Glutamate release mechanisms from megakaryocytes

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Abstract

Cardiovascular disease (CVD) is one of the main causes of death in western society. Platelet activation, thrombus formation and plaque rupture are all central events in the pathogenesis of acute coronary syndromes, therefore therapies targeted at controlling platelet numbers and aggregation are likely to be beneficial in the treatment of CVD. Megakaryocytes (MKs) which are the precursors to platelets are an ideal target for these therapies, however the intrinsic factors that regulate the production and shedding of platelet precursors are poorly understood. Recent studies identified that MKs express functional NMDA-type glutamate receptors similar to those found in the CNS and that antagonism of these receptors prevents normal MK differentiation and platelet function. This thesis investigates glutamate signalling within MKs further, focusing on the glutamate release capability of MK cells and the mechanisms involved. Using molecular and cellular techniques it was demonstrated that MK cells expressed numerous regulatory proteins required for vesicular glutamate release, including core SNARE proteins, VAMP, SNAP-23 and syntaxin; specific glutamate-loading vesicle proteins, VGLUT1 and VGLUT2; and glutamate transporters, EAAT1 and EAAT2. Active vesicle recycling was observed in MK cells using a fluorescent reporter and an enzyme-linked fluorimetric assay confirmed that MK cells constitutively released glutamate and that glutamate release levels increased significantly following MK differentiation. Transient transfection of the human cell line MEG-01 with tetanus toxin, which disables vesicle recycling, induced a 30% decrease ($P<0.001$) in released glutamate compared to empty vector controls. In contrast, over-expressing VGLUT1 caused a 41% increase ($P<0.001$) in glutamate release activity of MEG-01 cells compared to controls.

These data demonstrate that MK cells regulate glutamate exocytosis through specific vesicular proteins, indicating that glutamate signalling may be a potential target for CVD therapies. Also the observations that MKs both release and recycle glutamate indicates an important role for glutamate signalling from these cells in autocrine and paracrine interactions within the bone marrow microenvironment.

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This thesis is dedicated to all those who believed in me.

Declaration

I performed all the work presented in this thesis, with exception to the generation of the pcDNA/VGLUT1, pEGFP/VGLUT1 (Chapter 4: Dr. Gary Spencer, University of York), pBoNT/C1 (Chapter 5; Professor Robert Burgoyne, University of Liverpool) and TeNTLC (Chapter 5: Dr Sean Sweeney, University of York) constructs: the BHK-TPO cells (Baby Hamster Kidney cells engineered to constitutively release thrombopoietin (TPO) (Chapter 2; Professor Kenneth Kaushansky, University of San Diego); and preparation of samples for TEM (Chapter 5; Technology facility, University of York). All figures in this thesis were drawn by myself, figures not attributed to a source were adapted from multiple references. This thesis has not been submitted for any other degrees. Some of the data has been presented at scientific conferences, and invited talks (listed below). All the experiments were performed at the Department of Biology, University of York.

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Spencer, G.J., McGrath, C.J., and Genever, P.G. (2007). Current perspectives on NMDA-type glutamate signalling in bone. Int. J. Biochem. Cell Biol. 39, 1089-1104.

Chapter 1

Introduction

Chapter 1: Introduction

1.1 Cardiovascular disease

1.1.1 Prevalence and Mortality statistics

Statistics released by the British Heart Foundation in 2006 (www.heartstats.org) indicate that diseases of the heart and circulatory system, cardiovascular diseases (CVD), remain the main cause of death in the UK. Approximately four in every ten people die from CVD, and in 2004 it accounted for more than 216,000 deaths. The two main forms of CVD are coronary heart disease (CHD) which accounts for approximately half of all deaths by CVD, and stroke which accounts for about a quarter of all deaths from CVD.

CHD is the most common cause of death in the UK and it is estimated that more than one in six women and one in five men die from the disease. In total there were just over 147,000 deaths caused by heart disease in the UK in 2003, CHD by itself was responsible for over 114,000 of these deaths and approximately 33,500 deaths were caused by other forms of heart disease (Figure 1.1). Although there is a higher incidence of CVD in the elderly, it is also one of the main causes of premature death in the UK (death before the age of 75), leading to over 65,000 premature deaths in the UK in 2003.

Since the early 1970s the UK has seen a decrease in the death rates from CVD, and in the past 10 years they have fallen by 37% for people under 75 years. Since the late 1970s death rates from CHD have also fallen, and in the last 10 years have decreased by 44% for people under 65 years. However in recent years CHD death rates have been falling slower in the younger age groups, and fastest in the 55-64 year age group (Figure 1.2). In the last 10 years the death rate due to stroke has seen a decrease also, there has been a 23% decrease in mortality under the age of 65 years. In recent years death rates due to stroke have declined at a slower rate than previously, particularly in the younger age groups as seen with CHD.

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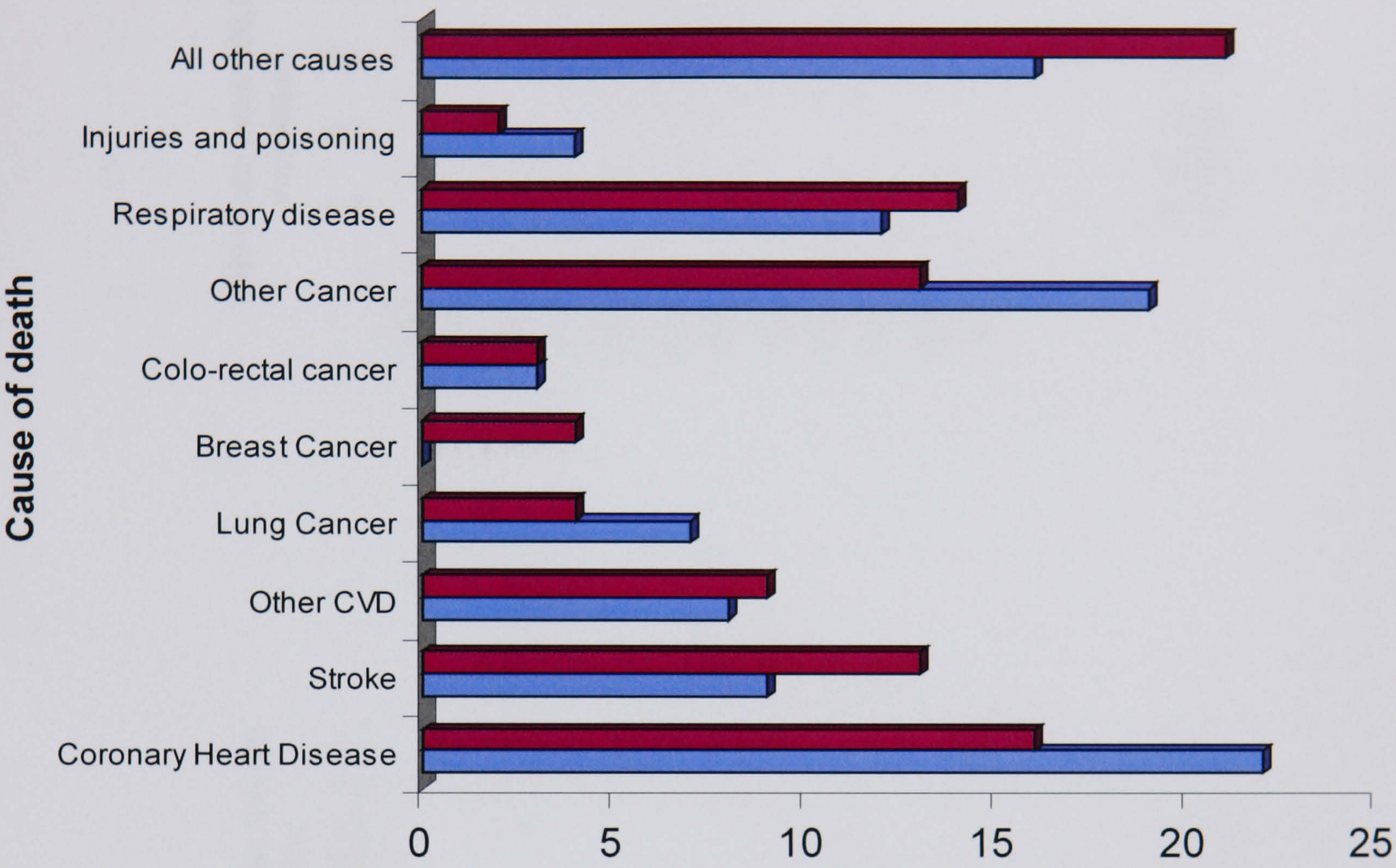

Figure 1.1: Causes of death in the United Kingdom in 2003. Coronary heart disease (CHD), stroke and other forms of cardiovascular disease (CVD) are the main causes of death un the United Kingdom for both males and females.

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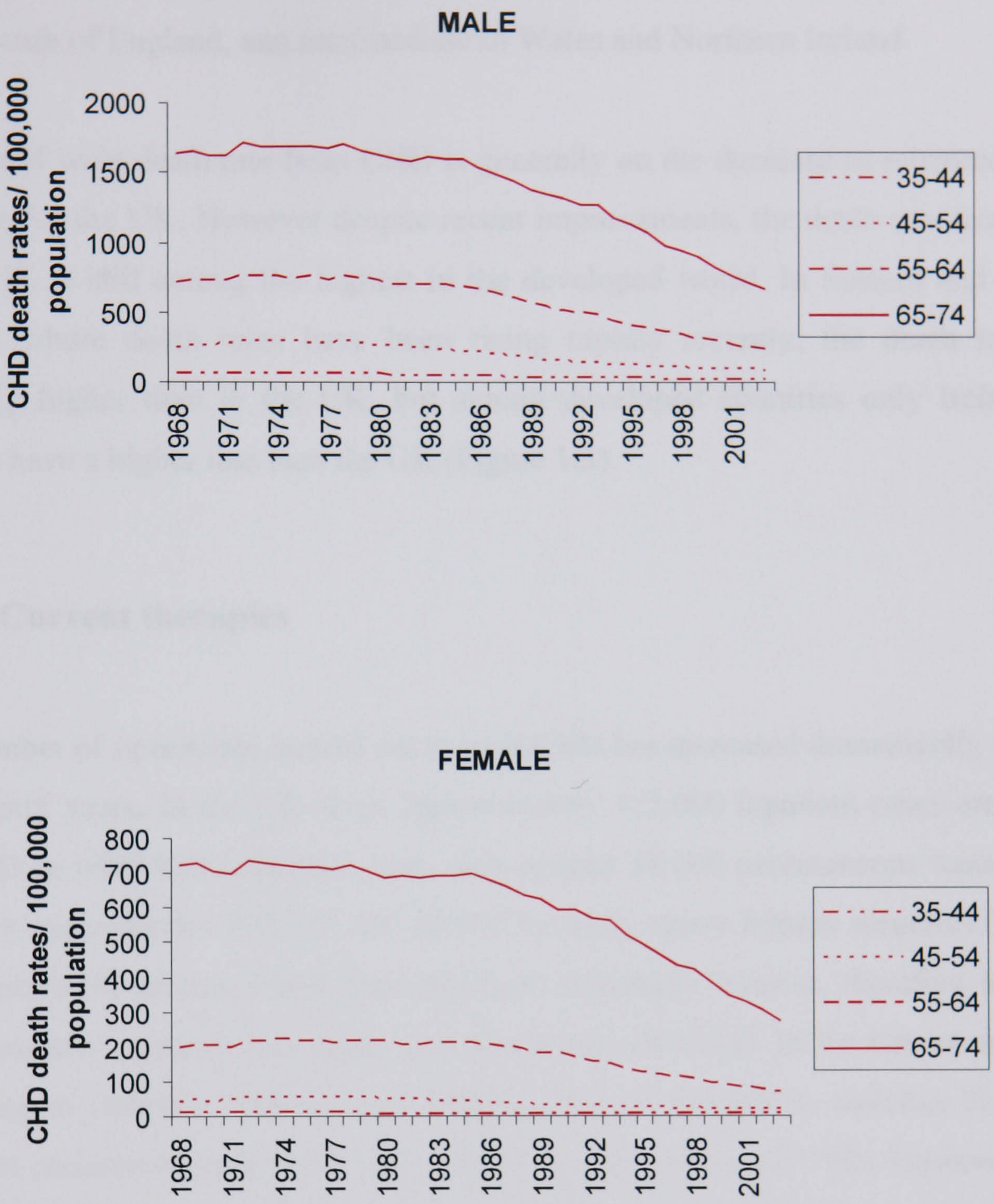

Figure 1.2: Male and female death rates per 100,000 population from coronary heart disease (CHD) in the United Kingdom, from 1968 until 2003. UK death rates from CHD remain higher in males than in females, although death rates in younger males are decreasing more than those in females of a similar age. Since the late 1970s death rates from CHD have fallen, and in the last 10 years have decreased by 44% for people under 65 years. However in recent years CHD death rates have been falling slower in the younger age groups, and fastest in the 55-64 year age group.

