+}, either directly or via VOCC. This promotes the activation of PKA in an apparently indispensable step for the nicotine-induced activation of the MEK-ERK1/2 cascade in SH-SY5Y cells and hippocampal cultures. PKA modulates the phosphorylated state and kinase activity of other targets: in SH-SY5Y cells at least, this leads to the inhibition of B-Raf. A positive effect of PKA on Raf-1 kinase activity and its preferential association with Ras could then lead to the activation of MEK and ERK1/2 signalling cascades.

One of the major roles for ERK is the regulation of gene expression. As an example, the transcription factor CREB can be phosphorylated and activated in an ERK dependent manner (Xing et al 1996). This constitutes a major target of both neurotrophin and neuronal Ca^{2+}. Interestingly, PKA has also been shown to use an ERK-dependent pathway to CREB in the hippocampus (Roberson et al., 1999), while the transcription factor CREB has a well-defined role in memory mechanisms (Sweatt, 2001) and has been implicated in nicotine-ERK1/2 signalling in the hippocampus (Dineley et al., 2001). In fact, the regulation of the ERK cascade using PKC and PKA as upstream transducers has been suggested as a prominent mediator in the short- and long-term regulation of synaptic strength in the hippocampus (Roberson et al., 1999). In addition, the nAChR-PKA-ERK relationship may have wider significance beyond the neuronal memory processes and the hippocampus, as both α7 nAChR and CREB can also regulate the transcription of tyrosine hydroxylase (Gueorguiev et al., 2000; Lim et al., 2000), and this enzyme can be regulated by nicotine in a manner dependent on adenylyl cyclase activity (and most probably PKA) in PC12 cells (Gueorguiev et al., 1999). Thus, ERKs have probably multiple roles in the stimulation of transcription and, in many instances, the translocation of ERKs from the cytoplasm to the nucleus, where they can directly phosphorylate and stimulate specific transcription factors, is a pre-requisite step (Grewal et al., 1999; Patterson et al., 2001). In summary, the results presented would indicate that brief stimulation of the α7 nAChR and the subsequent activation of PKA and ERK1/2 signalling pathways could provide a functional cellular mechanism for the long-term regulation of diverse neuronal processes.

CHAPTER 5

< Conclusions >

5.1 Specificity of neuronal signalling

It is now widely accepted that nAChR have a vital role in many of the diverse functional mechanisms that regulate CNS function, a concept that was illustrated in the course of the previous chapters. The experimental evidence presented here focused primarily on the specific and selective role of the α7 subtype of nAChR, demonstrating its mediation in neuroprotection, generation of intracellular Ca^{2+} signals and activation of signalling cascades.

In hippocampal primary cultures, the prevention of the Ca^{2+}-dependent and NMDA-evoked neuronal loss by the activation of α7 nAChR provided direct evidence for the existence of specific Ca^{2+} signals able to activate neuroprotective cellular mechanisms (Chapter 2). In effect, the existence of Ca^{2+} as a signalling messenger represents one of the key mechanisms in the transduction of synaptic currents into biochemical events, while the specificity of Ca^{2+}-activated cascades relies on the capacity to segregate diverse Ca^{2+} signals in the different contexts of space and time. In Chapter 2, the results presented demonstrated the versatility of Ca^{2+}-dependent neuronal processes, which mediated neuronal responses as apart as life or death. In accordance with the proposal that the spatio-temporal complexity of intracellular Ca^{2+} signals allows the simultaneous control of multiple Ca^{2+} dependent cellular functions (Johnson and Chang, 2000), the results obtained are consistent with the notion that Ca^{2+} signal-specificity is highly dependent on the source of Ca^{2+} influx (Ghosh and Greenberg, 1995).

The overall profile of a particular increase in cytoplasmic Ca^{2+} concentration is determined by the combination of extracellular and/or intracellular Ca^{2+} sources (Simpson et al., 1995). In turn, the generation of these Ca^{2+} signals is a highly regulated process, which would be selectively buffered and/or amplified in specific neuronal compartments. Taking into consideration the ability of nAChR to regulate diverse cellular functions through elevation of intracellular Ca^{2+} levels, it was thus suggested that stimulation of nAChR could generate complex intracellular Ca^{2+} signals through activation of different Ca^{2+} influx and/or release processes. By using the SH-SY5Y cell line as a model system, we were able to demonstrate the contribution of various Ca^{2+} sources to the nicotine-evoked elevation of cytoplasmic Ca^{2+} (Chapter 3). Besides the direct influx through the α7 nAChR channel and the activation of

VOCC, we demonstrated that the nicotine-evoked Ca^{2+} responses were highly reliant on the activation of Ca^{2+} induced- and IP_3-evoked Ca^{2+} release from internal stores. Interestingly, our results might also indicate that the generation of α7 nAChR mediated Ca^{2+} increase is specifically associated with ryanodine-dependent Ca^{2+} release. On the whole, the recruitment of intracellular Ca^{2+} stores provides further complexity to the Ca^{2+} signals activated by stimulation of nAChR, a phenomenon that probably underlies the ability of nAChR to regulate multiple neuronal functions.

The generation of specific and complex Ca^{2+} signals following activation of nAChR is only the initial step towards the modulation of neuronal function. Eventually, much of the capacity to regulate intracellular mechanisms depends on the activation of specific signalling molecules. In Chapter 4, we showed how the activation of α7 nAChR regulates the activity of ERK, through a novel signalling cascade that involves the activation of PKA. Considering that SH-SY5Y cells express various functional nAChR, the complete inhibition of ERK activation following application of α7 selective antagonists indicates that the nicotine evoked ERK signalling activity is specifically associated to the α7 nAChR. In hippocampal neurones, where nicotine-evoked Ca^{2+} responses were exclusively mediated by α7 nAChR, nicotine also stimulated ERK activity via activation of PKA. Overall, results presented demonstrate the specific association of α7 nAChR with PKA and ERK signalling cascades in both SH-SY5Y cells and hippocampal cultures. The existence of this cascade in both of the neuronal models studied might indicate that the specific association of the α7 with PKA and ERK signalling provides a general cellular mechanism for the regulation of diverse neuronal processes in various neuronal systems.

The ability to segregate specific intracellular signalling mechanisms is one of the crucial aspects of signal transduction. However, the understanding of the process that allows intracellular signals to be relayed from the cell membrane to specific cellular targets remains a daunting challenge. It is presently known that the relatively broad substrate specificity of many protein kinases and also protein phosphatases may be used in varying combinations to achieve distinct biological responses. As a result, mechanisms must exist to organize the correct repertoire of enzymes into individual signalling pathways. The post-synaptic density is the functionally specialised region of the neuron involved in the transduction of synaptic inputs into intracellular events (for a review on the organisation of proteins at synapses see Kim and Huganir, 1999). Emerging evidence indicates that specificity is achieved, in part, by the use of scaffolding or anchoring proteins that coordinate the specific binding of upstream and downstream effectors, and can thus control signal transduction pathways (Burack and Shaw, 2000). In fact, scaffolding can bring together proteins for their interaction and regulation, or may conversely sequester proteins so they do not interact with other proteins

(Garrington and Johnson, 1999). In practical terms, scaffolds are said to preserve the specificity of the signalling pathway, a function commonly described as "isolating" or "stabilising" the otherwise weak interactions between the kinases of a single cascade. Not surprisingly, the regulation of these scaffolding complexes provides a further level of control in the already complex system of intracellular signalling. Considering that the function of a scaffold is to bring together a variety of different proteins, the concentration of a scaffold needs to be titred closely to the concentration of the component to which it binds. Too much scaffold protein will dilute the pathway components, working to defeat the purpose of the scaffold by sequestering an activating kinase from its substrate. Interestingly, it is possible that signalling inhibitor proteins, which have been previously described in overexpression studies, are in fact scaffolding proteins (Burack and Shaw 2000). Overall, the relatively recent emergence of scaffolding complexes provided physical evidence for the ability of neurones to segregate specific signalling pathways. The presence of these localised protein complexes is also consistent with the existence of multidimensional Ca^{2+} microdomains able to activate specifically targeted molecules.

