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Association of NGF receptors with membrane rafts in PC12 cells

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ABSTRACT

Nerve Growth Factor (NGF) signal transduction is involved in the survival, differentiation and maintenance of neurons through the receptors TrkA and p75^{NTR}. These receptors activate downstream protein kinase cascades that regulate cell survival. NGF binding to TrkA promotes cell survival, however NGF binding to the low-affinity receptor, p75^{NTR} can lead to cell death in the absence of TrkA. Therefore the interaction of these two receptors and their downstream pathways are very important for determining cell survival. Recent studies have shown that many receptors and their associated downstream proteins have been found in membrane rafts, areas of the plasma membrane enriched in sphingolipids and cholesterol. To investigate the presence of the NGF receptors and downstream signalling proteins in these rafts, we have devised a method of cellular fractionation and detergent extraction quite different from those used previously. Mechanical permeabilisation separated the cytosolic components of PC12 cells. Non-ionic detergent extraction was used to solubilise the majority of the plasma membranes, leaving the detergent-insoluble membranes and cytoskeleton. Equilibrium flotation gradients were used to separate the membrane rafts from other detergent-insoluble material such as the cytoskeleton. Using these methods, we found that not only are TrkA and p75^{NTR} present in rafts, but also the downstream signalling protein ERK1 and the cytoskeletal protein, tubulin. In addition to plasma membrane rafts, we have isolated detergent-insoluble intracellular membranes from the endoplasmic reticulum and Golgi. NGF binding, *in vitro* reactions with an ATP regenerating system and the addition of ganglioside GM1 to the cells, have been found to have a large effect on the raft association of both TrkA and p75^{NTR}. These results indicate an important role for membrane rafts in NGF signalling through its receptors TrkA and p75^{NTR}, and suggest a model in which signalling centres form around rafts and microtubules.

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ABBREVIATIONS

Akt/PKB	Protein kinase B (serine/threonine kinase)
ATP	Adenosine triphosphate
BB	Bud buffer
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CNS	Central nervous system
CPM	Counts per minute
Da	Dalton
DAG	Diacylglycerol
ddH ₂ O	Double distilled water
DIGs	Detergent-insoluble glycolipid-rich domains
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRM	Detergent-resistant membranes, TX100-insoluble pellet (10,000xg)
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol-bis(β -aminoethyl ether N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular-signal regulated kinase (ERK1 = p44 MAPK, ERK2 = p42 MAPK)
Flot	Flotillin
FRET	Fluorescence resonance energy transfer
GD1	Disialoganglioside
GDP	Guanosine diphosphate
GM1	Monosialoganglioside
GM2	Monosialoganglioside
GM3	Monosialoganglioside
Gmix	Ganglioside standard mixture
GPI	Glycosylphosphatidylinositol
Grb-2	SHC binding protein

GSK-3	Glycogen synthase kinase-3
GSLs	Glycosphingolipids
GTP	Guanosine triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IP ₃	Inositol 1,4,5 trisphosphate
JNK	c-Jun amino-terminal kinase
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MβCD	Methyl-β-cyclodextrin
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NT	Neurotrophin
NT-3	Neurotrophin-3
P1	1,000xg pellet - cell membranes, cytoskeleton, large organelles
P1M	10,000xg supernatant - solubilised membranes
p70S6K	p70 ribosomal protein S6 kinase
p75 ^{NTR}	75 kDa neurotrophin receptor
PBS	Phosphate-buffered saline
PC12	Rat adrenal pheochromocytoma cell line
PEE	PBS with EDTA and EGTA
pERK	hyperphosphorylated ERK1 (~46 kDa)
PGB	PBS with glucose and BSA
PI 3,4-P ₂	Phosphatidylinositol 3,4-bisphosphate
PI 4,5-P ₂	Phosphatidylinositol 4,5-bisphosphate
PI3-kinase	Phosphatidylinositol 3'-kinase
PKBKs	Protein kinase B kinases
PKC	Protein kinase C
PLC-γ	Phospholipase C-γ
PM	Plasma membrane
PMSF	Phenylmethylsulfonyl fluoride
Raf	MAPK/ERK kinase kinase

Ras	GTP binding protein
RTA	anti-rat TrkA antibody
S1	1,000xg supernatant - small organelles, cytosol
SAPK	Stress-activated protein kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHC	TrkA binding protein, src homology containing protein
SOS	Guanine nucleotide exchange factor
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TrkA	140 kDa tyrosine receptor kinase A
Tween-20	Polyoxyethylenesorbitan monolaurate
TX100	Triton X-100
v/v	volume/volume
w/v	weight/volume

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CHAPTER 1: LITERATURE REVIEW

1.1 Nerve growth factor

Nerve Growth Factor (NGF) is a 26,000 Da polypeptide neurotrophic factor of the neurotrophin (NT) gene family, which acts as a survival factor for sympathetic and sensory neurons in the central and peripheral nervous systems (Levi-Montalcini, 1987). NGF has an important role in the maintenance and repair of the adult nervous system, and has been implicated as both an oncogenic agent and a tumour suppressor in various human tumours (Martin-Zanca *et al.*, 1986).

NGF also causes differentiation of rat pheochromocytoma (PC12) cells into sympathetic neuron-like cells (Greene and Tischler, 1976). PC12 cells are a single cell clonal line which have been established from a transplantable rat adrenal pheochromocytoma (Greene and Tischler, 1976). Prolonged NGF treatment, followed by its withdrawal, induces these cells to undergo programmed cell death closely resembling that seen in sympathetic neurons (Batistatou and Greene, 1993; Mesner *et al.*, 1992). Therefore, these cells have been used as a model for signal transduction events that occur in axon tips in nerve cells *in vivo*.

1.2 Nerve growth factor receptors

NGF signalling is mediated through specific cell surface receptors, gp140^{TrkA} (TrkA) (Kaplan *et al.*, 1991; Klein *et al.*, 1991) and p75^{NTR} (neurotrophin receptor). TrkA is a 140 kDa single transmembrane glycoprotein that has a tyrosine kinase cytoplasmic domain (Fig. 1). The 140 kDa plasma membrane-associated TrkA arises from a 110 kDa high-mannose precursor that is found in the endoplasmic reticulum (Grimes *et al.*, 1996). TrkA can bind to either neurotrophin-3 (NT-3) or NGF (Maisonpierre *et al.*, 1990), however, is generally known to bind with high affinity to NGF in most cell lines (Barker *et al.*, 1994). NGF has been found to be more efficient than NT-3 in mediating short-term TrkA activation in PC12 cells (Belliveau *et al.*, 1997). The other members of the receptor tyrosine kinase family, TrkB and TrkC, bind the neurotrophins brain-derived neurotrophic receptor (BDNF) and NT-3 respectively (reviewed in Grimes *et al.*, 1993).

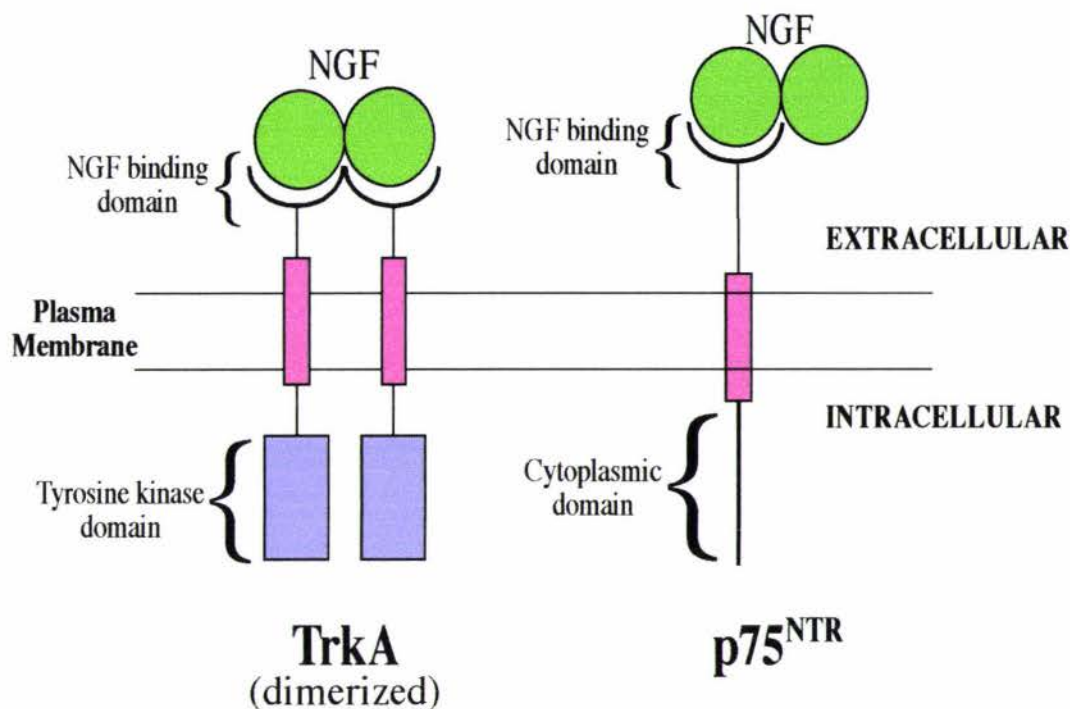


Figure 1: Schematic representation of the NGF-bound TrkA and p75^{NTR} receptors.

■ NGF, ■ transmembrane domain, ■ tyrosine kinase cytoplasmic domain.

This representation of TrkA and p75^{NTR}, shows that both TrkA and p75^{NTR} have similar transmembrane, and NGF binding domains. When TrkA binds NGF the single transmembrane receptor dimerizes to become active, this does not occur with NGF binding to p75^{NTR}. The main difference between the two receptors is the presence of a cytoplasmic tyrosine kinase domain on TrkA only. This is the domain where autophosphorylation occurs upon NGF binding. The p75^{NTR} cytoplasmic domain is believed to be involved in interactions with G-proteins and the activation of sphingomyelinase.

p75^{NTR} is a single transmembrane glycoprotein (Fig. 1) that lacks kinase activity but binds to all of the neurotrophins including NGF (Squinto *et al.*, 1991). p75^{NTR} has a lower affinity for NGF than the TrkA receptor (Rodriguez-Tebar *et al.*, 1992). NGF bound to TrkA dissociates 40-fold more slowly than NGF bound to p75^{NTR}, hence TrkA is known as a high affinity, or "slow", NGF receptor. p75^{NTR} on the other hand is known as a low affinity or "fast" NGF receptor (Schechter and Bothwell, 1981).

When NGF binds to TrkA, the receptor dimerizes and in doing so the cytoplasmic kinase domain becomes activated and autophosphorylates certain tyrosine residues on this intracellular domain (Kaplan *et al.*, 1991; Klein *et al.*, 1991). This autophosphorylation of the receptor creates sites for the binding and activation of signalling intermediates that initiate signal transduction through several pathways (Stephens *et al.*, 1994). These include the phospholipase C- γ (PLC- γ) pathway, Ras/Mitogen Activated Protein kinase (Ras/MAPK) pathway, and the phosphatidylinositol 3'-kinase (PI3-kinase) pathway (Stephens *et al.*, 1994). Each of these pathways play a role in neuronal survival and differentiation,

1.3 Signal transduction pathways

1.3.1 TrkA signalling

Activation of PLC- γ results in hydrolysis of phosphatidylinositol 4,5-bisphosphate and the subsequent formation of the second messengers inositol 1,4,5 trisphosphate (IP₃) and diacylglycerol (DAG) (Fig. 2). IP₃ is responsible for the liberation of calcium from intracellular organelles (Berridge, 1987). Increasing cellular calcium levels results in the activation of calmodulin, which forms a complex with calcineurin. This complex then leads to increased gene transcription (Berridge, 1993). DAG is involved in the activation of protein kinase C (PKC) which interacts with the Ras/MAPK pathway (Berridge, 1993).

The Ras/MAPK pathway involves the MAPK (mitogen activated protein kinase) cascade. This pathway is activated through SHC binding to phosphotyrosine residues on TrkA. SHC is recognised by the adapter protein Grb-2 which binds to SOS, a guanine nucleotide exchange factor that exchanges GDP for GTP on the small

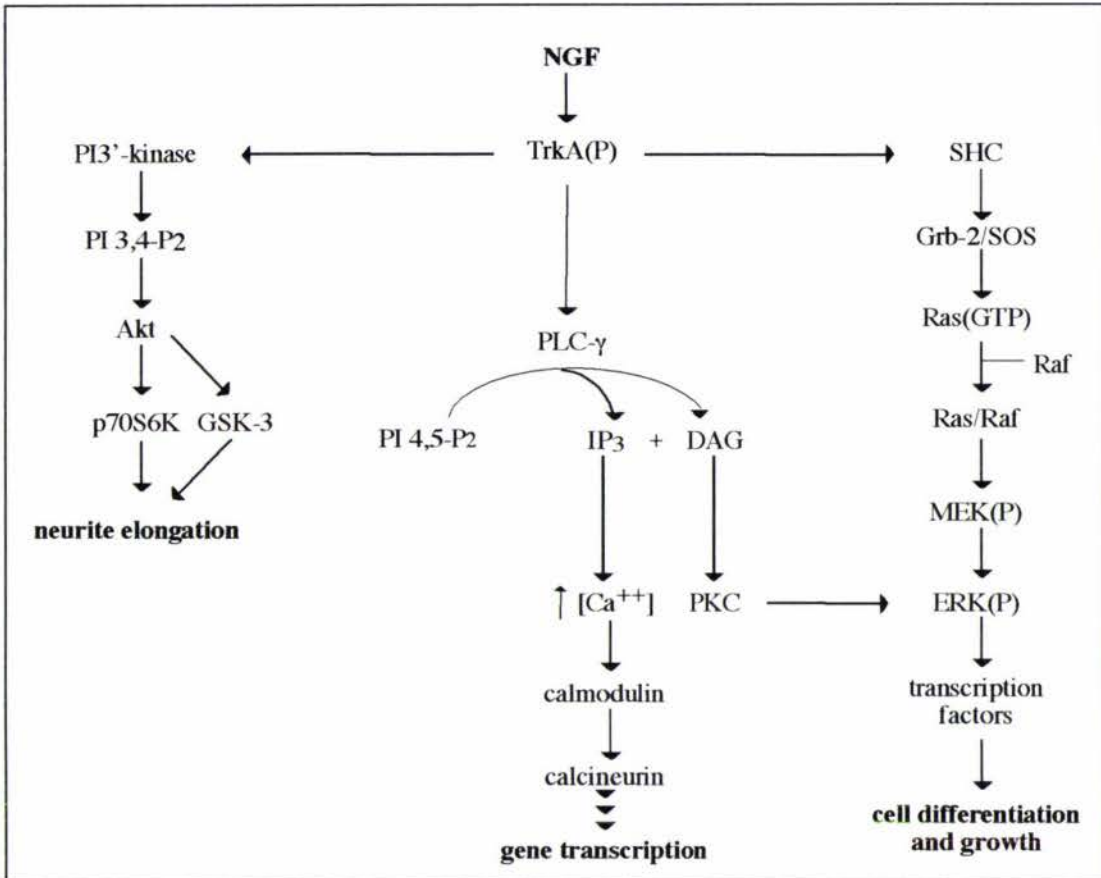


Figure 2: Signal transduction pathways activated by NGF binding to TrkA.

NGF binding to TrkA activates three pathways including the PLC- γ , Ras/MAPK, and the PI3-kinase pathways. The abbreviations are as follows: **(P)** (phosphorylated Protein), **PI3-kinase** (phosphatidylinositol 3'-kinase), **PLC- γ** (phospholipase C- γ), **SHC** (adapter protein), **PI 3,4-P2** (phosphatidylinositol 3,4-bisphosphate), **Akt** (protein kinase B), **PI 4,5-P2** (phosphatidylinositol 4,5-bisphosphate), **IP3** (inositol 1,4,5-trisphosphate), **DAG** (diacylglycerol), **PKC** (protein kinase C), **Grb-2** (adapter protein), **SOS** (guanine nucleotide exchange factor), **Ras** (small GTP binding protein), **Raf** (mitogen activated protein (MAP) kinase kinase kinase), **MEK** (MAP kinase kinase), **ERK** (MAP kinase), **p70S6K** (p70 ribosomal protein S6 kinase), **GSK-3** (glycogen synthase kinase-3).

membrane bound GTPase, Ras. Ras_(GTP) forms a complex with Raf, a MAPKKK (MAP kinase kinase kinase). Raf phosphorylates MEK (a MAPKK) which in turn phosphorylates ERK (a MAPK) (Boulton *et al.*, 1991). ERK then activates transcription factors leading to cell differentiation and growth (Kaplan and Stephens, 1994).

The PI3-kinase pathway involves the activation of Akt, a serine/threonine kinase also known as protein kinase B. Akt is recruited to the plasma membrane by phosphatidylinositol 3,4-bisphosphate, a phospholipid second messenger generated by PI3-kinase activity (Carpenter and Cantley, 1996; Hemmings, 1997). Once at the membrane, Akt is phosphorylated and activated by two protein kinase B kinases (PKBKs), that are also membrane localised (Kohn *et al.*, 1996). Akt is believed to be upstream of the p70 ribosomal protein S6-kinase, but the connection with this enzyme is likely to be indirect (Burgering and Coffey, 1995). Akt also goes on to activate glycogen synthase kinase-3 (GSK-3), which has a role in regulation of protein synthesis and modulation of transcription factors (Hemmings, 1997). Akt also phosphorylates and inhibits BAD, which is known to induce cell death (Datta *et al.*, 1997). Therefore, this pathway is believed to inhibit apoptosis and promote neurite elongation.

1.3.2 p75^{NTR} signalling

The effects of NGF binding to p75^{NTR} are not as well defined as they are for TrkA (Bothwell, 1996; Carter *et al.*, 1996). However, recent research has shown that the binding of NGF to p75^{NTR} in the absence of TrkA activates mechanisms leading to apoptosis of the cell (Casaccia-Bonofil *et al.*, 1996; Dobrowsky *et al.*, 1994; Kuner and Hertel, 1998). It has been proposed that ligand-bound p75^{NTR} activates a sphingomyelinase in the plasma membrane that hydrolyses sphingomyelin to form ceramide (Dobrowsky *et al.*, 1995; Dobrowsky *et al.*, 1994). An increase in ceramide concentration induces the activation of stress-activated protein kinase (SAPK), c-Jun kinase (JNK) (Verheij *et al.*, 1996) and nuclear factor- κ B (NF- κ B), resulting in apoptosis of the cell (Carter *et al.*, 1996; Kuner and Hertel, 1998). This p75^{NTR}-dependent cell death has been shown in some studies to be specific for NGF binding to p75^{NTR} (Carter *et al.*, 1996; Casaccia-Bonofil *et al.*, 1996). This is believed to be due to a distinct conformational change of p75^{NTR} induced only by NGF binding (Kuner and Hertel, 1998). However, a recent study of p75^{NTR} signalling in developing hippocampal

neurons has demonstrated that all neurotrophins can directly cause death through binding to p75^{NTR} (Friedman, 2000). Research has also indicated that the effect of NGF and other neurotrophins binding to p75^{NTR} could actually be cell-specific (Casaccia-Bonofil *et al.*, 1996). The interaction of neurotrophins with p75^{NTR} may be a general mechanism for normal developmental cell death and possibly for neuronal death associated with damage or disease in the CNS (Friedman, 2000).

TrkA and p75^{NTR} appear to work antagonistically. Co-expression of TrkA and p75^{NTR} has been shown to abolish the NGF-induced cell death seen in cells expressing p75^{NTR} alone (Dobrowsky *et al.*, 1995; Kaplan and Miller, 1997). The cellular ratio of p75^{NTR} to TrkA is a fundamental determinant of the signalling mediated by exposure to NGF (Friedman, 2000) (Fig. 3). p75^{NTR} has been found to increase the affinity of TrkA for NGF, therefore TrkA signalling is enhanced if both TrkA and p75^{NTR} are co-expressed in the cell (Hempstead *et al.*, 1991; Meakin *et al.*, 1992). This may be due to p75^{NTR} concentrating NGF locally in the microenvironment surrounding TrkA, and thus enhancing the ability of TrkA to bind and respond to NGF (Barker *et al.*, 1994). Therefore the survival of the cell depends on the level of expression of both TrkA and p75^{NTR} and how their signal transduction pathways interact; it is not an all-or-nothing response (Kaplan and Miller, 1997).

1.4 Retrograde transport of TrkA and p75^{NTR}

NGF is released from target tissues and acts on responsive neurons for regulation of gene expression to support survival, differentiation, and maintenance. Therefore it interacts with TrkA receptors at the distal axons of neurons and a signal must be conveyed to the cell body to cause alterations in gene expression (Grimes *et al.*, 1996).

In the presence of NGF in the short-term, there is an internalisation of TrkA receptors from the cell surface (Beattie *et al.*, 1996; Grimes *et al.*, 1996). The NGF-bound TrkA is then retrogradely transported from the nerve terminal to the cell body in order to promote neuronal survival (Grimes *et al.*, 1997; Reynolds *et al.*, 1998). Once the survival signal has been generated, NGF-bound TrkA is then degraded in the lysosomes (Beattie *et al.*, 1996), or recycled back to the plasma membrane (Kamal and Goldstein, 2000). Organelles derived from the endocytosis of the activated receptor, which are termed signalling endosomes or signalling vesicles (Beattie *et al.*, 1996), have

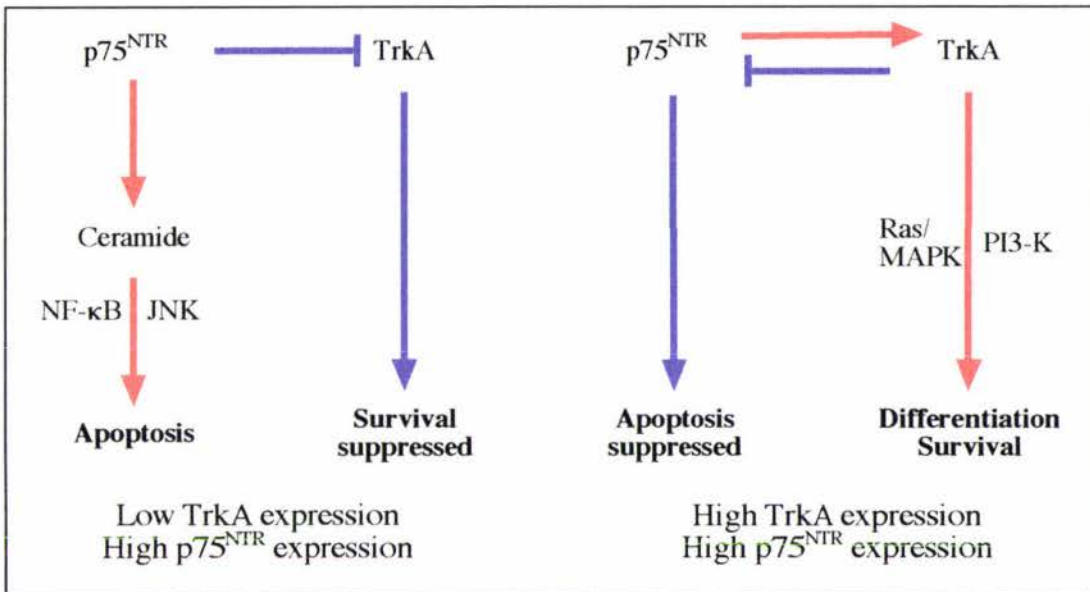


Figure 3: Interaction of TrkA and p75^{NTR} signalling pathways.

With high p75^{NTR} expression and low TrkA expression there is an inhibition of survival and promotion of apoptosis. With high TrkA expression accompanied by high p75^{NTR} expression, apoptosis is inhibited and survival and differentiation are promoted. In this state there is also an enhancement of TrkA signalling by activated p75^{NTR}. This figure was adapted from Kaplan and Miller (1997).

previously been isolated from PC12 cells (Grimes *et al.*, 1997). It is through the formation and retrograde transport of activated TrkA-containing signalling endosomes that the signal is transduced across a distance. As well as vesicles, it has also been demonstrated that microtubules are involved in the retrograde transport of NGF-bound TrkA (Kamal and Goldstein, 2000). The long-term effect of NGF stimulation is the up-regulation of expression of the TrkA receptors. This up-regulation is thought to replace the TrkA receptors that have been degraded after internalisation (Beattie *et al.*, 1996).

p75^{NTR} is also internalised and retrogradely transported, but it does not appear to be down-regulated from the cell-surface in response to NGF (Grimes *et al.*, 1996). Therefore p75^{NTR} does not appear to play a significant role in the retrograde transport of NGF.

Overall, the activation of TrkA and p75^{NTR} by NGF, retrograde transport and their signalling through multiple pathways, will lead to cell differentiation, growth and neurite elongation. It is noteworthy that many of the features of TrkA and p75^{NTR} activation, especially the downstream signalling, involves key steps where proteins are interacting with the plasma membrane.

1.5 Membrane rafts

The fluid mosaic model of the plasma membrane that was described over 20 years ago characterised the cell membrane as a "two-dimensional oriented solution of integral proteins in a viscous phospholipid bilayer" (Singer and Nicolson, 1972). Strong evidence has now accumulated indicating that this is not the full story. Most membrane proteins do not enjoy the continuous, unrestricted lateral diffusion as was once believed (Jacobson *et al.*, 1995; Vale *et al.*, 1985); instead, some proteins tend to be found in clusters within the plasma membrane (Cerneus *et al.*, 1993). These clusters have become known as 'glycosphingolipid microdomains' or 'membrane rafts' (Rietveld and Simons, 1998) which are present in many cell types, including PC12 cells (Huang *et al.*, 1999). These domains, which are likely to be less than 70 nm in diameter (Varma and Mayor, 1998), have been found to exist *in vivo* (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998). They are dynamic assemblies to which specific proteins are selectively sequestered while others are excluded (Rietveld and Simons, 1998). Membrane rafts are thought to consist of clusters of glycosphingolipids (GSLs) that

achieve a liquid-ordered state in the presence of cholesterol (Ahmed *et al.*, 1997; Schroeder *et al.*, 1998). Membrane rafts are likely to be most abundant in membranes that are rich in cholesterol and GSLs. This includes the plasma membrane, late secretory pathway, and the endocytic compartments (Brown and London, 2000).

1.5.1 Glycosphingolipids

GSLs consist of a sphingosine backbone with an amide linkage to a long chain fatty acid which is often hydroxylated. From this basic structure, the attachment of different types of sugar head groups to the terminal hydroxyl of the backbone then gives rise to different classes of GSL. GSLs that contain sialic acid structures as part of the sugar head group are known as gangliosides (Fig. 4), and are found largely in the central nervous system (Brown, 1998; Derry and Wolfe, 1967). One particular GSL, monosialoganglioside (GM1), which contains one sialic acid group, is found in high concentrations in membrane rafts (Huang *et al.*, 1999; Schnitzer *et al.*, 1995).

GSLs exhibit strong lateral cohesion that is thought to be a consequence of van der Waals forces and hydrogen bonding between the sugar head groups and between the sphingosine backbones of the GSLs (Harder and Simons, 1997; Simons and Ikonen, 1997). Also, because GSLs have mainly saturated acyl chains, they pack together tightly and can form separate gel-phase domains when mixed with phospholipids (Thompson and Tillack, 1985). These characteristics of GSLs were thought to be an important factor for membrane raft formation. It has actually been found, however, that GSL headgroup interactions are not required for raft formation; rafts can form even in the absence of GSLs (Ostermeyer *et al.*, 1999). This opens up the possibility that rafts may also exist in the cytoplasmic leaflet of the plasma membrane bilayer where the concentration of GSLs is usually low (Arni *et al.*, 1998). GSLs are usually found in the exoplasmic leaflet of the bilayer (Ghidoni *et al.*, 1986), and the nature of the phospholipids occupying the cytoplasmic side of the membrane rafts is unknown (Simons and Ikonen, 1997). Several observations suggest that rafts are present in the inner leaflet and that the rafts in the two leaflets are coupled (Brown and London, 2000), but so far it has been unclear how rafts could form in the cytoplasmic leaflet (Arni *et al.*, 1998).