In the UK death rates from CHD remain higher in males than in females, although death rates in younger males are decreasing more than those in females of a similar age. Also, as well as differences between sexes, there are also variations between regions. CHD mortality rates are highest in the North of England and Scotland, lowest in the South of England, and intermediate in Wales and Northern Ireland.

The World wide death rate from CHD is generally on the decrease as mirrored by the statistics for the UK. However despite recent improvements, the death rate from CHD in the UK is still among the highest in the developed world. In Eastern and Central Europe, where death rates have been rising rapidly recently, the death rates are generally higher than in the UK, but among developed countries only Ireland and Finland have a higher rate than the UK (Figure 1.3).

1.1.2 Current therapies

The number of operations carried out to treat CHD has increased dramatically over the last twenty years. In the UK alone approximately 415,000 inpatient cases are treated for CHD in NHS hospitals each year, with around 58,000 percutaneous transluminal coronary angioplasties (PTCA) and 30,000 coronary artery bypass surgeries (CABG) taking place per annum. These procedures are extremely invasive, therefore there has been extensive research into drugs to treat and prevent CVD. In the last twenty years according to statistics released by the British Heart Foundation, diuretics have been the most commonly prescribed drug used in the prevention of CVD. However, there has also been an increase in the number of prescriptions for drugs aimed at preventing clotting in the blood, namely, antiplatelet, and anticoagulant drugs. Megakaryocytes, among the rarest of haematopoietic cells, differentiate and produce platelets via intermediate cytoplasmic extensions known as proplatelets (Shivdasani, 2001; Italiano, Jr. and Shivdasani, 2003) (for a more in-depth review of platelets and megakaryocytes see Introduction 1.2). Platelets have an important, but sometimes under-estimated role in cardiovascular disease (Willoughby et al., 2002a). Plaque rupture exposes collagen and von Willebrand factor (vWf) resulting in platelet adhesion, platelet activation, clotting activation and aggregation leading to occlusive thrombus formation, and the pathogenesis of acute coronary syndromes (ACS) such as stable angina pectoris and

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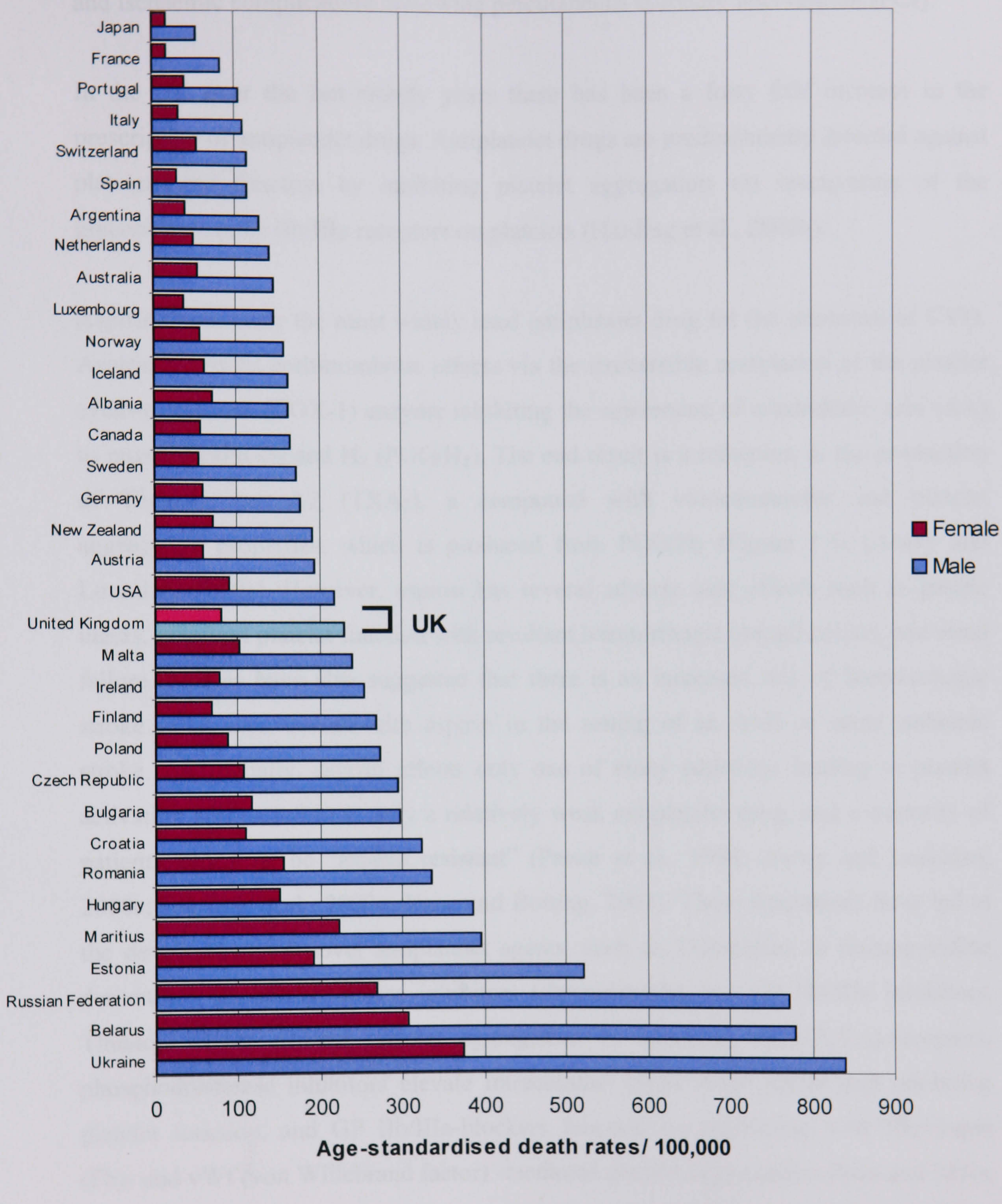

Figure 1.3: Male and female death rates per 100,000 population from coronary heart disease (CHD) in selected countries in the year 2000. The death rate from CHD in the UK is still among the highest in the developed world. However the death rates in eastern and central Europe are generally higher than the world wide average.

acute myocardial infarction (AMI) (Harding et al., 2002a; Willoughby et al., 2002b) and ischaemic complications following percutaneous coronary intervention (PCI).

In the UK over the last twenty years there has been a forty fold increase in the prescription of antiplatelet drugs. Antiplatelet drugs are predominantly directed against platelets and function by inhibiting platelet aggregation via inactivation of the glycoprotein (GP) IIb/IIIa receptors on platelets (Harding et al., 2002b).

Aspirin is currently the most widely used antiplatelet drug for the treatment of CVD. Aspirin exerts its antithrombotic effects via the irreversible acetylation of the platelet cyclo-oxygenase (COX-1) enzyme inhibiting the conversion of arachidonic acid (AA) to prostaglandin G_2 and H_2 (PGG/H_2). The end result is a reduction in the production of Thromboxane A2 (TXA_2), a compound with vasoconstrictor and platelet aggregation properties, which is produced from PGG/H_2 (Figure 1.4) (Awtry and Loscalzo, 2000a). However, aspirin has several adverse side effects such as gastric ulcers, impaired platelet function with resultant haemorrhagic complications, and renal failure. Studies have also suggested that there is an increased risk of haemorrhagic stroke in patients treated with aspirin in the setting of an AMI or acute ischemic stroke. Additionally, aspirin affects only one of many pathways leading to platelet activation and therefore is only a relatively weak antiplatelet drug, and a minority of patients appear to be "aspirin resistant" (Pawar et al., 1998; Awtry and Loscalzo, 2000c; Harding et al., 2002c; Vane and Botting, 2003). These limitations have led to the development of novel antiplatelet agents, such as Ticlopidine (a thienopyridine derivative), phosphodiesterase inhibitors (dipyridamole) and GP IIb/IIIa inhibitors. Thienopyridines prevent platelet aggregation by blocking ADP(P_2Y_{12})-receptors, phosphodiesterase inhibitors elevate intracellular cyclic AMP levels and inhibiting platelet function, and GP IIb/IIIa-blockers function by interfering with fibrinogen (Fb)- and vWf (von Willebrand factor) -mediated platelet aggregation (Born and Mills, 1969; Awtry and Loscalzo, 2000b; Harding et al., 2002d) (Figure 1.4). Further details about platelet function and their involvement in vascular disease is discussed in section 1.2.4.

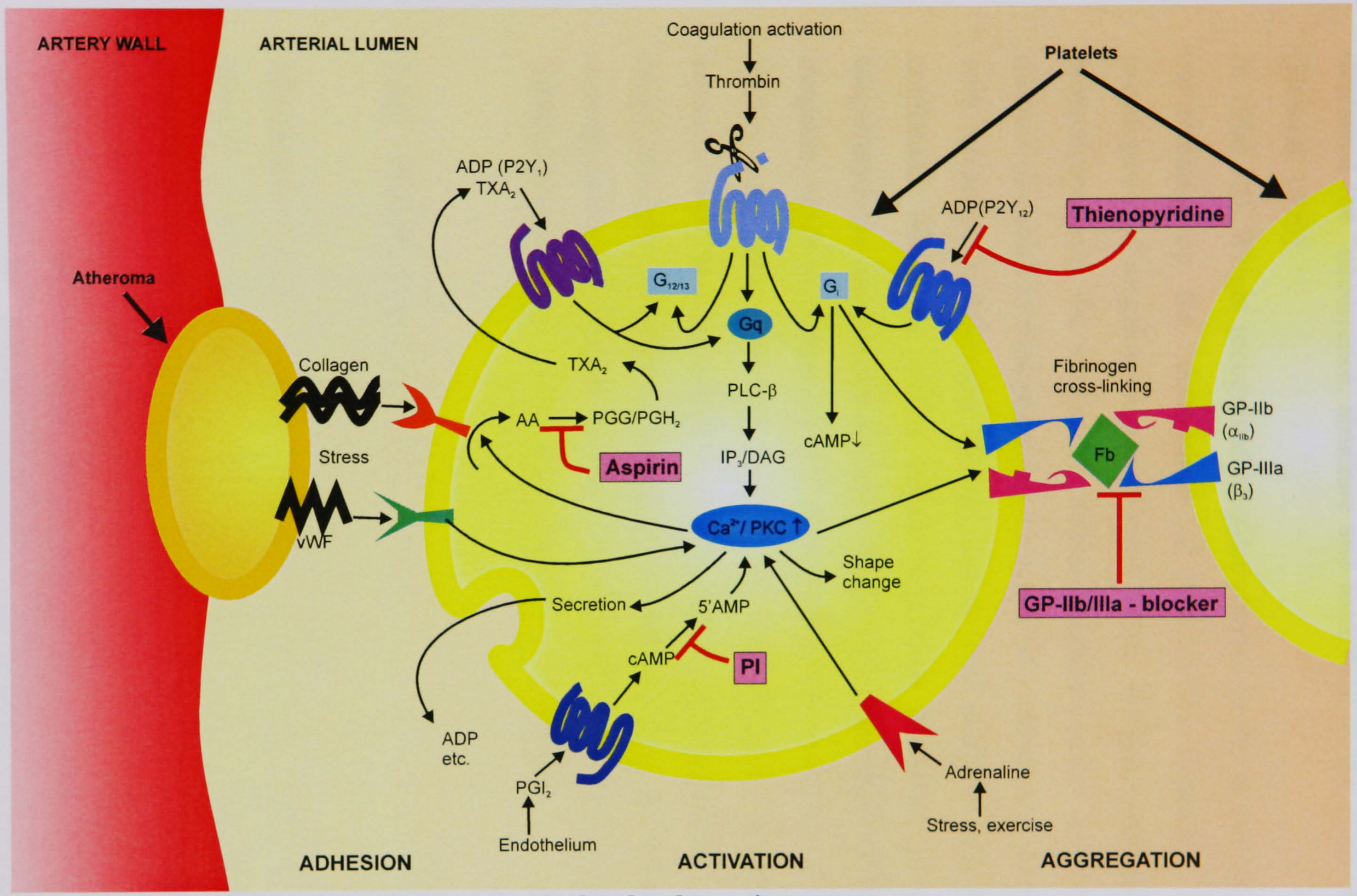

Figure 1.4: The role of platelets in thrombosis and current anti-platelet therapies

Adhesion of platelets to proteins (collagen, vWf), under conditions of high shear stress, and the action of platelet agonists (adrenaline, thrombin, ADP, thromboxane A2) lead platelet activation. Aspirin blocks thromboxane A2 synthesis by irreversibly acetylating cyclooxygenase-1; the thienopyridines (clopidogrel, ticlopidine) irreversibly inhibit the ADP receptor; and glycoprotein IIb/IIIa inhibitors block platelet activation inhibiting fibrinogen cross-linking of platelets and platelet aggregation. Phosphodiesterase inhibitors (dipyridamole, cilostazol) elevate intracellular cyclic AMP levels inhibiting platelet function.