In recent years, studies mainly carried out with glutamate receptors, started to unravel the extreme complexity of receptor associated protein complexes. One of the key features required for the clustering of ion channels and receptors in the plasma membrane and for spatially directing kinases and phosphatases toward their substrates appears to be the presence of PDZ domains in specific interacting proteins (Sheng and Pak, 2000 and references therein). The PDZ domain is a protein-protein interaction motif of approximately 90 aminoacids shown to be present in over 100 otherwise unrelated proteins (Ponting et al., 1997). In the particular case of LGIC, Grant and co-workers have shown that the 2000 kD complexes of the NMDA receptor and the PDZ containing PSD-95 molecule with associated proteins comprised at least 78 proteins divided into receptors, adaptors, signalling and cytoskeletal proteins (Husi et al., 2000; Husi and Grant, 2001, and references therein). Complementing these studies, Valtschanoff and Weinberg (2001), demonstrated the laminar organisation of the NMDA receptor complex within the postsynaptic density, and by using quantitative immunogold electron microscopy, they provided valuable information into the architecture of the NMDA receptor complex. Their data revealed that some proteins (like PSD-95) lay close to the plasma membrane, others (such as the guanylate kinase associated protein, GKAP, and Shank) lay markedly farther away, while some lay in a position intermediate between the other two (nitric oxide synthase, nNOS; Fig 5.1). Functional studies have also shown that some of the proteins in these complexes are associated with the specific coupling of receptor function. In effect, NMDA toxicity in cortical neurones was significantly reduced in PSD-95 deficient neurones (Sattler et al., 1999). Interestingly, the PSD-95 deficiency did not affect the NMDA-evoked Ca^{2+} loading or the

kainate-evoked neuronal toxicity, thus suggesting a selective coupling of NMDA and PSD-95 with a specific toxic pathway. Taking into consideration that the lack of PSD-95 blocked the Ca^{2+}-activated nitric oxide production by NMDA receptors selectively, without affecting neuronal nitric oxide synthase expression or function, Sattler et al. (1999) proposed that PSD-95 is required for efficient coupling of NMDA receptor activity to nitric oxide toxicity, imparting specificity to excitotoxic Ca^{2+} signalling. This report has particular relevance for the results described in Chapter 2, when Ca^{2+} signals evoked by NMDA or nicotine stimulation mediated totally opposed physiological outcomes.

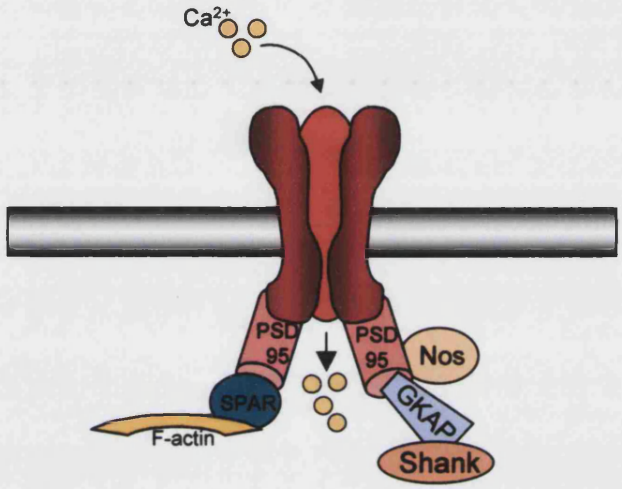

Fig 5.2 NMDA receptor and scaffolding proteins
The diagram shows the laminar organisation of the NMDA receptor complex. Based on data from immunogold labelling (Valtschnoff and Weinberg, 2001; Hering and Sheng, 2001) it was possible to establish that some proteins lay close to the plasma membrane (PSD-95), others lay markedly farther away (GKAP and Shank), whereas other lay in an intermediate position (Nos). In addition to the signalling processes, scaffolding proteins are fundamental in determining the structure of the dendritic spine by association with cytoskeletal proteins (i.e. interaction between SPAR, spine associated RapGAP, and F-actin.

In addition to the NMDA receptor complex, the AMPA subtype of glutamate receptors have been also analysed for their interactions with postsynaptic protein complexes. Similar to their NMDA counterparts, the C-terminal of AMPA receptor subunits has been identified as the main region to interact with cytoplasmic proteins. Studies using the yeast two-hybrid system demonstrated the interaction of AMPA receptors with glutamate receptor interacting proteins (GRIP), AMPA receptor binding protein (ABP) and protein interacting with C-kinase (PICK1), all containing the PDZ-domain (Braithwaite et al., 2000 and references therein). In addition, several non-PDZ domain-containing proteins have been also reported to associate with AMPA receptors. Most notably, the N-ethylmaleimide sensitive fusion protein (NSF) has been reported to bind to a seemingly unique recognition site within the C-terminus of AMPA receptors (Nishimune et al., 1998). This NSF protein is a well-characterised ATPase which plays a role in the membrane fusion processes of protein trafficking through the Golgi

apparatus and in the vesicular release of neurotransmitters. Interestingly, the interaction between NSF and AMPA has been demonstrated to regulate the surface expression of AMPA receptors in hippocampal neurones (Nishimune et al., 1998), while inhibition of NSF can lead to LTP induction blockade (Lledo et al., 1998). The AMPA receptors have been also reported to interact directly with, and signal via, a protein tyrosine kinase called Lyn (Hayashi et al., 1999). A member of the src-family non receptor protein kinase, the rapid activation of Lyn by the AMPA receptor does not requires Ca^{2+} or Na^{+} influx, and can lead to the stimulation of the MAPK signalling cascade.

Unlike the glutamate receptors, the data regarding neuronal nAChR scaffolding proteins is still scarce. It is very likely, however, that the large cytoplasmic domain of nAChR provides the molecular structure to interact directly with cytosolic proteins of different function. In effect, Anand and co-workers have used the cytoplasmic domain of the α4 nAChR as a bait to screen mouse brain cDNA yeast two-hybrid library (Jeanclos et al., 2001). Hitherto, they have described the isolation of a previously known protein (14-3-3η) that functions as intracellular regulator or scaffolding/adaptor protein in diverse cellular functions. In addition, a similar experimental approach using the cytoplasmic loop of the α7 nAChR, identified the PDZ containing PICK1 as one of the interacting molecules (Huh and Fuhrer, 2001). Interestingly, the interface seems to be specific for α7, as PICK1 did not interact with the cytoplasmic loops of α4, α3 and β2 nAChR subunits (Huh and Fuhrer, 2001). Although the physiological significance of PICK1-α7 interaction is presently unknown, it provides structural evidence for the existence of specific α7-mediated neuronal functions.

In recent years, the report and characterisation of diverse scaffolding proteins has provided a functional basis for efficient and specific intracellular signalling. However, some important questions remains still unanswered. In effect, if the role of scaffolding proteins is to reduce complexity, why has evolution conserved a complex system of interacting kinases? One of the possible explanations is given by the fact that multileveled signalling systems offer the opportunity for fine modulation, while signal amplification has also been postulated (Burack and Shaw, 2000). In fact, some of the results reported here suggest that the nAChR-mediated signalling cascade proceeds through a downstream amplification route, considering that a relatively modest activation of Raf-1 kinase (~15 %) gives rise to a more robust increase in ERK activity (~50 %). Nevertheless, there is still no strong evidence showing that the major reason for conserving the multiple steps levels of organisation is indeed amplification (Burack and Shaw, 2000).