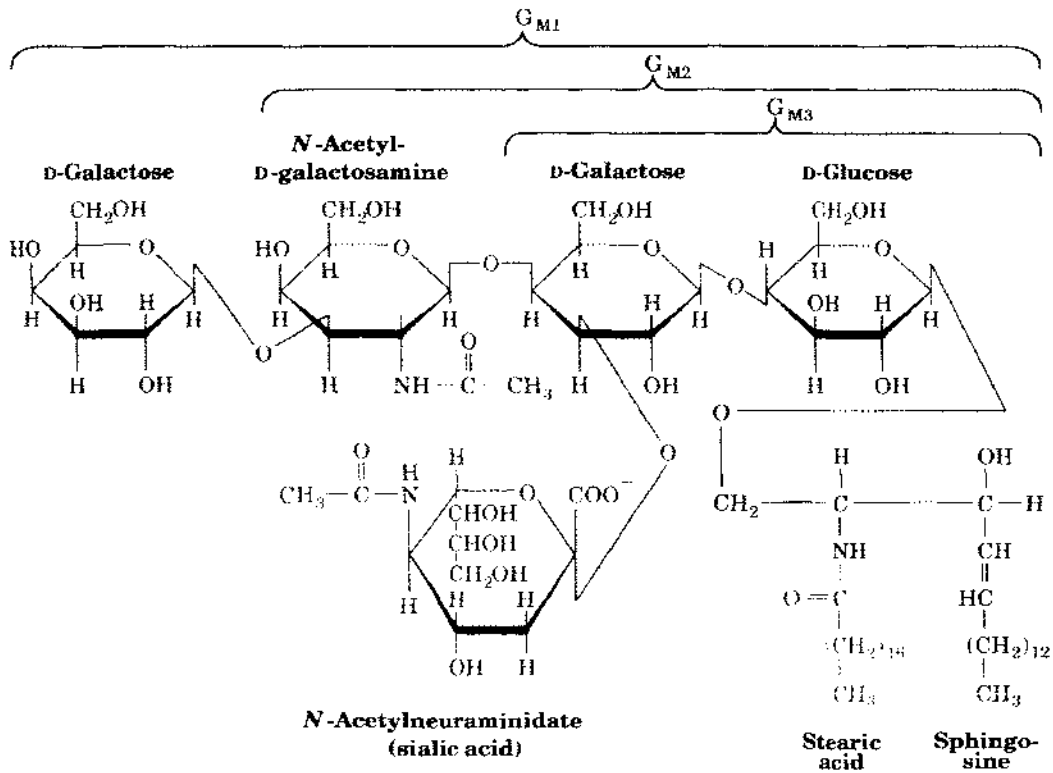


Figure 4: Structure of three common gangliosides.

The sphingosine backbone of these particular gangliosides is linked to the long chain fatty acid, stearic acid. The hydroxyl group of stearic acid is involved in the attachment of sugar head groups, D-glucose and D-galactose for GM3. In GM2 there is the attachment of another sugar group, N-acetyl-D-galactosamine and for GM1 an additional D-galactose. Note that these glycosphingolipids are classed as gangliosides due to the attachment of N-acetylneuraminidate or sialic acid to the sugar head groups. This figure was obtained from Voet and Voet (1995).

1.5.2 Cholesterol

Cholesterol is critically important for normal cellular function and for the integrity of rafts (Friedrichson and Kurzchalia, 1998; Rietveld and Simons, 1998). The cholesterol content of membranes in the secretory pathway increases continuously from the endoplasmic reticulum to the plasma membrane (Bretscher and Munro, 1993; Rietveld and Simons, 1998), thus the bulk of cellular cholesterol is localised in the plasma membrane. The plasma membrane cholesterol is further divided into two pools, one that is raft-associated and another that is found in the fluid, non-raft regions of the membrane (Simons and Ikonen, 1997).

Cholesterol is not essential for the actual formation of the membrane rafts, but it is important for maintaining the stability of these domains once formed (Brown, 1998). One of the functions of cholesterol is to maintain the liquid ordered state of the membrane rafts by filling any voids between associating GSLs, essentially acting as spacers (Rietveld and Simons, 1998; Simons and Ikonen, 1997). In this way, a rigid gel-like phase can be avoided (Ilangumaran and Hoessli, 1998). The actual physical properties of sphingolipid microdomains seem to arise from this interaction of sphingolipid hydrocarbon chains with the intercalating cholesterol (Rietveld and Simons, 1998).

1.6 Isolation of rafts

Glycosphingolipid-cholesterol rafts are insoluble in the non-ionic detergent, Triton X-100 at 4°C (Brown and Rose, 1992; Schroeder *et al.*, 1994) due to the interaction between GSLs and cholesterol (Hanada *et al.*, 1995). In a tightly packed state, lipid-lipid interactions are more stable than lipid-detergent interactions (Brown and London, 2000). Upon detergent extraction, the majority of the membrane phospholipids are solubilised, leaving behind a core of "detergent-insoluble" GSL domains (Ilangumaran and Hoessli, 1998). Once the detergent extraction has taken place, the rafts can be separated from other insoluble material such as cytoskeletal components, by sucrose equilibrium or density gradient centrifugation (Brown and Rose, 1992; Weimbs *et al.*, 1997). The detergent-insoluble protein-lipid complex will float to a position on the sucrose density gradient characteristic of the original membrane density (Stephens, 1985) due to their high lipid content (Brown and Rose, 1992).

The choice of detergent has a strong influence on the protein and lipid composition of the isolated final material (Fiedler *et al.*, 1993). Also, when using this detergent-isolation method, the temperature is very important. With a higher temperature (greater than 4°C) it is possible to get solubilisation of rafts with Triton X-100, whereas at 4°C, they will be insoluble (Schroeder *et al.*, 1994). This is because the lateral mobilities of lipids in the bilayer are enhanced therefore decreasing their tendency to engage in horizontal interactions (Schroeder *et al.*, 1994).

It has been suggested that rafts may not actually be present in the membrane prior to detergent treatment (Fiedler *et al.*, 1993). In this view, upon treatment with Triton X-100, the insoluble components found in the low-density gradient fractions are actually only remnants that once were independent and randomly dispersed in the membranes (Brown and London, 1998b). Also, with the detergent approach, it is not possible to determine the size and subcellular localisation of rafts, or to distinguish between rafts of different composition (see section 1.10) (Rietveld and Simons, 1998). Detergent-extraction has actually been found to underestimate raft association of both proteins and lipids (Arni *et al.*, 1998; Schroeder *et al.*, 1998). This has led to the generation of detergent-free methods for the isolation of membrane rafts. These include the colloidal silica particle coating procedure (Schnitzer *et al.*, 1995), immunoisolation methods (Stan *et al.*, 1997) and chemical crosslinking (Friedrichson and Kurzchalia, 1998). With this last method, it has been found that cross-linking of proteins actually stabilises their association with membrane rafts, leading to increased temperature resistance to Triton X-100 solubilisation (Harder *et al.*, 1998). Other methods to investigate membrane rafts such as fluorescence resonance energy transfer (FRET), to detect whether two raft components are spatially close, have also been recently used to investigate proteins that could possibly be associated with membrane rafts (Simons and Toomre, 2000).

Through the use of these alternative methods, it is now known that the presence of proteins in detergent-resistant fractions is a useful indication of raft association in intact membranes (Brown and London, 2000; Melkonian *et al.*, 1999; Schroeder *et al.*, 1998). However, it has also been shown that the detergent-extraction method can not be used as a quantitative measure of preferential raft association (Arni *et al.*, 1998; Brown and London, 2000). Therefore, detergent-extraction is a useful method for determining a proteins affinity for rafts, it is just not useful for the quantification of this association.

To further ensure that the detergent-extraction method is indeed isolating rafts, there are several methods that can be used. One of these methods is the use of methyl- β -cyclodextrin, a water soluble compound which, when added to the cells, will deplete cholesterol from the plasma membranes (Rietveld and Simons, 1998; Yancey *et al.*, 1996). Cholesterol, as mentioned earlier, is critical for the integrity of the membrane rafts, thus once cholesterol is depleted the membrane rafts should be disrupted. Therefore, when treated with Triton X-100 the proteins, which were once raft-associated, will no longer have the same buoyant density on an equilibrium or density gradient (Friedrichson and Kurzchalia, 1998; Harder *et al.*, 1998; Ilangumaran and Hoessli, 1998; Peiro *et al.*, 2000; Rodal *et al.*, 1999). Another method to test for membrane rafts is to use a slightly different non-ionic detergent. Octylglucoside, a gentle non-ionic detergent, has been found to completely solubilise raft-associated proteins in some cell types (Brown and Rose, 1992), indicating that this detergent is indeed disrupting membrane rafts. The use of octylglucoside is also an important test to show that Triton X-100 insolubility of certain proteins is not due to cytoskeletal attachment rather than membrane raft association (Brown and Rose, 1992).

1.7 Raft-associated proteins and lipid modifications

Some of the first proteins that were identified to be associated with membrane rafts were GPI-anchored proteins such as alkaline phosphatase (Brown and Rose, 1992; Cerneus *et al.*, 1993). These proteins are anchored to the outer leaflet of the plasma membrane by a covalently attached glycosylphosphatidylinositol (GPI)-anchor. This anchor consists of a complex oligoglycan linked to a phosphatidylinositol molecule located in the lipid bilayer (Low, 1989). Alkaline phosphatase is synthesised as a detergent-soluble precursor, but becomes detergent-insoluble during transport to the cell surface. It remains at the cell surface in a detergent-insoluble form and undergoes limited endocytosis (Cerneus *et al.*, 1993). Therefore alkaline phosphatase is believed to be associated with membrane rafts.

Several proteins of interest that have been found to associate with membrane rafts are the small G-protein, Ras (Song *et al.*, 1996), the adaptor protein SHC, and PLC- γ (Huang *et al.*, 1999), which are all involved in the NGF signal transduction pathway (Fig. 2). Also, a novel protein named flotillin, has been discovered that can now be used as a marker for membrane rafts (Bickel *et al.*, 1997; Volonte *et al.*, 1999). Flotillin

is detectable in the Triton X-100-insoluble membrane fractions of PC12 cells, which is suggestive that neurons may also express flotillin (Bickel *et al.*, 1997).

As well as a GPI-anchor, proteins can also be targeted to membrane rafts through other post-translational modifications such as palmitoylation (Arni *et al.*, 1998; Caron, 1997; Melkonian *et al.*, 1999). Palmitoylation generally occurs by a thioester bond between an acyl chain and one or more cysteine residues (Barker *et al.*, 1994), however, serine, threonine and lysine residues may also be palmitoylated (Ozols and Caron, 1997). So far, no consensus sequence for palmitoylation has been identified (Barker *et al.*, 1994; Mumby, 1997). Palmitoylation of proteins is reversible leading to the belief that this modification plays a regulatory role in a manner analogous to phosphorylation (Caron, 1997; Mumby, 1997).

Multiple palmitoylation seems to enhance membrane raft association (Arni *et al.*, 1998; Melkonian *et al.*, 1999). Indeed, dually-palmitoylated proteins are the main form that tend to be found in membrane rafts (Arni *et al.*, 1998; Melkonian *et al.*, 1999). As well as dual-palmitoylation, there is also a N-terminal methionine-glycine-cysteine motif where glycine is myristoylated and the cysteine is palmitoylated (Shenoy-Scaria *et al.*, 1994).

All of these membrane raft targeting signals contain two closely spaced acyl chains. Palmitate, myristate and most of the acyl chains on GPI-anchored proteins are saturated therefore they should fit well into the ordered lipid domain of the raft (Melkonian *et al.*, 1999). The contribution of the lipid modification to membrane raft association is not simply due to the addition of a hydrophobic moiety, as prenylated proteins are not enriched in rafts (Melkonian *et al.*, 1999; Moffett *et al.*, 2000).

It is also important to note that not all palmitoylated proteins are targeted to membrane rafts, although this modification can increase the affinity of proteins for the rafts, this effect is not always strong enough to mediate stable association (Melkonian *et al.*, 1999). The transmembrane domain sequences play an important role in determining the affinity for rafts (Melkonian *et al.*, 1999; Scheiffele *et al.*, 1997). Other than palmitoylation, little is known of the requirements for transmembrane proteins to be associated with rafts (Ilangumaran *et al.*, 1999).

It is not yet known whether individual raft proteins are randomly distributed between different rafts, or whether they are each grouped in specialised rafts (Simons and Toomre, 2000).

1.8 Caveolae and rafts

Caveolae are flask-shaped 50-100 nm invaginations of the plasma membrane which are found in many, but not all cell types (Lisanti *et al.*, 1994; Stan *et al.*, 1997). The caveolar membrane specialisation is also detergent-resistant, therefore it was initially believed that caveolae and membrane rafts were one and the same thing. However, it is now known that rafts can exist both inside and outside of caveolae (Chigorno *et al.*, 2000; Fra *et al.*, 1994; Schnitzer *et al.*, 1995; Stan *et al.*, 1997). Caveolae are believed to be formed from lipid rafts by polymerisation of caveolins, which are a hair-pin like, palmitoylated integral membrane protein (Parton, 1996). The biochemical analysis of caveolin suggests that it organises raft lipids and in this way influences raft dynamics, making rafts more stable (Harder and Simons, 1997). Thus, the plasma membrane is envisaged as a moving mosaic of raft and non-raft regions, of which only caveolae are large and stable raft domains (Harder and Simons, 1997).

Rafts have been found in the membranes of cells that do not express the major caveolar protein, caveolin (Fra *et al.*, 1994). However, if a cell does not express detectable amounts of caveolin, this does not mean that they do not express a protein that fulfills an equivalent function (Volonte *et al.*, 1999). There has been some controversy over the expression of caveolin and the presence of caveolae in PC12 cells. Until recently it was believed that there was little or no expression of caveolin in this cell line (Bilderback *et al.*, 1997; Huang *et al.*, 1999). However, recent observations suggest that PC12 cells do express caveolin and caveolae are indeed present in the plasma membranes of these cells (Galbiati *et al.*, 1998; Peiro *et al.*, 2000).

1.9 Raft-association vs. cytoskeletal-association

Originally, insolubility of proteins in detergent was believed to be an indication that the protein was associated with the cytoskeleton (Payraastre *et al.*, 1991; Schechter and Bothwell, 1981; Vale *et al.*, 1985; Venkatakrishnan *et al.*, 1991). However, Brown and

Rose (1992) discovered that GPI-anchored proteins also exhibited detergent insolubility. These proteins could not be associated with the cytoskeleton as they are limited to the extracellular side of the cell. This is one of the first studies that used a detergent extraction method in combination with sucrose gradient centrifugation to identify membrane raft-associated proteins. The cytoskeleton has a high buoyant density on these gradients as the cytoskeleton remains intact following treatment with Triton X-100 (Wiegant *et al.*, 1986). Membrane rafts, however, have a lower buoyant density (Brown and Rose, 1992), so the two types of detergent insoluble material, cytoskeleton and rafts, can be effectively separated. Therefore an equilibrium density gradient is required to distinguish whether the detergent-insoluble proteins are associating with the cytoskeleton or with membrane rafts (Roper *et al.*, 2000).

1.10 Other membrane domains

As well as membrane rafts and caveolae, new evidence suggests that more than one kind of membrane raft may exist on the cell surface (Iwabuchi *et al.*, 1998; Madore *et al.*, 1999; Roper *et al.*, 2000). Two types of raft structures have been found in addition to caveolae. These have been termed the detergent-insoluble glycolipid-rich domains (Schnitzer *et al.*, 1995) and low density microdomains (Waugh *et al.*, 1999). The detergent-insoluble glycolipid-rich domains or DIGs appear to lack detectable GM1 (a typical ganglioside present in membrane rafts), although are rich in GPI-anchored proteins. These membrane domains are believed to flank the caveolae region of the plasma membrane (Fig. 5) (Schnitzer *et al.*, 1995). Waugh *et al.* (1999) used a detergent-free method to show that the epidermal growth factor receptor was not in caveolae or in DIGs but was in a novel plasma membrane domain which possessed a similar buoyant density to caveolae. Roper *et al.* (2000) discovered that their protein of interest, prominin, resided in a distinct plasma membrane microdomain from the well-characterised alkaline phosphatase. These two types of domain mainly differed in their protein-lipid interactions. They concluded that there are multiple, distinct types of raft-like assemblies of lipids and proteins that co-exist in one biological membrane.

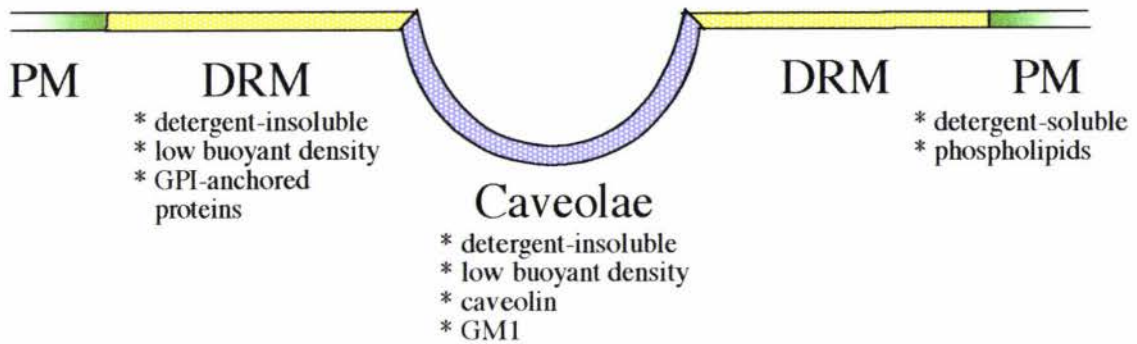


Figure 5: Representation of different membrane domains.

Two different membrane domains within the plasma membrane are illustrated. Caveolae are generally invaginated, have the protein, caveolin and are enriched in the ganglioside, GM1. The other domains, which are known as detergent-resistant membranes (DRMs), are also detergent-insoluble and have a low buoyant density. These regions have been found to be enriched in GPI-anchored proteins. The plasma membrane (PM) regions, represent the traditional plasma membrane formation, soluble in detergent and composed of phospholipids rather than glycosphingolipids. It must be noted that this is only one representation of the possible formation of the membrane domains as discussed in Schnitzer *et al* (1995).

1.11 Raft function

1.11.1 Golgi to plasma membrane transport and endocytosis

The role of rafts probably differs for different proteins and cell types (Brown and London, 1998a). However, several functions for rafts have been proposed. One of these roles is the transport of specific proteins from the Golgi complex to the plasma membrane, which has been well characterised in epithelial cells (Brown and Rose, 1992; Keller and Simons, 1998; Zheng *et al.*, 1999). It has been postulated that the axonal sorting machinery of neurons may be similar to that of apical transport in epithelial cells (Ledesma *et al.*, 1998; Rietveld and Simons, 1998). Therefore it is possible that the model of raft function proposed in epithelial cells may also apply to neurons.

Recently, neurons have been found to sort a subset of axolemmal proteins by a mechanism that requires formation of protein-lipid rafts (Ledesma *et al.*, 1998). Also, rafts have been found to be platforms that directly function in the lateral recruitment of certain types of proteins while trafficking through the Golgi (Brown, 1998). Raft formation in neurons could serve as one of the mechanisms that guarantees precise sorting to membrane subcompartments such as the axon hillock, nodes of Ranvier, presynaptic terminals or the axonal shaft (Ledesma *et al.*, 1998).

Lipid raft trafficking does not end with surface delivery, rafts are continually endocytosed from the plasma membrane. The role of rafts in the endocytic pathway is quite well defined and it is readily accepted that rafts have a role in endocytic sorting (Rodal *et al.*, 1999). From early endosomes, rafts either recycle directly back to the cell surface or return indirectly through recycling endosomes, which could also deliver rafts to the Golgi (Puri *et al.*, 1999).

1.11.2 Rafts and signal transduction

Rafts are believed to have a role in signal transduction because many signalling molecules are found to be raft-associated (Simons and Ikonen, 1997). Rafts may provide sites for the assembly of cytoplasmic signalling complexes that go on to

transduce a particular signal. Recently it has been observed that TrkA and p75^{NTR} associate with membrane rafts in PC12 cells (Huang *et al.*, 1999). In addition, TrkA has been shown to co-localise with caveolin in caveolae (Peiro *et al.*, 2000). The presence of these receptors, along with the signalling molecules SHC, Ras, and PLC- γ (Huang *et al.*, 1999; Song *et al.*, 1996), indicates that membrane rafts and caveolae will possibly have a role in regulating neuronal signalling.

Peiro *et al* (2000) have demonstrated ERK association with caveolae in PC12 cells, and found that the presence of intact caveolae inhibited basal ERK phosphorylation and activation. However, despite this effect, caveolae were still required for the propagation of the NGF-induced signal (Peiro *et al.*, 2000). It appears that the potential role of membrane rafts and caveolae in cell signalling is only just being revealed.

Rafts are also believed to be involved in prion diseases, Alzheimers disease and cancer (Brown and London, 1998a). However, the precise physiological significance of 'rafting' still requires further experimental confirmation (Weimbs *et al.*, 1997).

1.12 Research aims and strategy

The main aim of this thesis was to investigate the association of NGF receptors, TrkA and p75^{NTR}, and downstream signalling proteins with membrane rafts. During the production of this thesis, research was published indicating that TrkA and p75^{NTR} associate with membrane rafts in PC12 cells (Huang *et al.*, 1999). For this thesis however, a novel method of detergent-extraction was used to investigate the association of these receptors with membrane rafts. The cells were treated very gently, with mechanical permeabilisation and cellular fractionation carried out in a cytosol-like buffer. As with previously published research, the non-ionic detergent, Triton X-100 was used to separate the detergent-insoluble membrane rafts from the detergent-soluble plasma membranes. Iodixanol equilibrium gradient centrifugation was used to isolate the membrane rafts from other detergent-insoluble material. Many researchers tend to use a discontinuous sucrose gradient for their isolation of the membrane rafts. For this thesis, a continuous gradient was used which allows a better separation of the detergent-insoluble material. To investigate the presence of downstream signalling proteins in membrane rafts, SDS-PAGE and Western blotting of the gradient fractions was carried out. Lipid extraction of the gradient fractions and thin layer chromatography was used

to investigate the lipid content of the isolated detergent-insoluble material. Also, cholesterol depletion using methyl- β -cyclodextrin was employed to investigate the effect that this had on TrkA and p75^{NTR} association with membrane rafts.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

Most general chemicals were purchased from Sigma (St. Louis, MO). NGF was a kind gift of Professor William Mobley (University of California, San Francisco). Horse serum and foetal calf serum were from Life Technologies (Gaithersburg, MD). RPMI-1640 media, lysoganglioside-GM1 and methyl- β -cyclodextrin were purchased from Sigma. Iodixanol (OptiprepTM) was from Nycomed Pharma, Inc. (Oslo, Norway). ¹²⁵I radioisotope was obtained from NENTM Life Science Products Inc. (Boston, MA). The anti-rat TrkA antibody (RTA) was a kind gift of Dr. Louis Richardt (University of California, San Francisco). Anti-p75^{NTR} (H-137) and anti-ERK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-flotillin was purchased from Transduction Laboratories (Lexington, KY) and anti- α -tubulin was obtained from Sigma. The anti-rabbit and anti-mouse secondary antibodies were purchased from Amersham (Buckinghamshire, UK). For thin layer chromatography, foil-backed and glass-backed plates were obtained from MERCK (Germany). The GM1 ganglioside standard was obtained from Calbiochem (La Jolla, CA). The GM2 standard and ganglioside mixture were from Matreya, Inc. (Japan). Solutions were made using ddH₂O unless otherwise stated.

2.2 Cell culture

Rat pheochromocytoma (PC12) cells were obtained from Lloyd Greene (Columbia University, NY). These cells were grown on collagen-coated dishes in RPMI-1640 medium, supplemented with 10% Horse Serum and 5% foetal calf serum, as described (Greene and Tischler, 1976). Fresh media was supplied three times a week and when 80-100% confluent, cells were harvested and used for experiments and/or passaged onto new plates (1 onto 4).

2.3 Iodination

NGF was iodinated with ^{125}I using lactoperoxidase and hydrogen peroxide (Grimes *et al.*, 1996). This involved mixing 1 mCi of ^{125}NaI , 5 μg NGF, 0.01 mg/ml Lactoperoxidase and H_2O_2 diluted 1:10,000 in 0.1 M K_2HPO_4 . The reaction was then allowed to proceed for 30 minutes before being stopped by the addition of KI. The reaction mix was then added to a KwikSepTM ExocelluloseTM desalting column from Pierce (Rockford, Illinois) and 0.5 ml fractions were collected once the first radioactive drop was detected. The reaction mix and the first fraction were then diluted 1:100 for trichloroacetic acid (TCA) precipitation, along with 5 μl directly from fractions 2-5 from the column. The precipitates were centrifuged 3,500xg for 35 minutes at 4°C and the supernatant removed into a separate tube. The radioactivity in the supernatants and pellets was counted using an LKB 1271 RiaGamma automatic Gamma counter. The amount of ^{125}I -NGF needed to obtain 1 nM of iodinated NGF in 5 ml of cell suspension was determined.

2.4 Cell harvesting and ^{125}I -NGF incubation

Six to eight plates of 80-100% confluent PC12 cells were harvested for each fractionation experiment. Growth media was removed, each plate was rinsed with 10 ml of warm PBS (phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , 1.8 mM KHPO_4 , to pH 7.4), and cells were harvested in 10 ml of warm PBS by titration. The number of cells present was measured using a Neubauer improved hemacytometer, usually around 4×10^7 cells/ml. Cells were then centrifuged at 100xg for 3 minutes and washed with 5 ml of ice cold PEE (PBS, 1mM EDTA, 1mM EGTA). All following washes were ice cold with the cells maintained on ice, and all centrifugation steps carried out at 4°C. Cells were washed with PGB and resuspended in 5 ml PGB. 1 nM ^{125}I -NGF was added to the cells and the test tube rotated for 1 hour at 4°C.

The cells were centrifuged 100xg for 3 minutes and washed with 5 ml PGB to remove any unbound ^{125}I -NGF (see Fig. 6 for overview of cell fractionation protocol). After resuspending the cells with 5 ml PGB, the cells were left on ice or warmed to 37°C for 2, 10 or 30 minutes, to allow ^{125}I -NGF-bound receptor internalisation and initiation of

signal transduction. Cells were then chilled rapidly in ice-water for 3-5 minutes, centrifuged 100xg for 3 minutes and washed with 5 ml PEE, followed by a wash with 5 ml bud buffer (BB), which mimics the composition of the cytosol (BB: 38 mM aspartic acid, 38 mM gluconic acid, 38 mM glutamic acid, 20 mM MOPS, 10 mM potassium bicarbonate, 0.5 mM magnesium carbonate, 1 mM EDTA, 1 mM EGTA, adjusted to pH 7.4 at 37°C with KOH, and 5 mM glutathione was added fresh.

2.5 Cell permeabilisation and *in vitro* reactions

After washing in BB, the cells were centrifuged at 100xg for 3 minutes and resuspended in 0.4 ml - 1.0 ml BB containing protease inhibitors (depending on cell pellet volume) using an 18 gauge needle and syringe. Protease inhibitors were added to the BB to a final concentration of 174 µg/ml PMSF, 1 µg/ml o-phenathroline, in anhydrous ethanol, and 10 ng/ml pepstatin, 10 ng/ml chymostatin, 10 ng/ml leupeptin, 10 ng/ml aprotinin, in DMSO. Cells were then permeabilised (or cracked) by passing them once through a stainless-steel ball homogeniser (or cell cracker) obtained from European Molecular Biology Laboratory (Heidelberg, Germany). The ball homogeniser had been assembled prior to cell cracking in a ddH₂O water bath with the removal of any air bubbles that may impede the passage of cells. The cell cracker was placed on ice, and 1 ml of ice-cold BB was passed through the apparatus before the application of the cells. The homogeniser was disassembled, washed, and reassembled in between samples.

After cell cracking, the volume of each sample was noted. Samples were split into either '+ATP' or '- ATP' samples. The '-ATP' samples were maintained on ice while the '+ATP' samples were subjected to an *in vitro* reaction with an ATP regenerating system (1 mM ATP, 8 mM creatine phosphate, 5 mg/ml [240 units/mg] creatine kinase) and warmed for 15 minutes at 37°C. The '+ATP' samples were quenched in ice water for 3-5 minutes for rapid cooling.