AA=arachiodonic acid; ADP = adenosine diphosphate; cAMP = cyclic adenosine monophosphate; GP = glycoprotein; Fb = fibrinogen; PGG2 = prostaglandin G2; PGH2 = prostaglandin H2; PGI2 = prostacyclin; PI = phosphodiesterase inhibitor; TXA2 = thromboxane A2; vWF = von Willebrand factor.

1.2 Megakaryocytes and Platelet production

The term megakaryocyte was first introduced by W.W. Howell in 1890, who described the "occurrence, structure and formation of the giant cells of the marrow" (Howell, 1890).

In 1906 J.H. Wright recognised megakaryocytes as the precursors to blood platelets, commenting on the 'detachment of platelet-like fragments or segments from pseudopods' of megakaryocytes (Wright, 1906). Wright hypothesised that pseudopod projections from the surface of mature megakaryocytes penetrated into the bone marrow sinusoids to shed platelets (Wright, 1910). However it was not until the 1970s, with advances in molecular biology and in vitro methods, that this hypothesis was confirmed. Since the 1970s, which have been described as the modern era of megakaryocyte and platelet research, insights into the stages of normal and abnormal megakaryocyte development and its regulation by numerous cytokines and growth factors have been achieved. One of the most important advances in this area of research was the identification of the C-mpl proto-oncogene as the receptor for megakaryocyte development and platelet production, and the subsequent isolation and cloning of the *c-mpl* ligand, thrombopoietin (TPO) (Wendling et al., 1994b; Lok et al., 1994b; Kaushansky et al., 1994b; de Sauvage et al., 1994b).

1.2.1 Megakaryocytopoiesis

Megakaryocytes are the precursors to platelets and are primarily located in the bone marrow, where they represent approximately 0.1% of the marrow cell population (Nurden et al., 1997d). The production of an adequate number of platelets is vital in everyday life to repair minute vascular damage, to initiate thrombus formation in response to vascular injury and to aid wound healing. Megakaryocytes produce approximately 10^{11} platelets each day and in response to platelet demand can increase production of circulating platelets by more than 10-fold (Gewirtz et al., 1995b; Kaushansky, 2003; Kaushansky, 2005).

Within the bone marrow megakaryocytes are derived from common haematopoietic stem cells (HSCs) (see Figure 1.5). HSCs are responsible for producing all of the circulating blood cells required for haemostasis (Ogawa, 1993). HSCs give rise to the early common myeloid progenitors (CMPs). Megakaryocyte and erythroid lineages are produced by a common megakaryocyte-erythroid progenitor (MEP) that is derived from the CMPs. In response to transcription factors such as GATA-1 which drive differentiation of MEP, cytokines and growth factors (see section 1.2.2 for further details) MEP develop into BFU-MK (burst-forming units of megakaryocytes) and CFU-MK (colony-forming units of megakaryocytes), which have the ability to proliferate, but are committed to the megakaryocyte lineage (Briddell et al., 1989). Alternatively, MEP can progress down the early or late erythroid lineage giving rise to BFU-E or CFU-E (Schulze and Shivdasani, 2004c). During megakaryocyte development, proliferating diploid megakaryocyte progenitors (megakaryoblasts) stop dividing and give rise to mature polyploid megakaryocytes by a process of cell endoreduplication and cytoplasmic maturation (Nurden et al., 1997c; Shivdasani, 2001; Ravid et al., 2002a) (see section 1.2.1.1 and Figure 1.6 for more details). Mature megakaryocytes have a large abundant cytoplasm, and a unique set of organelles including alpha granules, dense bodies and a distinctive demarcation membrane system (DMS).

1.2.1.1 Megakaryocyte endomitosis

The main hallmarks of megakaryocyte maturation are the formation of a polyploid nucleus and the expansion of cytoplasmic mass. A cell is described as being polyploid when it has a greater than diploid content of DNA, and is thought to occur in megakaryocytes by endomitosis. Endomitosis is a cell cycle in which the number of normal haploid chromosomes in the cell is multiplied (e.g., 2N in diploid cells, 4N or greater in polyploid cells) (for review see Ravid et al., 2002b and Figure 1.2.2). Polyploidy has also been identified in other mammalian cells including hepatocytes (Kudryavtsev et al., 1993), cardiac myocytes (Sandritter and Scomazzoni, 1964), and arterial smooth muscle cells (Barrett et al., 1983) all of which become polyploid at some point during their normal life cycle. Polyploidisation has also been observed in

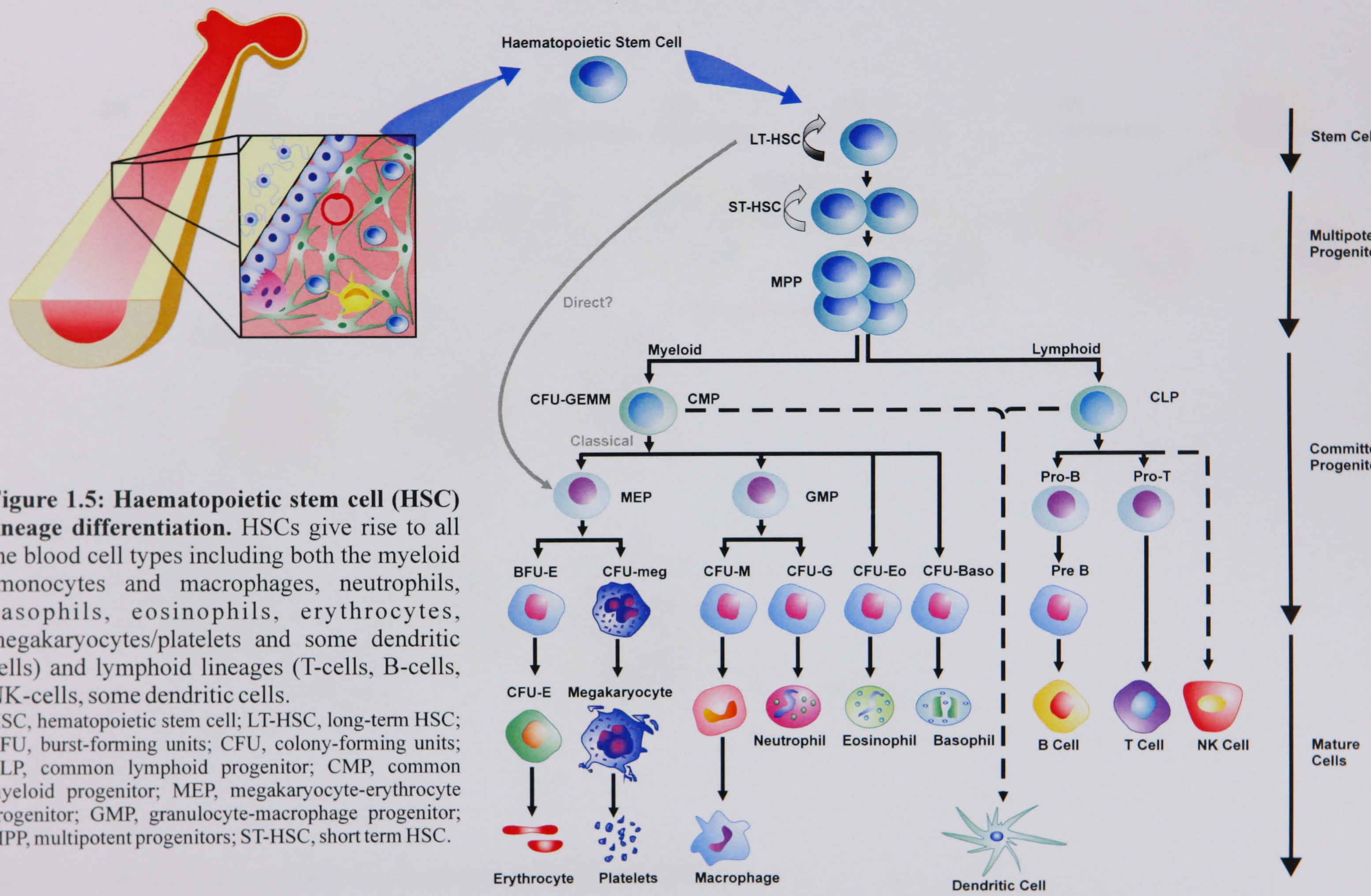

Figure 1.5: Haematopoietic stem cell (HSC) lineage differentiation. HSCs give rise to all the blood cell types including both the myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets and some dendritic cells) and lymphoid lineages (T-cells, B-cells, NK-cells, some dendritic cells.

HSC, hematopoietic stem cell; LT-HSC, long-term HSC; BFU, burst-forming units; CFU, colony-forming units; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage progenitor; MPP, multipotent progenitors; ST-HSC, short term HSC.

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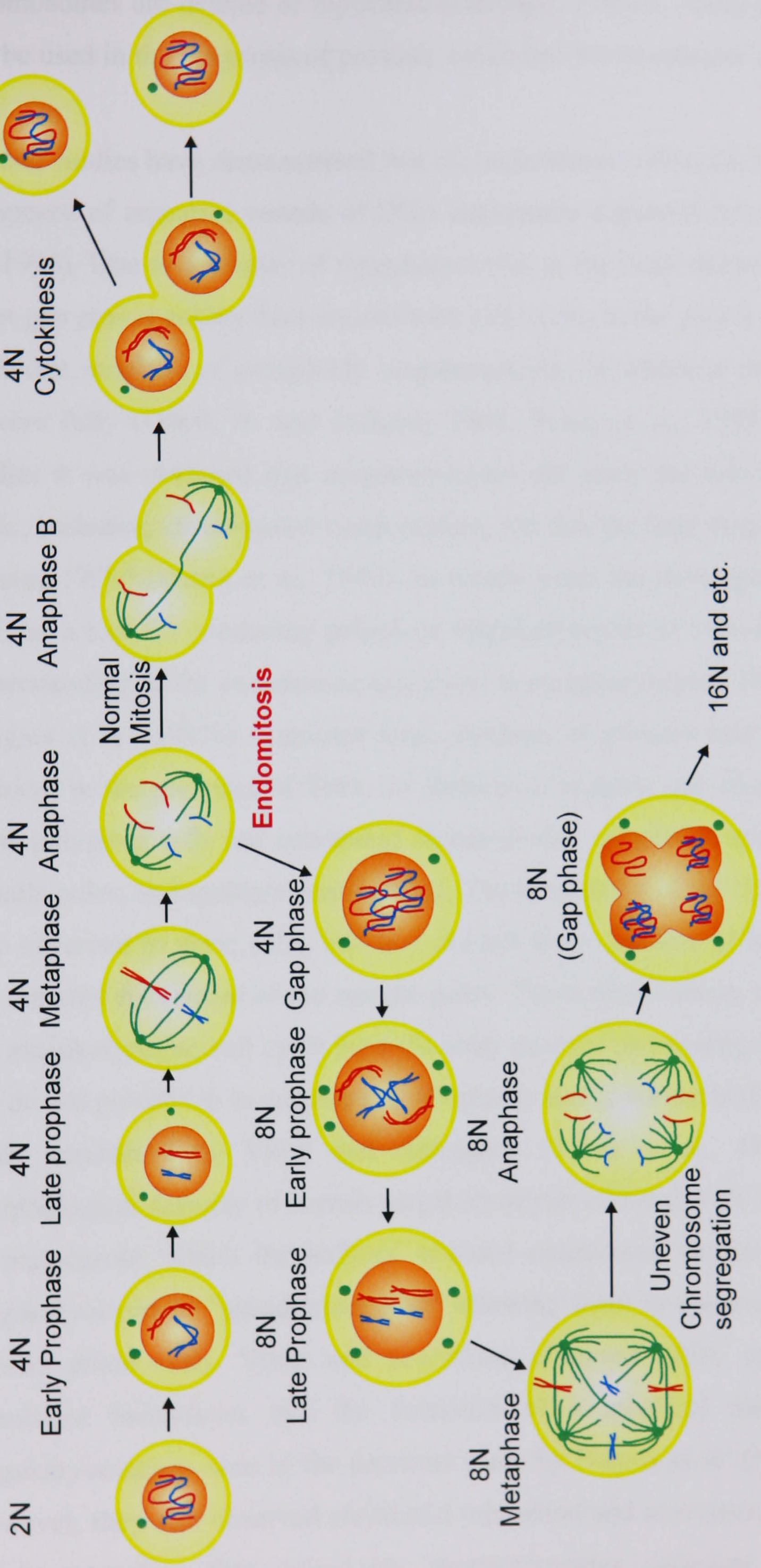

Figure 1.6: Megakaryocytic cell cycle (adapted from Ravid et al.2002).

cancerous tumours, caused by a process called aneuploidy, in which specific chromosomes are deleted or replicated (Barlogie, 1984a). Analysis of aneuploid cells can be used in the prognosis of prostate, colon and breast cancers (Barlogie, 1984b).