In summary, the transduction of synaptic currents into biochemical changes of neuronal function involves the integration of a multilayered system, highly, although not exclusively

dependent on Ca^{2+} as the main trigger. The existence of various membrane receptors (i.e. LGIC, VOCC) provides an initial set of diversity, allowing the activation of selective Ca^{2+} sources. Ultimately, the final level of integration is provided by the activation of Ca^{2+} dependent signalling cascades. It has been demonstrated that individual pathways transmit signals along linear tracts that result in the regulation of discrete cell functions. However, as increasingly larger numbers of cell signalling components and pathways are being identified and studied, it has become apparent that these linear pathways are not free-standing entities but parts of larger networks. The ability of a cell to regulate spatially resolved multiple functions in a coordinate manner seems to arise from the organisation of signalling pathways into networks, which result from the interconnections between signalling pathways that occur when the same signalling component is capable of receiving signals from multiple inputs. An early example of signal integrators was adenylyl cyclase, which was shown to produce cAMP in response to signals from G-coupled receptors, as well as Ca^{2+} (Poser et al., 2001; Hanoune et al., 2001). As a result, cAMP levels in the cell could serve as an indicator of the balance of signals between many pathways (Pieroni et al., 1993), while the subsequent activation of PKA can be routed to regulate numerous physiological events. Consistent with this mechanism, the signalling pathway that follows the stimulation of α7 nAChR appears to depend on PKA as the intermediate connector to the ERK cascade. However, many other signalling pathways are probably simultaneously activated. In this regard, although results presented here do not support the notion of a nicotine-dependent PKC, CaMK II or PI3-kinase link to ERK, it remains possible that these kinases are activated after nicotine stimulation in SH-SY5Y cells or hippocampal neurones, their activation leading to other, non-ERK, signalling systems. To add more complexity into an already intricate process, any cellular response generated by systems of kinases will be subjected to the balance determined by stimulation of phosphatase systems, which in many cases can be also activated subsequent to Ca^{2+} dependent processes (Stull, 2001). In the end, models of signalling networks and their interface with cellular mechanisms are likely to be extremely complex, and although linear representations are useful for the process of understanding the basic principles, they are probably far from reality.

5.2 α7 nAChR and neuronal signaling

Consolidated synaptic inputs can produce changes in cellular function, and is the combination of these altered functions that gives rise to a final physiological response. For example, stimulation of the CA1 glutamatergic neuron can result in an immediate increase in synaptic efficiency, altered patterns of gene expression (Malenka and Siegelbaum 2000),

stimulation of local protein synthesis (Frey et al., 1993) and dendritic outgrowth (Maletic-Savatic et al., 1999).

In the case of α7 nAChR, we have demonstrated its involvement in the various levels of signalling integration in neurones (see Fig 5.2). They can generate the activation of complex Ca^{2+} signals, stimulate key signalling molecules and promote neuroprotective Ca^{2+} processes. With the α7 nAChR, the cholinergic system has a precise and selective tool for the activation of specific Ca^{2+} signals, and as a direct result, the regulation of neuronal function. In the hippocampus in particular, the recent report by Fabian-Fine et al., (2001) has highlighted the putative relevance of nAChR in hippocampal function, after reporting a near-ubiquitous presence of α7 nAChR in hippocampal synapses, where 96 % of synaptic contacts were positive for α7 labelling. Double labelling for both α7 nAChR/GABA and α7 nAChR/glutamate also revealed that most of the postsynaptic neurones with α7 nAChR were glutamatergic, presumably pyramidal neurones (Fabian-Fine et al., 2001). Remarkably, the mean immunogold particle density at these α7 nAChR labelled synapses was extremely close to the reported particle density for NMDA and AMPA glutamate receptor labelling at synapses in similarly prepared tissue (Fabian Fine et al., 2001; Racca et al., 2000). In effect, the intensity of the detected signal has further indicated that the importance of α7-mediated nicotinic cholinergic signalling may be far greater than currently recognised.

As reviewed in the present work, activation of nAChR can lead to the modulation of diverse cellular tasks. In the particular case of the α7-ERK signalling cascade, we still do not know the exact function of ERK activation following stimulation of α7 nAChR. The results obtained do not support a role for ERK signalling in nicotine-evoked neuroprotection of hippocampal neurones. Alternatively, the activation of ERK following stimulation of α7 nAChR is most probably related to the proposed function of nAChR in memory processing (see Chapter 2). In effect, given the role that ERK has in the cellular processes of synaptic plasticity, and the evidence showing the involvement of nAChR in memory processes, it is probable that α7 nAChR and ERK signalling systems are cellular mediators of neuronal plasticity processes in the CNS.

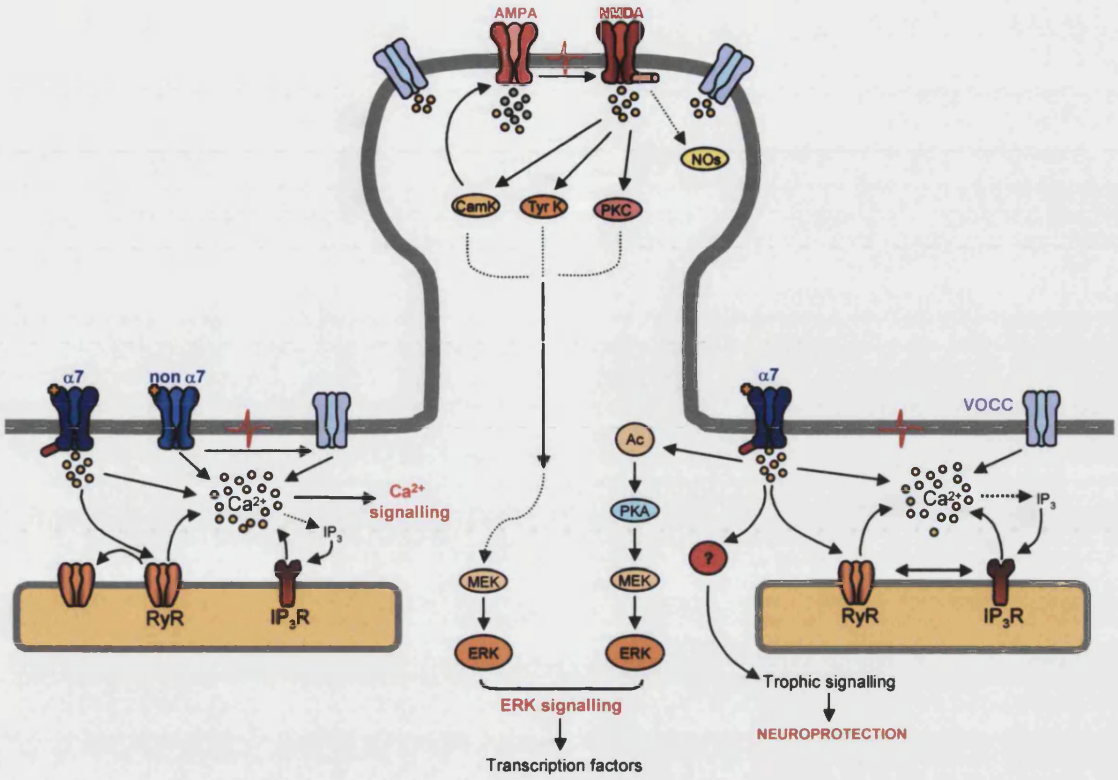

Fig 5.2 Cell signalling processes activated by stimulation of nAChR in neuronal systems. Schematic diagram summarising the results obtained, with the three main topics investigated shown in red. The generation of complex Ca^{2+} signals following the activation of nAChR is shown on the left side of the panel. Besides the direct influx through α7 nAChR and VOCC, the nAChR can generate the activation of Ca^{2+}-induced and IP_3-mediated Ca^{2+} release from internal stores. Interestingly, the results might also indicate that α7 nAChR are functionally associated with ryanodine dependent stores. On the right side, the diagram shows the specific activation of Ca^{2+} dependent intracellular signalling mechanisms following stimulation of α7 nAChR. These cellular processes are capable of activating trophic cascades that will lead to neuroprotection against excitotoxicity. However, the precise signalling cascade is still unknown. Although the nAChR mediated ERK signalling might not be involved in the neuroprotective properties of nicotine, it may have a role in the processing of long term signalling in neurones (note: the location of nAChR in the neuron diagram is arbitrary).