2.6 Differential centrifugation

All samples were centrifuged at 1,000xg for 10 minutes to separate the cell ghosts and cytoskeleton (pellet=P1) from the cytosol and small vesicles (supernatant=S1). The P1 pellet was then resuspended in 200 µl BB containing protease inhibitors. Triton X-100

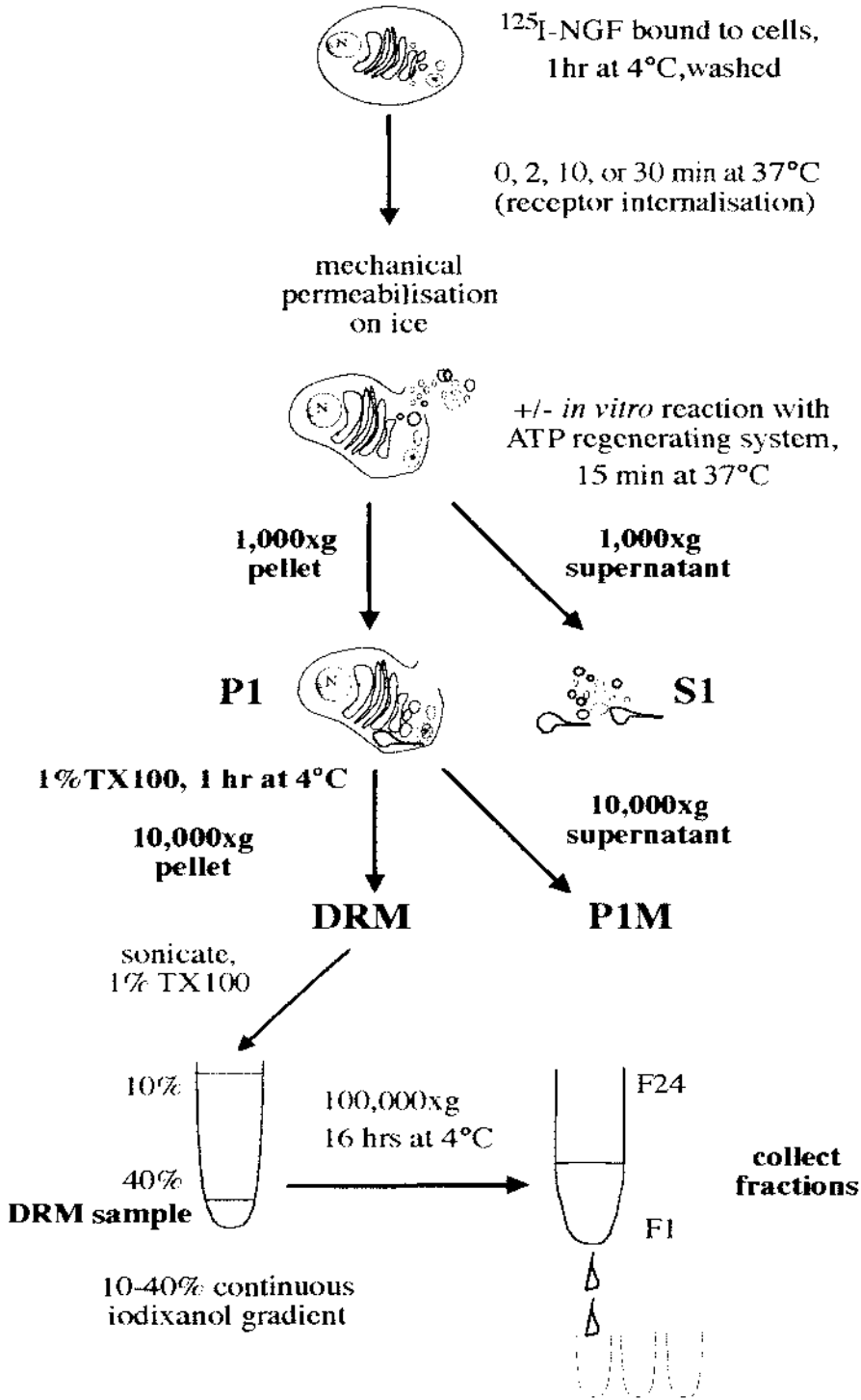


Figure 6: Overview of the cell fractionation and equilibrium gradient protocol.

Cytosol and small organelles and vesicles (S1). Cell membranes, cytoskeleton and large organelles (P1). Triton X-100-soluble membranes (P1M). Triton X-100-insoluble membranes, cytoskeleton, large organelles (DRM).

was added to a final concentration of 1% and the sample was vortexed and left on ice for at least 1 hour. After 1 hour the sample was centrifuged at 10,000xg for 10 minutes to separate the detergent-solubilised membranes (supernatant=P1M) from the detergent-insoluble particles, cytoskeleton and DNA (pellet=DRM). This pellet was then resuspended in 180 µl BB with protease inhibitors and Triton X-100 was again added to a 1% concentration. Iodixanol (OptiPrep™) was added to a 40% concentration and the samples sonicated for 2 x 5-second pulses. This step was necessary as in preliminary experiments without sonication, DNA and cytoskeletal elements often clogged the needle of the gradient fraction collector.

2.7 Equilibrium gradients and fraction collection

Sonicated samples were placed into 5 ml ultracentrifuge tubes and 10-40% continuous iodixanol gradients were poured over the top of the samples at 4°C. For this a Hoefer Scientific SG15 gradient maker was used with 2.3 ml 10% and 2.0 ml 40% iodixanol in BB. Gradients were centrifuged to equilibrium at 100,000xg for 16-18 hours. Approximately 200 µl fractions were collected from the bottom of the ultracentrifuge tube using a collection apparatus with a hollow needle that pierced a hole in the bottom of the tube. Fractions were collected manually by counting drops. Radioactivity in each fraction was determined using an LKB 1271 RiaGamma automatic Gamma counter. The refractive index of each gradient fraction was measured and the density calculated based on the formula: density = $n \cdot 3.1518 - 3.2158$, where n = refractive index. TCA was added to each fraction to a final concentration of 10%, and left overnight at 4°C to precipitate protein. Protein precipitates were recovered by centrifugation at 3,500xg for 35 minutes, washed in ice-cold acetone and re-centrifuged (3,500xg for 35 minutes). Precipitates were air dried at room temperature to remove all traces of acetone. 7 M urea sample buffer (7 M urea, 125 mM Tris HCl pH 6.95, 0.1% w/v bromophenol blue with 100 mM DTT added immediately before use) was added and samples were heated to 55°C for 15 minutes and vortexed before loading on SDS gels or freezing at -20°C for later analysis by SDS-PAGE.

2.8 Exogenous GM1 experiments

Cells were harvested, separated into two equal aliquots and incubated in either serum-free media with 65 μM GM1, or serum-free media alone, for 5 hours at 37°C in a 5% CO₂ incubator. After this incubation period the cells were washed, a small sample removed to test for apoptosis (see section 2.10), and ¹²⁵I-NGF was bound to the cells (as in section 2.4). No internalisation step (warming to 37°C after NGF binding) was carried out in these experiments. Both 'no ATP' and '+ATP' *in vitro* reactions were carried out on the samples. Cells were fractionated and applied to equilibrium gradients (as in sections 2.5 - 2.7).

2.9 Depletion of cholesterol with methyl- β -cyclodextrin

Harvested cells were evenly divided into two tubes. The cell pellets were then resuspended in serum-free RPM1-media containing 50 mM HEPES, pH 7.4. To one of the tubes, methyl- β -cyclodextrin was added to a 50 mM final concentration, mixed well, and both tubes rotated for 1 hour in a 37°C temperature-controlled room. A small sample of cells was removed after the treatment period to test for apoptosis (see section 2.10). ¹²⁵I-NGF was bound to the cells after the cyclodextrin treatment (section 2.4). Again, no internalisation step was carried out and the samples were subjected to both 'no ATP' and '+ATP' *in vitro* reactions. The fractionation procedure was then carried out as in sections 2.5 - 2.7.

2.10 Detection of apoptosis

A small sample of cells was removed at the washing step, before NGF binding. These were stained with 10 $\mu\text{g}/\text{ml}$ Hoechst 33342 (Molecular Probes, Inc.) for 30 minutes at room temperature in the dark, washed in PBS, resuspended in 10% Glycerol and examined using a Zeiss Axioskop fluorescence microscope. The presence of apoptotic bodies was assessed and scored as brightly stained, clumped chromatin in the cells. These were photographed with 35 mm film.

2.11 SDS-PAGE and Western blotting

Frozen samples in 7 M urea sample buffer, were reheated to 55°C for 15 minutes and separated on a 10% (37:1 acrylamide/bisacrylamide) sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) according to Laemmli (1970). Each gradient was run on a separate gel along with a sample of S1 and P1M fractions from the same experiment. BenchMark™ prestained protein markers (Life Technologies, Gaithersburg, MD) were run on every gel along with biotinylated protein markers (New England Biolabs Inc., Beverly, MA). The proteins were transferred to nylon-reinforced nitrocellulose (Optitran™, Schleicher and Schuell, Dassel, Germany) using the Western blotting method of Towbin *et al.* (1979). After transfer, the blot was washed in ddH₂O and stained for proteins with Ponceau S (0.2% Ponceau S in 3.0% TCA) for 10 minutes. The blot was then rinsed in ddH₂O several times to remove excess stain and photocopied to visualise transfer of proteins. To prevent non-specific binding of antibodies the blot was blocked for 60 minutes in TBS (Tris buffered saline: 10 mM Tris, 150 mM NaCl, to pH 7.7 with HCl) with 5% w/v non-fat milk powder and 0.05% v/v Tween 20.

After blocking, blots were probed with primary 1:4,000 anti-TrkA (RTA) in TBS with 0.05% Tween 20, 0.5% bovine serum albumin (BSA), and 0.5% non-fat milk powder, 1:2,000 anti- α -tubulin and 1:2,000 anti-ERK in TBS with 0.05% Tween 20, 1% BSA and 1% non-fat milk powder. The blots were also probed with 1:1,000 anti-p75^{NTR} or 1:1,000 anti-flotillin antibodies in blocking buffer and agitated overnight at 4°C. After removing the primary antibody, blots were washed three times for 15 minutes each in TBS with 0.05% Tween 20. Secondary antibody conjugated to horseradish peroxidase was applied to the blots for 2 hours. The secondary antibodies were 1:5,000 anti-rabbit (RTA, ERK), 1:2,000 anti-rabbit (p75^{NTR}), 1:5,000 anti-mouse (α -tubulin) or 1:2,000 anti-mouse (flotillin) in TBS with 0.05% Tween 20 and 2% w/v non-fat dry milk powder. An anti-biotin secondary antibody (1:5,000, New England Biolabs Inc., Beverly, MA) was also added to the secondary antibody mixture. After removing the secondary antibody the blot was again washed as described above. An enhanced chemiluminescent reaction was then performed on the blot using an Amersham ECL™ western blotting detection kit. The blot was either exposed to x-ray film (Fuji medical x-ray film, HR-G 30) or exposed directly in a Fujifilm Intelligent Dark Box II with a cooled CCD camera (LAS-1000, Fuji Photo Film Co. Ltd, Japan).

2.12 Stripping and reprobing membranes

To strip antibodies from blots for subsequent reprobing, the nitrocellulose membranes were washed in TBS pH 2.0 for 10 minutes exactly, followed by a wash with ddH₂O for 5 minutes. The blots were then blocked and the appropriate primary antibody added as described above.

2.13 Image analysis and calculations

X-ray films, digitally scanned using the Fujifilm Intelligent Dark Box II, and chemiluminescent data captured directly by the LAS-1000 image analyzer, were analyzed using Image Guage 3.4 software (Fuji Film Co. Ltd). The signal intensity of each band of interest was measured in the pixels of a defined area (a box encompassing the desired band). A profile of peaks was created and the area under the peak corresponding to the band of interest was determined. With the background subtracted, the area values obtained were used as a measurement of band intensity. The protein band intensities for the S1 and P1M samples were calculated by taking into account the volume that was loaded onto the gel compared with the original S1 and P1M sample size. For each protein these area values were added together to give a 'total DRM' or 'total whole cell' (including S1 and P1M) protein value. To take into account variations in protein loading between experiments and to plot the % of DRM or % of whole cell gradient profiles (which show the changes in intensity of the bands throughout the gradient), each bands area value was expressed as a percentage of that particular protein DRM or whole cell total.

2.14 Interpretive calculations

¹²⁵I-NGF counts in each fraction were plotted against fraction number to obtain an NGF profile of the gradient. NGF which had floated up the gradient to a low density was then defined as the 'floating peak'. To calculate the percentage of protein from the whole cell which was associating with the 'floating peak' the band intensities for the protein in the DRM, S1 and P1M fractions were summed. The percentage of this total whole cell value which was associating with the 'floating peak' was then determined.

The amount of protein in the 'floating peak' was also calculated as a percentage of the total DRM protein. P values were calculated using the students t-test.

2.15 Lipid extraction and thin layer chromatography

Lipids were extracted from gradient fractions using a method adapted from Tettamanti *et al.* (1973). 8 volumes of tetrahydrofuran (THF) was added to the samples, vortexed well, and left overnight at room temperature. The samples were centrifuged at 12,000xg for 10 minutes at 15°C. The supernatant was removed into a fresh tube and the pellet resuspended in 4 volumes of THF using a glass rod. The samples were again centrifuged at 12,000xg for 10 minutes at 15°C. This re-extraction was repeated, retaining the supernatant in a fresh tube each time. The supernatants of all extractions were pooled, 0.3 volumes of diethyl ether added, and the samples centrifuged 600xg for 20 minutes at 15°C. The aqueous THF phase, containing lipids, was then removed and concentrated by freeze-drying. The dried lipids were re-dissolved in chloroform:methanol (1:2) and spotted on to heat-activated foil-backed or glass-backed thin layer chromatography (TLC) plates. Ganglioside standards were also applied to the plate. Plates were developed (method of Muthing, 1998), in an equilibrated tank containing chloroform:methanol:0.22% aqueous CaCl₂ (45:45:10) until the solvent front reached 1 cm from the top of the plate. Plates were air-dried and subjected to either char stain (10% CuSO₄ in 8% H₃PO₄) to detect all lipids and cholesterol, or resorcinol spray (2% aqueous resorcinol in 80% HCl and 0.25 mM CuSO₄) for the detection of gangliosides. In both cases, after application of stain, the plate was heated at 100°C for several hours. Once the lipid bands/spots were visible, plates were cooled and scanned using image analyzer (LAS-1000) software (mentioned in section 2.13). The positions of standard and sample bands on the plate (Rf), were calculated by the distance that the band had moved from the point of origin, compared with the distance that the solvent front had moved up the plate.

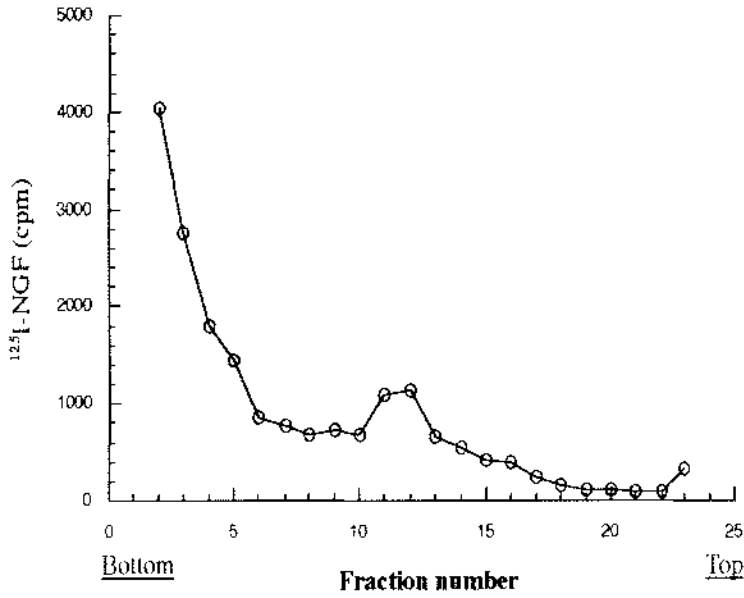
CHAPTER 3: RESULTS

3.1 NGF, TrkA and p75^{NTR} are detergent-insoluble and float on an equilibrium density gradient

When PC12 cells are treated with non-ionic detergent, 30% of bound ¹²⁵I-NGF remains insoluble and associates with the pellet upon centrifugation (Grimes *et al.*, 1997; Grimes *et al.*, 1996). This indicates that NGF, presumably due to its interaction with TrkA and p75^{NTR}, associates with either detergent-insoluble membranes or cytoskeletal elements (Roper *et al.*, 2000). These two types of association can be distinguished by using a flotation equilibrium density gradient, where the sample is mixed with high density medium and layered under the gradient. The cytoskeleton and associated proteins have a high buoyant density (Schechter and Bothwell, 1981; Simons and Toomre, 2000; Wiegant *et al.*, 1986), therefore remain at the bottom of the gradient where the sample was layered. Detergent-resistant membranes, such as membrane rafts, have a low buoyant density due to their high concentration of glycosphingolipids and cholesterol. Therefore proteins associated with membrane rafts usually float to their low density on the gradient (Brown and Rose, 1992; Parton, 1996).

The first objective of this thesis was to investigate if NGF, bound to its receptors TrkA and p75^{NTR}, was associated with detergent-resistant membranes that floated on an equilibrium density gradient. For this, a novel method was devised that incorporated the use of an equilibrium gradient. The cells were permeabilised using a ball homogeniser (cell cracker), which forces the cells through a narrow gap that splits the plasma membrane but does not damage the contents of the cells. During this process the cytosol and small organelles leak out of the cells and the nucleus and large organelles such as the Golgi remain attached to the plasma membranes and cytoskeleton (Grimes and Kelly, 1992; Grimes *et al.*, 1996). Centrifugation was used to separate the small organelles and cytosol (S1) from the permeabilised cells (cell ghosts, P1) containing plasma membranes, cytoskeleton and large organelles (Fig. 6). The cell ghosts were treated with 1% Triton X-100 and the detergent-insoluble material pelleted by centrifugation (pellet=DRM). The detergent-soluble membranes remain in the supernatant (P1M). The DRM sample was brought to 40% iodixanol, sonicated, underlayered beneath a 10-40% iodixanol gradient and centrifuged to equilibrium.

7a



7b

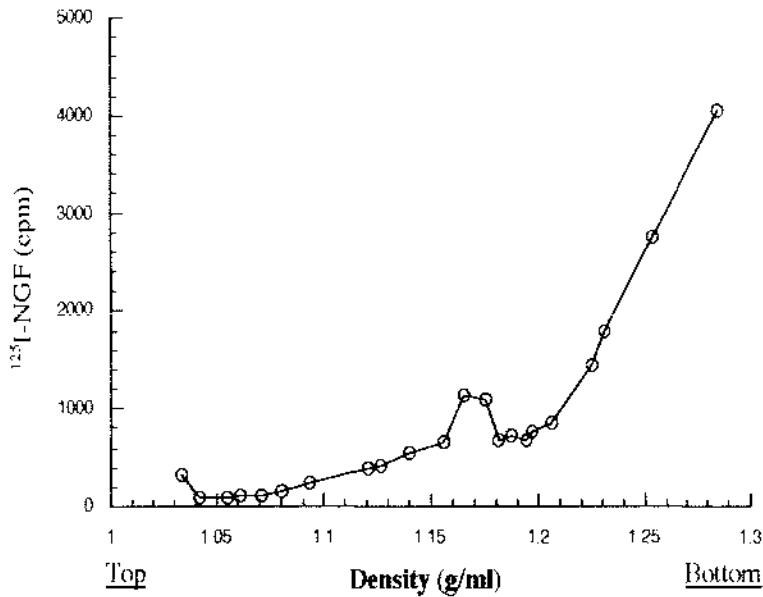


Figure 7: NGF floats on an iodixanol gradient to a density of 1.16-1.17 g/ml.

PC12 cells, incubated with 1 nM ¹²⁵I-NGF at 4°C, were washed, permeabilised and fractionated. The detergent-resistant fraction was made to 40% iodixanol, underlayered beneath a 10-40% iodixanol gradient and centrifuged 16 hrs, 100,000xg. The counts per minute (cpm) emitted by each fraction from the gradient is plotted against fraction number (7a) or density (calculated from the refractive index) of each fraction (7b). The orientation of the gradient is indicated below each graph. Graphs are representative of nine experiments.

Using this procedure approximately 15-20% of ^{125}I -NGF bound to PC12 cells was found to associate with the detergent-resistant fraction (DRM). 10-15% of this DRM fraction floated up the gradient (fractions 11-12, Fig. 7a). Fractions were collected from the bottom of the gradient so that fraction 1 had the highest density and fraction 25, at the top of the gradient, had the lowest density.

The refractive index of each fraction can be used to calculate the density (see section 2.7) due to the chirality of iodixanol. Plotting the radioactivity in each fraction against density (Fig. 7b) allows direct comparison between experiments. The floating peak of NGF was reproducibly found to have a density of 1.16-1.17 g/ml (Fig. 7b). The average density of the NGF peak over nine experiments was 1.16 ± 0.02 g/ml. The NGF at the bottom of the gradient (below fraction 5, Fig. 7a; above 1.2 g/ml, Fig. 7b) did not float or migrate into the gradient beyond what would be expected for diffusion of the sample layered under the gradient, which could be due to NGF that had dissociated from its receptors during the course of the experiment. The majority of free NGF at the bottom of the gradient had probably dissociated from p75^{NTR} as this is the low affinity receptor that has a fast dissociation rate.

The gradient fractions from these experiments were Western blotted and probed with an antibody against TrkA (Fig. 8a). Mature, glycosylated TrkA was detected in the fractions where the NGF peak was observed (as indicated below the blot). TrkA could also be seen in the lower fractions (3-6) of the gradient.

The 110 kDa, immature form of TrkA was predominantly found at the bottom of the gradient, not in the floating fractions (Fig. 8a). In these floating fractions, a single sharp band between the 110 kDa and 140 kDa forms of TrkA was also detected by the TrkA antibody. The identity of this protein is unknown.

Protein signals on the Western blots were quantified (see section 2.13) and plotted as a percentage of the total DRM (% of DRM), (Fig. 8b). In addition to the floating TrkA peak at ~ 1.16 g/ml, there appeared to be another peak of TrkA at the bottom of the gradient, which had a density above 1.2 g/ml. Initially, it was thought that protein at the bottom of the gradient was due to cytoskeletally attached or free protein. However, there appeared to be some movement of TrkA up the gradient, which indicates that, rather than free or cytoskeletally attached protein, TrkA was associated

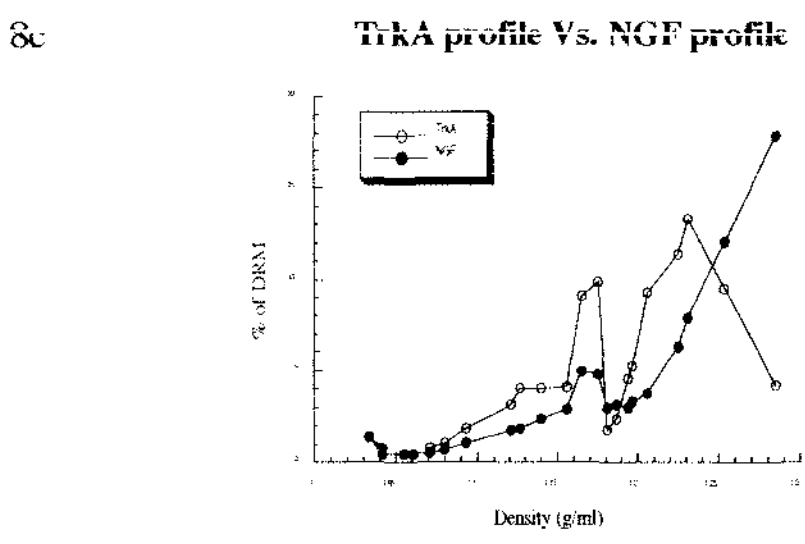
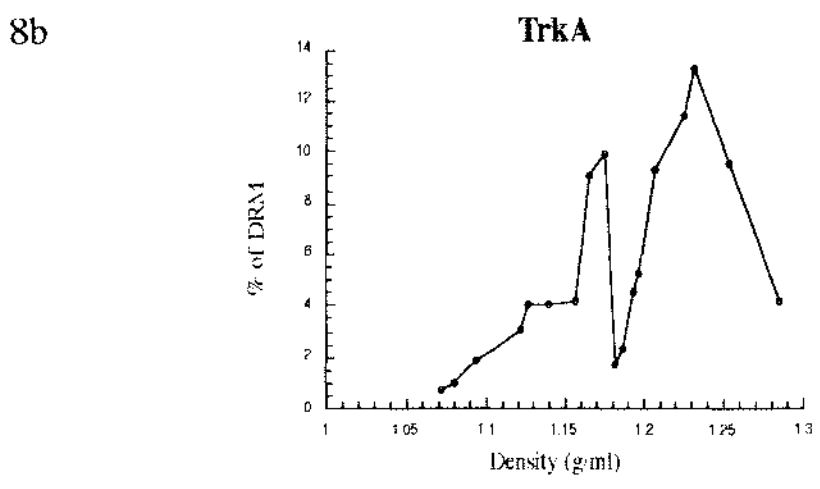
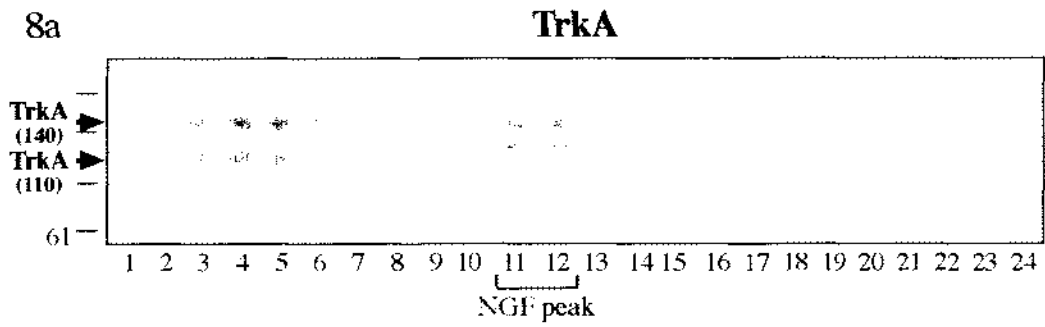


Figure 8: TrkA floats up the gradient to two peaks rather than one.

Gradient fractions from the same experiment as in Fig. 7 were run on a SDS gel, Western blotted and probed with an antibody against TrkA (8a). The position of 140 kDa and 110 kDa TrkA is indicated to the left of the blots. The position of the floating peak of NGF is indicated below the fraction numbers of the blot. 140 kDa TrkA bands are plotted as a percentage of the total in the DRM against the density of each fraction (8b). The TrkA profile is compared with the NGF profile (expressed as a percentage of total TrkA or NGF in the DRM), to observe whether all of the TrkA on the gradient is NGF-bound (8c). The blots and profiles are representative of nine experiments.

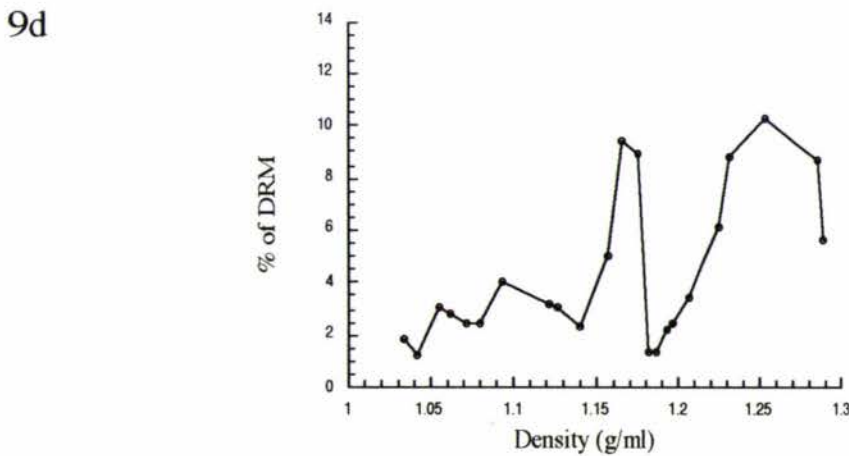
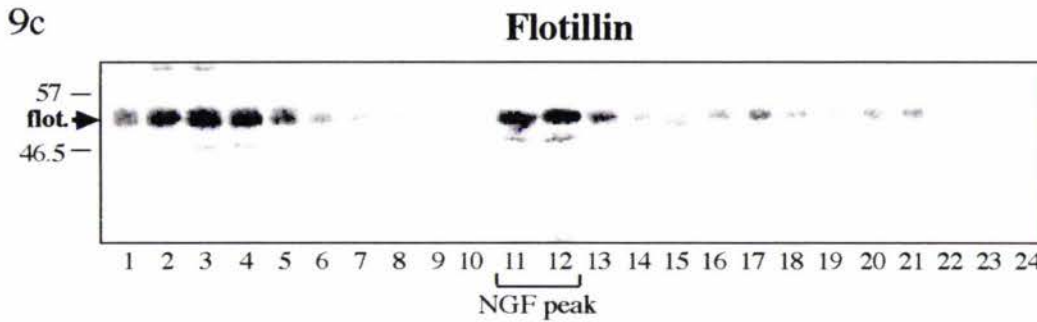
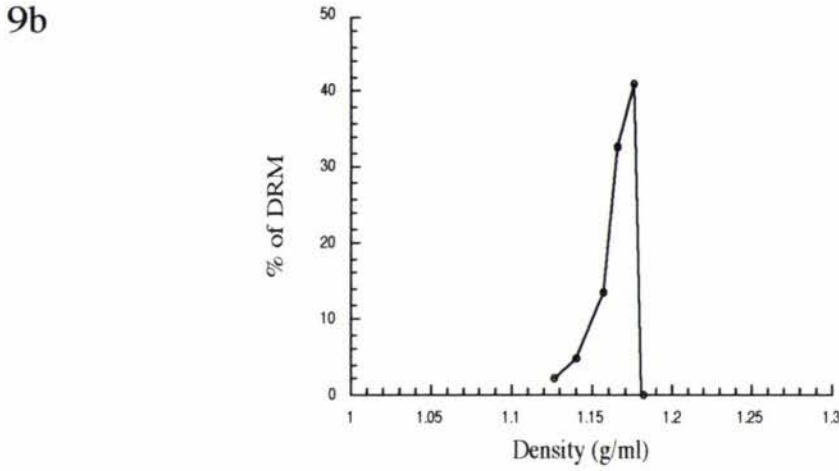
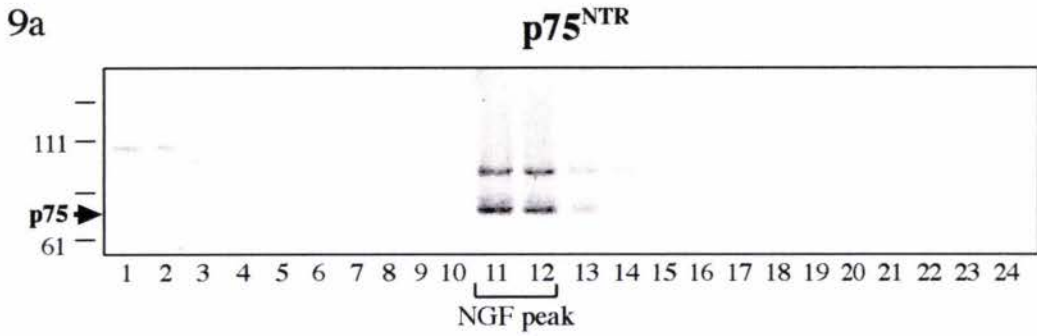


Figure 9: There was one peak of p75^{NTR} and two peaks of flotillin in the gradient.