Several studies have demonstrated that the endomitotic cell cycle in megakaryocytes is composed of repeating rounds of DNA replication separated by short gaps (Zhang et al., 1996). Due to the rarity of megakaryocytes in the bone marrow, and the relatively short gap period during their endomitotic cell cycle, in the past it was difficult finding sufficient numbers of polyploidy megakaryocytes in which to study the endomitosis process fully (Odell, Jr. and Jackson, 1968; Wang et al., 1995). However in early studies it was observed that megakaryocytes did enter the mitotic phase of the cell cycle, including chromosome condensation, but that the later stages of this phase were missing (Winkelmann et al., 1987). In recent years the development of recombinant TPO as a tool for producing polyploid megakaryocytes in vitro has lead to a greater understanding of the endomitotic cell cycle in megakaryocytes. Nagata and coworkers (Nagata et al., 1997c) examined large numbers of primary murine megakaryocytes, cultured in the presence of TPO, for features of mitosis and observed that several of these polyploid cells had condensed chromosomes, spindles originating from multiple spindle poles, and multiple centrosomes. The breakdown of the nuclear membrane was also observed in these cells, but they did not show features of late mitosis including the outward movement of the spindle poles. These observations suggested that during the megakaryocytic cell cycle the cells enter mitosis, proceeding as far as anaphase A, but do not proceed in to anaphase B, telophase, and cytokinesis (Figure 1.6). A similar study conducted by Vitrat and colleagues (Vitrat et al., 1998b) examined the morphological features of human megakaryocytes cultured with TPO, in the presence of nocodazole, which the authors' claimed maintained approximately 20% of the megakaryocytes in "pseudometaphase" allowing them to observe a larger number of mitotic phase cells. Vitrat and coworkers observed DNA condensation, nuclear membrane breakdown, and the formation of a spherical mitotic spindle in the megakaryocytes as seen in the previous study by Nagata et al. (Nagata et al., 1997b). However, they also observed chromatid separation and movement towards the spindle poles, suggesting that polyploidy megakaryocytes complete anaphase but lack cytokinesis, contradicting the hypothesis by Nagata et al. that megakaryocytic endomitosis is due to a block at the metaphase-anaphase transition. The precise events

surrounding the mitotic events of polyploid megakaryocytes are still debatable, however it is clear that these cells undergo a process of abortive mitosis, possibly caused by alterations in the regulation of mitotic exit (Nagata et al., 1997a; Vitrat et al., 1998a).

1.2.2 Platelet formation

Platelet formation is a complex process and due to the extreme rarity of the megakaryocytes in normal bone marrow (<0.1% of total cells) the mechanisms underlying this process are poorly understood. The current model for platelet production from megakaryocytes is based on the observation that megakaryocytes extrude long cytoplasmic projections (proplatelets) which contain swellings encompassing platelet organelles that are later delivered into the blood circulation as platelets (see Figure 1.7) (Italiano, Jr. et al., 1999d; Hartwig and Italiano, Jr., 2003).

Platelets are small anucleate particles that are found in close proximity to blood vessel walls during laminar flow. In response to vascular injury, platelets undergo morphological changes allowing them to aggregate and cover the site of injury (see section 1.2.4 for a more detailed review of platelet activation and thrombus formation). Platelets are assembled along intermediate pseudopodial extensions called proplatelets that are created from the escape and evagination of the extensive internal membrane system of mature megakaryocytes (Radley and Haller, 1982). Proplatelet formation generally commences from one site on the megakaryocyte, where one or more pseudopodial extensions form. These extensions continue to elongate and widen for 4 to 10 hours, forming proplatelets of approximately 2 to 4 μM in diameter. Proplatelets continue to form from the original site until the entire megakaryocyte cytoplasm is transformed into a complex network of proplatelets (Italiano, Jr. et al., 1999c).

The reorganisation of microtubules, which are polymers of αβ-tubulin dimers, are essential for proplatelet elongation (Tablin et al., 1990). Microtubules consolidate in a mass underneath the plasma membrane in the area where the proplatelet is to be formed. Microtubules then align into bundles and fill the cortex of the first blunt process to be formed by the megakaryocyte. The proplatelet shaft is thickened and extended by the merger of microtubules into linear bundles. Platelet-sized swellings

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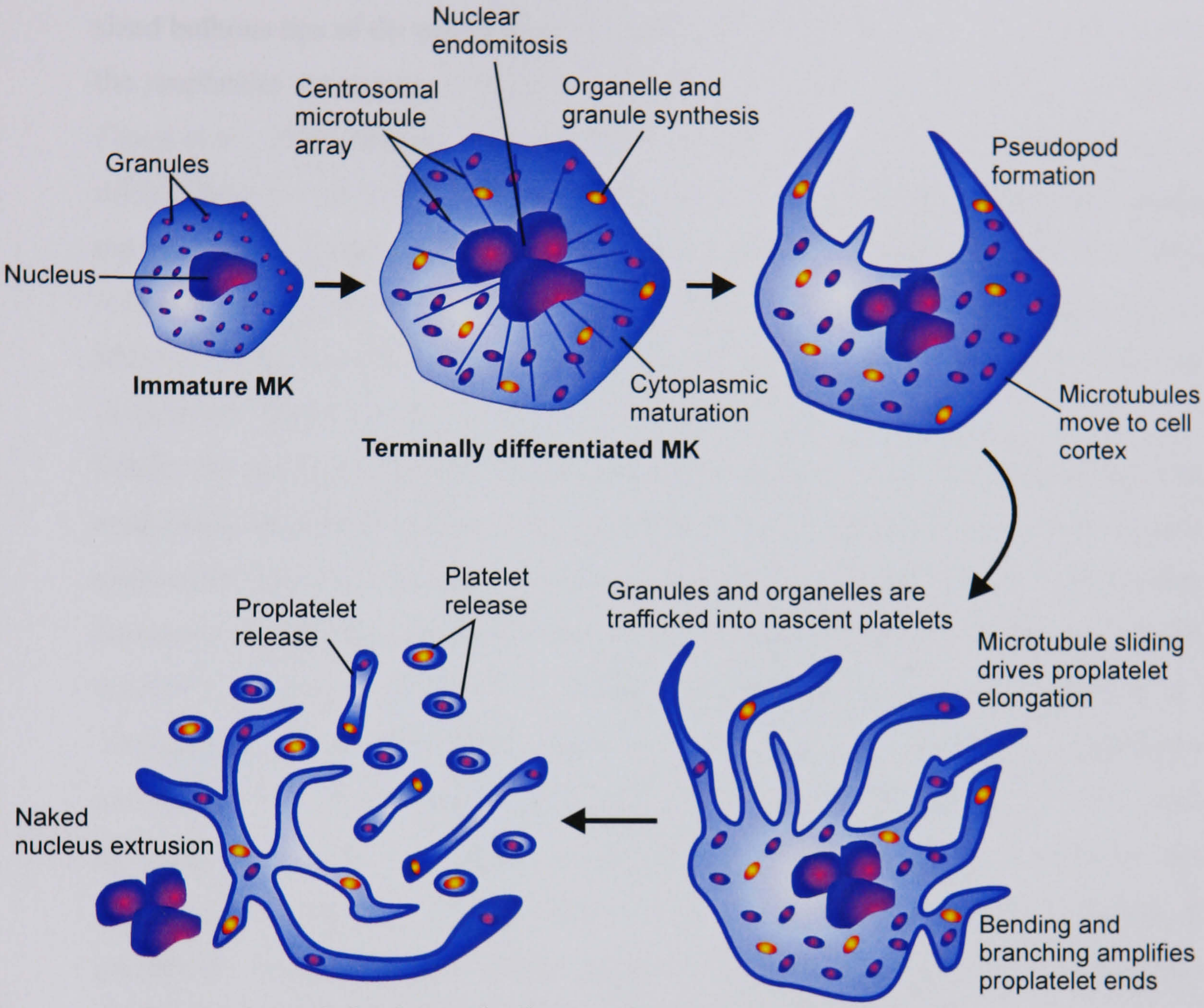

Figure 1.7: Platelet production from megakaryocytes.
Initially the immature megakaryocyte undergoes endomitosis, followed by organelle and granule synthesis, centrosomal microtubule array and cytoplasmic maturation. Prior to proplatelet formation centromes disassemble and microtubules move to the cell cortex, leading to the formation of thick pseudopods. Proplatelet formation is driven by the sliding of over-lapping microtubules at the cell cortex, organelles and granules are trafficked to the tips of these proplatelet structures where nascent platelets develop. Finally the entire megakaryocyte cytoplasm is converted into a mass of proplatelets and released from the cell. The nucleus is then extruded from the mass of proplatelets and platelets are released from the ends of the proplatelet structures (figure adapted from Patel et al. 2005).

develop along the length and at the ends of the proplatelets. These swellings along the proplatelet have been identified as regions where microtubule bundles with in the shaft diverge for a short distance before reconvening to thicken the proplatelet. At the free end of the proplatelet, microtubule bundles form loops which give rise to the platelet-sized bulbous tips of the proplatelets. Coiling of microtubules only occurs at the tips of the proplatelet extensions, indicating that this is the primary site for platelet formation (Topp et al., 1990; Italiano, Jr. et al., 1999b; Schwer et al., 2001a). The importance of microtubule assembly in platelet formation has been highlighted in experiments using transgenic mice lacking β1-tubulin. These mice develop thrombocytopenia (15 to 30% reduction in platelet numbers) and form spherocytic circulating platelets, due to defective marginal band formation and poor assembly of microtubules within the proplatelets (Schwer et al., 2001b).

While the physical properties of proplatelet formation are well established, the underlying intracellular factors and conditions involved in this process are not yet well understood. Recent studies into megakaryocytic signalling pathways and transcription factors have implicated protein kinase C, serine/threonine phosphatases, NF-E2 and GATA-1 in proplatelet formation and normal platelet production (Shivdasani et al., 1995b; Leven et al., 1997; Shivdasani et al., 1997c; Lecine et al., 1998c; Rojnuckarin and Kaushansky, 2001; Raslova et al., 2006). Other proteins regulated by NF-E2, such as Rab27b, caspase-12, 3β-hydroxysteroid dehydrogenase, and β1-tubulin (as discussed previously), are also thought to play an important role in the regulation of proplatelet formation and/or platelet release (Lecine et al., 2000; De Botton et al., 2002; Nagata et al., 2003; Tiwari et al., 2003; Kerrigan et al., 2004).

The actual process of platelet release in vivo has not yet been identified due to the rarity of megakaryocytes within the bone marrow; however, it has been proposed that in vivo the proplatelets extend into the bone marrow vascular sinusoids, where they can be released in to the blood stream (Italiano, Jr. et al., 1999a). However, evidence is accumulating to suggest that the bone marrow stroma contributes to proplatelet development and platelet release. In recent studies chemokine SDF-1 and the growth factor FGF-4 have been shown to be necessary for megakaryocyte recruitment to sinusoid endothelial cells (Avecilla et al., 2004). Studies have also shown fibrinogen, supports proplatelet development in vitro and in vivo is localized to the vascular sinusoids (Larson and Watson, 2006b). Further it has been demonstrated that

fibrinogen regulates proplatelet formation via integrin αIIbβ3 in vitro a finding supported by the use of thrombopoietin-stimulated αIIbβ3 knockout mice, where the absence of αIIbβ3 lead to reduced platelet counts (Larson and Watson, 2006a). Other matrix proteins such as vitronectin and collagen have also been shown to promote proplatelet formation (Leven and Tablin, 1992; Sabri et al., 2004).

1.2.3 Regulation of megakaryocytopoiesis

Megakaryocytes are derived from haematopoietic stem cells (HSCs) via a tightly regulated process involving specific cytokines, haematopoietic growth factors and interactions with the bone marrow microenvironment. The primary function of megakaryocytes is to produce blood platelets, which play a vital role in haemostasis and thrombosis.