5.3 Future Perspectives

The study of the complex organisation of neuronal function should be closely associated with the understanding of the specific signals that are generated following synaptic activity. The understanding of how neurones are able to assimilate these signals constitutes one of the main objectives for the future. In the case of α7 nAChR the integration of synaptic inputs is further complicated by the ability of choline to activate and/or inactivate the receptor. It is in fact possible that ACh release from an activated cholinergic terminal could result in α7 mediated facilitation of synaptic transmission at simultaneously activated glutamatergic synapses close to the activated terminal, where the local ACh/choline concentration would be transiently high. As a result of diffusion, synapses farther from the cholinergic terminal would experience inactivating choline concentrations. However, the real significance of this

hypothetical cholinergic transmission is still far from being understood. In order to obtain a more precise understanding of nAChR function, it will be critical to determine the specific localisation of α7 dependent Ca^{2+} signals in synaptic dendritic boutons, and to analyse their interaction with other excitatory transmitter systems. The ability to integrate various Ca^{2+} sources must be also explored in the context of signalling activation. In particular, it would be important to address if the singular Ca^{2+} influx through α7 nAChR is sufficient to mediate ERK activation, or if Ca^{2+} release from internal stores is part of the signalling process (i.e. can stimulation of α7 nAChR activate ERKs after blockade of ryanodine dependent Ca^{2+} stores?). Furthermore, a crucial step in the understanding of nAChR signalling cascades must be given by the study (co-immunoprecipitations, yeast two-hybrid screens) of the specific scaffolding proteins that are associated to the nAChR.

Several of the cellular mechanisms that regulate neuronal function were investigated during the course of this work. In the process of understanding α7-nAChR functions we have probably generated more questions than answers. Ultimately, this must be a clear indication that the actual definition of the nAChR role in CNS function is still being actively shaped.

APPENDIX 1

< Ca^{2+} sensitive fluorescent dyes >

A1.1 Fluorescent indicators

The development of fluorescent indicators of free Ca^{2+} that could be loaded into cells in a non-disruptive manner initiated a revolution in the studies of cellular Ca^{2+} homeostasis. In terms of ease of use and calibration, fluorescent Ca^{2+} indicators are the most straightforward and accessible of the available techniques for measuring Ca^{2+}.

Fluorescence is the molecular process in which emission of electromagnetic radiation results from the absorption of radiation from an extra-molecular source. The excited state of the molecule generally has a lifetime in the order of 10^{-8} s, and as such, fluorescence does not persist once exposure to the excitation source is terminated. Fluorescent radiation has a longer wavelength (lower energy) than that of the absorbed radiation (for review on fluorescent indicators of cytosolic free Ca^{2+} see Thomas and Delaville 1991; Tsien 1989) Under conditions of constant illumination and in a fixed chemical and physical environment, the intensity of fluorescence from molecular species is proportional to its concentration; thus, fluorescence intensity can be used as a quantitative tool. However, in order for a fluorescent probe to provide useful information about its environment, it is necessary that its fluorescence properties be altered in a suitable manner by the parameter to be measured. In general, any of the following three property changes are appropriate:

- A change in fluorescence yield (fluorescence output as a function of excitation intensity)
- A shift in the excitation spectrum
- A shift in the emission spectrum

The measurements of fluorescence intensity changes resulting from alterations in fluorescence yield at a single wavelength are the most straightforward to carry out. However, calibration of these signals requires some measure of the absolute amount of probe present. In general, these dyes do not allow the determination of intracellular Ca^{2+} concentrations. This problem is overcome by carrying out measurements with dyes that give shifts of excitation or emission spectra in response to the parameter to be measured, and allow fluorescence measurements at two wavelengths (see chapter 1 and Thomas and Delaville, 1991, for review). Wavelengths are selected to give signals that change inversely in response to the variable being measured, while one of the wavelengths can be also selected to be an "iso-fluorescent" point, where fluorescence intensity does not change in response to

the measured parameter. Using the dual wavelength approach (either dual emission or dual excitation), it is possible to calculate the proportion of the dye in one of two forms (e.g. Ca^{2+}-bound versus Ca^{2+}-free) from the ratio of fluorescence measured at the two wavelengths. This then becomes independent of dye distribution or concentration (see Grynkiewicz et al 1985).

The available fluorescent indicators for measuring Ca^{2+} have been modelled on the widely used Ca^{2+}-selective chelator EGTA (see Tsien, 1989). While the tetracarboxylic acid Ca^{2+} binding site is largely unchanged in the fluorescent Ca^{2+} indicators, additional groups have been added to act as fluorescent reporters. Due to the fact that Mg^{2+} is too small to contact more than about half the ligand groups simultaneously, the octacoordinate binding site binds Mg^{2+} about five orders of magnitude more weakly than Ca^{2+}. In the case of monovalent cations, they do not form detectable specific complexes probably because their charge is inadequate to organise the binding pocket in the face of the electrostatic repulsion of the negative carboxylates. In general, most of the indicators are used in an esterified form (acetoxymethyl esters being the most commonly employed), which by being uncharged and hydrophobic allows the permeation of cell membranes (for review see Thomas and Delaville, 1991). Once in the cytosol, endogenous esterases "activate" the Ca^{2+} indicator by releasing the free acid form which does not permeate the membrane. This results in the accumulation of trapped indicator in the cell cytosol.

A1.2 Fura-2

Fura-2 is currently the most widely used of the fluorescent Ca^{2+} indicators dyes and is the one most commonly employed for imaging studies. Fura-2 is about 30 times more fluorescent than its predecessor, quin-2, has a higher Kd for Ca^{2+}, less Mg^{2+} sensitivity, and more importantly, it can be used in the ratio mode (Grynkiewicz et al., 1985). In addition, fura-2 is normally used at intracellular concentrations in the tens of micromolar as opposed to the millimolar range used for quin-2, and as a result, has much less Ca^{2+} buffering effect. With a Kd for Ca^{2+} of about 225 nM, fura-2 is suitable for Ca^{2+} measurements up to several micromolar. The fluorescence excitation maximum of fura-2 shifts to a lower wavelength on Ca^{2+} binding, with negligible shift in the emission maximum (Grynkiewicz et al., 1985). This allows fura-2 to be utilised as a dual excitation indicator. For optimal separation of fluorescence due to the two forms of the indicator, Ca^{2+}-free fura-2 is usually monitored at 380 nm and Ca^{2+}-bound dye at 340 nm (for more details and experiments using Fura-2, see Chapter 2). Fura-2 has an iso-fluorescence point at 360 nm, which is useful for monitoring Ca^{2+}-independent fluorescence or for following quenching by heavy metal ions. Perhaps the only problems that have been encountered with fura-2 relate to the incomplete hydrolysis of

the acetoxymethyl ester form and the propensity of this dye to become compartmentalised in subcellular organelles during loading (Thomas and Delaville, 1991).