The Western blot in Fig. 8a was reprobated with antibodies against p75^{NTR} (9a) and flotillin (9c). The position of these proteins is indicated to the left of the blots. The bands were quantified and expressed as a percentage of the total in the DRM for both p75^{NTR} (9b) and flotillin (9d). Blots and profiles are representative of nine experiments.

with something that had a low buoyant density. The presence of the immature, 110 kDa form of TrkA indicated that membranes from the endoplasmic reticulum (ER) may be present in this higher density floating peak. A direct comparison of the NGF counts and the DRM profile of TrkA (Fig. 8c), showed that the TrkA in the floating peak at ~1.16 g/ml was NGF-bound. However, the TrkA in this second, higher density peak (above 1.2 g/ml), did not appear to be NGF-bound, indicating that this mature TrkA had not yet reached the plasma membrane. Therefore this high-density peak may also contain membranes from the Golgi.

The Western blots were probed with an antibody against p75^{NTR} (Fig. 9a) and the membrane raft protein, flotillin (Fig. 9c). Like TrkA, p75^{NTR} was detected in the floating fractions around 1.16 g/ml where the NGF peak was seen. The p75^{NTR} signal was quantified and plotted as a percentage of the total in the DRM (Fig. 9b). In contrast to the two peaks of TrkA, p75^{NTR} was predominantly found in the fractions containing the floating peak of NGF (~1.16 g/ml).

Flotillin has been shown previously to associate with membrane rafts in PC12 cells (Bickel *et al.*, 1997; Volonte *et al.*, 1999). In our experiments, flotillin was detected in the same fractions as NGF, TrkA and p75^{NTR}, in the peak at 1.16-1.17 g/ml (Fig. 9c,d), suggesting that these proteins, which are detergent-resistant and floating, may be associated with membrane rafts in PC12 cells. This peak of proteins around 1.16 g/ml is referred to here as the floating raft peak.

Flotillin was also associated with the high density fractions (above 1.2 g/ml) (Fig. 9d) where the second peak of TrkA was observed. This suggested that this second peak may also consist of membrane rafts, perhaps from intracellular organelle membranes from the ER and Golgi. This second peak is referred to here as the high-density peak.

3.2 Detection of gangliosides in gradient fractions

Gangliosides are one particular marker for membrane rafts, especially monosialoganglioside (GM1) (Huang *et al.*, 1999; Schnitzer *et al.*, 1995). In the previous section, some TrkA and p75^{NTR} were shown to be detergent-insoluble, float to a low density upon equilibrium centrifugation, and co-localise with the membrane raft protein flotillin. To detect gangliosides in these gradient fractions, a lipid extraction of

the fractions and thin layer chromatography was performed. Four gradient fractions around the density of 1.16 g/ml were pooled and the lipids extracted (see section 2.15). This was carried out for four separate gradients and the extracted lipids were pooled. The lipids were spotted on to TLC plates and stained with resorcinol (Fig.10). Resorcinol stains to a blue colour any lipids containing sialic acid. The ganglioside-mix, GM1 and GM2 standards were stained blue with resorcinol and showed the final position that these lipids migrated on the plate. In the lane where the sample was applied, a dark yellow smear was observed (as indicated in Fig. 10) due to the presence of glycolipids that were also extracted during the lipid extraction procedure. A faint, blue-stained ganglioside band was also observed (Fig. 10). This band did not co-migrate with any ganglioside standards used. The Rf values for the unknown band and the standard bands were calculated (Table 1). The unknown blue band migrated somewhere between GD1a and GM1, suggesting that this ganglioside was slightly more polar than GM1 but less polar than GD1. The GD1 ganglioside has two sialic acid residues and these can be attached at different positions. This results in slight differences in polarity. Therefore it was possible that the faint ganglioside observed was a form of GD1.

To investigate the presence of gangliosides in the high-density peak (above 1.2 g/ml), a lipid extraction of four pooled gradient fractions from the bottom of the gradient (fractions 2-6) was carried out as above. The extracted lipids from four separate gradients were pooled, spotted on to TLC plates and the developed plate was sprayed with resorcinol to detect gangliosides. The ganglioside standards appeared as in Fig. 10, however the sample lane appeared completely blank - no faint blue bands or yellow smears due to glycolipids (data not shown). This indicated that there were no detectable gangliosides in these high-density peak fractions.

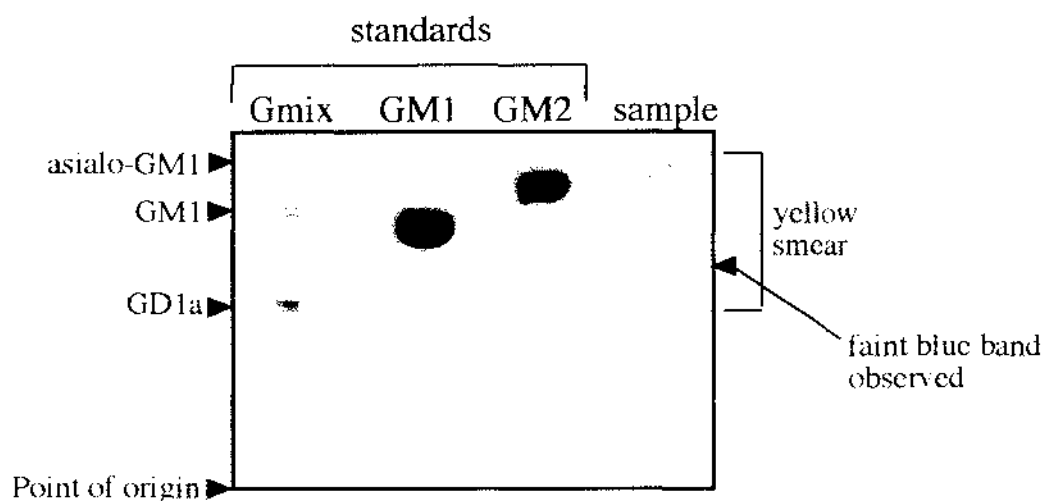


Figure 10: Unknown gangliosides were detected in the floating raft peak by thin layer chromatography.

Lipid extraction was carried out on four pooled gradient fractions from around the density of 1.16 g/ml. This was repeated for four separate gradients, then the resulting lipids were pooled into one sample. The lipid sample was then developed on a glass-backed thin layer chromatography plate along with the standards, ganglioside-mix (Gmix), GM1, and GM2. The plate was stained with resorcinol so all gangliosides present were stained blue. Three blue bands were detected in the Gmix lane, as shown on the far left. GM1 and GM2 standards produced single blue bands. The sample lane contained mainly a dark yellow smear, however a faint blue band could be observed by eye as indicated on the right. This plate is representative of a single experiment.

Table 1: Rf values for standard and unknown ganglioside bands.

		Band	Rf
Standards	Gmix	asialo-GM1	0.714
		GM1	0.623
		GD1	0.481
		GM1	0.627
		GM2	0.693
Samples		yellow smear	0.787
		blue band	0.560

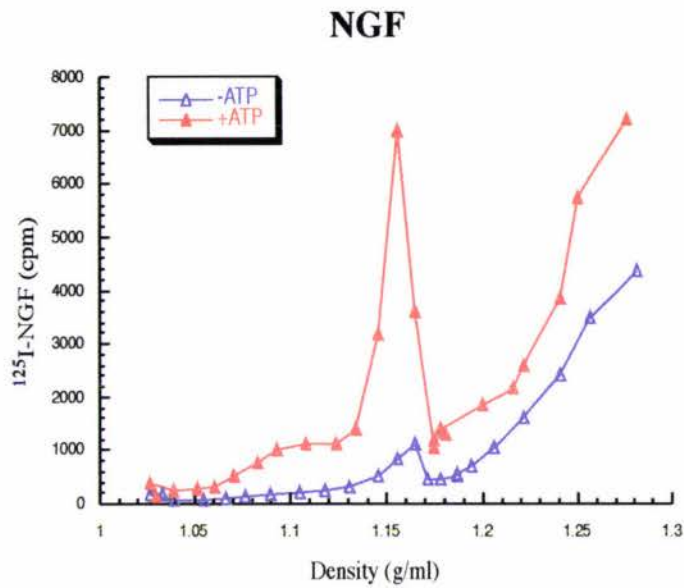
Rf values for the position of both standard and sample bands on the TLC plate in Fig. 10 were calculated. These showed that the faint ganglioside band in the sample lane runs between GD1a and GM1 indicating that it was possibly a form of GD1 ganglioside.

3.3 Effect of *in vitro* reactions with ATP

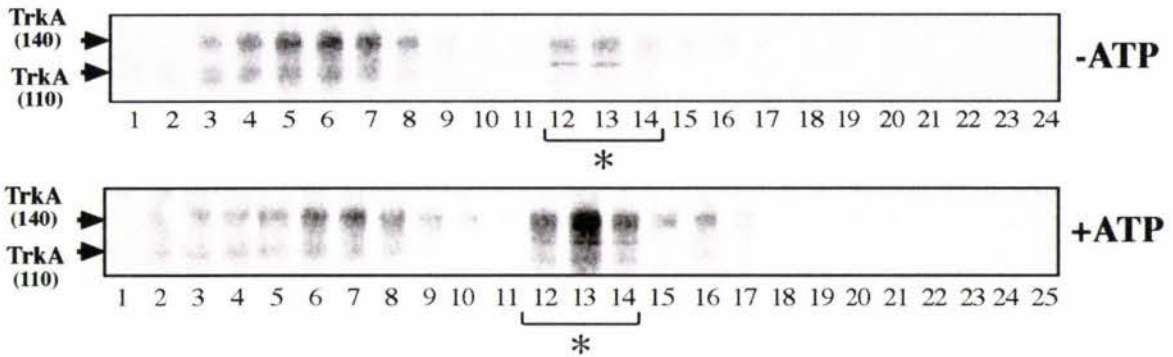
In vitro reactions with an ATP regenerating system involved a 15 minute, 37°C incubation of permeabilised cells. ATP, creatine kinase and creatine phosphate were also added to the incubation to regenerate ATP. This may actually have resulted in 'normalisation' of the lysed cell, restoring ATP to physiological levels and allowing processes that require ATP to occur. To investigate the effect that these reactions may have on the two types of membrane domain that had been isolated, *in vitro* reactions with ATP were carried out after the NGF-bound cells had been permeabilised. Cells that had been subjected to *in vitro* reactions (+ATP), showed a marked increase in the amount of NGF associated with the floating raft peak at ~1.16 g/ml (Fig. 11a). This increase in NGF in the floating raft peak coincided with an increase in TrkA in these same fractions (Fig. 11b). There was only slightly more 110 kDa TrkA in the floating raft peak with the *in vitro* ATP reactions.

The 140 kDa TrkA bands from the gradient and the S1 and P1M fractions were quantified and the amount of TrkA in the gradient fractions was expressed as a percentage of total TrkA in the whole cell (Fig. 11c). The TrkA in the high-density peak (~1.2 g/ml) was slightly decreased with the *in vitro* reactions. There was a very large increase in TrkA association with the floating raft peak. A decrease in the amount of TrkA in the P1M (solubilised membrane) fraction was also observed with the *in vitro*

11a



11b



11c

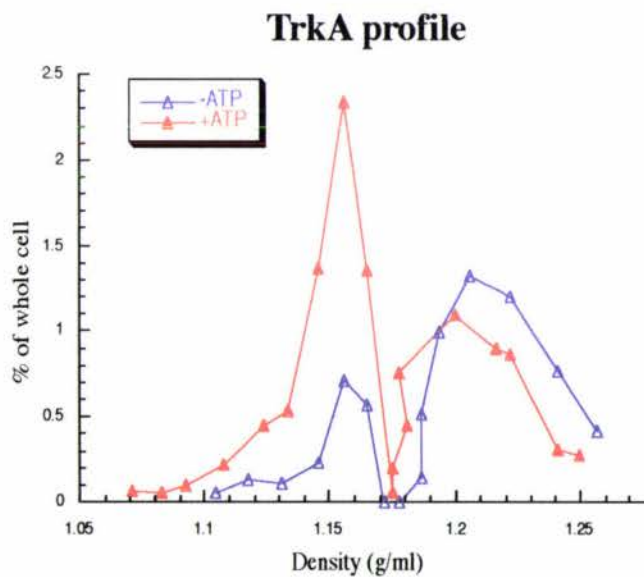


Figure 11: *In vitro* reactions with ATP caused increased NGF and TrkA association with the floating raft peak.

Cells incubated with 1 nM ^{125}I -NGF at 4°C, were washed, permeabilised and fractionated. After permeabilisation, the cracked cells were evenly divided and one sample subjected to an *in vitro* reaction with ATP (+ATP). The detergent-insoluble material was layered beneath a 10-40% iodixanol gradient and centrifuged 16hrs, 100,000xg. Gradient fractions were measured for radioactivity (cpm) and plotted against fraction density for both control '-ATP' and '+ATP' reactions (11a). These fractions were also Western blotted and probed with an antibody against TrkA (11b). The '*' below the fraction numbers of the blots indicates where NGF is in the floating raft peak. The 140 kDa TrkA bands were quantified and expressed as a percentage of the total in the whole cell (11c). Data are representative of nine (-ATP) and five (+ATP) experiments.

reaction. Therefore the increase in TrkA associated with the floating raft peak was probably due to the movement of TrkA from the non-raft regions of the plasma membrane (P1M) to the detergent-insoluble membrane rafts (DRM) with the *in vitro* ATP reaction. Also, the slight decrease observed in the high-density peak indicated that there may have been a small amount of movement from these high-density intracellular membranes to the lower density membrane rafts in the floating raft peak.

The Western blots were reprobed with antibodies against p75^{NTR} (Fig. 12a) and flotillin (Fig. 12b). The proteins signals were quantified and plotted as a percentage of the total in the whole cell (Fig. 12c). This showed that there was a single peak of p75^{NTR} at ~1.16 g/ml and two peaks of flotillin, both with and without the *in vitro* ATP reactions.

The percentage of total whole cell NGF, TrkA, p75^{NTR} and flotillin that were associated with the floating raft peak was calculated. There was a 4.5-fold increase in NGF ($p < 0.001$) and a 4-fold increase in TrkA ($p < 0.01$) in the floating raft peak with the *in vitro* ATP reactions (Fig. 13a). In contrast, the percentage of whole cell p75^{NTR} in the floating raft peak was decreased by ~10% ($p < 0.2$) after *in vitro* reactions (Fig. 13b). Flotillin was not significantly affected (Fig. 13b).

Interestingly, the percentage of total DRM p75^{NTR} that was in the floating raft peak was not significantly affected by this *in vitro* ATP reaction (Fig. 13c). This suggested that there must have been a movement of p75^{NTR} out of the detergent-insoluble membrane rafts and into another region of the cell with the *in vitro* reaction. The amount of p75^{NTR} in the P1M fractions of the cell was also measured. There was an increase in the amount of p75^{NTR} in the P1M fractions with the *in vitro* ATP reaction, indicating that p75^{NTR} was moving out of the membrane rafts into the non-raft regions of the plasma membrane (P1M).

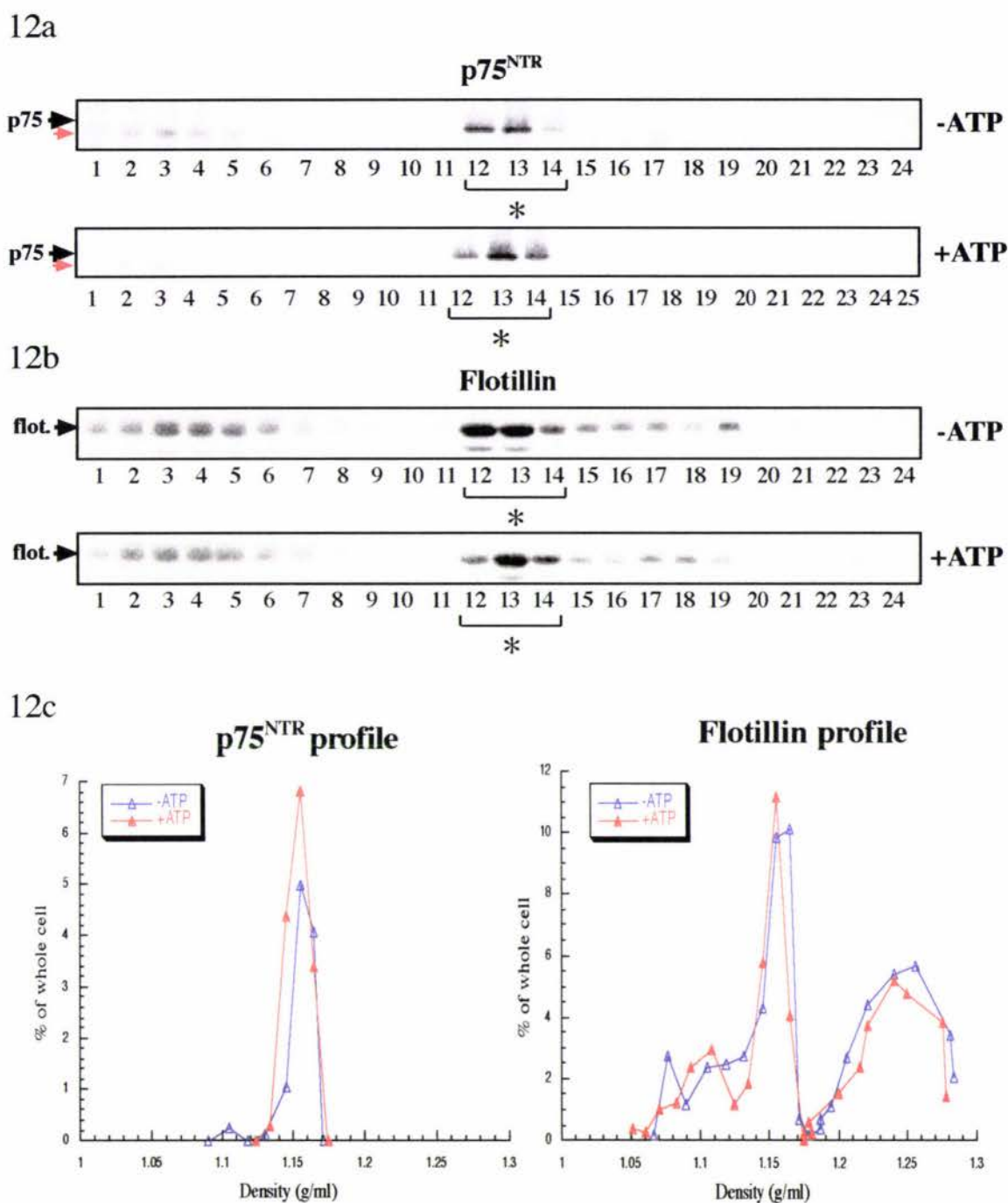


Figure 12: Flotillin did not change but p75^{NTR} appeared increased in the floating raft peak with an *in vitro* ATP reaction.

The Western blots in Fig. 11b were reprobbed with antibodies against either p75^{NTR} (12a) or flotillin (12b). The red arrows to the left of the p75^{NTR} blots indicate an unknown protein band. The ‘*’ below the fraction numbers indicates where NGF is associated with the floating raft peak. Fig. 12c shows the quantification of both the p75^{NTR} and flotillin bands, expressed as a percentage of the total in the whole cell. Data are representative of nine (-ATP) and five (+ATP) experiments.

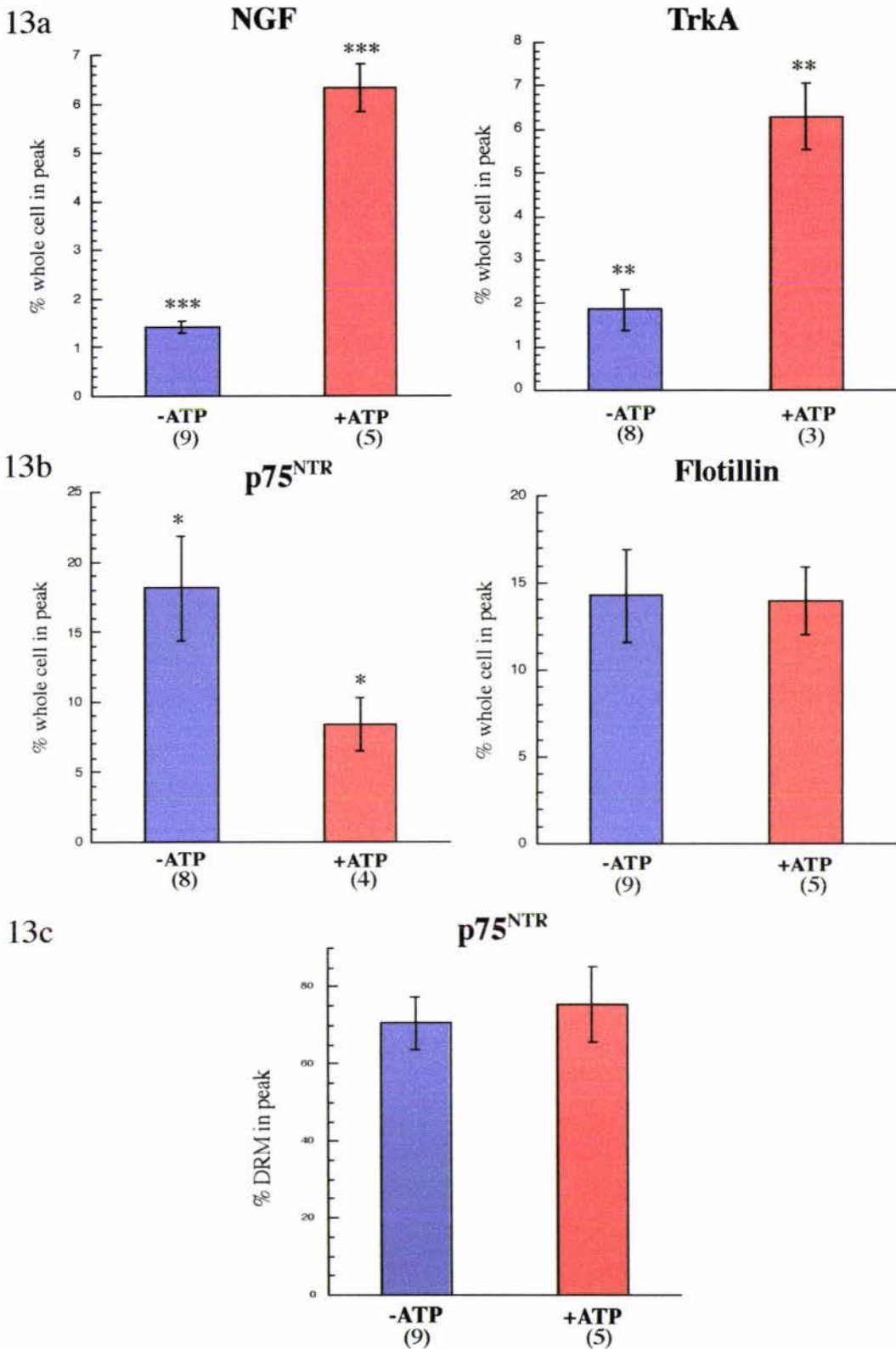


Figure 13: NGF, TrkA and p75^{NTR} association with the floating raft peak was affected by an *in vitro* ATP reaction. Flotillin was not affected.

The proteins on Western blots in Fig. 11 and 12 were quantified. The percentage of whole cell protein associating with the floating raft peak was determined for each protein (13a,b). Also, the percentage of total whole cell NGF that was in the floating raft peak was determined (13a). The amount of p75^{NTR} in the floating raft peak was also plotted as a percentage of the total in the DRM (13c). * indicates $p < 0.2$, ** $p < 0.01$ and *** $p < 0.001$. The number of experiments used for these calculations are indicated below the bars of each graph.

3.4 Other signalling proteins in the gradient

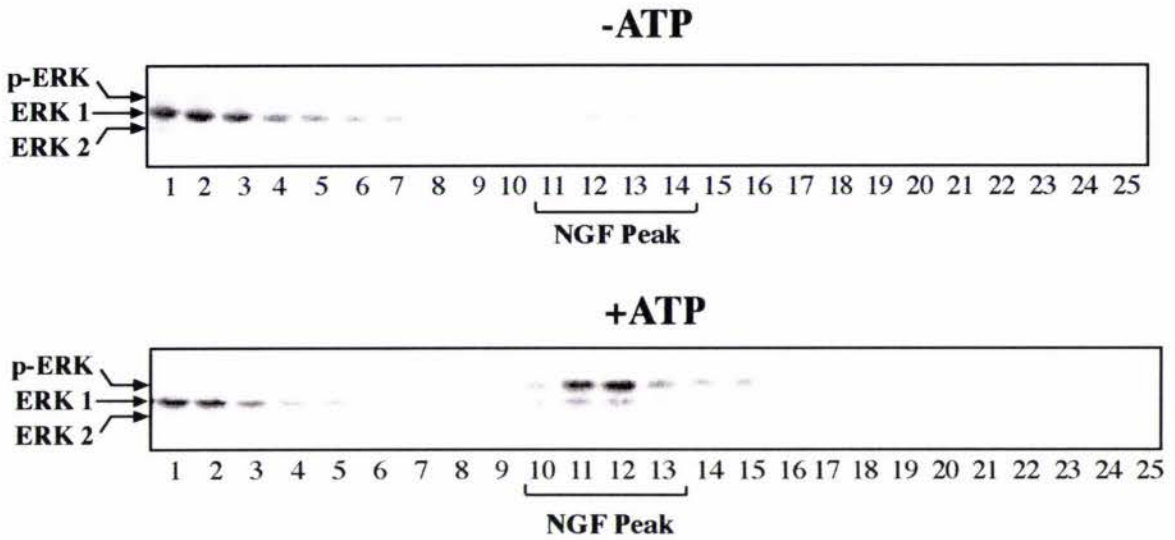
3.4.1 ERK

Membrane rafts are thought to be sites for bringing together signalling proteins, so we investigated the membrane raft association of ERK, a signalling protein downstream from TrkA. The Western blots from an experiment with or without *in vitro* reactions with ATP were probed with an antibody against ERK (Fig. 14a). This antibody detected three forms of ERK, hyperphosphorylated ERK1 (phospho-ERK), ERK1 and ERK2. Without the *in vitro* ATP reaction, the majority of ERK1 was found in fractions 1-4 at the bottom of the gradient (Fig. 14a). A small amount was also spread throughout the gradient and in the fractions corresponding to the floating raft peak (as indicated below the blots). Very faint amounts of phospho-ERK were detected throughout the gradient without the *in vitro* ATP reaction (-ATP). After *in vitro* reactions with ATP, there was an increase in ERK1 association with the floating raft peak and a very large increase in phospho-ERK in these fractions. Very small amounts of ERK2 were found throughout the gradient both with and without the ATP reaction, however, very little was detected in the floating raft peak.

The phospho-ERK and ERK1 bands were quantified and plotted as a percentage of the total of each in the whole cell (Fig. 14b). Very little phospho-ERK was found at the bottom of the gradient (highest density) and it was difficult to observe whether it was associated with the high-density peak or whether it was free protein. A marked increase in phospho-ERK association with the floating raft peak was seen after *in vitro* reactions with ATP, suggesting that upstream kinases were active during the reaction and phospho-ERK became associated with the detergent-insoluble membrane rafts.