Thrombopoietin (TPO) is the major hormone controlling megakaryocyte development. It was first identified as the as the ligand for the c-mpl proto-oncogene receptor, which is present on the surface of both megakaryocytes and platelets. The importance of TPO in megakaryocytopoiesis and thrombopoiesis was confirmed using mice deficient in the TPO gene and the c-mpl receptor. These mice showed an 85% reduction in peripheral platelet, marrow and spleen megakaryocytes, however the megakaryocytes and platelets that remained were functionally normal. Therefore TPO seems to be important in the development of an adequate number of megakaryocytes and platelets, but other factors are also involved (see Figure 1.8) (Gurney et al., 1994a; Kaushansky, 1995c; de Sauvage et al., 1996b).

There are several haematopoietic growth factors which regulate proliferation, polyploidisation and maturation of megakaryocytes *in vivo* and *in vitro* (Figure 1.8). These include cytokines, such as interleukin-1 (IL-1), IL-3, IL-6, IL-11, granulocyte macrophage-colony stimulating factor (GM-CSF), and stem cell factor (SCF). It has been reported that SCF acts on the late stages of megakaryocytopoiesis by inducing polyploidisation and improving the maturity of the final megakaryocytes. IL-3 and GM-CSF are stimulators of megakaryocyte colony formation, however IL-3 is more potent than GM-CSF as it promotes the production of a greater number of colonies containing a higher number of megakaryocytes. IL-6 has been shown to promote megakaryocyte maturation by increasing the cell size, polyploidisation, number and

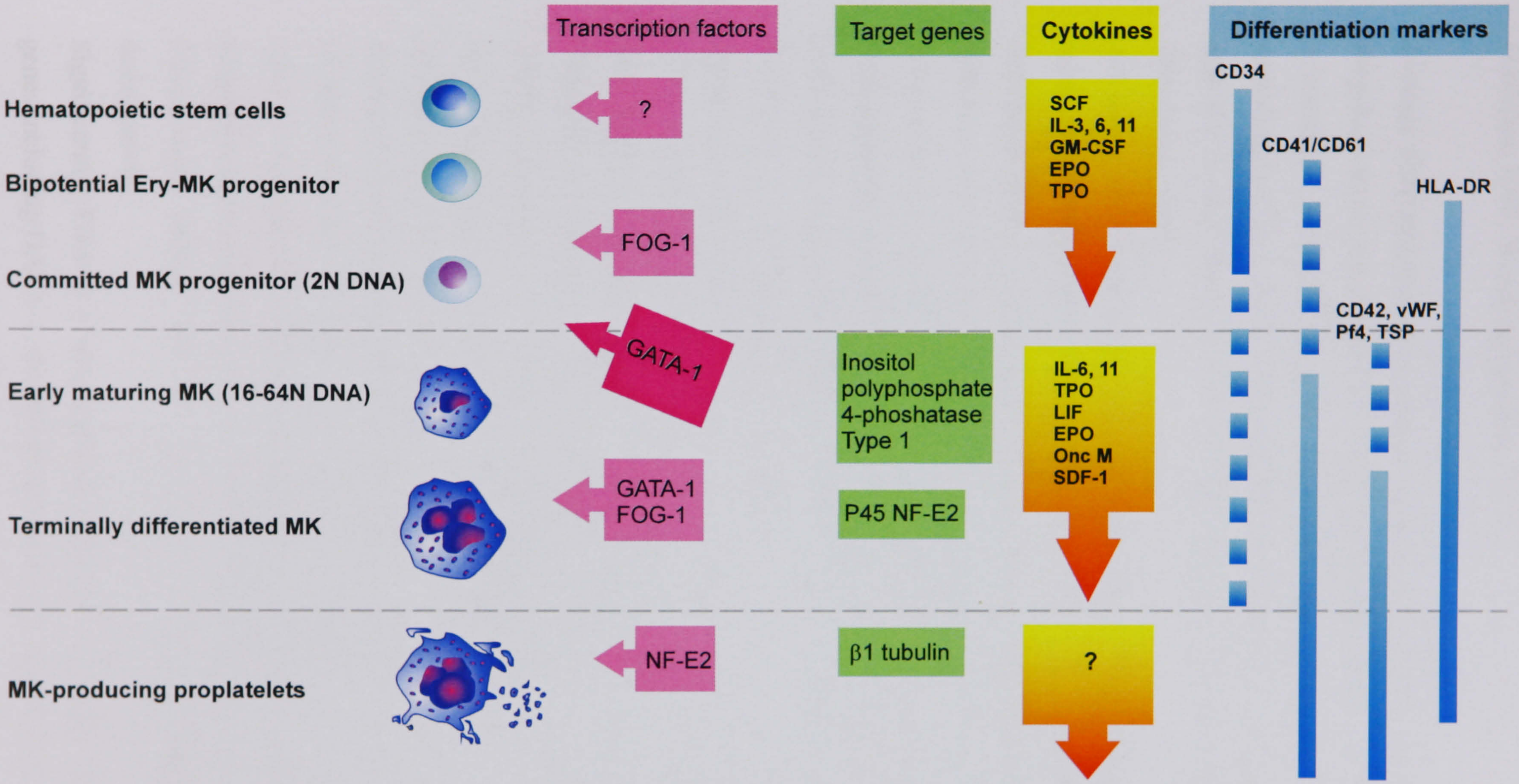

Figure 1.8: Diagram summarising the cytokine, chemokines and transcription factors regulating megakaryocytopoiesis, and the expression differentiation markers during this process. Megakaryocytes originate from HSCs and undergo a complex maturation process to form mature megakaryocytes which produce platelets. Numerous cytokines positively regulate megakaryocytopoiesis (light pink) whilst some appear to have a negative effect (dark pink). Cytokines such as TPO, IL-3, -6, -11, -12 and GM-CSF have an important role in megakaryocytopoiesis acting at different stages during megakaryocyte maturation. The blue lines indicate the expression of differentiation markers during megakaryocytopoiesis, dashed lines indicate a lower expression of these markers, CD34 expression is highest in HSCs and early megakaryocyte progenitors, where as CD41, CD61 and CD42 are markers of mature megakaryocytes.

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synthesis of platelet proteins and proplatelets, and IL-11 induces the maturation of early megakaryocytes by increasing megakaryocyte ploidy and size (Gordon and Hoffman, 1992; Wendling and Han, 1997).

Lineage differentiation of megakaryocytes is governed by many transcription factors. megakaryocytic and erythroid lineages, as discussed in section 1.2.1. are derived from a common precursor and share many early lineage-restricted transcription factors including GATA-1, FOG-1 (Friend Of Gata-1), Fli-1 (Friend leukaemia integration-1). and the nuclear factor 2 (NF-E2) (see Figure 1.8) (Shivdasani and Orkin. 1996; Shivdasani, 2001).

Ets genes are a large family of transcriptional regulators consisting of a conserved winged helix-loop-helix DNA binding (ETS) domain that recognises purine-rich DNA sequences with a GGA(A/T) consensus, named the Ets binding sequence (EBS) (Oikawa and Yamada, 2003; Seth and Watson, 2005). Fli-1 (Friend leukaemia integration-1), a member of the Ets transcription family. is preferentially expressed in haematopoietic lineages and vascular endothelial cells where it has a vital role in maintaining normal development and oncogenesis (Truong and Ben David, 2000). Evidence is accumulating to suggest that Fli-1 is a positive regulator of megakaryocytopoiesis (Hart et al., 2000c; Spyropoulos et al., 2000c; Kawada et al., 2001d; Jackers et al., 2004a). Fli-1 expression is increased in the megakaryocytic cell line. K562. after phorbol-ester induced differentiation promoting the expression of megakaryocytic markers CD41 and CD61 (Athanasiou et al., 1996; Holmes et al., 2002a; Eisbacher et al., 2003b). Targeted disruption of the Fli-1 gene in mice leads to severe dysmegakaryopoiesis and vascular defects, embryos of these homozygous mutants (Fli-1 -/-) showed hemorrhage from the dorsal aorta into the lumen of the neural tube and ventricles of the brain beginning on embryonic day 11 and were dead by embryonic day 12 (Hart et al., 2000b; Spyropoulos et al., 2000b; Kawada et al., 2001c). Analysis of cultured cells from day 10 embryos revealed an absence of megakaryocytes and aberrant red blood cell development (Kawada et al., 2001b). These results indicate that Fli-1 has a vital role in haematopoiesis and embryonic development.

Significantly. Fli-1 is a transcriptional activator of several megakaryocytic-specific genes including GATA-1. thrombopoietin receptor (c-mpl). glycoprotein-IIb (GP-IIb). GP-VI. GP-IX. and GP-Ib (Watson et al., 1992; Lemarchandel et al., 1993; Seth et al.,

1993; Zhang et al., 1993; Deveaux et al., 1996; Wang et al., 2002; Holmes et al., 2002b; Eisbacher et al., 2003a).

The zinc-finger proteins GATA-1 and GATA-2 have been shown to have important roles in erythro-megakaryocyte transcription (Martin and Orkin, 1990; Romeo et al., 1990). GATA-1 has a vital role in both the early and late stages of megakaryocyte differentiation and regulates megakaryocyte proliferation (Shivdasani et al., 1997b). Mice lacking the GATA-1 gene in megakaryocytes and humans with critical point mutations have platelet counts 15% less than that of normal, with increased platelet size, spherical platelets instead of discoid, and increased bleeding times (Shivdasani et al., 1997a; Nichols et al., 2000; Freson et al., 2001). GATA-1 mutations in humans lead to severe diseases involving both megakaryocytes and erythroid cells (Schulze and Shivdasani, 2004b; Muntean and Crispino, 2005a). GATA-1 mutations, which produce a truncated form of GATA-1 (GATA-1s), precede the transition to megakaryocyte-lineage acute leukaemia (AMKL) in patients with Down's syndrome (trisomy 21), indicating a role for GATA-1 in the proliferation of megakaryocyte progenitors (Wechsler et al., 2002; Crispino, 2005).

FOG-1 (Friend of GATA-1) is a zinc finger transcription factor, initially discovered as a GATA binding protein, whose expression pattern mirrors that of GATA-1 in both erythroid and megakaryocytic cells. The interaction between GATA-1 and FOG-1 is vital for embryonic haematopoiesis and megakaryocyte development (Shimizu et al., 2004). Patients with GATA mutations that interfere with this interaction have severe thrombocytopenia and dyserythropoietic anaemia, indicating that the GATA-1/FOG-1 interaction is clinically significant in megakaryocytopoiesis (Muntean and Crispino, 2005b). Targeted disruptions in FOG-1 have a phenotype similar to that observed in GATA-1 null mice, however FOG-1 knockout mice lack megakaryocytes indicating that FOG-1 has a critical role in early megakaryocyte development (Tsang et al., 1998).

The transcription factor NF-E2, a heterodimeric basic-leucine zipper, comprises of a megakaryocytic-erythroid specific 45-kDa subunit and a non-specific p18 Maf family subunit which control megakaryocyte maturation, proplatelet formation and platelet release (Shivdasani, 1996; Lecine et al., 1998a; Schulze and Shivdasani, 2004a). NF-

N2 regulates the transcription of megakaryocytic genes, including Rab27b, β-tubulin. and thromboxane synthase. (For more information about the role of NF-N2 target genes in megakaryocyte differentiation please refer to a recent review by Schulze and Shividasani, 2005 (Schulze and Shivdasani, 2005)). NF-N2 knockout mice. either lacking the megakaryocytic-erythroid specific 45-kDa subunit or both the alternative p18 subunits, have severe thrombocytopenia (<5% of normal). These megakaryocytes have hallmarks of maturity, including an enlarged cytoplasm. and polyploidy DNA. but also have disorganised internal membranes, reduced granule numbers and fail to produce proplatelets in culture (Shivdasani et al., 1995a; Shivdasani, 1996; Lecine et al., 1998b; Shiraga et al., 1999; Onodera et al., 2000). These findings indicate that NF-N2 functions at the point where megakaryocytes prepare to produce proplatelet extensions and release platelets.

Recent studies have provided evidence that the Rho/ Rho Kinase (ROCK) pathway. a regulator of actin cytoskeleton, is a negative regulator of proplatelet formation (Chang et al., 2007a). Rho is expressed at high levels during all stages of megakaryocyte differentiation and is activated by TPO. Overexpression of spontaneously active or a dominant negative RhoA leads to a decrease or increase in proplatelet formation respectively, indicating that Rho activation inhibits proplatelet formation. Inhibition of ROCK leads to an increase in proplatelet formation, indicating that the inhibitory effect of Rho is mediated through ROCK (Chang et al., 2007b).