A1.3 Fluo-3

The fluorophore component of fluo-3 is based on fluorescein and as a result it is excited in the visible range of light rather than the UV required for previously developed indicators, such as fura-2 (Minta et al., 1989). Fluo-3 does not undergo a wavelength shift on Ca^{2+} binding and so must be used in a single wavelength mode, being therefore not suitable for fluorescence ratio measurements. The Ca^{2+} affinity (Kd 400 nM) of fluo-3 is two to tenfold weaker than those of their UV-excited predecessors. This decrease improves resolution of micromolar and higher levels of intracellular Ca^{2+} concentration, surprisingly, without sacrificing resolution of low Ca^{2+} concentrations. This is due to the fact that Ca^{2+} free fluo-3 is almost non-fluorescent, with a fluorescence enhancement upon Ca^{2+} binding of about 40-fold, the largest change of any of the available indicators (Minta et al., 1989). As a result, it allows the detection of significant signal changes for even relatively small Ca^{2+} variations at the low end of the intracellular Ca^{2+} concentration range. In addition, the comparatively low affinity for Ca^{2+} means that bound Ca^{2+} could be expected to dissociate relatively rapidly and not artificially prolong the Ca^{2+} signal that is observed. This property is particularly useful when measuring sustained Ca^{2+} signals (see results of Chapter 3). Fluo-3 is similar to fura-2 in its selectivity for Ca^{2+} over Mg^{2+} and is essentially insensitive to pH above 7.0 (Minta et al., 1989). As with the other Ca^{2+} indicators, heavy metal ions such as Mn^{2+} and Zn^{2+} bind the dye with high affinity. Apart from the lack of ratio capability, the only drawback of fluo-3 is the rather small separation between excitation and emission wavelengths, which necessitates the use of relatively narrow band filters or monochromators. In general fluo-3 can be excited at ~490 nm and fluorescence monitored at ~525 nm.

APPENDIX 2

< nAChR mediated dopamine release in the substantia nigra >

A2.1 Introduction

A2.1.1 The basal ganglia

The basal ganglia consist of five extensively interconnected subcortical nuclei: the caudate nucleus, putamen, globus pallidus, subthalamic nucleus and the midbrain structure called substantia nigra. The caudate nucleus and putamen develop from the same telencephalic structure and as a result they are composed of identical cell types throughout. The two nuclei are called the neostriatum (or striatum) and serve the role of input nuclei for the basal ganglia. The globus pallidus (or pallidum) is divided into external and internal segments and derives from the diencephalon, lying medial to the putamen and lateral to the internal capsule, while the subthalamic nucleus lies below the thalamus at its junction with the midbrain. The substantia nigra lies in the midbrain and has two zones, the pars compacta and pars reticulata. Together with the globus pallidus they constitute the major output nuclei of the basal ganglia.

Almost all afferent connections to the basal ganglia terminate in the striatum, which receives input from two major sources outside the basal ganglia: the cerebral cortex and the intralaminar nuclei of the thalamus. The corticostriatal projection contains fibres arising from the entire cerebral cortex, including motor, sensory, association, and limbic areas. The basal ganglia, together with the cerebellum, constitute the major components of two important subcortical loops of the motor system. Both receive major projections from the cerebral cortex and both project back to the cortex via the thalamus. However, there are three important differences between the connections of the basal ganglia and those of the cerebellum. First, the basal ganglia receive inputs from the entire cerebral cortex, in contrast with the cerebellum, which receives inputs only from the sensorimotor cortex. Second, the output of the cerebellum is directed back to the premotor and motor cortex, whereas the output of the basal ganglia is directed not only to the motor cortices but also to the prefrontal association cortex. Finally, the cerebellum receives somatic sensory information directly from the spinal cord and has major connections with many brain stem nuclei. In contrast, the basal ganglia have relatively few connections to the brain stem and no direct connections at

all to the spinal cord. These differences suggest that the cerebellum directly regulates execution of movement, whereas the basal ganglia are involved in higher-order, cognitive aspects of motor control. In spite of the good anatomical knowledge of the basal ganglia connectivity, this does not answer several questions, such as: what do the basal ganglia do? or why do lesions of the basal ganglia and the motor cortex or cerebellum result in distinctively different motor disturbances? A great insight into the function of the basal ganglia is provided by the study of several diseases affecting the neurons of these nuclei. In this regard, it has been proposed that the basal ganglia selectively facilitate some movements and suppress others, analogous to the inhibitory surround characteristic of receptive fields in the sensory systems. It would do this by focusing activity from widespread regions of the cortex onto the motor areas. Alternatively, the basal ganglia may compare commands for movement from the pre-central motor fields with propioceptive feedback from the evolving movement. Finally, the basal ganglia may be involved in the initiation of internally generated movements. This possibility is consistent with the striking inability to initiate movement (akinesia) exhibited by patients with Parkinson's, one of the major diseases of the basal ganglia.

A2.1.2 Substantia Nigra

The substantia nigra (SN) lies in the midbrain and can be divided in two zones. A ventral pale zone called pars reticulata that resembles the globus pallidus cytologically. A dorsal, darkly pigmented zone, the pars compacta, comprised of dopaminergic neurons whose cell bodies contain neuromelanin. This dark pigment, a polymer derived from dopamine, gives the substantia nigra its name (Latin: black substance), considering that in humans this part of the brain appears black in cut sections. Because of the striking similarities in cytology, connectivity, and function of the internal segment of the globus pallidus and the substantia nigra pars reticulata, these two nuclei can be considered as a single structure arbitrarily divided by the internal capsule, serving the role of major output nuclei of the basal ganglia.

In spite of its role as output nucleus, the SN plays a fundamental role in the internal basal ganglia circuitry. The projecting axons from dopaminergic neurons that constitute the main cell type of the substantia nigra give rise to the nigrostriatal pathway. These neuronal path ends by synapsing in the striatum, where it has a fundamental role in the modulation of basal ganglia connectivity. In effect, the loss of this pathway gives rise to severe motor disruptions and the clinical manifestations of Parkinson's syndrome in humans. This disease may include numerous motor impairments, including the difficulty in the initiation of movement and paucity of spontaneous movements (akinesia), slowness in the execution of movement (bradykinesia) and postural reflex impairment.

A2.1.3 Somatodendritic release of neurotransmitters in the substantia nigra

As described above, the nigrostriatal DA pathway is an important component of basal ganglia circuitry, which has been postulated to play a fundamental role in the initiation and maintenance of motor behaviours. When studying the function and activity of the basal ganglia, much of the emphasis is placed on the actions of dopamine in the striatum, where it can regulate the descending pathways to both the substantia nigra and globus pallidus (Gerfen, 1992). However, it is believed that DA may also contribute to basal ganglia output through its actions within basal ganglia nuclei other than the striatum. The notion that nigrostriatal dopamine neurons might release transmitter at the somatodendritic level as well as from axonal nerve terminals emerged more than 20 years ago, when early biochemical investigations provided evidence that the DA localised to dendrites was certainly stored and released (Geffen et al., 1976; Cheramy et al., 1981). Indeed, a functional role for somatodendritic DA release in the autoregulation of the electrophysiological activity of SN dopaminergic neurones was suggested by early studies (Bunney, 1973). More recently, it has been shown that somatodendritically released DA can modulate SNc cell firing and subsequent DA output in the striatum (Santiago and Westerink, 1991). In addition, through the modulation of γ-aminobutyric acid and/or glutamate release within the SN pars reticulata, DA may regulate nigral output to the thalamus and cortex (Abarca, 1995; Aceves, 1995).

At present, the potential functional importance of somatodendritic DA release in the SN are not matters of dispute, and several investigators have implicated this phenomena as a key factor in the regulation of various motor behaviours (Robertson and Robertson, 1989 and references therein). However, due to the small size and low tissue content of the SN, the measurement of somatodendritic release has been a difficult task. As a result, there are still many aspects of SN dopamine release that remain unanswered. Some investigations into the mechanisms of DA release within the SN have led to the suggestion that the somatodendritic release of DA differs from the release observed at striatal synapses (Hoffman, 1999). These observations strengthen early studies by Groves and Linder (1983), which indicated that much of the DA stored within the SN is not contained within classical synaptic vesicles and may involve an important Ca^{2+} independent component. However, other authors have suggested that the biochemical properties of somatodendritic DA in the SN are indistinguishable from those of the dopaminergic nerve terminal in the striatum (Heeringa and Abercrombie, 1995). As a whole, it appears that somatodendritic release is dependent on vesicular accumulation (Heeringa and Abercrombie, 1995; Jaffe, 1998), sensitive to depolarisation (Geffen, 1976; Cheramy, 1981; Robertson et al., 1991), and that it

is mostly Ca^{2+} dependent, consistent with an exocytotic mechanism (Geffen, 1976; Cheramy, 1981).