In the ERK1 profile (Fig. 14b), both with and without the *in vitro* reaction, there was more ERK1 at the bottom of the gradient than in the floating raft peak fractions. ERK1 did not appear to be associated with the high-density peak. Its distribution was similar to that of NGF at the bottom of the gradient (Fig. 7), suggesting that it may be free protein, or possibly associated with the high density cytoskeleton. With the *in vitro* ATP reaction, there was an increase in the amount of ERK1 associated with the floating

14a



14b

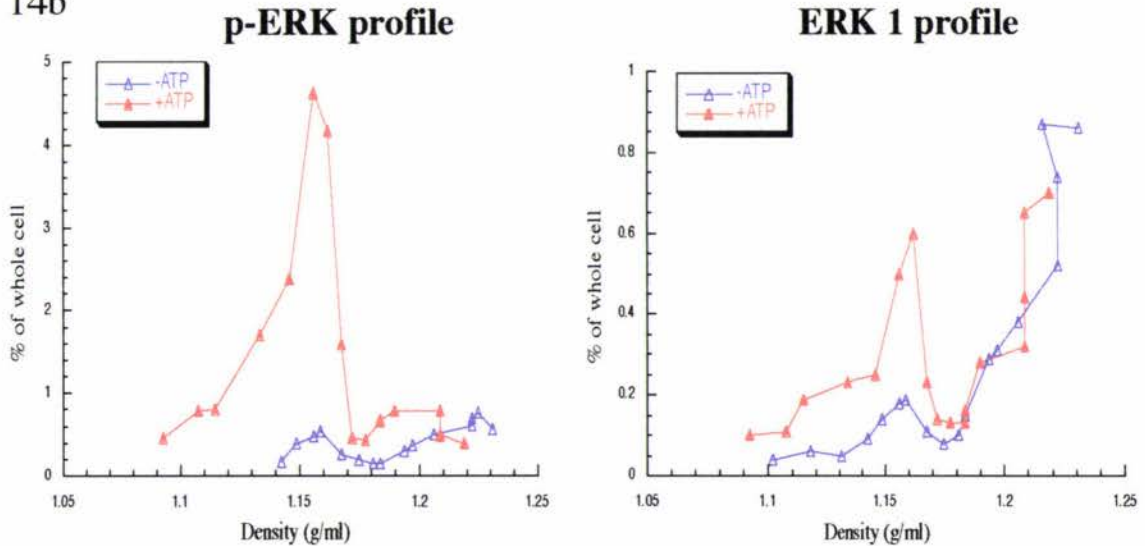


Figure 14: Phospho-ERK and ERK1 were increased in the floating raft peak with an *in vitro* ATP reaction.

Cells incubated with NGF were permeabilised and either retained on ice (-ATP) or subjected to an *in vitro* reaction with ATP for 15 minutes at 37°C (+ATP). The cells were fractionated and detergent-insoluble material centrifuged to equilibrium in a continuous iodixanol gradient as in Fig. 7. The collected fractions were Western blotted and probed with an antibody against ERK (14a). This antibody detected hyperphosphorylated ERK1 (p-ERK) at ~46 kDa, ERK1 at 44 kDa and ERK2 at 42 kDa as indicated to the left of the blots. The position of NGF in the floating raft peak is indicated below the fraction numbers for each blot. The p-ERK and ERK1 bands were quantified and plotted as a percentage of the total p-ERK and ERK1 in the whole cell (14b). Data are representative of nine (-ATP) and five (+ATP) experiments.

raft peak, however this was not as pronounced as the effect observed with phospho-ERK.

The total amount of ERK1 in the S1 and P1M was measured and a decrease was observed in the amount of ERK1 in the P1M fraction after *in vitro* reactions, suggesting that ERK1 was becoming associated with the detergent-insoluble membrane rafts under these conditions.

3.4.2 Tubulin

Previous work has demonstrated that ERK is associated with microtubules in PC12 cells (Morishima-Kawashima and Kosik, 1996), so Western blots from Fig. 14a were reprobbed with an antibody against α -tubulin (Fig. 15a). Without the *in vitro* ATP reaction, the majority of tubulin was found in fractions 1-5 at the bottom of the gradient. There was also a small amount of tubulin in the fractions where the floating raft peak was observed (as indicated below the blots). The tubulin bands were quantified and plotted against density (Fig. 15b). The tubulin in the lower fractions of the gradient appeared to be associated with the high-density peak, where both TrkA and flotillin were also found. With the *in vitro* ATP reaction, there was a marked redistribution of tubulin from this high-density peak to the lower density, floating raft peak. Although both TrkA and flotillin had been found in this high-density peak, only tubulin showed this redistribution from the high-density peak to the floating raft peak with the *in vitro* ATP reaction.

The percentage of whole cell tubulin associated with either the high-density peak or the floating raft peak was calculated (Fig. 15c). With the *in vitro* ATP reaction there was a 9-fold increase ($p < 0.001$) in the amount of tubulin associated with the floating raft peak. A 2.3-fold decrease in tubulin was observed in the high-density peak with the ATP reaction. The decrease in the amount of tubulin in the high-density peak did not seem to correspond with the increase in tubulin in the floating raft peak. There was little change observed in the amount of tubulin in the P1M with ATP. This indicated that tubulin may polymerise during the *in vitro* reactions with ATP, and these microtubules associate with membrane rafts in the plasma membrane. There also may be a shift of microtubules from the high-density peak with the *in vitro* reactions.

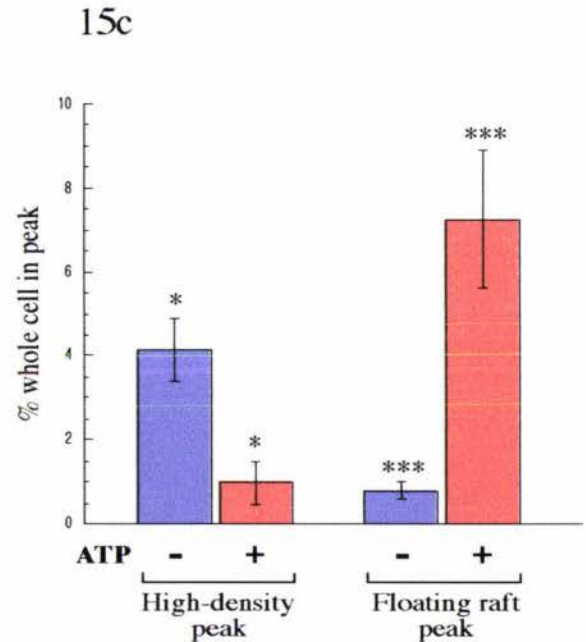
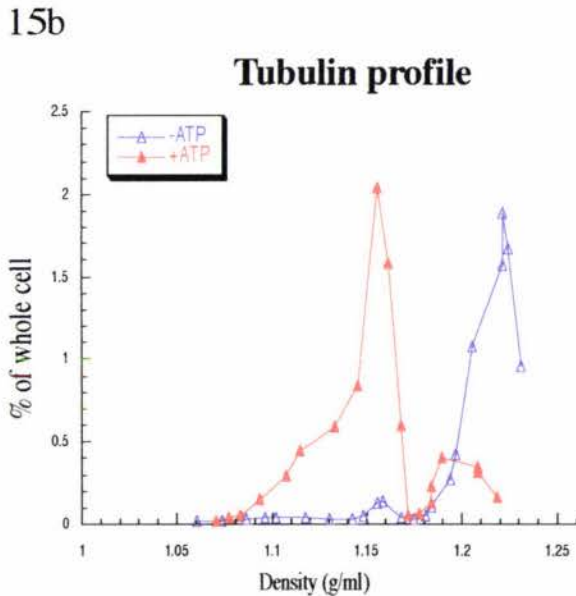
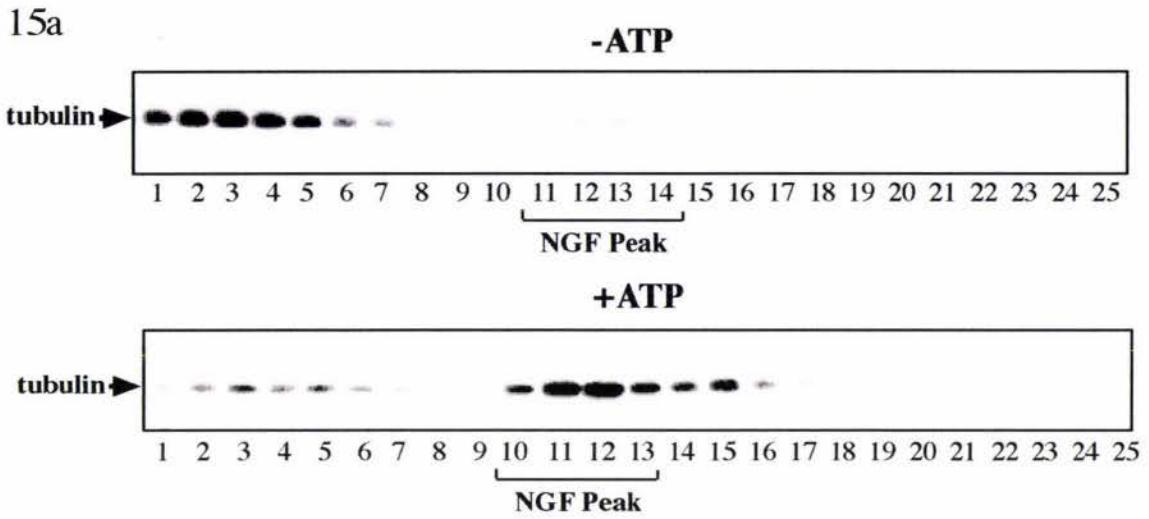


Figure 15: Tubulin has increased co-localisation with the floating raft peak with an *in vitro* ATP reaction.

The Western blots in Fig. 14a were reprobed with an antibody against α -tubulin (15a). The position of tubulin (55 kDa) is indicated to the left of the blots, also the position of NGF in the floating raft peak is noted below the fraction numbers. The tubulin bands were quantified and plotted as a percentage of the total tubulin in the whole cell (15b). The percentage of whole cell tubulin that was found in either the floating raft peak (~1.16 g/ml) or the high-density peak (above 1.2 g/ml) was plotted (15c). * indicates $p < 0.1$, *** indicates $p < 0.001$. Data are representative of, and the graphs are an average of nine (-ATP) or five (+ATP) experiments. The high-density peak data in Fig. 15c are an average of six (-ATP) and three (+ATP) experiments.

3.5 Internalisation of receptors

3.5.1 2, 10 or 30 minutes internalisation

When a cell is warmed to 37°C, NGF-bound receptors are normally internalised into clathrin-coated vesicles, endosomes and endosome-derived signalling vesicles (Grimes *et al.*, 1997; Reynolds *et al.*, 1998). To investigate the effect of internalisation on TrkA and p75^{NTR} association with membrane rafts, the cells were bound to NGF, washed, then incubated for 2, 10, or 30 minutes at 37°C, or maintained on ice (0 min). The cells were permeabilised, fractionated and the detergent-insoluble material separated on an equilibrium gradient. The radioactivity in each fraction was measured and plotted against density for each incubation time period (Fig. 16a). There was very little difference in the NGF profiles with no internalisation or, 2, 10, or 30 minutes internalisation. Each of the floating raft peaks had approximately 300 cpm of radioactivity at their highest point.

The average floating raft peak density for each internalisation period was calculated (Fig. 16b). There was a slight increase in the peak's density at 2 minutes internalisation compared with no internalisation (0). The transient increase in the peak's density seen with 2 and 10 minutes internalisation, appeared to be abolished by 30 minutes.

Like NGF association with the floating raft peak with different internalisation times, there was very little difference in the amounts of TrkA and p75^{NTR} associated with the floating raft peak with time of internalisation. The degree of association of TrkA, flotillin and tubulin with the high-density peak was also not affected by any of the internalisation times. It appeared that internalisation of NGF-bound receptors had little effect on the amounts of TrkA and p75^{NTR} associated with the two types of detergent-resistant membrane domains that had been isolated.

3.5.2 10 minutes internalisation with an *in vitro* ATP reaction

10 minutes of internalisation at 37°C was found to be the optimum time to study TrkA internalisation into signalling vesicles, whose production was enhanced by *in vitro* reactions with ATP (Grimes *et al.*, 1996). Western blots of cells with 0 or 10 minutes

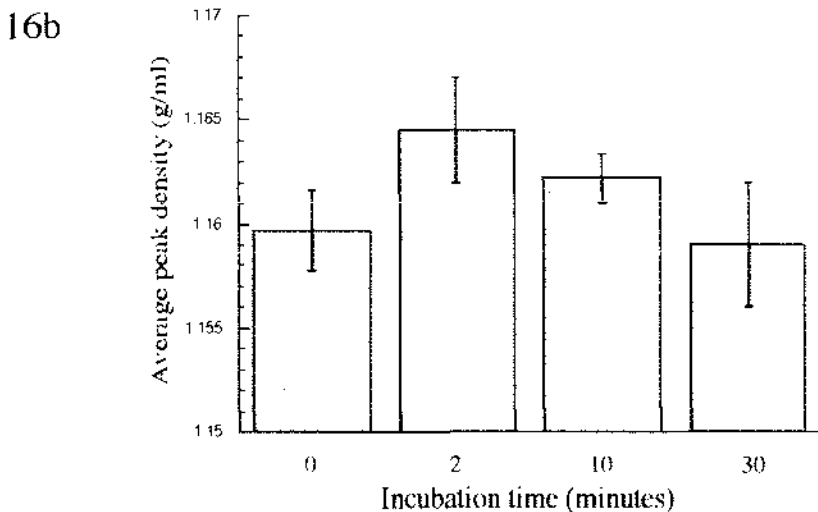
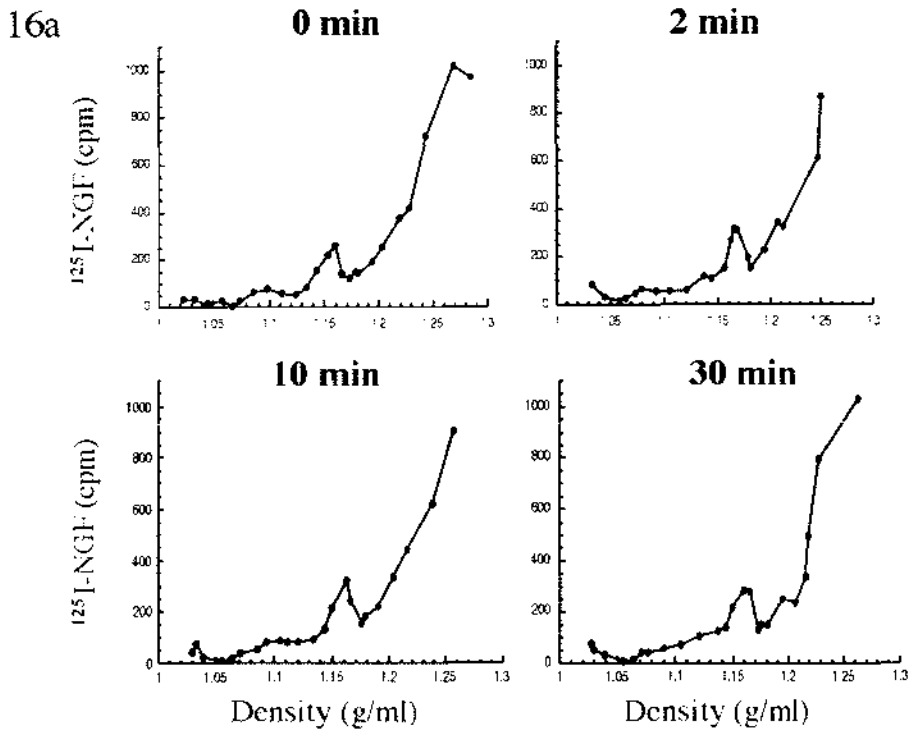


Figure 16: Internalisation of receptors after NGF binding had a slight effect on the average density of the floating raft peak.

Directly after $^{125}\text{I-NGF}$ binding, cells were washed and warmed to 37°C , for either 2, 10, or 30 minutes, to allow internalisation of NGF-bound receptors. They were then chilled rapidly in ice-water. One sample (0 min) was kept on ice during this process. The cells were permeabilised, fractionated, and the detergent-insoluble material separated on a gradient. The radioactivity of each fraction was determined and plotted against the density of each fraction (16a). From these NGF profiles the average density of the floating raft peak could be calculated (16b). No p-value could be calculated for the difference between 0 and 2 min internalisation as there were only two data points for the 2 min incubation. Data are representative (16a), or an average (16b) of nine (0 min), six (10 min), or two (2, 30 min) experiments.

internalisation at 37°C, with or without *in vitro* reactions with ATP, were probed with an antibody against TrkA (Fig. 17a). The blots for 10 minutes internalisation can be compared with those of no internalisation in Fig. 11b. TrkA association with the floating raft peak (fractions 12-14) after *in vitro* ATP reactions and 10 minutes internalisation (Fig. 17a) was similar to the increase observed in TrkA association with no internalisation (Fig. 11b).

The amount of TrkA in the whole cell that was associated with the floating raft peak was calculated (Fig. 17b). Without internalisation there was a 4-fold increase ($p < 0.01$) in TrkA association with the floating raft peak after *in vitro* ATP reactions. With 10 minutes internalisation the effect observed with the ATP reaction was reduced to a 3-fold increase ($p < 0.01$). The percentage of whole cell flotillin associated with the floating raft peak was also calculated (Fig. 17c). There was no significant difference in the amount of flotillin in the floating raft peak under any of the conditions used.

In section 3.3 it was shown that there was a 10% decrease in the amount of whole cell p75^{NTR} associated with the floating raft peak with the *in vitro* ATP reaction. After 10 minutes internalisation, this effect was enhanced; there was a 14% decrease in p75^{NTR} associated with the floating raft peak (data not shown).

An effect of 10 minutes internalisation at 37°C was observed for tubulin (Fig. 18a). Without internalisation or *in vitro* ATP reactions, there was a small amount of tubulin associated with the floating raft peak. This association was increased 9-fold with the *in vitro* ATP reaction ($p < 0.001$). However, with 10 minutes internalisation, this was reduced to a 6-fold increase in the amount of tubulin associated with the floating raft peak with ATP ($p < 0.001$).

The Western blots were also probed with an antibody against ERK. 10 minutes internalisation had little effect on the association of phospho-ERK with the floating raft peak after *in vitro* ATP reactions (Fig. 18b). However, there appeared to be slightly less than half the amount of phospho-ERK associated with the floating raft peak after 10 minutes internalisation and no *in vitro* ATP reactions.

The percentage of whole cell ERK1 associated with the floating raft peak was also calculated (Fig. 18c). Like phospho-ERK, without the *in vitro* ATP reaction, there was

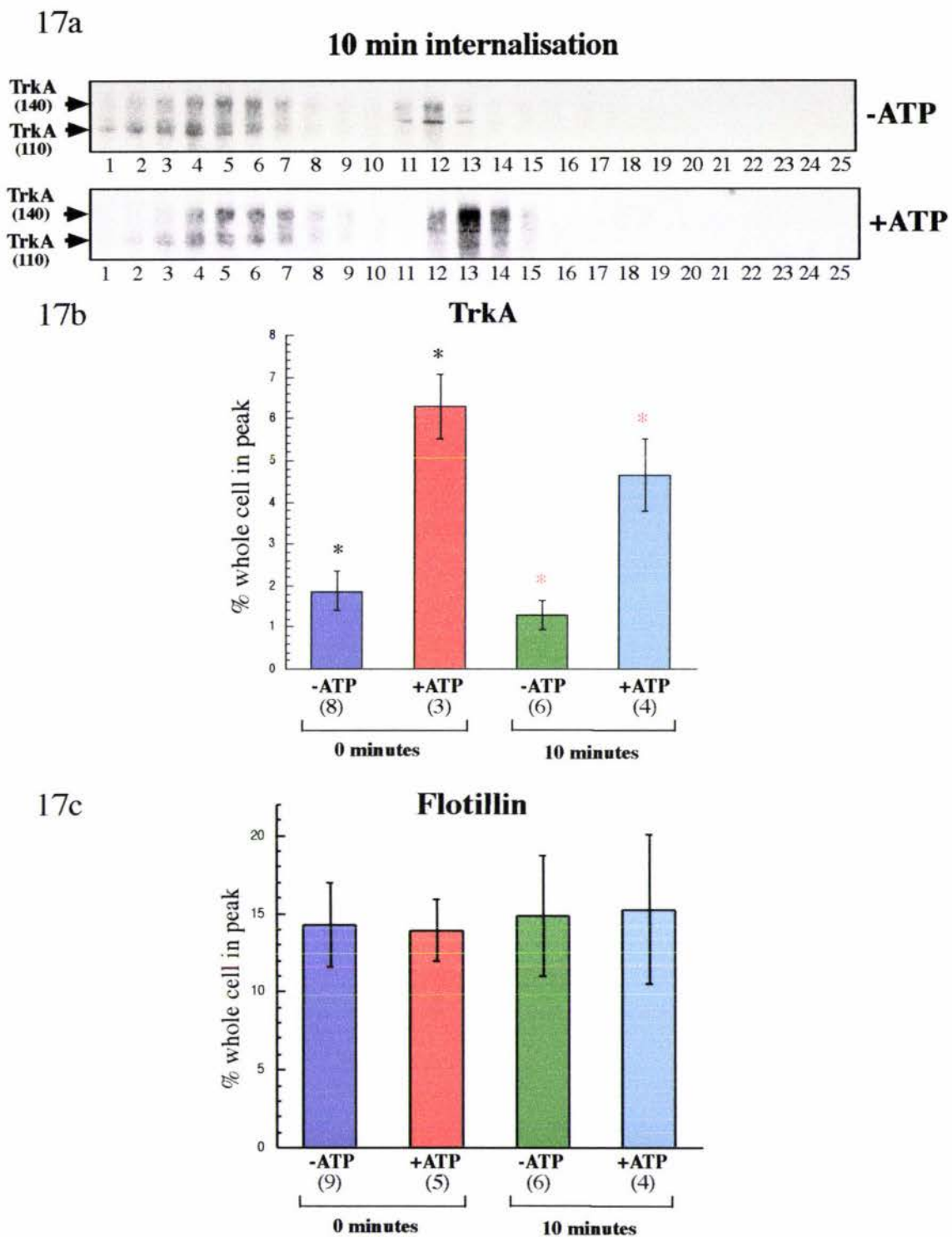


Figure 17: 10 minutes internalisation with an *in vitro* ATP reaction had a small effect on the association of TrkA with the floating raft peak but had no effect on flotillin.

After NGF binding, cells were either warmed to 37°C for 10 minutes or kept on ice (0 min). The cells were permeabilised and either subjected to an *in vitro* reaction with ATP (+ATP) or maintained on ice (-ATP). After fractionation and equilibrium gradient centrifugation, gradient fractions were Western blotted and probed with an antibody against TrkA (17a). These blots can be compared with TrkA blots under the same conditions but with no internalisation in Fig. 11b. The TrkA bands in this blot, and also flotillin bands from a subsequent reprobe, were quantified. The percentage of whole cell TrkA (17b) and flotillin (17c) that were associated with the floating raft peak was calculated and compared with the results for no internalisation. * and * indicate $p < 0.01$. The blots are representative of nine (0min, -ATP), five (0min, +ATP), six (10min, -ATP) and four (10min, +ATP) experiments. The graphs are an average of the number of experiments noted below each bar.

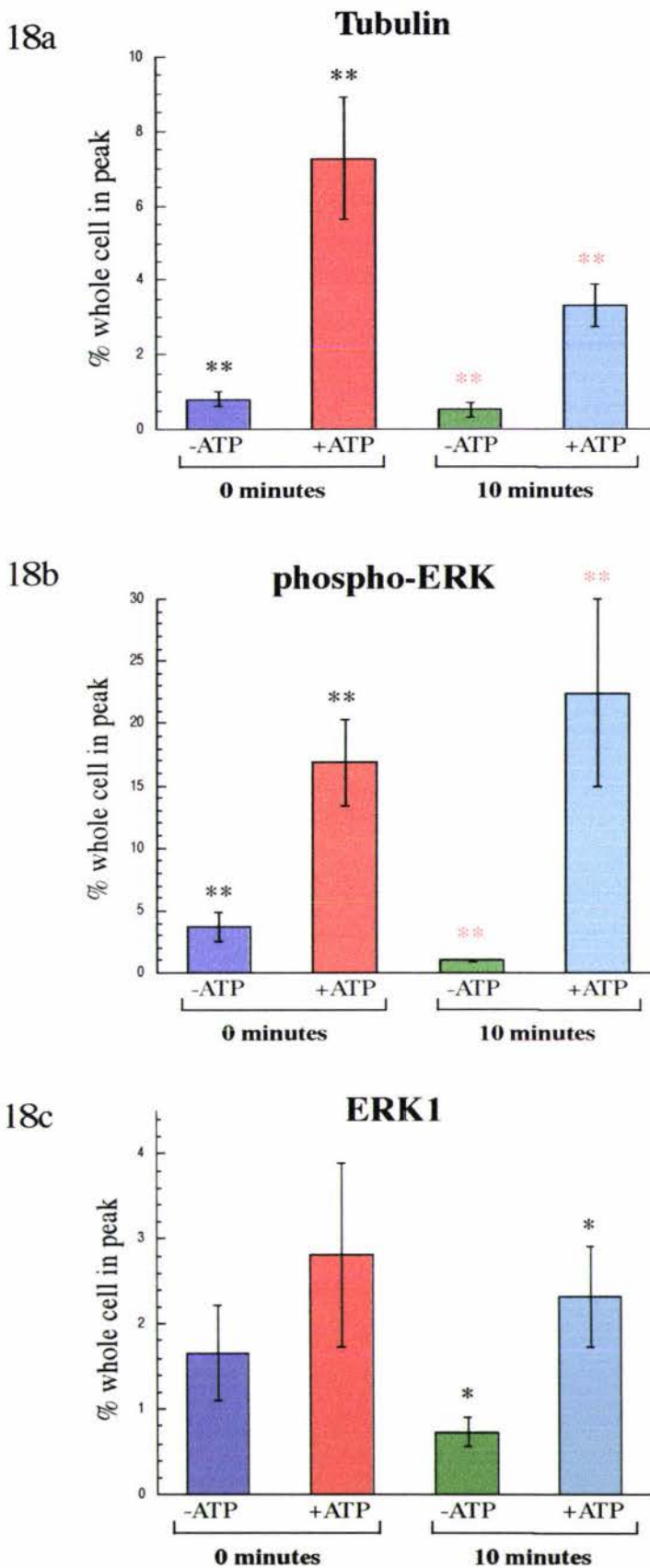


Figure 18: 10 minutes internalisation had an effect on tubulin, phospho-ERK and ERK1 association with the floating raft peak.

Western blots from Fig. 17a were reprobbed with antibodies against tubulin and ERK. The tubulin (18a), phospho-ERK (18b) and ERK1 (18c) bands were quantified and plotted as a percentage of the whole cell that was associated with the floating raft peak. ** and * indicate $p < 0.001$, $p < 0.01$. Data are an average of nine (0min, -ATP), five (0min, +ATP), six (10min, -ATP) and four (10min, +ATP) experiments.

a decrease in the amount of ERK1 in the floating raft peak with 10 minutes internalisation. Again, there was no difference in ERK1 association with the floating raft peak with no or 10 minutes internalisation and *in vitro* reactions with ATP.

Therefore, with 10 minutes internalisation and *in vitro* reactions with ATP, there was a reduction in tubulin association with the floating raft peak. However, for both phospho-ERK and ERK1, only with no *in vitro* reactions was there decreased raft-association after 10 minutes internalisation.

3.6 Effect of NGF binding on TrkA and p75^{NTR} association with membrane rafts

Previously, addition of ligand to cells has been found to cause clustering of membrane rafts and their associated proteins (Viola *et al.*, 1999). This effect is believed to have a role in enhancing the signalling of this receptor by clustering the activated receptors into domains containing the downstream signalling proteins (Ahmed *et al.*, 1997; Liu *et al.*, 1997). To investigate whether NGF binding has this clustering effect on TrkA and p75^{NTR}, cells were incubated with or without ¹²⁵I-NGF, washed and divided into two samples, one was left on ice (0 min), the other was incubated for 10 minutes at 37°C to allow internalisation of receptors. Cells were then permeabilised and the incubated sample divided and subjected to an *in vitro* reaction with ATP or left on ice.

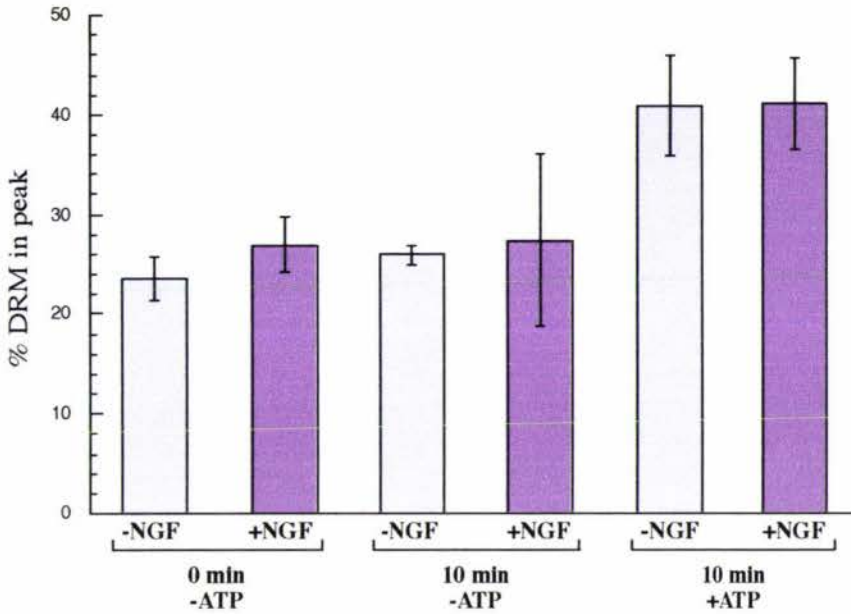
3.6.1 TrkA

There was no significant difference in the amount of DRM TrkA in the floating raft peak with or without NGF for any of the conditions used (Fig. 19a). Indicating that there was no redistribution of TrkA from the high-density peak to the floating raft peak with NGF binding.