1.2.4 Role of megakaryocytes and platelets in vascular disease

Platelets are activated in response to vascular injury, acting to control bleeding and maintaining normal haemostasis. Platelets are normally heterogeneous with respect to their size, density and reactivity. Changes in these characteristics may be involved in the pathogenesis of vascular diseases such as acute myocardial infarction, unstable angina and sudden cardiac death (Thompson et al., 1982; Falk, 1983). During normal haemostasis, platelet activation and adhesion to the blood vessel wall is initially controlled by two adhesion receptors. glycoprotein (GP) Ib-IX-V and GPVI which bind to von Willebrand factor (vWf) and collagen respectively under conditions of high shear stress (Kroll et al., 1996b; Jackson et al., 2003; Nieswandt and Watson.

2003a: Andrews et al.. 2004; Farndale et al.. 2004: Gawaz. 2004). Platelet adhesion is also mediated by several platelet integrin receptors and their ligands. The major platelet integrins involved in platelet adhesion are αIIbβ3 (GPIIb/IIIa: CD41/CD61) and α2β1 integrin (GPIa/IIa). which interact with vWf and fibrinogen. and collagen respectively (Nieuwenhuis et al.. 1985: Kroll et al.. 1996a; Ruggeri. 1997b: Savage et al., 1998; Xiong et al.. 2003b).

GPVI consists of two Ig domains. a mucin-rich stalk and a cytosolic sequence coupled to a disulfide linked Fc receptor (FγR) γ-chain homodimer (Moroi et al., 1989: Clemetson et al., 1999; Nieswandt et al.. 2000: Nieswandt and Watson. 2003b). The FcR γ-chain is required for the expression of GPVI on platelets. and the cross-linking of collagen with the GPVI–FγR-chain complex initiates a signalling pathway via tyrosine phosphorylation of an ITAM (immunoreceptor tyrosine-based activation motif) present on the FγR chain. resulting in the phosphorylation and activation of PLCγ 2 (phospholipase Cγ 2). Activation of PLCγ 2 leads to a subsequent increase in the levels of the messengers inositol 1,4,5-trisphosphate, 1,2-diacylglycerol, phosphatidylinositol 3,4,5-trisphosphate, and intracellular calcium resulting in platelet activation (Gibbins et al., 1996; Poole et al., 1997; Tsuji et al., 1997; Gibbins et al., 1997; Asselin et al., 1997).

The GPIb-IX-V complex consists of four transmembrane glycoproteins, GPIbα (CD42bα) disulfidelinked to GPIbβ (CD42bβ/CD42c), noncovalently complexed with GPIX (CD42a) and GPV (CD42d) in the ratio 2:2:2:1, all of which are members of the leucine-rich protein family (Andrews et al.. 1997: Lopez et al.. 1998b; Berndt et al.. 2001b). It is the N-terminal domain of GPIbα that contains the binding sites for vWf (Berndt et al., 2001a). Platelet activation, which is required for platelet aggregation and subsequent thrombus formation, is stimulated by the interaction of GP Ib-IX-V and vWf. Platelet activation leads to a conformational change in the αIIbβ3 (GPIIb/IIIa) receptor on the platelet surface enabling it to bind to plasma fibrinogen, leading to the reorganisation of the platelet cytoskeleton and cross-linking of this complex with adjacent activated platelets resulting in platelet aggregation and the formation of a haemostatic plug (Isenberg et al., 1987; Frojmovic, 1998; Shattil, 1999; Ruggeri, 2000). Several platelet agonists including adenosine diphosphate (ADP), thrombin, collagen, thromboxane A2 and epinephrine have been identified as activators of platelet aggregation (Wagner and Burger, 2003). ADP and thrombin

activate platelets via G-coupled purinergic receptors $P2Y_1$ and $P2Y_{12}$ and G-coupled protease-activated receptors PAR-1 and PAR-4 respectively, enabling αIIbβ3 to bind to its ligands fibrinogen or vWf resulting in platelet aggregation and thrombus formation (Coughlin, 2000; Dorsam and Kunapuli, 2004).

Under normal conditions of haemostasis platelets circulate close to the endothelial cells of the blood vessel lining but do not attach to it, however under pathological conditions platelets respond to changes in the endothelial lining, such as fat deposits and plaque rupture, and exposed subendothelial structures by adhering to the site of the lesion leading to thrombus formation and cardiovascular ischaemic events (Frishman et al., 1995; Body, 1996; Ruggeri, 1997a). Further studies have demonstrated platelet hyperaggregatibly in stable angina pectoris, unstable angina pectoris, non-Q-wave myocardial infarction, and in patients with coronary artery disease risk factors such as smoking, hypertension, hypercholesterolaemia, hyperhomocysteinemia and diabetes mellitus (For review see Willoughby et al., 2002c).

1.2.5 Megakaryocyte and platelet disorders

Qualitative disorders of platelets and megakaryocytes are a large group of rare inherited diseases which give rise to excessive mucocutaneous bleeding due to defects in megakaryocyte development and platelet formation and function (For review see Nurden, 2005a). It has been known since the mid 1970s that both cell surface proteins such as the glycoproteins (GP) on the platelet surface, and intracellular proteins have a major role in platelet function, and the pathogenesis of inherited platelet and megakaryocyte disorders (Nurden et al., 1997a).

Glanzmann's thrombasthenia (GT) is the most common platelet disorder, it is an autosomal recessive disorder characterised by deficient platelet aggregation caused by an absence or non-functioning αIIbβ3 (GPIIb/IIIa) complex (George et al., 1990b; Caen and Rosa, 1995; Nurden, 1999). As mentioned in section 1.2.4 during normal platelet activation, αIIbβ3 binds fibrinogen and vWf allowing cross-linking of platelets and thrombus formation, however in GT patients this does not occur and as platelets fail to respond to physiological agonists, such as ADP, thrombin or collagen leading to

the impaired platelet aggregation. The disease is caused by mutations in the genes encoding GPIIb or GPIIIa that result in qualitative or quantitative abnormalities of the proteins, to date over 70 mutations have been identified which give rise to this autosomal recessive disease (these can be found on an internet data base; http://med.mssm.edu/glanzmanndb) (Tomiyama, 2000). GT patients are divided into two groups according to the amount of GPIIb/IIIa complex expressed on their platelet surface: type I patients have less than 5% of GPIIb/IIIa; type II patients 10-20% and type III (variant) patients 50–100% (For reviews see George et al., 1990a; French and Seligsohn, 2000; Bellucci and Caen, 2002).

Another qualitative megakaryocyte and platelet disorder is Bernard–Soulier syndrome (BSS) which is an autosomal recessive disorder first described in 1948 by Bernard and Soulier (For review see Bellucci, 1997) and is characterised by prolonged bleeding times, moderate to severe thrombocytopenia, and enlarged (giant) platelets. BSS platelets aggregate normally in response to ADP and collagen but show a lack of platelet response to ristocetin or botrocetin (Howard et al., 1973; Lopez et al., 1998a). The underlying defect causing BSS is an abnormal GPIb-V-IX complex, required as described previous in section 1.2.4, for normal haemostasis by binding vWf, allowing platelet adhesion and thrombus formation at sites of vascular injury (Clemetson, 2003). There are four distinct transmembrane proteins required for the assembly of the functional receptor on the cell-surface of bone marrow megakaryocytes, GPIbα, GPIbβ, GPIX and GPV (Andrews et al., 1999). GPIbα, GPIbβ, and GPIX are closely associated and the lack of a single subunit dramatically decreases the surface expression of the GPIb-V-IX complex (Ulsemer et al., 2001). The majority of these mutations are in the GPIbα region and decrease the expression of GPIbα on the platelet surface, whereas mutations of GPIX and GPIb generally decrease expression of the entire complex on the platelet surface leading to the abnormal functioning of the complex and the impaired platelet function seen in BSS patients (Lopez et al., 1998c; Ware et al., 2000; Poujol et al., 2002; Strassel et al., 2003; Kato et al., 2004).

Familial thrombocytopenias are an extensive group of bleeding disorders caused by quantitative and qualitative defects in megakaryocyte development and platelet formation. In patients suffering from this disorder the genetic defect can lead to profound changes being observed in platelet morphology, and other blood cells and/ or

organs (Geddis and Kaushansky, 2004; Drachman, 2004a). An example of a familial thrombocytopenia is Paris-Trousseau syndrome, where large platelets with giant α-granules and an increased number of small immature megakaryocytes with an arrested maturation are observed in the bone marrow. It is thought that a mutation of the Ets family transcription factor gene, *FLI1*, may be the potential cause of Paris-Trousseau thrombocytopenia because of similar abnormalities observed in the megakaryocytes of mouse embryos in which *fli1* has been genetically deleted, including immature megakaryocytes with hypolobulated nucleus, abnormal α-granule synthesis, and an increased number of small CFU-MK colonies. To support this evidence it has also been shown that the *FLI1* gene is present on human chromosome 11q24, a region deleted in Paris-Trousseau thrombocytopenia (Spyropoulos et al., 2000a; Hart et al., 2000a; Kawada et al., 2001a; Jackers et al., 2004b).

In patients suffering from congenital amegakaryocytic thrombocytopenia, another familial thrombocytopenia, the numbers of megakaryocytes are low from birth, with aplastic bone marrow evolving with age (Drachman, 2004b; Nurden, 2005b). An example of this type of thrombocytopenic condition is X-linked thrombocytopenia, caused the substitution of a single amino acid caused by a 2-base mutation on the X-chromosome. This substitution inhibits the interaction between GATA-1 and FOG-1, resulting in severe thrombocytopenia and large platelets (Mehaffey et al., 2001). In this disorder, mutations in the c-mpl gene, which encodes the TPO receptor, prevent TPO from carrying out its normal thrombopoietic roles in megakaryocytic cells. Complete loss of the C-mpl receptor lead to a more severe disease with plasma TPO levels being 10-fold less than that observed in normal cells (Ihara et al., 1999b; Ballmaier et al., 2001a; Ding et al., 2004). This is the only known megakaryocytic disease thought to be as a direct result of abnormal TPO/c-Mpl signalling (Muraoka et al., 1997; Ihara et al., 1999a; Ballmaier et al., 2001b).

Impaired TPO signalling has been implicated only in congenital amegakaryocytic thromboeytopenia suggesting that other factors may be involved in the pathogenesis of other megakaryocyte disorders. One such factor recently identified to have a key role in megakaryocyte differentiation and platelet production is glutamate signalling (Genever et al., 1999m; Hitchcock et al., 2003m). Functional N-methyl-D-aspartate (NMDA)-type glutamate receptors were shown to be expressed by bone marrow

megakaryocytes, similar to those identified at neuronal synapses responsible for mediating excitatory neurotransmission in the central nervous system (CNS) (Genever et al., 1999l), and their inhibition using an NMDA receptor antagonist MK-801, lead to impaired megakaryocytopoiesis and platelet production suggesting a fundamental role for glutamate signalling in megakaryocytes (Hitchcock et al., 2003l).

1.3 Glutamate signalling in the CNS

The amino acid L-glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). It is believed to be involved in aspects of normal brain function including learning, cognition and memory (Fonnum, 1984a; Ottersen and Storm-Mathisen, 1984c; Collingridge and Lester, 1989c; Headley and Grillner, 1990c). Glutamate is also involved in neuronal differentiation, cell migration and cell death, as well as formation or removal of synapses in the developing brain (Foster and Fagg, 1984; Mayer and Westbrook, 1987; Komuro and Rakic, 1993).

Neurotransmission of glutamate at glutamatergic synapses can be divided into four stages (see Figure 1.9); the first is the passage of an action potential along the axon of the pre-synaptic cell leading to the influx of Ca^{2+}, the fusion of the synaptic vesicle carrying the neurotransmitter to the presynaptic membrane, and finally, the release of glutamate in to the synaptic cleft. During the second stage the released glutamate diffuses across the synaptic cleft where it is free to bind to the glutamate receptors on the post-synaptic cell. The third stage involves the binding of glutamate agonists to their receptors leading to receptor activation, leading to the influx of charged ions such as Ca^{2+} and Na^{+} (or activation of coupled G-proteins) into the post-synaptic cell, leading to the depolarisation of the post-synaptic cell, allowing the action potential from the pre-synaptic cell to be continued activating of a variety of downstream signalling events. In the fourth stage glutamate is removed from the synaptic cleft by glutamate transporters expressed on neighbouring astrocyte and glial cells, leading to the termination of synaptic transmission.