Nicotinic receptors in the CNS are localised both on cell bodies and/or their dendrites, as well as on axon terminals (Sorenson, 1998 and references therein). In the past, most of the attention regarding nAChR has been focused on the presynaptic type of receptors and its role in regulating neurotransmitter release (for review see Wonnacott, 1997). This was partly due to the scattered distribution of nAChR-containing targets through cortical, limbic and central autonomic structures and the complexity of stimulating specific cholinergic nerves in the brain. In spite of these difficulties, there is now evidence for the presence of functional somatodendritic receptors in the SN and ventral tegmental area (VTA), where the cell bodies of the two major dopaminergic pathways (nigrostriatal and mesolimbic) are located (Yin and French, 2000; Reuben, 2000a, b).

nAChR have been extensively studied in their role of modulators of the basal ganglia circuitry. Much of this function has been attributed to the capacity of nAChR to modulate dopamine release at the striatum. On the contrary, their role as modulators in the substantia nigra has been largely overlooked. In spite of the limited number of studies reported in the literature, it has been shown that systemic or ionotrophic application of nicotinic agonists increases the firing rate of SN DA neurons (Lichtensteiger, 1982; Clarke, 1985; Grenhoff, 1986) and stimulates the release of dopamine in the striatum (Blaha and Winn, 1993). Immunohistochemical studies complemented the mentioned reports, indicating that SN neurons make synapses with cholinergic terminals (Sorenson, 1998). On the whole, there is strong evidence suggesting an active role for nAChR at the SN. However, there are almost no data reporting the putative action of these receptors in regulating the release of DA at the somatodendritic level.

It has been previously proposed that nAChR can have a modulatory role in the function of the CNS, and this has been mainly associated with their presynaptic location and ability to promote neurotransmitter release at nerve terminals. The fact that nAChR can also be found at cell bodies and dendrites suggested other functional aspects of the activation of nAChR. Some of the intracellular mechanisms triggered by nAChR stimulation where illustrated and discussed in the previous chapters of this work. However, an important modulatory aspect of nAChR function could involve the release of neurotransmitters at the somatodendritic level, where they can also act as modulators. Considering the importance of DA release in the SN for the control of basal ganglia circuitry, and pondering the lack of data regarding the role of nAChR in the modulation of this phenomenon, we decided to investigate the action of nicotinic agonists upon DA release in SN superfused slices.

The particular aims of this part of the project were:

- To establish and characterise an in vitro experimental assay for the study of somatodendritic dopamine release in the SN.
- To analyse the role of nAChR in the regulation of dopamine release in the SN.

A2.2 Methods

A2.2.1 Materials

Male Sprague-Dawley rats were obtained from the University of Bath Animal House breeding colony. Nomifensine, citalopram and 4-aminopyridine were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Epibatidine and (±)-anatoxin-a (AnTx-a) were obtained from Tocris Cookson (Bristol, Uk). Culture media and media supplements were purchased from Gibco BRL (Paisley, UK). All other chemicals used were of analytical grade and obtained from standard commercial sources.

A2.2.2 Superfusion of substantia nigra slices for [^{3}H]DA release

Male Sprague-Dawley rats (weighing 240-250 g) were killed by cervical dislocation and decapitated. After rapidly removing the brain, a coronal section from the mesencephalon was obtained and the left and right substantia nigra (15-20 mg of wet tissue weight per rat) were rapidly dissected for the preparation of the slices. Superfusion experiments were performed in Krebs bicarbonate buffer of the following composition: 118 mM NaCl, 2.4 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM K_2HPO_4, 25 mM $NaHCO_3$ and 10 mM glucose, buffered to pH 7.4 with 95 % O_2/5% CO_2 and supplemented with 1mM ascorbic acid and 8 μM pargyline.

Substantia nigra slices were prepared based on the method previously described by Soliakov and Wonnacott (1996) for striatal slices, using a McIlwain tissue chopper. SN slices from four rats were washed once with 3 ml of Krebs buffer and loaded with [^{3}H]DA (0.1μM, 0.132 MBq/ml) for 15 min at 37°. After two washes, slices were resuspended in Krebs buffer and loaded into six closed superfusion chambers (200 μl and ~15 mg of slices per chamber). Superfusion experiments were performed as previously described for striatal slices (Soliakov and Wonnacott, 1996), in a Brandel apparatus using Krebs bicarbonate buffer at a flow rate of 0.5 ml/min. After 24 minutes of perfusion wash, 2-min fractions were collected (3 samples before and 12 samples after the stimulus). All drugs were applied for 40 s, separating the pulse of drug from the bulk buffer flow by 10 s air bubbles.

Fractions were counted for radioactivity in a Tri-Carb 1600 liquid scintillation counter (counting efficiency, 48%). Stimulation-induced overflow of radioactivity in excess of baseline efflux was calculated and expressed as the percentage of total radioactivity present in the nigral slices at the onset of the stimulation. To estimate the radioactivity remaining in slices at the end of the experiment, superfusion was continued for 30 min with 10 mM HCl,

to lyse the slices. Aliquots (1ml) of the lysates were counted for radioactivity. Total radioactivity was calculated as the sum of released [^{3}H] DA plus that found in the lysate.

A2.2.3 Superfusion data analysis

The baseline was derived by fitting the following double exponential decay equation to the data, using the Sigma Plot (v. 2.0) program for Windows: $y = ae^{-bx} + ce^{-dx}$, where a, b, c and d are the curve parameters and x is the fraction number. Evoked [^{3}H] DA release was calculated as the area under the peak after subtraction of the baseline. Data are presented as percentages of the corresponding controls, assayed in parallel, and are mean ± SEM values of at least three independent experiments, each consisting of two replica chambers for each condition. For comparison between different experiments, superfusion profiles were normalised as percentages of the fitted baselines. Student's paired t test was used to determine the significance of differences from the control value.

A.4 Results

A2.4.1 Dopamine release from SN slices

Each dissected SN weighed 8 mg and tissue from four rats was needed in order to attain a clear baseline of transmitter release in six independent perfusion chambers. This animal requirement more than doubles the one for striatal slices experiments, where striata from three rat brains allows a 12-chamber superfusion experiment.

The basal release of the SN slices was determined after 24 minutes of washout perfusion and previous to the addition of the stimulus. On average, each fraction represented 1.8 ± 0.2 % of the total [^{3}H] DA present at the beginning of the experiment (Fig A1).

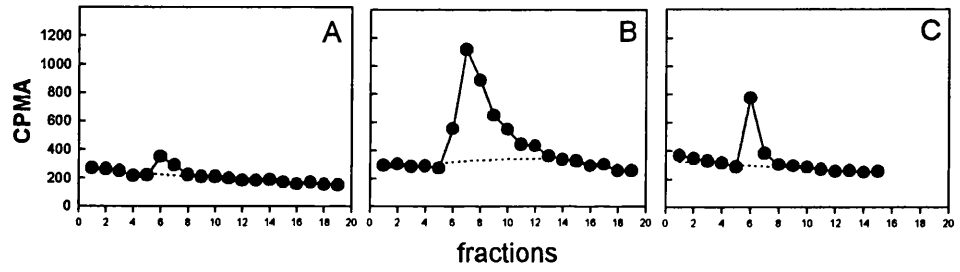

Fig A.1 Time course and profile of [^{3}H]-dopamine release in the substantia nigra Representative results of fractional release of [^{3}H]-dopamine after stimulation with (A) buffer, (B) 4-AP (1mM), and (C) KCl (20 mM). All drugs were perfused for 40 seconds, starting at fraction 4, and fractions were collected every 2 min, with a 0.5 μl/min perfusion flow.