The percentage of whole cell TrkA associated with the floating raft peak showed slightly different results (Fig. 19b). Again there was no significant difference either with or without NGF when there was no *in vitro* reaction. However, with this reaction and 10 minutes internalisation, there was 2-fold more TrkA in the floating raft peak when NGF was bound to the cells ($p < 0.1$). This indicated that the amount of TrkA in the whole cell that was detergent-insoluble and associated with the floating raft peak

TrkA

19a



19b

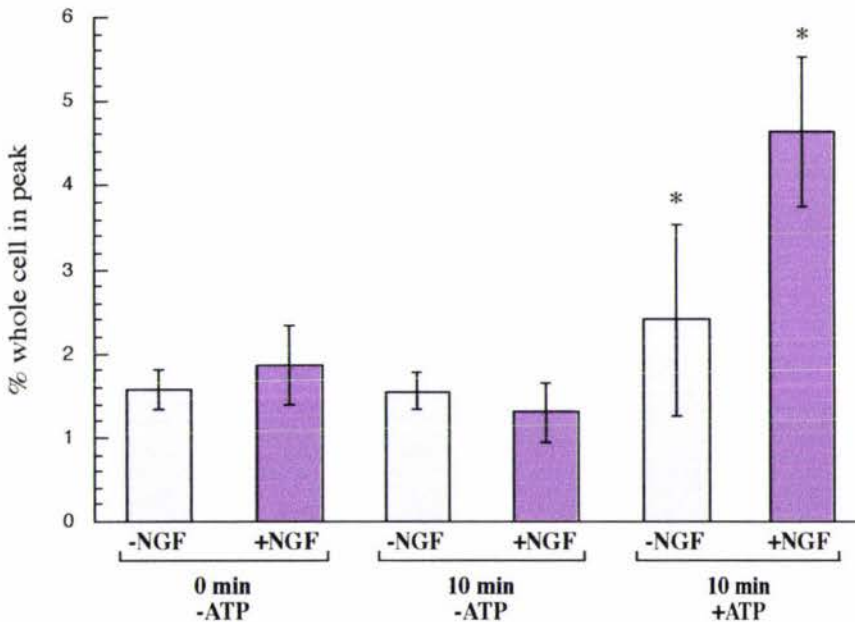


Figure 19: With *in vitro* reactions, the percentage of TrkA in the whole cell that was associated with the floating raft peak was affected by NGF binding.

Harvested cells were divided and one sample incubated with ^{125}I -NGF at 4°C (+NGF), the other was incubated without NGF (-NGF). Cells were washed, incubated at 37°C for 10 minutes, or left on ice (0 min) and permeabilised. The 10 minute samples were either left on ice (-ATP) or subjected to an *in vitro* ATP reaction (+ATP). The detergent-insoluble material was separated on an equilibrium gradient and gradient fractions were Western blotted and probed with an antibody against TrkA. The TrkA bands were quantified and plotted as a percentage of TrkA from the DRM (19a) or the whole cell (19b) that was in the floating raft peak at ~ 1.16 g/ml. * indicates $p < 0.1$. Data are an average of two experiments (all -NGF data), or nine (0min, -ATP), six (10min, -ATP) and four (10min, +ATP) experiments for the +NGF data.

was increased with NGF binding and *in vitro* ATP reactions. Under these conditions, NGF must be causing TrkA to move from the non-raft regions of the plasma membrane to the detergent-insoluble membrane rafts in the floating raft peak, but only after *in vitro* reactions.

With NGF having this effect on TrkA, the next step was to investigate the effect that NGF binding had on the raft association of p75^{NTR}, the low affinity NGF receptor.

3.6.2 p75^{NTR}

The Western blots from the NGF binding experiments were probed with an antibody against p75^{NTR} (Fig. 20a). Only the fractions showing p75^{NTR} association with the floating raft peak are presented. The percentage of whole cell p75^{NTR} in the floating raft peak was calculated (Fig. 20b). With no internalisation or 10 minutes internalisation with no *in vitro* reactions, there was no significant difference in the amount of p75^{NTR} associated with the floating raft peak. With 10 minutes internalisation and *in vitro* reactions with ATP, there was a decrease in p75^{NTR} with NGF binding. This is similar to the raft association of TrkA, where 10 minutes internalisation and an *in vitro* reaction were required to see an effect of NGF binding. However, with TrkA there was an increase in raft-association with NGF binding, whereas with p75^{NTR} a decrease was observed.

To investigate the effect of NGF binding on the integrity of the membrane rafts in this floating raft peak, the gradient distribution of the membrane raft protein, flotillin was analysed.

3.6.3 Flotillin

The main difference in the flotillin blots and percentage of whole cell profiles (Fig. 21a,b,c) was that with NGF binding there appeared to be a slight increase in the density of the floating raft peak. Also, with no internalisation (Fig. 21a) or 10 minutes internalisation and *in vitro* reactions (Fig. 21c), there appeared to be an increase in flotillin association with the high-density peak (above 1.2 g/ml) with NGF binding. The percentage of whole cell flotillin that was associated with the floating raft peak was

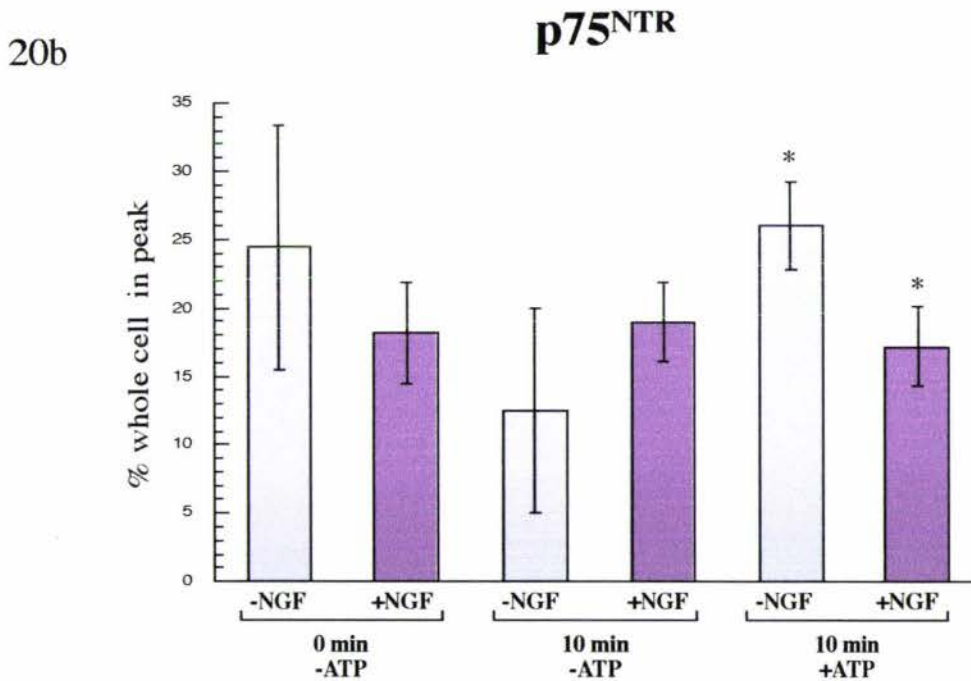
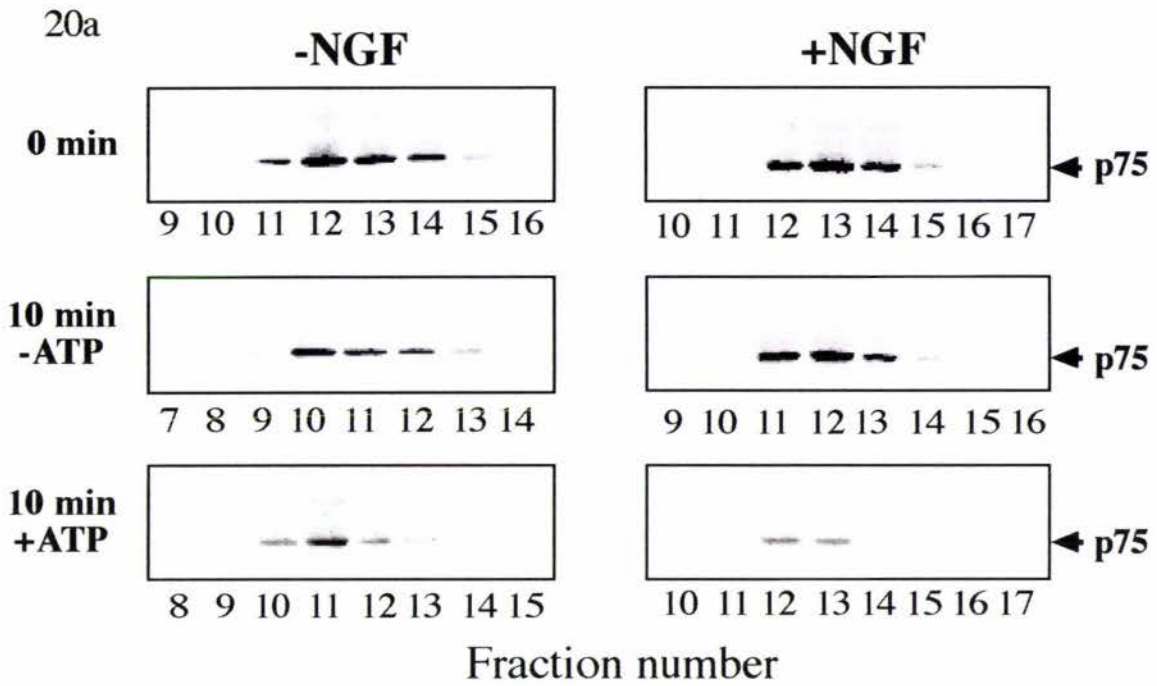


Figure 20: With the *in vitro* ATP reaction, the percentage of whole cell p75^{NTR} in the floating raft peak was decreased by NGF binding.

Western blots from the experiment described in Fig. 19 were probed with an antibody against p75^{NTR} (20a). For presentation purposes, only the fractions with the floating raft peak of p75^{NTR} are displayed. The p75^{NTR} bands were quantified and expressed as a percentage of the total p75^{NTR} in the whole cell (20b). * indicates p < 0.05. Data are an average of two experiments (all -NGF data), or nine (0min, -ATP), six (10 min, -ATP) and four (10min, +ATP) experiments for the +NGF data.

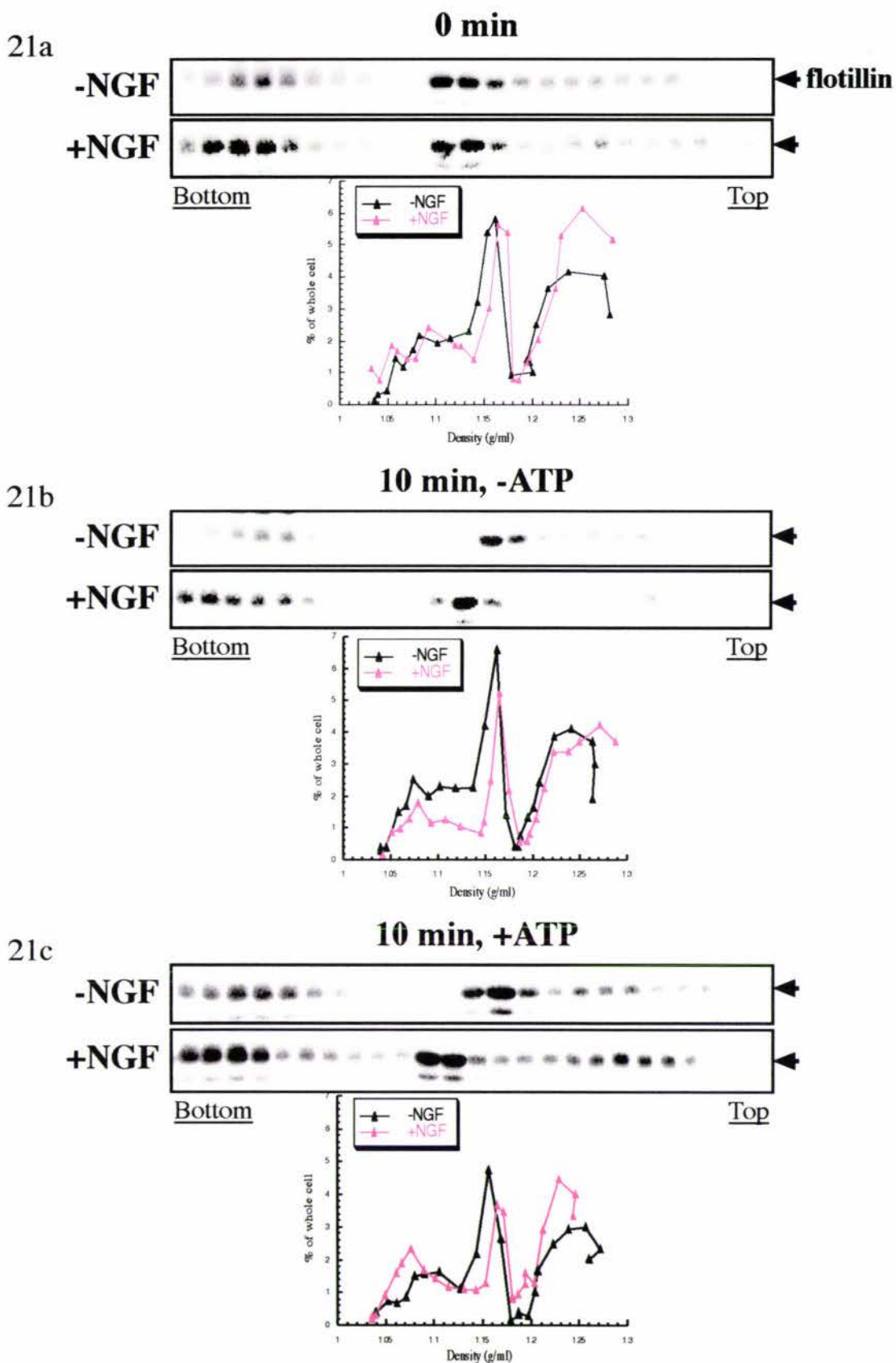


Figure 21: The floating raft peak appeared to shift to a higher density and flotillin appeared to be redistributed from the floating raft peak to the high-density peak with NGF binding.

Western blots from Fig. 20 were reprobed with an antibody against flotillin. The orientation of the gradients is noted below the blots. The flotillin bands were quantified and expressed as a percentage of the whole cell total. These are shown below each corresponding blot. Data are representative of two experiments (-NGF data) or nine (0min, -ATP), six (10min, -ATP) and four (10min, +ATP) experiments for the +NGF data.

calculated (Fig. 22a). There was no significant difference in flotillin association with the floating raft peak with NGF binding, either with no internalisation or 10 minutes internalisation and *in vitro* reactions. With 10 minutes internalisation but no *in vitro* reactions with ATP, there appeared to be a slight increase in flotillin association with the floating raft peak when NGF was bound to the cells.

Overall, the main effect common to all conditions used was that NGF binding resulted in a slight shift of the floating raft peak to a higher density when NGF was bound to the cells.

3.6.4 ERK and tubulin

To investigate if NGF binding was also affecting the association of other proteins with the floating raft peak, the blots were probed with antibodies against α -tubulin and ERK. The tubulin, phospho-ERK and ERK1 bands were quantified and expressed as a percentage of whole cell protein associated with the floating raft peak. NGF binding had no effect on phospho-ERK association with the floating raft peak with 10 minutes internalisation, with or without the *in vitro* ATP reaction (Fig. 22b). However, NGF caused an increase in phospho-ERK in the floating raft peak with no internalisation ($p < 0.02$).

The percentage of whole cell ERK1 associated with the floating raft peak was significantly increased by NGF (Fig. 22c). This was seen for both no internalisation and 10 minutes internalisation. However, with 10 minutes internalisation and *in vitro* reactions with ATP, there was no significant difference in the amount of ERK1 in the floating raft peak as was also seen with phospho-ERK.

NGF binding caused a significant increase in tubulin association with the floating raft peak for all conditions used (Fig. 22d).

Overall it appeared, that under some conditions NGF caused increased association of TrkA, phospho-ERK, ERK1 and tubulin with the membrane rafts in the floating raft peak. However NGF binding resulted in displacement of p75^{NTR} from these rafts. The amount of flotillin associating with the floating raft peak was not substantially affected

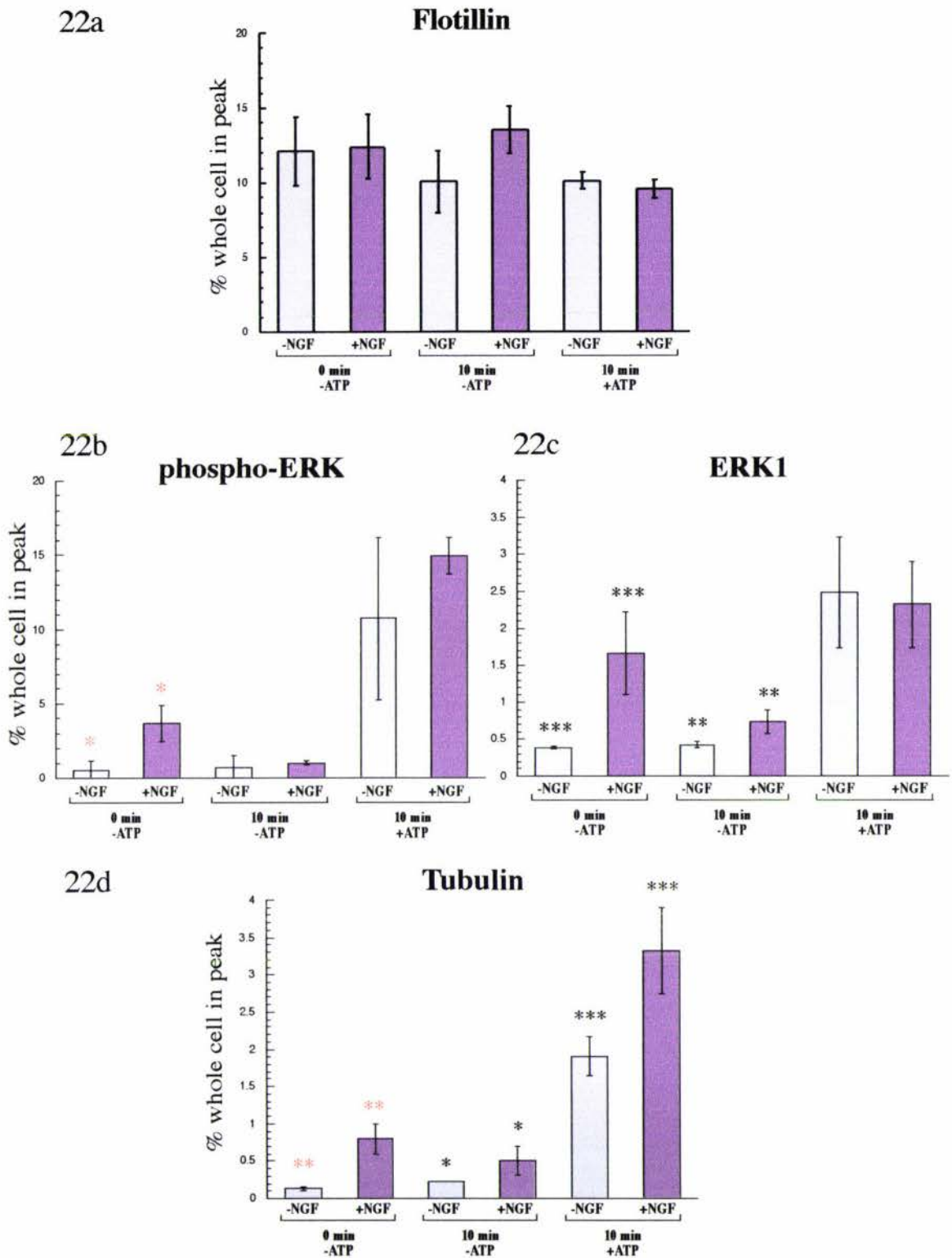


Figure 22: NGF binding did not substantially effect flotillin, but tubulin, phospho-ERK and ERK1 association with the floating raft peak was increased.

Flotillin bands from Fig. 21 were quantified and plotted as a percentage of whole cell flotillin in the floating raft peak (22a). The blots from Fig. 21 were stripped and reprobbed with antibodies against ERK or tubulin. The phospho-ERK, ERK1 and tubulin bands were quantified and plotted as a percentage of whole cell phospho-ERK (22b), ERK1 (22c) and tubulin (22d) that were associated with the floating raft peak. The tubulin -NGF, 10min, -ATP bar does not have a standard error as the data points were identical. The symbols above the bars of the graphs indicate the significant difference between the two bars with the same symbol. * indicates $p < 0.2$, ** $p < 0.1$, *** $p < 0.05$, * $p < 0.02$ and ** $p < 0.01$. Data are an average of two experiments (-NGF data), or nine (0min, -ATP), six (10min, -ATP) and four (10min, +ATP) experiments for the +NGF data.

by NGF, however the flotillin profiles showed that there may be a slight increase in density of the membrane rafts with NGF binding to the cells.

3.7 Effect of exogenous GM1 on proteins in the detergent-insoluble membrane domains

GM1 is known to be abundant in membrane rafts of PC12 cells (Huang *et al.*, 1999; Schnitzer *et al.*, 1995). The addition of exogenous GM1 to cells could conceivably result in stabilisation of these membrane domains. However, the addition of exogenous GM1 to cells has been previously used as a method of disrupting membrane rafts, causing raft-associated proteins to become detergent-soluble (Simons *et al.*, 1999). To investigate the effect of adding exogenous GM1 on TrkA and p75^{NTR} association with the membrane domains in the two floating peaks, cells were incubated with or without 65 μ M GM1 for 5 hours at 37°C. The cells were washed and a small sample removed to test for apoptosis. This was a long period of time for cells to be incubated in serum-free media, so it was important to test that the cells were still viable. Both with and without GM1, the number of apoptotic cells was less than 1%. The cells were then bound to NGF, washed, permeabilised and fractionated. One sample was subjected to an *in vitro* reaction with ATP (+ATP), the other was left on ice (-ATP).

3.7.1 NGF and TrkA

Exogenous GM1 caused no change in raft density; NGF was still associated with the floating raft peak at ~1.16 g/ml (Fig. 23a). However, the 5-fold increase normally seen in NGF association with the floating raft peak after *in vitro* reactions was abrogated by the addition of GM1 to the cells.

The percentage of whole cell NGF that was associated with the floating raft peak was calculated and averaged for several experiments (Fig. 23b). Without *in vitro* reactions there was 2.4-fold more NGF in the floating raft peak when GM1 had been added to the cells ($p < 0.001$).

Western blots were probed with an antibody against TrkA (Fig. 24a,b) and the percentage of whole cell TrkA associated with the floating raft peak calculated (Fig. 24c). There was only a very small amount of 110 kDa TrkA present in the

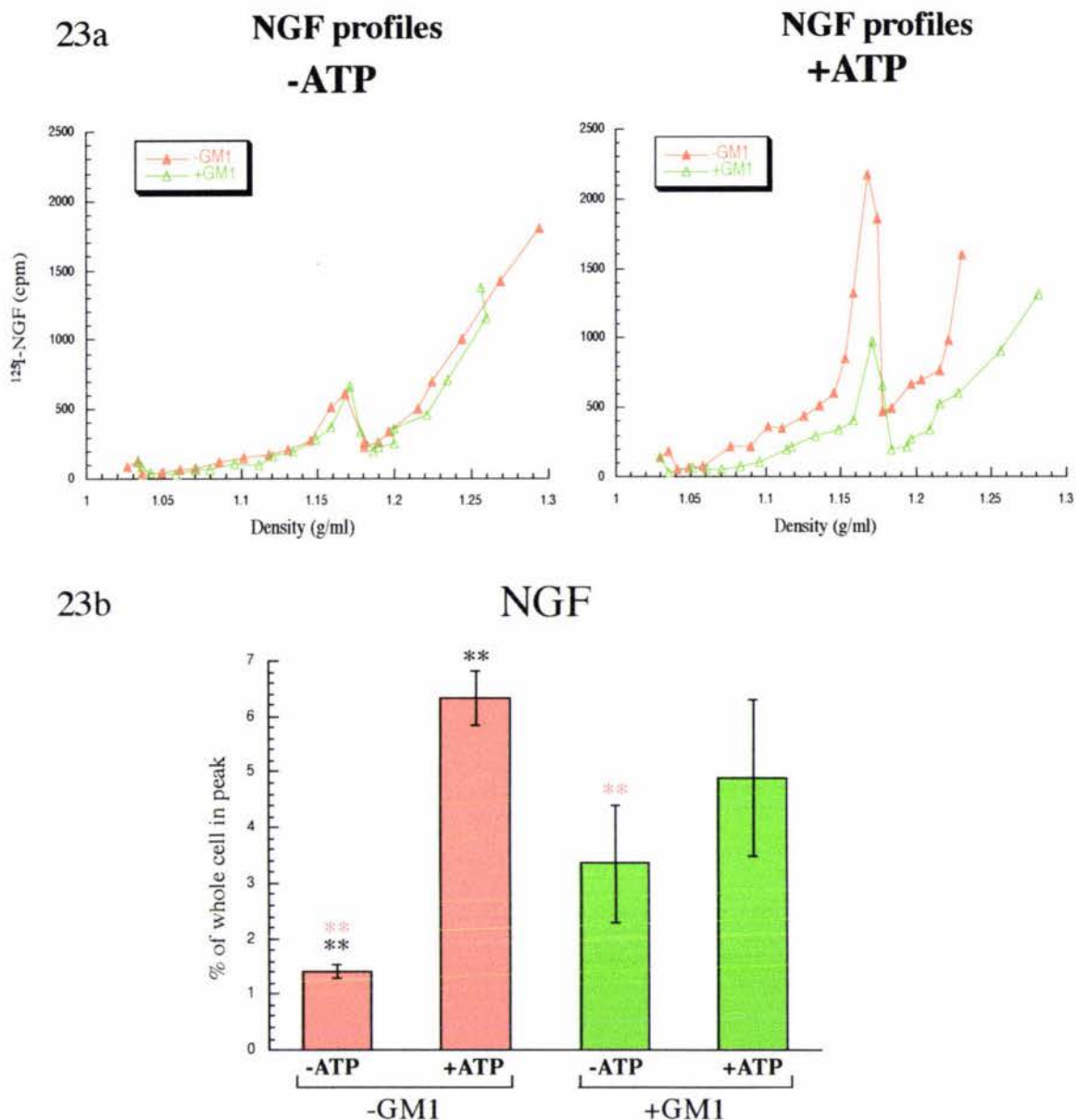


Figure 23: Addition of exogenous GM1 increased NGF association with the floating raft peak without an *in vitro* ATP reaction.

Cells were incubated in either serum-free media with 65 μ M GM1 (+GM1), or serum-free media alone (-GM1), for 5 hours at 37°C. The cells were washed and then incubated with 1 nM 125 I-NGF. After permeabilisation cells were divided and one sample left on ice (-ATP) and the other subjected to an *in vitro* ATP reaction (+ATP). The detergent-insoluble material was separated by equilibrium gradient centrifugation and the radioactivity of each gradient fraction plotted against density (23a). The percentage of total NGF in the whole cell that was associated with the floating raft peak was calculated (23b). The symbols above the bars of the graphs indicate the significant difference between the two bars with the same symbol. ** and ** p<0.001. Data are representative (23a), or an average of (23b), two (+GM1 data), nine (-GM1, -ATP) and five (-GM1, +ATP) experiments.