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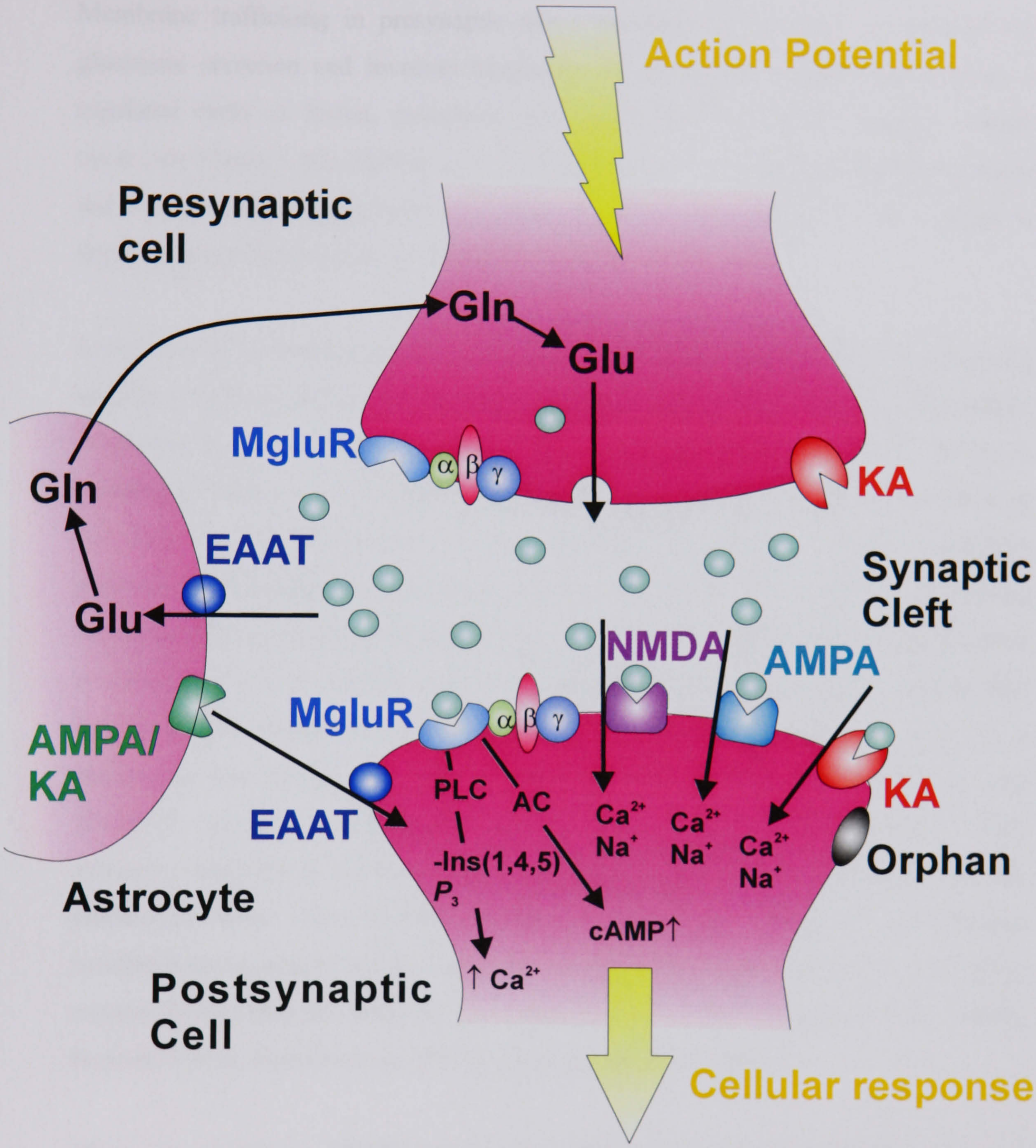

Figure 1.9: Glutamate signalling at CNS synapses.
In response to an action potential glutamate-containing vesicles dock with the presynaptic cell membrane and release glutamate into the presynaptic cleft. Glutamate then binds to glutamate receptors situated on the postsynaptic cell, resulting in numerous cellular responses. Glutamate transporters, such as EAAT, remove glutamate from the synaptic cleft terminating the signal and recycling the glutamate back to the presynptic cell via astrocytes or glial cells.

1.3.1 Glutamate release from pre-synaptic neurons

Membrane trafficking in presynaptic nerve terminals in the CNS is essential for glutamate secretion and involves biogenesis of specialised vesicles that perform a regulated cycle of fusion, exocytosis and regeneration, called the synaptic vesicle cycle (see Figure 1.10) (Sollner et al., 1993d; Calakos and Scheller, 1996b; Robinson and Martin, 1998a; Richmond and Broadie, 2002a). See section 3.1 for a more in depth review of exocytosis mechanisms.

Exocytosis of neurotransmitter is a complex and highly regulated process involving specific protein-protein interactions. The current model involves the SNARE (soluble N-ethylmaleimide-sensitive factor [NSF] attachment protein [SNAP] receptor) hypothesis (Figure 1.11), which was first proposed in 1993 to explain vesicle docking and fusion in molecular terms. It proposes that vesicles have specific v-SNARE proteins which recognise and interact with the target protein t-SNAREs on the plasma membrane of presynaptic cells (Sollner et al., 1993c). This membrane protein complex is composed of the vesicle associated membrane protein, VAMP, a single-pass transmembrane protein located primarily on the vesicle membrane; syntaxin 1a, a single-pass transmembrane protein residing primarily on the plasma membrane; and SNAP-25 (synaptosomal-associated protein, 25kDa), which contains two SNARE domains bordering a region of palmitolyated cysteines which associate with the plasma membrane. These proteins interact with each other in a structurally conserved parallel 4-helix bundle which brings the vesicle and plasma membranes into close contact so that they are ready to fuse (Sollner et al., 1993b; Chapman et al., 1994e; Bennett, 1995a; Hanson et al., 1997b; Chen and Scheller, 2001b).

There are numerous SNARE-interacting proteins that regulate SNARE complex assembly and disassembly (Figure 1.11). SNARE assembly is triggered by the binding of calcium to a putative calcium sensor, synaptotagmin. This induces the fusion of synaptic vesicles docked at the active zone close to calcium channels. Following membrane fusion, disassembly of the SNARE complex is regulated by two proteins, the ATPase NSF (N-ethylamide sensitive fusion protein) and an adapter protein α-SNAP (soluble NSF attachment protein). SNARE formation is also regulated by

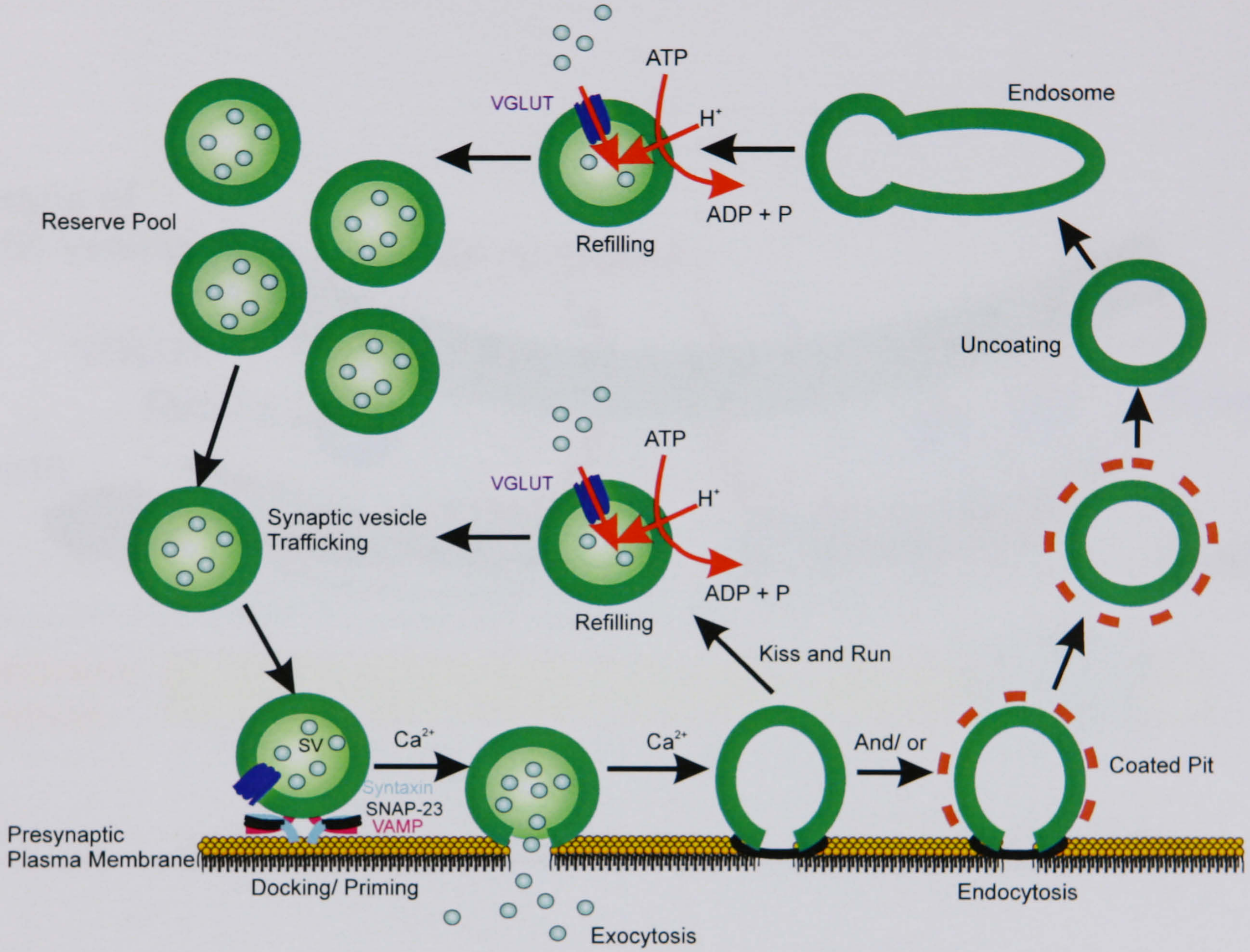

Figure 1.10: The synaptic vesicle cycle.
Membrane trafficking in presynaptic nerve terminals in the CNS is essential for glutamate secretion and involves biogenesis of specialised vesicles that perform a regulated cycle of fusion, exocytosis and regeneration, called the synaptic vesicle cycle. Vesicular transporters such as the VGLUTs are responsible for packaging glutamate into synaptic vesicles, from where it is released at the presynaptic cell membrane into the presynaptic cleft via exocytosis involving the SNARE complex. Once the neurotransmitter has been released vesicles are regenerated via endocytosis completing the cycle (adapted from Li and Chin, 2003).

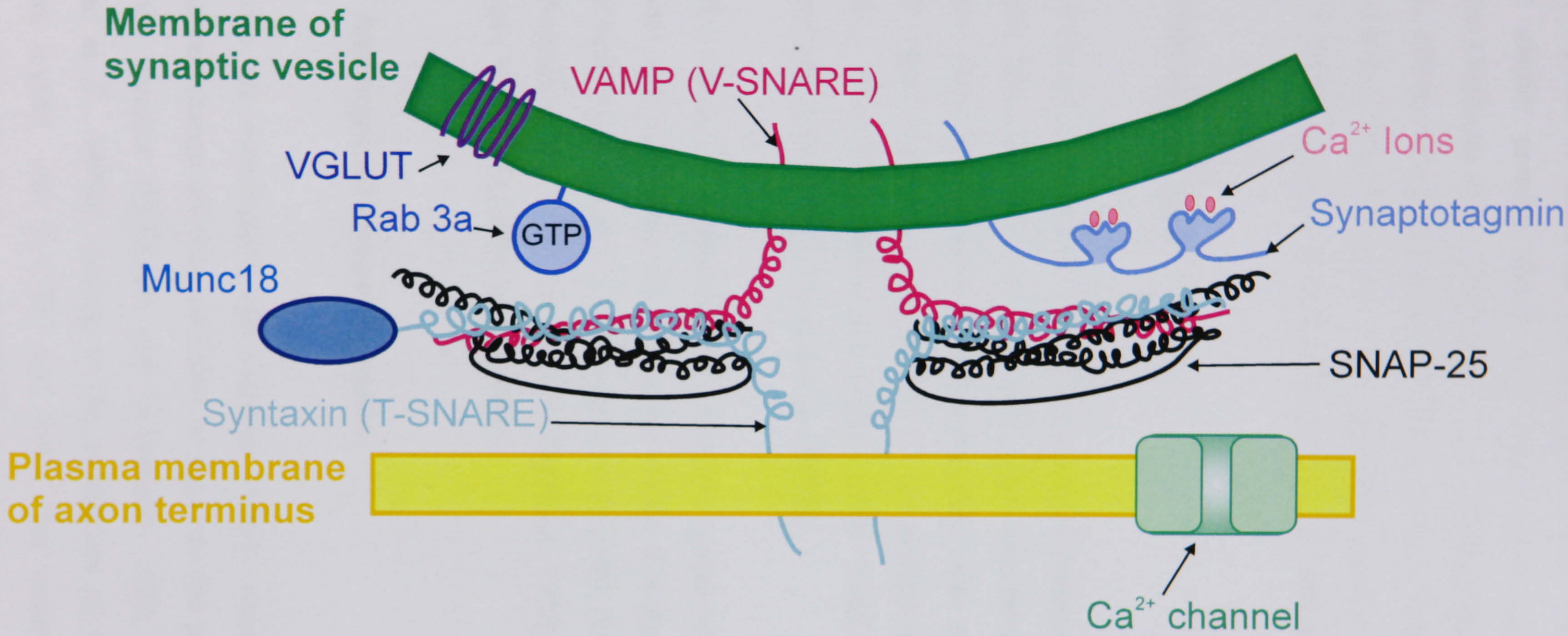

Figure 1.11: The SNARE complex and the main exocytotic proteins.
The SNARE complex is composed VAMP/synaptobrevin (vSNARE protein) and the tSNARE proteins syntaxin and SNAP-25 (synaptosomal-associated protein, 25kDa). These proteins form a 4-helix bundle which bring the synaptic membrane and vesicle membrane into close proximity so that they can fuse allowing the neurotransmitter to be released into the synaptic cleft.Other proteins such as synaptotagmin a putative calcium sensor, Munc18 and the GTPase Rab 3a are also key regulators of SNARE complex formation.