Since our experiments regarding the DA release from SN slices were performed after loading the tissue with [^{3}H] DA, it was important to characterise the relative contribution of the different catecholaminergic uptake systems at the SN. As well as the dopaminergic neurons containing the DA transporter molecule, the SN receives an important input of serotonergic terminals from the Raphe nucleus. Therefore, in order to demarcate the relative contribution of the serotonin transporter in our experiments we used citalopram, a selective inhibitor of the serotonin uptake molecule. When the [^{3}H] DA loading and superfusion experiments were carried out in the presence of citalopram (50 nM), the basal release of transmitter was reduced in nearly 30 % (Fig A2), indicating that in the presence of the inhibitor a decrease in the uptake of transmitter was observed. The further addition of nomifensine (5 μM), a typical selective inhibitor of the DA transporter, produced a significant reduction in the basal release of approximately 75 % (Fig A2). This latter result showed that, in spite of the relatively significant influence of the serotonin uptake system, the main

contribution to the [^{3}H] DA being taken up and released comes from the dopaminergic cell bodies.

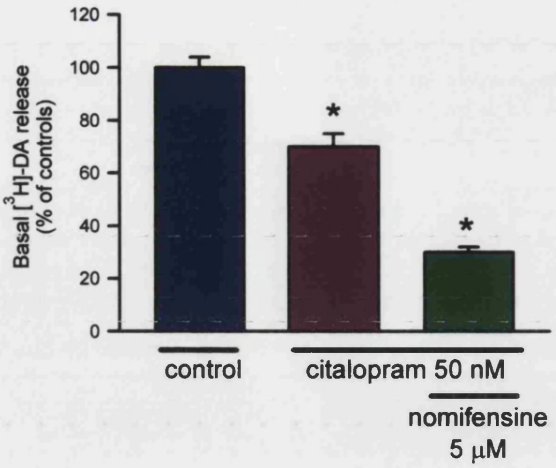

Fig A.2 Effect of uptake inhibitors on the basal release of [^{3}H]-dopamine
Two cathecolaminergic uptake inhibitors, citalopram (50 nM) and nomifensine (5 μM), were present in the 15 min incubation with [^{3}H]-dopamine. Subsequent basal release was estimated as described in the methods section. Data represent the mean ± SEM of at least 3 independent experiments, each consisting of two replica chambers for each condition. Significantly different from control (*, $p < 0.05$, Student's t test).

After elucidating the relative contribution of the main cathecholaminergic uptake systems reported to be present in the SN, we initiated the study of [^{3}H] DA release after different depolarising stimulus. The perfusion of KCl 20 mM generated a significant increase of DA release when compared with the effect of buffer, which normally produces a small non-specific increase (Fig A1). When 1 mM 4-AP was perfused, an even more pronounced effect was observed, nearly doubling the increase in DA release evoked by KCl (Fig A1, A3).

Considering the significant input of serotonergic axon terminals to the SN, and the observation that a significant part of the [^{3}H] DA may be uptaken by these terminals, the depolarisation-evoked release of [^{3}H] DA was examined in the presence of citalopram. When the inhibitor was added to the incubation and perfusion buffer, the increase in [^{3}H] DA release after 4-AP was reduced to 50 % of the original effect (Fig A3). Interestingly, the addition of citalopram did not affect the KCl evoked [^{3}H] DA increase (Fig A3). In addition, it is important to mention that the relative increase in [^{3}H] DA after KCl or 4-AP, expressed as a percentage of total [^{3}H] DA uptake, was not significantly different from that observed in striatal slices (Fig A4).

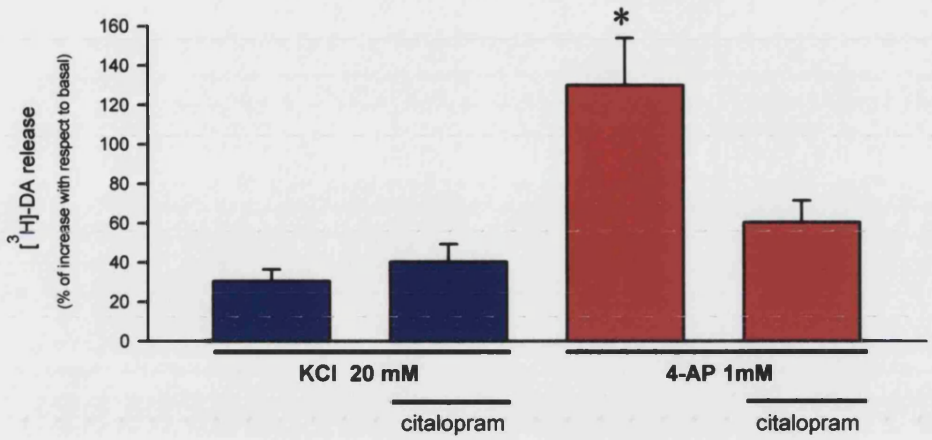

Fig A.3 Effect of depolarising agents on [^{3}H]-dopamine release in the substantia nigra. Substantia nigra slices were stimulated with KCl (20 mM) or 4-AP (1 mM). Where indicated, stimulated slices were incubated with [^{3}H]-dopamine in the presence of citalopram. Data represent the mean ± SEM of at least 3 independent experiments, each consisting of two replica chambers for each condition. Significantly different from 4-AP and citalopram (*, $p < 0.05$, Student's t test).

To test the action of nicotinic receptors in the modulation of DA release from the SN, two different nicotinic agonists were tried. The perfusion with (±)-AnTx-a (1 uM), an agonist of the nicotinic receptor, failed to give any significant response on [^{3}H]DA release (Fig A4). When epibatidine (100 nM), a more potent and more efficacious nicotinic agonist of striatal release was tried, it produced only a small augmentation in the [^{3}H] DA release at the SN (Fig A4). However, after several experimental repeats (n = 8, with 2 replicates each) the increase observed with epibatidine was relatively inconsistent, with low and variable results. Unlike KCl and 4-AP stimulations, the effect of nAChR agonists differed markedly when comparing results from SN versus striatal slices (Fig A4). The lack of a clear effect of nicotinic agonists on the DA release in the SN precluded a quantitative and pharmacological approach, and as a result, this line of research was not pursued further.

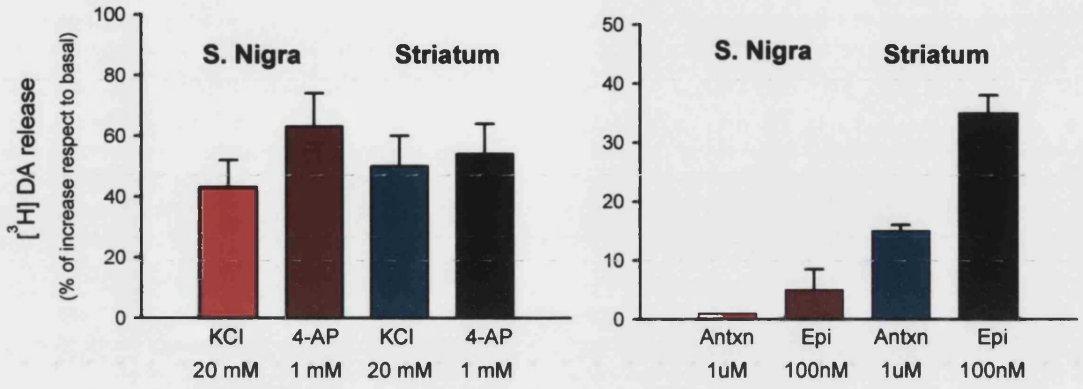

Fig A.4 Effect of depolarising agents and nAChR agonists on [^{3}H]-dopamine release in the substantia nigra.