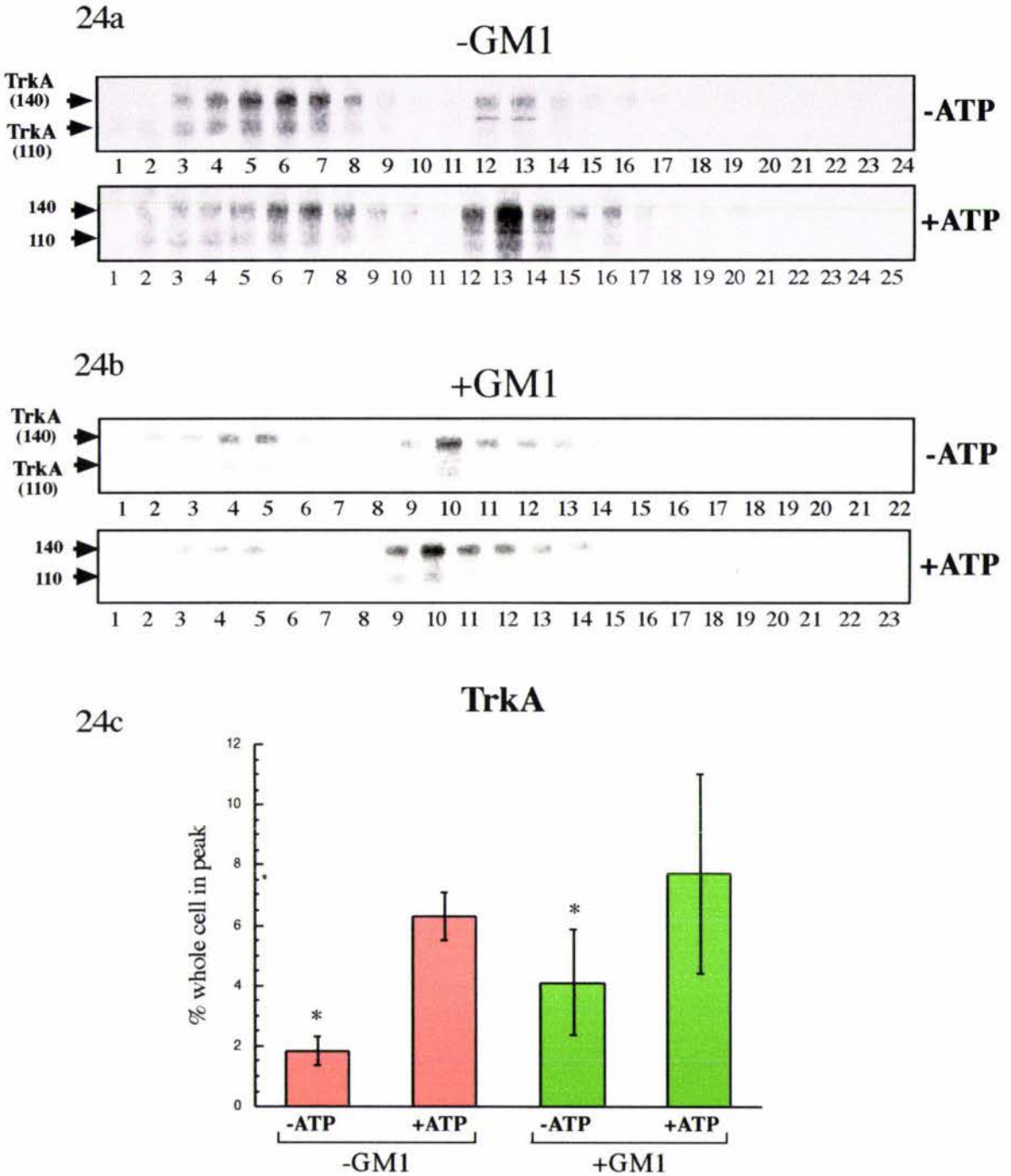


Figure 24: The effect of GM1 on TrkA association with the floating raft peak was similar to that seen with NGF.

The gradient fractions from the experiment described in Fig. 23 were Western blotted and probed with an antibody against TrkA (24a,b). The percentage of TrkA in the whole cell that was in the floating raft peak is plotted (24c). The * indicates $p < 0.1$. Data are representative (24a,b), or an average of (24c), two (+GM1, +/-ATP), nine (-GM1, -ATP) and five (-GM1, +ATP) experiments.

gradients after the cells had been exposed to GM1 (Fig. 24b). Similar to the effect of GM1 on NGF, without the *in vitro* reaction there was a 2-fold increase in TrkA association with the floating raft peak with GM1 ($p < 0.1$) (Fig. 24c). The 3-fold increase in raft-association of TrkA after *in vitro* ATP reactions, was abrogated by the addition of GM1 to the cells.

Overall, it appeared that, rather than the GM1 causing disruption of the rafts, it was promoting the association of NGF and TrkA with the floating raft peak *in vivo*, almost to levels seen after *in vitro* reactions.

To investigate the effect of GM1 on the association of p75^{NTR}, flotillin, tubulin and ERK with the floating raft peak, the Western blots were reprobbed with antibodies against these proteins. The protein bands were quantified and plotted as a percentage of total whole cell protein that was associated with the floating raft peak.

3.7.2 p75^{NTR} and flotillin

Exogenous GM1 appeared not to affect p75^{NTR} association with the floating raft peak (Fig. 25a), however, the variability of the results allowed no firm conclusion to be made.

The percentage of whole cell flotillin associated with the floating raft peak was decreased when the cells had been incubated with GM1 (Fig. 25b) both before and after *in vitro* reactions with ATP. Therefore, flotillin association with the membrane rafts in the floating raft peak was decreased, as would be expected if the membrane rafts were being disrupted by the addition of exogenous GM1.

3.7.3 Tubulin, phospho-ERK and ERK1

When the cells were incubated in GM1, there was increased tubulin ($p < 0.1$), (Fig. 25c), phospho-ERK ($p < 0.01$), (Fig. 25d) and ERK1 ($p < 0.01$), (Fig. 25e) in the floating raft peak (without *in vitro* reactions). When the *in vitro* reaction was performed there was no significant difference in tubulin or phospho-ERK association with the floating raft

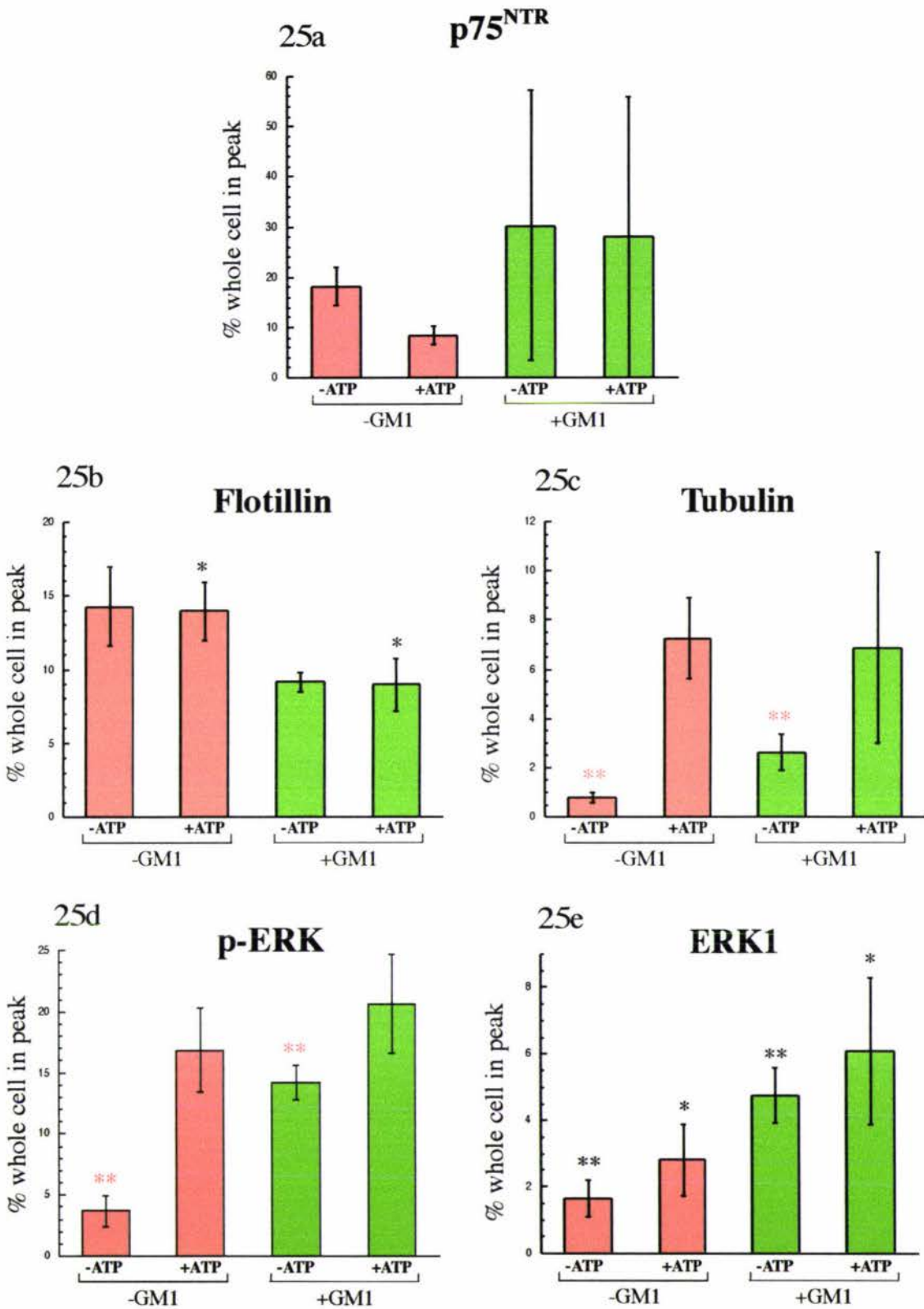


Figure 25: p75^{NTR}, flotillin, tubulin and ERK association with the floating raft peak was affected by exogenous GM1.

Western blots from Fig. 24 were reprobbed with antibodies against p75^{NTR}, flotillin, tubulin and ERK. The percentage of p75^{NTR} (25a), flotillin (25b), tubulin (25c), phospho-ERK (25d) and ERK1 (25e) from the whole cell that were associated with the floating raft peak is plotted. The error bars in 25a are very large due to variable results in only two experiments. The * indicates $p < 0.2$, ** $p < 0.1$ and *** $p < 0.01$. Data are an average of two (+GM1, -/+ATP), nine (-GM1, -ATP) and five (-GM1, +ATP) experiments.

peak with GM1. For ERK1 however, there was a significant increase in the floating raft peak even after *in vitro* reactions ($p < 0.2$).

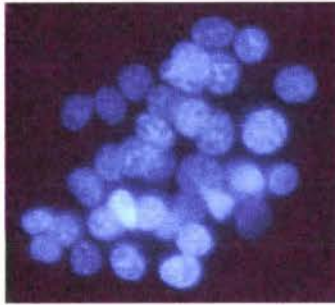
Addition of GM1 has previously been shown to disrupt membrane rafts, solubilising the raft-associated proteins. Here we observed that, although flotillin was decreased in the floating raft peak, suggesting the rafts were being disrupted, the increase in raft association of TrkA, tubulin, phospho-ERK and ERK1 suggested that the addition of GM1 was promoting the association of these proteins with membrane rafts.

3.8 Depletion of cholesterol using methyl- β -cyclodextrin

Cholesterol is very important for maintaining the integrity of membrane rafts (Friedrichson and Kurzchalia, 1998; Rietveld and Simons, 1998). Depletion of cholesterol with methyl- β -cyclodextrin (M β CD) has been shown to disrupt these rafts, causing raft-associated proteins to become detergent-soluble. The membrane rafts are also observed to shift to a higher density in the gradient when M β CD is used (Ilangumaran and Hoessli, 1998; Rodal *et al.*, 1999). To investigate the effect of M β CD on the isolated membrane domains, cells were incubated either with or without 50 mM M β CD, for 1 hour. The cells were washed and a small sample removed to investigate the viability of the cells. Fig. 26a shows a photo of Hoechst 33342-stained healthy cells compared with an example of an apoptotic cell with condensed chromatin (Fig. 26b). The cells were scored for apoptosis by counting the cells that had this apoptotic morphology. Less than 1% of the cells in the control (-M β CD) and treated (+M β CD) samples were apoptotic. Interestingly, the M β CD-treated cells appeared shrunken and deformed compared to the control cells, and most of their plasma membranes had become stretched out into long filaments (data not shown). An additional difference in these M β CD-treated cells was the centrifugation speed required to pellet the cells after washing. Cells normally pellet at 100xg, however these treated cells required 500xg to be pelleted.

Both control and M β CD-treated cells were incubated with NGF for 1 hour at 4°C, washed, permeabilised and half of the sample subjected to an *in vitro* reaction with ATP. The detergent-insoluble material was separated on an iodixanol equilibrium gradient. When the cells had been incubated in M β CD, the NGF associated with the

26a



26b

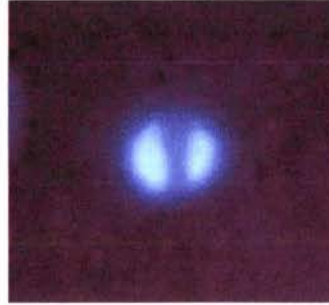


Figure 26: An example of healthy cells and an apoptotic cell that have been stained with Hoechst 33342.

A small sample of cells were removed after M β CD treatment to examine whether this procedure caused the cells to undergo apoptosis. Fig. 26a shows normal, healthy cells using the Hoechst 33342 stain (630x magnification). Fig. 26b is an enlarged example (1000x magnification) of an apoptotic cell with condensed chromatin. The number of apoptotic cells on the slides were counted. The M β CD-treated cells showed no signs of apoptosis but had a deformed cell shape. Unfortunately the photo taken of these deformed cells was not presentable.

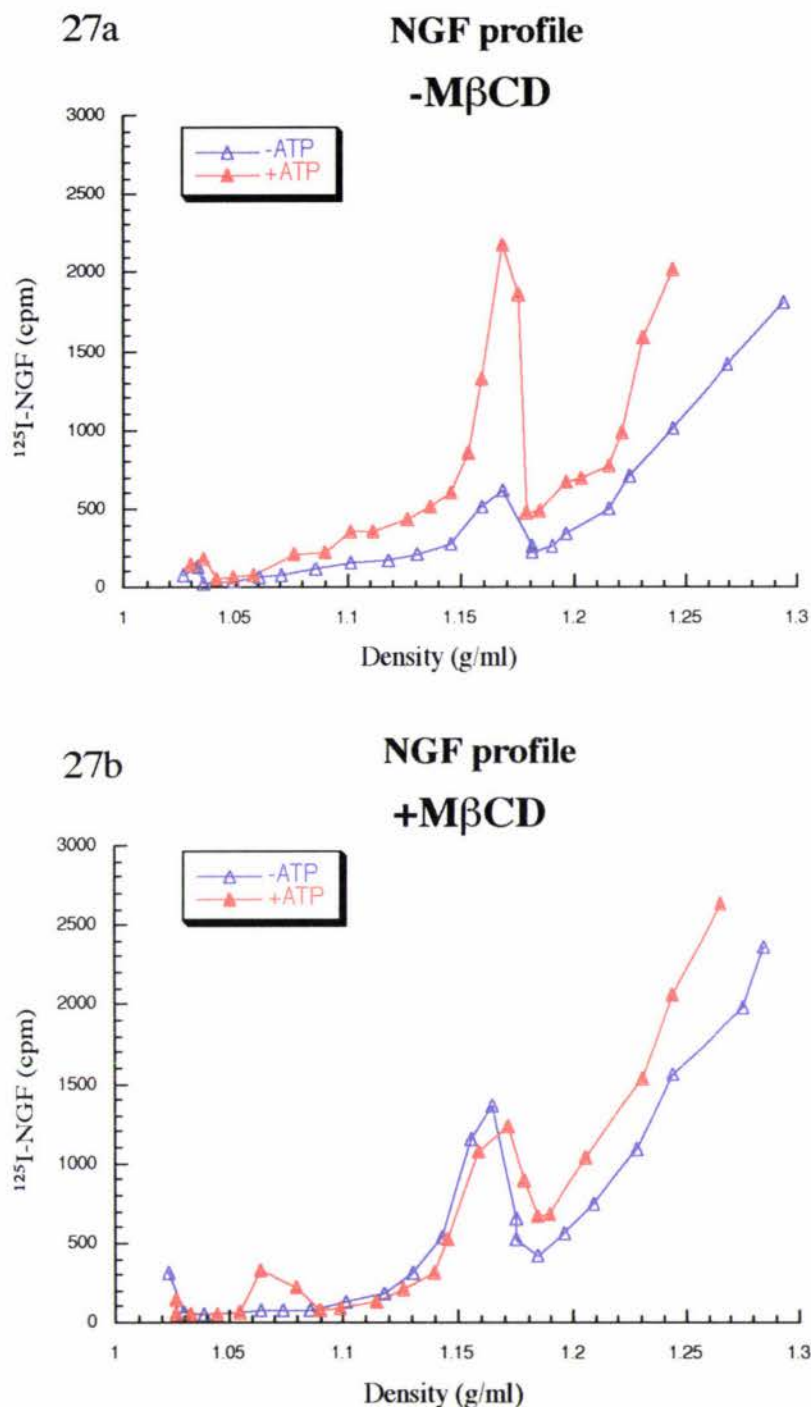


Figure 27: Methyl- β -cyclodextrin affected the increase in NGF association with the floating raft peak after an *in vitro* ATP reaction.

Cells were incubated in either serum-free media with 50 mM M β CD or serum-free media alone (-M β CD), for 1 hour at 37°C. They were then washed and incubated in 1 nM ^{125}I -NGF for 1 hour at 4°C. Cells were permeabilised and divided, one sample was left on ice (-ATP), the other had an *in vitro* ATP reaction (+ATP). The detergent-insoluble material was separated on an equilibrium gradient. The radioactivity of each gradient fraction was plotted against density for both without (27a) and with (27b) M β CD. The profiles where M β CD was used are representative of a single experiment.

floating raft peak was still found at ~1.16 g/ml (Fig. 27b), indicating that M β CD had not caused these membrane rafts to shift to a higher density.

Without M β CD treatment, there was normally a large increase in NGF association with the floating raft peak with the *in vitro* ATP reaction (Fig. 27a). With depletion of cholesterol, this effect of ATP was abrogated (Fig. 27b).

Apart from this effect with the *in vitro* ATP reaction however, the NGF profiles had changed very little with the M β CD treatment, suggesting that the depletion of cholesterol may also not have affected TrkA and p75^{NTR} association with the membrane rafts in the floating raft peak.

3.8.1 TrkA

In contrast to the NGF distribution, M β CD treatment had a very large effect on TrkA distribution in the gradient, both without (Fig. 28a) and with (Fig. 28b) the *in vitro* ATP reaction. There was a complete disappearance of 140 kDa TrkA from the high-density peak (fractions 3-8) and only a very small amount was found in the floating raft peak fractions with M β CD treatment.

Interestingly, with M β CD, there was a very high amount of 110 kDa TrkA in the floating raft peak and none in the high-density peak where it was normally found. This indicated that there was a total redistribution of 110 kDa TrkA from the high-density peak to the floating raft peak with depletion of cholesterol. Very little 110 or 140 kDa TrkA was detectable in the S1 and P1M fraction of the cell.

When the *in vitro* ATP reaction was carried out after the cells had been treated with M β CD, there was very little change in the 140 kDa TrkA association with the floating raft peak. This was similar to what was observed with NGF (Fig. 27b).

The 140 and 110 kDa TrkA bands were quantified and plotted as a percentage of the total in the DRM (Fig. 28c). Only the gradients without the *in vitro* ATP reaction are shown as they are almost identical to those where these *in vitro* reactions were used. Without M β CD treatment, 140 kDa TrkA was found in both the high-density peak

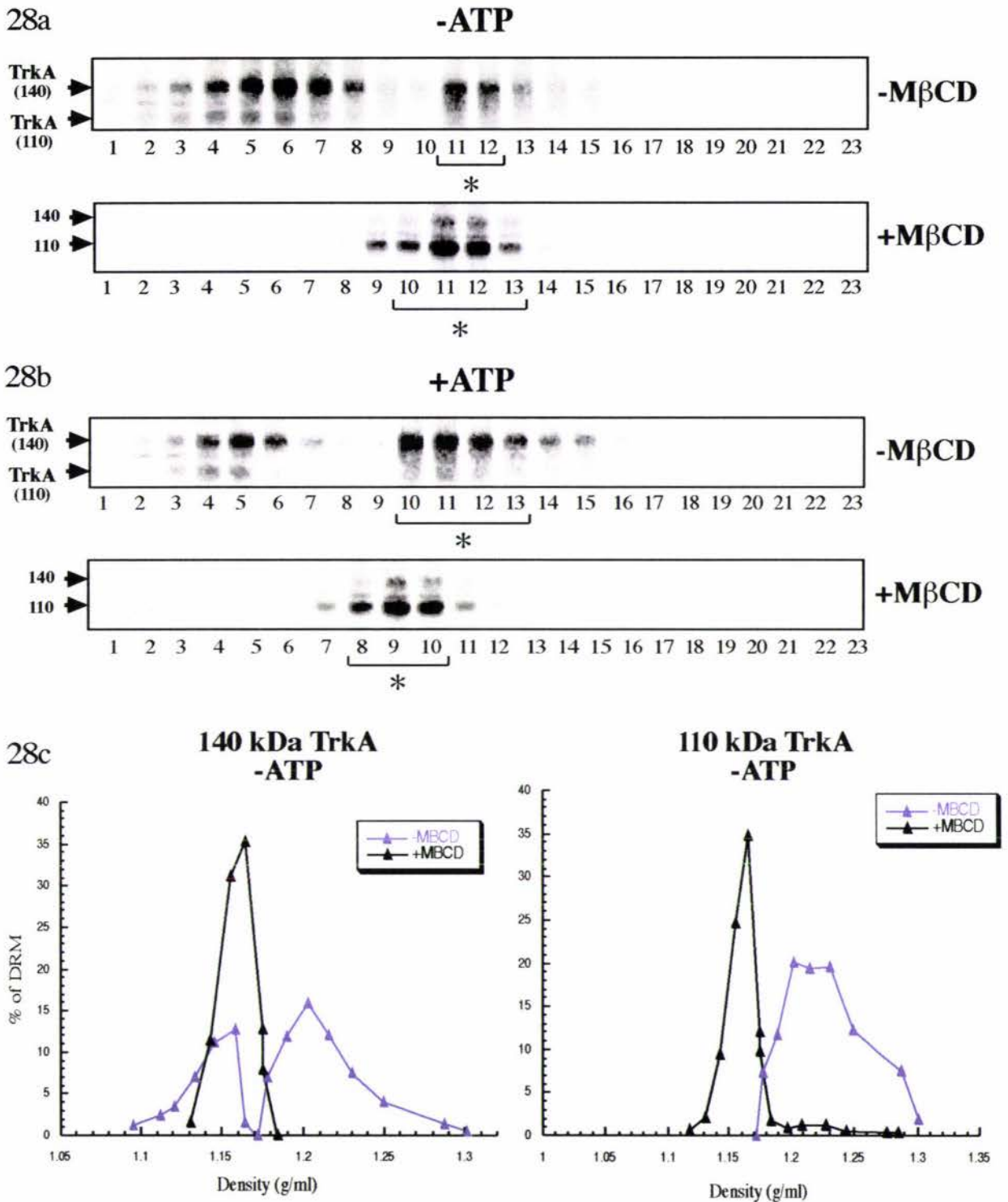


Figure 28: Methyl- β -cyclodextrin had a large effect on the distribution of both 140 kDa and 110 kDa TrkA in the gradient.

Gradient fractions from the experiment described in Fig. 27 were Western blotted and probed with an antibody against TrkA (28a,b). This antibody detected both the 140 kDa-mature and the 110 kDa-immature form of TrkA as indicated to the left of the blots. The '*' below the fraction numbers indicates where NGF was associated with the floating raft peak. The 140 and 110 kDa TrkA bands were quantified and expressed as a percentage of the total protein in the DRM (28c). The -M β CD blots and profiles are representative of nine (-ATP) and five (+ATP) experiments. The +M β CD blots and profiles are representative of only a single experiment.

(above 1.2 g/ml) and the floating raft peak as described in earlier sections. 110 kDa TrkA was mainly found in the high-density peak. Treatment with M β CD however, disrupted this high-density peak of 110 and 140 kDa TrkA so that only the floating raft peak remained, with increased association of both of these forms of TrkA.

3.8.2 p75^{NTR} and flotillin

The blots were probed with antibodies against either p75^{NTR} (Fig. 29a) or flotillin (Fig. 29b). Only the blots for the M β CD-treated cells are shown here, they can be compared with Western blots of control cells with the same conditions in Fig. 12a and 12b.

Without M β CD treatment, p75^{NTR} was concentrated in the floating raft peak fractions. This association did not appear to be affected by the depletion of cholesterol.

The flotillin blots (Fig. 29b) showed that the distribution of flotillin in the gradient was not altered by M β CD-treatment, unlike TrkA there was still flotillin associated with the high-density peak, however less flotillin was associated with both the floating raft peak and the high-density peak when the cells had been treated with M β CD (Fig. 29c). The decrease in flotillin with M β CD treatment was most marked in the high-density peak. Also, unlike TrkA and p75^{NTR}, flotillin could still be detected in the P1M fractions of the cell after depletion of cholesterol.

Flotillin has been shown to be a membrane raft protein, therefore if the membrane rafts were being disrupted by cholesterol depletion, then flotillin would have been affected. With these results there appeared to be less flotillin association with both of the floating peaks, indicating that the membrane domains in these peaks may have been partially disrupted.

3.8.3 Tubulin and ERK

Treatment with M β CD also affected tubulin (Fig. 30a), phospho-ERK and ERK1 (Fig. 30b) distribution in the equilibrium gradient. Only the Western blots of cells that had been treated with M β CD are shown; these can be compared with tubulin and

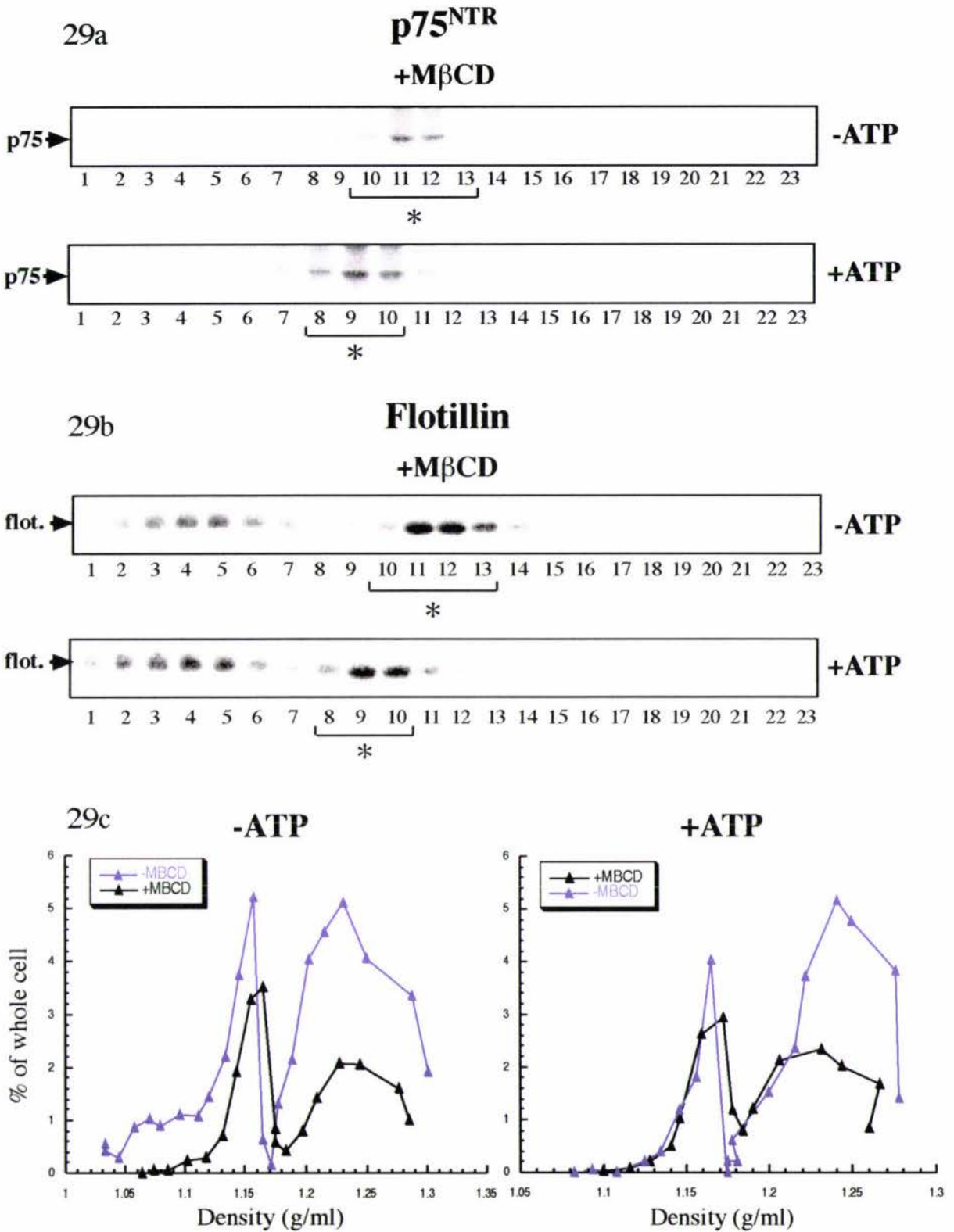


Figure 29: Methyl- β -cyclodextrin did not effect p75^{NTR}, but did effect flotillin association with the high-density peak and the floating raft peak.