The effects of depolarising agents (as described in previous figure) and nAChR agonists, anatoxin-a (Antx, 1 μM) or epibatidine (Epi, 100 nM) on [3H]-dopamine release, were compared in the substantia nigra and striatum, as estimated using the superfusion technique. Data represent the mean ± SEM of at least 3 independent experiments, each consisting of two replica chambers for each condition.

A2.5 Discussion

The existence, importance and potential functional relevance of somatodendritic DA release in the SN are well-accepted facts. Various authors have reported that DA containing cell bodies within the SN can synthesise, store and release locally, thereby regulating nigral output (Bjorklund and Lindvall, 1975; Cheramy et al., 1981). It has been shown that DA released at the SN can act through D_2 receptors to regulate nigrostriatal output and may act via D_1 and D_2 receptors when regulating the nigrothalamic tone (Elverfors and Nissbrandt, 1992; Abarca et al., 1995; Aceves et al., 1995; Martin and Waszczak, 1996). Accordingly, and considering the SN as part of the basal ganglia circuitry, several investigators implicated the release of DA within the SN as a key factor in the regulation of various motor behaviours (Robertson and Robertson 1989 and references therein).

In spite of the growing interest in nigral DA release, the regulation of DA levels at the SN has not been fully elucidated. Moreover, some contradictory results have been found regarding the role of the DA transporter molecule (DAT) in the SN. The DAT, the serotonin transporter molecule (SERT) and the noradrenaline transporter (NAT) are related members of a superfamily of Na^+ and Cl^{2-} dependent neurotransmitter transporters. In fact, biochemical measurements of [^{3}H]DA transport have suggested that a significant amount of DA may be taken up by other transporter systems (Kelly et al., 1985; Simon and Ghetti, 1993), and predominantly the SERT. On the contrary, in vivo electrochemical studies have demonstrated that DA within the SN is primarily removed by DAT, and not by the SERT or NAT (Hoffman and Gerhardt, 1998). The contradictory results observed might be partially due to the different target accessibility and physiological states that results of comparing *in vivo* versus *in vitro* results. Moreover, the relatively high concentration of tritiated transmitter utilised for [^{3}H]DA loading in *in vitro* experiments may saturate the DAT, then allowing other transporter systems to play a larger role in the uptake of [^{3}H] DA. Our results seem to confirm this latter hypothesis. We have found that the incubation of SN slices with [^{3}H]DA in the presence of citalopram, a selective inhibitor of SERT, diminished the basal release of DA in 30 %. Even considering the lack of full specificity for the SERT, thus opening the possibility for DAT inhibition with citalopram, it has to be said that a significant proportion of the DA taken up in our system seem to be loaded into serotonergic terminals. This latter result emphasizes the particular relevance of employing selective inhibitors of SERT when studies involving [^{3}H]DA loading into dopaminergic neurons are carried out in the SN. Finally, it is important to note that after being loaded into the cell, studies using reserpine-induced DA depletion have shown that the dopaminergic cell bodies of the SN are capable of storing DA at vesicles and release it via a vesicular mechanism (Heeringa and Abercrombie, 1995).

The release of DA from the striatal axon terminals has been extensively characterised, being well accepted that it occurs through depolarization-induced exocytosis (Wonnacott, 1997). In the SN, the somatodendritic release of DA also appears to be sensitive to depolarization, according to reports by different authors (Cheramy et al., 1981; Robertson et al., 1991). This was confirmed in the present work with the use of two different depolarising agents that increased the release of [^{3}H] DA in the SN. In spite of producing the same qualitative effect, KCl and 4-AP largely differ when considering the functional ways in which their assert their goals. While K^+ acts in a direct way, depolarising the cell body by affecting the fine balance of charges inside the cell, 4-AP acts in a more indirect way, blocking the K^+ channels present in the cell membrane thus increasing the number of intracellular positive charges and causing depolarisation. When experiments were carried out in the absence of citalopram the effect of 4-AP was higher than the one observed with KCl. However, when citalopram was added to the perfusion buffer the increase observed after 4-AP was dramatically decreased, while leaving almost invariant the KCl evoked release of [^{3}H] DA. This was an unexpected result and it probably indicates that both depolarising agents have divided targets depending on the cellular mechanisms that they affect. Depolarisation by KCl seems to be influencing mainly the dopaminergic cell bodies, in view of the similar results observed with or without citalopram. On the other hand, the effect produced by 4-AP seems to have a divided target, affecting the [^{3}H] DA stored at either dopaminergic cell bodies and/or the serotonergic axon terminals.

The significant increase in somatodendritic DA release after stimulation with depolarising agents clearly demonstrates that [3H] DA is loaded into neurones and can be released upon depolarisation, most probably through an exocytotic process. Of particular importance is the fact that the relative increases over basal levels of DA release after KCl or 4-AP were quantitatively similar in both the striatum and the SN (Fig A4). It appears that, although the absolute amount of transmitter is higher in the striatum, the dopaminergic cell bodies in the SN and the axon terminals in the striatum are capable of storing and releasing DA in a surprisingly similar manner.

Nicotine has been shown to increase the firing rate (Lichtensteiger et al., 1982) and bursting activity of mesenchephalic DA neurons (Grenhoff et al., 1986). Moreover, nicotine injected in the SN can modulate the release of DA in nigrostriatal axon terminals (Blaha and Winn, 1993). Also in the SN, Kayadjanian et al. (1994) demonstrated that nicotine can increase the release of GABA and that a partial blockade of this evoked-effect was observed after the addition of a D1-like DA receptor blocker. Although the certain involvement of ACh was not established, these authors postulated that cholinergic terminals were the most probable candidate for the evoked increase of DA in the SN, and the subsequent modulation of GABA

release through DA receptors. Indeed, it was previously demonstrated that substantia nigra neurones receive cholinergic innervation mainly from the pedunculopontine nucleus in the rat (Clarke et al., 1987). The results reported here do not support the hypothesis of a strong influence of nicotinic receptors in modulating the release of DA at the SN level. Contrary to the effects observed in the striatum, where a potent and clear modulation is observed, the role of nicotinic receptors in somatodendritic DA release seem, at least, less convincing. This is clearly demonstrated by comparing the release data from the present study with results of striatal [3H] DA release obtained in this laboratory with an identical methodology (Fig A4). In effect, the clear and pronounced action of nAChR agonists in striatal preparations contrast radically with the effect observed with the same agonists in the SN.

During the course of this study, Reuben et al (2000a, b) reported a very modest increase in dopamine release from SN dendrosomes after nicotine stimulation. Although similar to the synaptosomal preparation in the methodological prodecure, dopaminergic dendrosomes are obtained from the SN and ventral tengmental area, instead of the striatal terminal region. Although their study shows a small significant increase in DA release after nicotine stimulation, it still demonstrates the apparently minor role of nAChR in the modulation of somatodendritic dopamine release. In accordance with our results, Reuben et al. (2000a) also reported a considerable difference when comparing nicotine's effects in the striatum and SN.

The lack of effect after nicotine stimulation in SN slices can be argumented as the incapability of reaching the methodological requirements for detecting very small levels of somatodendritic DA release. However, the fact that a dendrosomes preparation also gave a very modest increase of DA release after nicotine, suggests that, unlike their counterparts in the striatum, nAChR are not major regulators of DA release in the SN. It appears as if the role of nAChR will be determined not only by the receptor per se, but also by its location on the structure of the cell. Accordingly, when located at the axon terminals the nAChR can have a fundamental role in modulating the release of a variety of neurotransmitters in different brain areas. However, the function of nAChR should not be solely circumscribed to that role. In effect, the presence of nAChR in somatodendritic areas seems to be fulfilling a different and far more complex purpose. The results presented in the main body of this thesis support this theory, and demonstrate the complexity of nAChR signalling in neurones.

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