Western blots from Fig. 28 were reprobbed with antibodies against either p75^{NTR} (29a) or flotillin (29b). Only the blots for the M β CD-treated samples are shown here, but they can be compared with blots under the same conditions without M β CD in Fig. 12a (p75^{NTR}) and Fig. 12b (flotillin). The position of NGF associated with the floating raft peak is indicated by an '*' below the fraction numbers. The flotillin bands were quantified and expressed as a percentage of the total in the whole cell (29c). The blots with M β CD treatment are representative of one experiment.

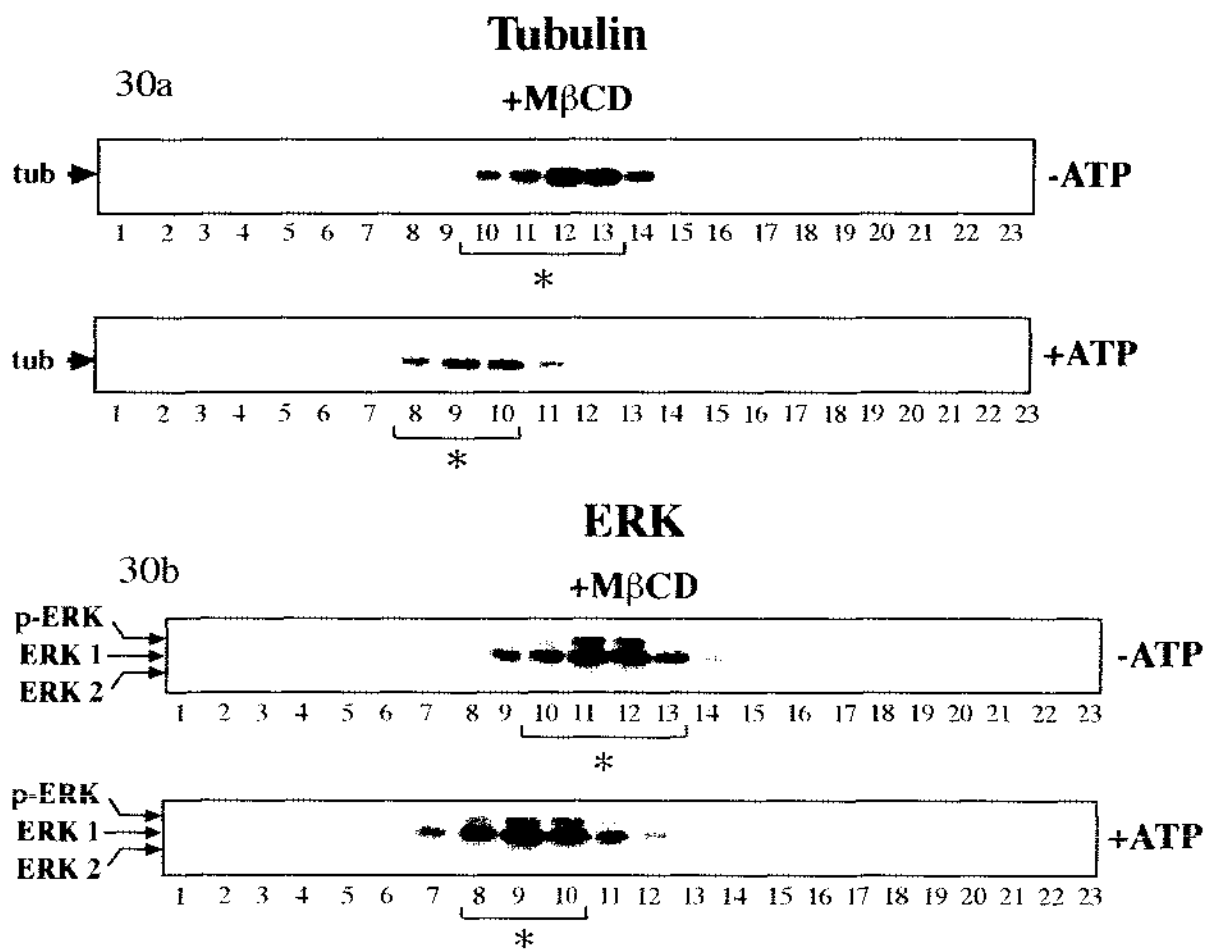


Figure 30: Tubulin, phospho-ERK and ERK1 were concentrated in the floating raft peak fractions with methyl- β -cyclodextrin.

Western blots from Fig. 29 were stripped and reprobed with antibodies against either tubulin (30a) or ERK (30b). The positions of the different forms of ERK detected by this antibody are indicated to the left of the blots. Only the blots for the M β CD-treated samples are shown here, however they can be compared with blots with similar conditions but without M β CD treatment in Fig. 14a (ERK) and Fig. 15a (tubulin). The position of the floating peak of NGF is indicated by an ‘*’ below the fraction numbers for each blot. These blots are representative of a single experiment.

ERK blots under the same conditions but without M β CD in Fig. 15a and Fig. 14a respectively. Without M β CD, tubulin was found mainly in either the high-density peak or the floating raft peak, depending on whether *in vitro* reactions were used. When the cells were treated with M β CD, the high-density peak of tubulin completely disappeared and tubulin was concentrated in the floating raft peak, regardless of whether an *in vitro* reaction was used (Fig. 30a). It actually appeared that with the *in vitro* ATP reaction, there was a decrease in tubulin associated with the floating raft peak after the cells had been treated with M β CD.

The use of M β CD had a similar effect on both phospho-ERK and ERK1 distribution in the gradient (Fig. 30b). Like tubulin, ERK1 and phospho-ERK were normally found in the lower fractions of the gradient, but not associated with the high-density peak (Fig. 14a). With the *in vitro* ATP reaction, there was normally a large increase in phospho-ERK and a smaller increase in ERK1 association with the floating raft peak. When cells were treated with M β CD, phospho-ERK and ERK1 could no longer be detected in the lower gradient fractions but were concentrated in the floating raft peak at ~1.16 g/ml. If these two proteins were not associated with the high-density peak, i.e., if they were indeed free protein, they should not have been affected by depletion of cholesterol, they should have maintained a distribution similar to that seen for NGF (Fig. 27b). Tubulin, phospho-ERK and ERK1 could not be detected in the S1 and P1M fractions of the cell when M β CD had been used.

Overall, it appeared that cholesterol depletion with M β CD, although deforming the cell shape, did not fully disrupt the floating raft peak that was believed to consist of membrane rafts similar to those studied previously. However, apart from flotillin, no other proteins of interest were found in the high-density peak once cholesterol had been depleted. This was an interesting and unexpected result that needs to be repeated before conclusions can be made.

CHAPTER 4: DISCUSSION AND CONCLUSIONS

4.1 Discussion

NGF, TrkA and p75^{NTR} were reproducibly observed to float on a continuous iodixanol equilibrium gradient to a density of ~1.16 g/ml. The membrane raft protein, flotillin, was also found in these floating fractions, suggesting that TrkA and p75^{NTR} are associated with membrane rafts. NGF therefore associates with these rafts due to its interaction with TrkA and p75^{NTR}. Glycolipids were detected in these floating raft peak fractions along with a ganglioside thought to be similar to GD1.

Two peaks of TrkA and flotillin were observed rather than just one, as was seen for p75^{NTR}, suggesting that two different types of detergent-resistant membranes had been isolated. This second peak had a higher density (around 1.2 g/ml) than the floating raft peak (~1.16 g/ml) and did not contain either p75^{NTR} or NGF. The absence of NGF from this peak indicated that the TrkA in these membranes had probably not been exposed to NGF on the cell surface. The immature, 110 kDa TrkA was also found in this high-density peak. 110 kDa TrkA moves from the endoplasmic reticulum (ER) to the Golgi, where alterations are made to its attached carbohydrate moiety. 140 kDa, mature TrkA emerges from the trans Golgi and is then transported to the plasma membrane. The presence of both 110 kDa TrkA and 140 kDa TrkA that is not NGF-bound, indicates that these detergent-resistant membrane domains may not have originated from the plasma membrane. They may instead consist of membrane domains from both the ER and cis Golgi (containing 110 kDa TrkA) as well as from the trans Golgi (containing 140 kDa TrkA).

No gangliosides or glycolipids could be detected in the fractions corresponding to this high-density peak. Resorcinol, which stains any lipids with sialic acid residues, may not have detected the sphingolipids in these rafts as they would not yet have sialic acid attached to their carbohydrate moiety. This does not occur until the trans Golgi (sphingolipids are called gangliosides when they have an attached sialic acid residue - see section 1.5.1). Therefore, if these membrane domains are from the ER and Golgi then they will not have sialic acid-containing sphingolipids.

Detergent-resistant membrane rafts have previously been shown to exist in intracellular membranes such as the Golgi, late secretory pathway, and endocytic compartment membranes (Brown and London, 1998b; Brown and Rose, 1992; Simons and Ikonen, 1997). The GPI-anchored protein, alkaline phosphatase, was found to be Triton X-100 insoluble while being transported through the Golgi. The Golgi contains sphingolipids and cholesterol, therefore it is quite likely that rafts can form in this intracellular organelle. It has actually been suggested that there are two routes of membrane traffic from the Golgi to the plasma membrane. One transports membrane proteins that have sorting signals in their cytoplasmic domains, the other handles membrane rafts and the proteins associated with them (Simons and Ikonen, 1997).

There has been some controversy over the presence of membrane rafts in the ER. Some proteins have been found to be in ER membranes with properties similar to membrane rafts (Sutterlin *et al.*, 1997). However, it is also thought that the formation of these domains in ER membranes is unlikely due to the low levels of sphingolipids and cholesterol (Brown and London, 1998).

Therefore, it is possible that the detergent-extraction procedure used in this thesis has isolated detergent-resistant membranes from both the plasma membrane, containing NGF-bound TrkA, p75^{NTR} and flotillin, and intracellular membranes that contain 110 kDa TrkA from the ER and 140 kDa TrkA from the trans Golgi, in addition to flotillin.

After *in vitro* reactions with an ATP regenerating system, there appeared to be movement of NGF-bound TrkA from the non-raft, detergent-soluble plasma membranes to the membrane rafts found in the floating raft peak at ~1.16 g/ml. Interestingly, this *in vitro* reaction had an opposite effect on p75^{NTR}, causing movement of this receptor out of the membrane rafts and into non-raft regions of the membrane.

With the *in vitro* reaction, there was also an increase in the amount of ERK1, phospho-ERK and tubulin associated with the floating raft peak. Without this reaction these proteins were normally found at the bottom of the gradient, ERK1 and phospho-ERK as free protein, and tubulin associated with the high-density peak containing intracellular membranes.

ERK1 is a downstream kinase in the Ras signalling pathway (Fig. 2). When NGF binds to TrkA, ERK1 becomes phosphorylated and activated, which in turn activates transcription factors in the nucleus of the cell. This alters the expression of proteins resulting in cell differentiation and growth. There has been no proposed mechanism explaining ERK association with membrane rafts. Often raft-associated proteins are palmitoylated or have a related lipid anchor which targets their association with the ordered membrane raft (Melkonian *et al.*, 1999), (see section 1.7), however, no previous studies have revealed ERK to be palmitoylated.

ERK is known to interact with the microtubules which make up the cytoskeleton of the cell (Morishima-Kawashima and Kosik, 1996; Reszka *et al.*, 1995). Therefore the apparent association of both ERK1 and phospho-ERK (which I shall collectively call ERK) with the floating raft peak may be due to their association with microtubules.

Tubulin, which polymerises to form microtubules, was also found to be associated with the membrane rafts in the floating raft peak after *in vitro* reactions with ATP. It was mentioned earlier, that the cytoskeleton should have a high buoyant density, therefore remain at the bottom of the gradient. However, since the detergent-insoluble material (DRM) was sonicated before layering beneath the gradient, the complex, polymerised microtubules that make up the cytoskeleton were probably broken into smaller fragments of polymerised and monomeric tubulin. With the *in vitro* reaction, ATP can be converted to GTP, which is required for microtubule polymerisation to occur (Stryer, 1995). Although tubulin has been known to associate with the plasma membrane (Caron, 1997), it has only recently been discovered that tubulin is also associated with membrane rafts via an attached lipid anchor which interacts with GM1 (Palestini *et al.*, 2000). Thus, polymerisation of microtubules would result in the observed increase in tubulin association with the floating raft peak, because some of the microtubule fragments bind to membrane rafts.

Tubulin also appeared to be associated with the detergent-resistant intracellular membrane rafts found in the high-density peak. Microtubules are well known to be involved in maintaining the normal intracellular locations and organisation of the ER (Terasaki *et al.*, 1986) and the Golgi (Turner and Tartakoff, 1989). Therefore, it is not unlikely that microtubules are also associated with the membranes of these organelles. It seems likely that after sonication, microtubule fragments remain attached to these

intracellular membranes, which are possibly some type of membrane raft, because they are detergent-insoluble.

Interestingly, the increase in tubulin association with the floating raft peak with the *in vitro* reaction was accompanied by a decrease in tubulin in the high-density, intracellular membrane peak. Therefore, in addition to polymerisation of microtubules attached to the detergent-resistant plasma membrane rafts, there seemed to be movement of microtubules away from intracellular membranes. ERK did not appear to be associated with this high-density peak, therefore it is unlikely that it is interacting with intracellular membrane-associated microtubules.

Thus, ERK1 and phospho-ERK interact with microtubule fragments, which are associated with the plasma membrane rafts by a lipid anchor. With *in vitro* reactions, microtubule polymerisation occurs, resulting in an observed increase in tubulin association. Phospho-ERK and ERK1 can then interact with these newly polymerised microtubules that are associated with the plasma membrane rafts.

4.1.1 Internalisation of activated receptors

It has been suggested that ligand-activated receptors such as NGF-bound TrkA, are associated with membrane rafts so that they can directly be internalised into clathrin coated vesicles (Simons and Ikonen, 1997). Once internalised, the receptors can continue to transduce their signal until their return to the plasma membrane or degradation in lysosomes (Beattie *et al.*, 1996; Kamal and Goldstein, 2000). Therefore, if the cell is incubated at 37°C after NGF binding, a decrease in the raft association of NGF-bound TrkA would be observed as it is internalised into vesicles. In these experiments, little change in TrkA or p75^{NTR} association with the membrane rafts was observed with internalisation times of 2 or 30 minutes. However, the optimum time for TrkA internalisation into vesicles (10 minutes), without *in vitro* reactions, appeared to slightly reduce the amount of TrkA, tubulin, phospho-ERK and ERK1 that were associated with the membrane rafts in the floating raft peak. This suggested that with 10 minutes incubation at 37°C, NGF-induced TrkA internalisation was occurring. Both tubulin and ERK appeared to follow TrkA out of these plasma membrane rafts. After 10 minutes internalisation and an *in vitro* reaction however, the amount of raft-associated TrkA was increased back to levels that were observed with no internalisation

and *in vitro* reactions. This effect may be due to the recruitment of TrkA from non-raft regions of the membrane to replace receptors that have been internalised from the membrane rafts.

For p75^{NTR}, 10 minutes internalisation appeared to result in a larger decrease in the amount of p75^{NTR} in the membrane rafts of the floating raft peak, than the decrease observed with no internalisation and *in vitro* reactions.

Overall, a small amount of internalisation of raft-associated TrkA appeared to be occurring, and this internalised TrkA appeared not to be associated with rafts. During the *in vitro* reactions non-raft plasma membrane TrkA was recruited into rafts.

4.1.2 NGF binding

NGF binding to the cell increased TrkA, tubulin, phospho-ERK and ERK1 association with the membrane rafts in the floating raft peak. For TrkA, this increase with NGF was only observed with 10 minutes internalisation and *in vitro* reactions. p75^{NTR} was actually found to be decreased by NGF binding after 10 minutes internalisation and an *in vitro* ATP reaction. Phospho-ERK and ERK1 however, were increased in rafts by NGF without or with 10 minutes internalisation and no *in vitro* reactions. Tubulin was increased in rafts by NGF under all conditions.

The amount of flotillin associated with the rafts in the floating raft peak was not significantly effected by NGF binding, however the flotillin profile showed that the rafts in the floating raft peak shifted to a slightly higher density with NGF. This could indicate that, with NGF binding to the cells, there is an increase in the amount of protein associated with these membrane rafts, which increases the density of these rafts because of the higher protein to lipid ratio.

Huang et al (1999) used a non-detergent extraction method to show that TrkA and p75^{NTR} were associated with membrane rafts in PC12 cells. They also investigated NGF binding, but found that both TrkA and p75^{NTR} association with the membrane rafts was not affected by NGF. This is probably because they did not use *in vitro* reactions in their experiments, and this is where the effect of NGF binding was observed for both TrkA and p75^{NTR}.

4.1.3 Addition of exogenous GM1

The GM1 experiments were carried out to investigate the possible effect of addition of exogenous GM1 on TrkA and p75^{NTR} association with membrane rafts. Because GM1 is abundant in membrane rafts, it is possible that the addition of GM1 would stabilise the rafts. However, previous studies using other cell types have shown that the addition of GM1 leads to disruption of rafts and the displacement of raft-associated proteins into non-raft regions of the plasma membrane (Simons *et al.*, 1999). In this thesis, the addition of GM1 resulted in decreased raft association of flotillin for all conditions, indicating that GM1 may be having a disruptive effect on the plasma membrane rafts. In contrast, increased raft association of TrkA, tubulin, phospho-ERK and ERK1 was observed when GM1 had been added to the cells. These effects were observed without the *in vitro* ATP reaction. After this reaction there was no additional stimulation of protein association with rafts compared to without GM1. The fact that the GM1 and ATP effects were not additive perhaps indicates that there is a maximum amount of these proteins permissible in the rafts, so even if two promoters of raft association are used, there is only so many proteins that can 'fit' in the membrane rafts.

Unfortunately the results for p75^{NTR} were inconclusive. p75^{NTR} is not known to interact with GM1 however, therefore with the addition of GM1 to the cells it is likely that this receptor, like flotillin, gets displaced from membrane rafts.

4.1.4 Depletion of cholesterol has an unexpected effect

So far, all of the results have suggested that the floating raft peak contains detergent-resistant rafts from the plasma membrane that are similar to those that have been studied previously. The final experiment carried out for this thesis, was to investigate the effect of depletion of cholesterol on these membrane rafts. For this, methyl- β -cyclodextrin was used to selectively extract cholesterol from the plasma membrane (Rodal *et al.*, 1999). M β CD has been used many times previously to show that a protein is associated with rafts. In other cell types when cholesterol is depleted, rafts are disrupted and associated proteins become soluble in Triton X-100 (Harder *et al.*, 1998; Rietveld and Simons, 1998).

In this thesis, a particularly high concentration of M β CD was used to deplete cholesterol. Unfortunately, no test was used to measure the cholesterol levels in the plasma membrane before and after treatment but the M β CD treatment appeared to reduce and deform the cell shape. The floating raft peak however, which was believed to contain typical plasma membrane rafts, was still found to float to ~1.16 g/ml after depletion of cholesterol. This indicated that these membrane rafts may not be the typical, cholesterol depletion-sensitive membrane raft that others had been studying previously. Flotillin was still found in both the high-density peak and the floating raft peak, although the levels of flotillin in each were considerably reduced compared with untreated cells. Therefore, there may have been slight disruption of both intracellular membrane rafts and plasma membrane rafts. It must be noted that, due to time constraints, this experiment has only been carried out once, so firm conclusions cannot be made until these results are found to be reproducible.

Interestingly the high-density peak of TrkA and tubulin, which was believed to consist of intracellular detergent-resistant membranes, completely disappeared with M β CD treatment. Phospho-ERK and ERK1 also disappeared from the fractions at the bottom of the gradient. Earlier it was mentioned that these proteins did not appear to be associated with the high-density peak, however they were effected by cholesterol depletion, contradicting the data above that suggested these proteins were not associated with these high density, intracellular membrane rafts.

TrkA, tubulin and ERK were highly concentrated in the membrane rafts of the floating raft peak even without the *in vitro* reaction (normally required to see tubulin and ERK in this peak). p75^{NTR} did not seem to be effected by M β CD treatment, it was still found in the floating raft peak. Interestingly, 110 kDa TrkA, which was normally found in the intracellular membranes of the high density peak, was present in the floating raft peak in much higher amounts than 140 kDa TrkA whose overall amounts were drastically reduced in all cell fractions.

This effect of M β CD was completely opposite of what was expected. It has been shown previously that M β CD treatment altered the flotation behaviour of a cell-surface protein, but not that of its ER form (Roper *et al.*, 2000). In contrast, here there was little disruption of the cell-surface rafts in the floating raft peak, but the intracellular

membrane domains, although not disrupted, had no apparent association of 110 kDa TrkA, 140 kDa TrkA or tubulin.

A possible explanation could be that such a high concentration of M β CD was used that cholesterol was depleted throughout the whole cell and this depletion of cholesterol affected the trafficking of proteins through the ER and the Golgi. Therefore there could be increased movement of 110 kDa TrkA to the plasma membrane without having its carbohydrate moiety altered on transit through the Golgi. This would explain both the large increase in 110 kDa TrkA observed in the plasma membrane rafts, and also the very low amount of 140 kDa TrkA detected throughout the cell (barely detectable in the S1 and P1M), because TrkA would remain in the 110 kDa form.

Overall, cholesterol depletion did not have the expected effect, it did not disrupt the plasma membrane rafts in the floating raft peak that were believed to be typical plasma membrane rafts. However, no firm conclusions can be made until this experiment is repeated, and the possible profound effects on membrane traffic make interpretation of this experiment difficult.

4.1.5 Proposed model

From the results of the *in vitro* reactions, internalisation, NGF binding, and GM1 experiments, a model can be built of the possible interactions occurring between proteins in these plasma membrane rafts of the floating raft peak.

Generally, if TrkA association with the floating raft peak was increased, then the association of tubulin, phospho-ERK and ERK1 was also increased. As discussed earlier, both phospho-ERK and ERK1 were believed to be indirectly associated with the membrane rafts through their interaction with microtubules. Microtubules in turn, interact with the membrane rafts through a lipid anchor on tubulin that interacts with GM1 in the rafts. It is possible then that there is also an interaction between TrkA and the microtubules within the plasma membrane rafts. A large complex may be formed containing, TrkA, tubulin, phospho-ERK and ERK, but excluding p75^{NTR}, flotillin and other typical membrane raft proteins such as GPI-anchored proteins. This complex would then be anchored in membrane rafts by two means, firstly TrkA, a transmembrane protein that must contain raft-targeting signals as it has been shown

previously to be targeted to membrane rafts (Huang *et al.*, 1999; Peiro *et al.*, 2000). Also, the complex will be attached to rafts through interactions between the lipid anchor on tubulin and GM1.

With the *in vitro* reactions, microtubule polymerisation occurs, recruiting more phospho-ERK and ERK to these raft-associated microtubules, leading to the observed increase in tubulin, phospho-ERK and ERK1 associated with the floating raft peak. Microtubules may also recruit additional TrkA into these membrane rafts from non-raft regions of the plasma membrane, which would explain the ATP- and NGF-dependent effect on TrkA association with rafts. Curiously, with this increase in TrkA in the rafts, p75^{NTR}, which is not part of this complex, is displaced into the non-raft regions of the membrane

Because TrkA seems to be in this complex with microtubules, it also provides an explanation for the results seen with internalisation. With 10 minutes internalisation, a slight decrease was observed in TrkA and also tubulin, phospho-ERK and ERK1 levels in the floating raft peak. This indicated that TrkA internalisation affected the amounts of microtubules, with their associated ERK, in the plasma membrane rafts. Then, with the *in vitro* reaction, more TrkA, tubulin and ERK moved into the membrane rafts as non-raft plasma membrane TrkA replaced the internalised receptors. Whether TrkA nucleates microtubule binding to rafts or vice versa is not clear, but the order of events provides a hint. NGF binding caused the raft association of tubulin to increase before TrkA was increased in rafts. Somehow, NGF must have been initiating a signal to cause polymerisation of microtubules in the membrane rafts. Again, with movement of TrkA into the rafts with NGF binding and *in vitro* reactions, p75^{NTR} was displaced into the non-raft regions. These data suggest that signalling events affect microtubules which then recruit TrkA into rafts.

The addition of GM1, as mentioned earlier, may have disrupted membrane rafts slightly (indicated by the apparent reduction in flotillin association), however, the association of signalling proteins was enhanced. Perhaps the answer to this discrepancy with flotillin and the other raft-associated proteins lies in GM1 itself. Exogenous GM1 will insert into the plasma membrane of the cells (Ghidoni *et al.*, 1986), and preferentially associate with the ordered membrane rafts. This could cause displacement of some membrane raft-associated proteins, such as GPI-anchored proteins (Simons *et al.*, 1999)

and flotillin, as seen here. However, GM1 has also been shown to have an interesting relationship with TrkA. The addition of GM1 to cells has been found to prevent cell death after serum deprivation (Ferrari *et al.*, 1993) through inducing phosphorylation of TrkA in a manner similar to NGF (Rabin and Mocchetti, 1995). The exact mechanism for this action remains unknown, however. It has been suggested that GM1 exhibits tight binding to TrkA (Mutoh *et al.*, 1995). If this is indeed the case, the addition of GM1 may displace other raft-associated proteins such as flotillin, but simultaneously keep TrkA in the rafts through its interaction with GM1. Also, as discussed earlier, tubulin has a lipid anchor that interacts with GM1 in membrane rafts. This could be another interaction that stabilises and even increases tubulin and ERK association with the membrane rafts when additional GM1 is added to the cells.

Unfortunately the effect of GM1 on p75^{NTR} association is inconclusive. However, the results with both *in vitro* reactions and NGF binding indicate that if TrkA raft association is increased, then p75^{NTR} association will be decreased. Therefore it is very likely that GM1 will also cause displacement of p75^{NTR} into the non-raft regions of the plasma membrane.

4.2 Conclusions

NGF-bound TrkA and p75^{NTR} are associated with plasma membrane rafts that contain flotillin. TrkA is possibly in a complex containing microtubules, which have phospho-ERK and ERK1 associated with them. This complex is strongly anchored in these plasma membrane rafts

Overall, this complex has increased membrane raft association with *in vitro* reactions, NGF binding and the addition of GM1, indicative that this complex, and its association with membrane rafts, is a key signalling centre for the transduction of the NGF signal.

This detergent-extraction procedure also resulted in the extraction of additional detergent-resistant membranes, which appear to originate from intracellular organelles.

4.3 Future work

The results so far are very exciting. However, a lot of work is still required to fully characterise both the high-density and the floating raft peak. The first objective however, would be to repeat the M β CD experiment, which had only been carried out once, and the internalisation, NGF binding and GM1 experiments that have been done twice but need to be repeated due to variable results. Also it would be interesting to observe the effects of NGF binding without internalisation and with and without *in vitro* reactions, which unfortunately was not carried out here.

The next objective would be to fully resolve the high-density peak found around 1.2 g/ml. This would involve altering the concentrations of iodixanol used so that this peak will float higher up the gradient. The results so far have indicated that the lower peak could correspond with membrane rafts from intracellular membranes. It would be useful to investigate the presence of intracellular membrane markers in these gradient fractions that are believed to contain intracellular membranes.

Also, to investigate the rafts in the floating raft peak, the presence of other known GPI-anchored proteins, such as alkaline phosphatase, could be investigated. This could be done either by Western blotting or perhaps by enzymatic assay. Another protein of interest is caveolin-1. It would be a good idea to probe the blots with an antibody against this protein, to investigate if the floating raft peak may actually contain caveolae.

Another attempt needs to be made to investigate the ganglioside composition of both of these types of membrane raft isolated. The lipid extraction protocol needs to be repeated to see whether better results are obtained and perhaps used in combination with mass spectrometry to give a more sensitive approach. Failing this, the use of cholera toxin-B conjugated to horse-radish peroxidase, or antibodies against particular gangliosides, could be used to detect the type of gangliosides present in both the high-density and the floating raft peak fractions. Another important marker for membrane rafts is cholesterol; its presence needs to be investigated, perhaps by using TLC and a FeCl₃ stain.

An additional experiment that was planned but, due to time constraints, could not be completed, was the solubilisation of membrane rafts with the non-ionic detergent octyl-glucoside. This has been used previously to indicate a proteins raft association by its solubilisation in this detergent but not in Triton X-100. This could also indicate whether the plasma membrane rafts that had been isolated have characteristics in common with typical membrane rafts that have been studied previously.

With these additional experiments, it may be possible to fully characterise the types of membrane rafts found in both the high-density peak and the floating raft peak. This may then help to explain the effects seen with the *in vitro* reactions, NGF binding, addition of GM1 and depletion of cholesterol with M β CD.

For the *in vitro* reactions, a further control could be useful, where the cells are incubated at 37°C after cracking without any ATP regenerating system. This would test whether the effects seen with this reaction are due to ATP regeneration or the incubation of the samples.

It would also be beneficial to examine the actual fractionation method used. In particular, the effect that sonication has on the membrane rafts. Also, to see whether detergent-extraction followed by sonication are both necessary to isolate the membrane rafts.

Through further characterisation of the two types of membrane raft isolated here, along with some additional experiments as mentioned, a more clear picture of the types of membrane domain involved should begin to emerge. This will hopefully lead to a better understanding of how these plasma membrane rafts, and perhaps the TrkA, tubulin and ERK complex, are involved in the NGF signalling pathway.

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