syntaxin-interacting proteins such as Munc-18, GTPases Rab 3, Rab 3 binding proteins Rabphilin 3, which are involved in exocytosis via hydrolysis of GTP, and vesicular adapter proteins Doc-2 and Mint, which regulate Munc-18/syntaxin interactions (Malhotra et al., 1988; Clary et al., 1990b; Calakos and Scheller, 1996a; Betz et al., 1997a; Novick and Zerial, 1997b; Sassa et al., 1999b; Biederer and Sudhof, 2000; Mochida, 2000; Nakajima et al., 2001; Richmond et al., 2001; Gerst, 2003). Section 3.1 provides a more in depth review of SNARE and associated proteins.

1.3.2 Glutamate receptors

The glutamatergic system in the CNS is composed of glutamate receptors (GluRs) and transporters. The majority of neuronal and glial cells have GluR on their plasma membranes, however glutamate signalling has also been shown to have a role in peripheral organs and tissues (see section 1.4 for details) (Hosli and Hosli, 1993a; Vernadakis, 1996a; Steinhauser and Gallo, 1996a; Conti and Weinberg, 1999a; Shelton and McCarthy, 1999b; Bergles et al., 2000b).

The GluRs, which are responsible for glutamate uptake in neuronal cells, can be divided into two categories: the ionotropic GluRs (iGluRs) and the metabotropic GluRs (mGluRs) (see Figure 1.12) (Monaghan et al., 1989; Hollmann and Heinemann, 1994; Schoepfer et al., 1994; Borges and Dingledine, 1998; Nakanishi et al., 1998; Ozawa et al., 1998a; Dingledine et al., 1999e).

1.3.2.1 Ionotropic Glutamate Receptors

The iGluRs are ligand-gated ion channels, which when activated undergo a conformational change allowing an influx of ions into the postsynaptic cell directly through the receptor (Hollmann and Heinemann, 1994; Ozawa et al., 1998b; Dingledine et al., 1999d), leading to the production of the intracellular second messengers cAMP, -ins (1,4,5)P3 and DAG (For reviews see Hollmann and Heinemann, 1994; Dingledine et al., 1999f). The iGluRs can be subdivided into three

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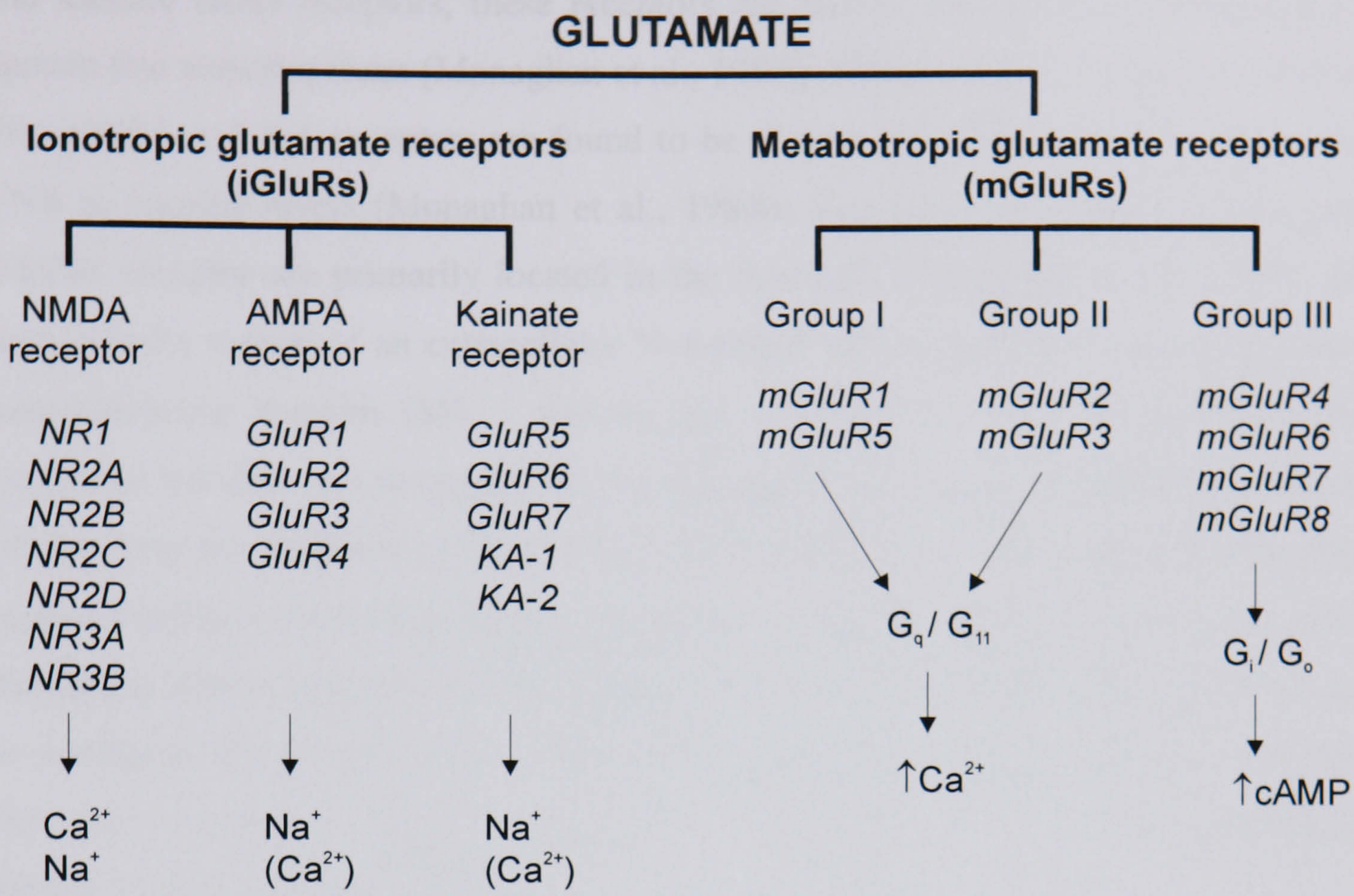

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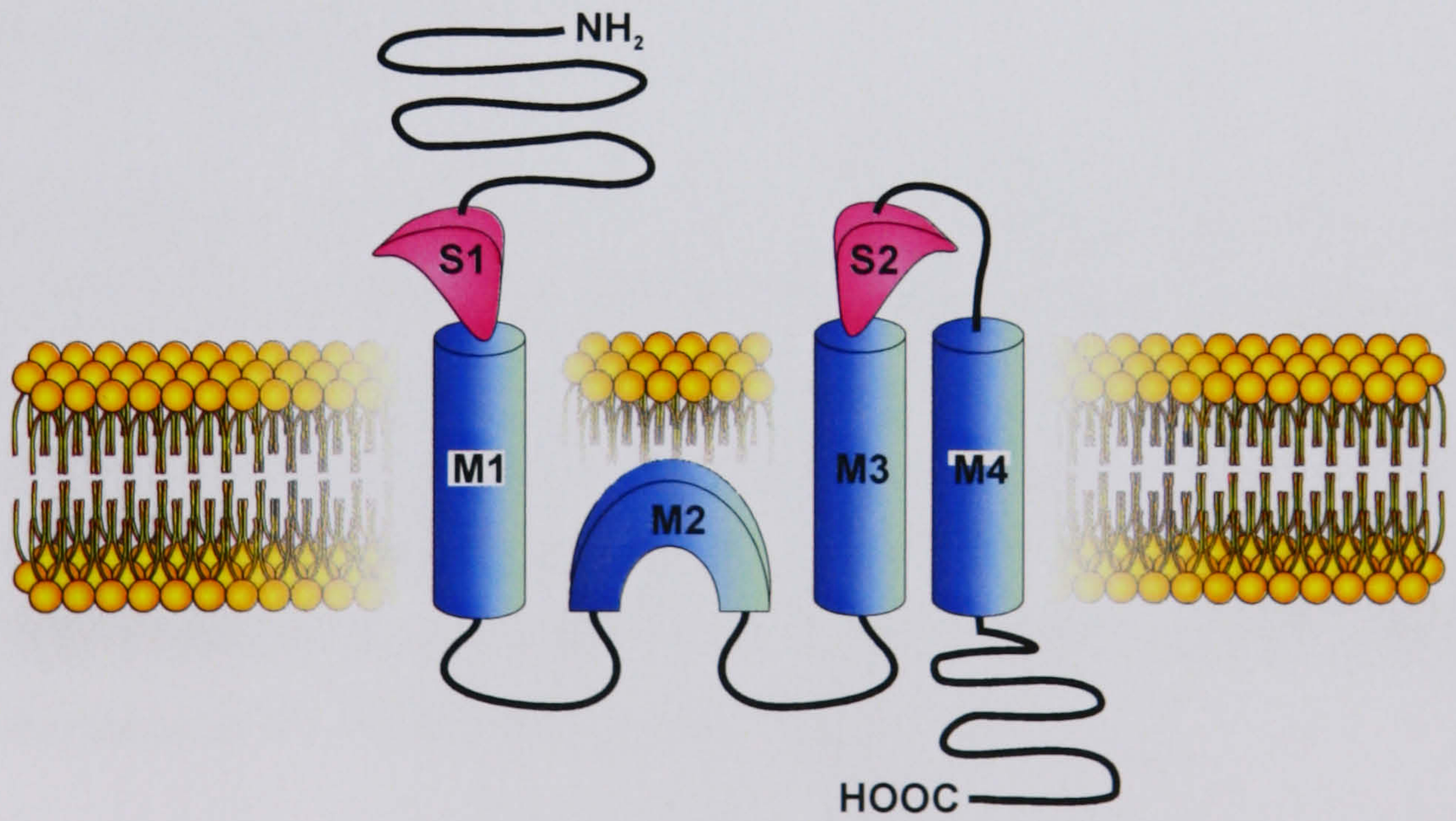


Figure 1.12: Ionotropic and metabotropic glutamate receptors.
(A) List of all the members of the iGluR and mGluR gene families based on amino acid sequence (adapted from Das et al, 1998). (B) A schematic diagram showing the generalised structure of iGluR subunits, demonstrating the 3 transmembrane (M1, 3 and 4) and the re-entreant loop (M2), and the agonist binding sites (S1 and 2).

groups based on their structural and pharmacological similarities: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate (KA) receptors, these receptors are named after their pharmacological agonist that activates them (Monaghan et al., 1984a; Hollmann and Heinemann, 1994). The AMPA and KA receptors are found to be ubiquitously expressed throughout the CNS at varying levels (Monaghan et al., 1984b; Represa et al., 1987) whereas the NMDA receptor are primarily located in the forebrain (Monaghan et al., 1989). All three iGluRs consist of an extracellular N-terminal and intracellular C-terminal, three transmembrane domains (M1, 3 and 4), and a re-entrant loop (M2) that faces the cytoplasm but does not transverse the membrane. The amino acids comprising the re-entrant loop are proposed to control the ion permeability of each receptor type. The agonist-binding sites for the iGluRs are located in the S1 region, located before M1, and the S2 region, located after M3 (Figure 1.12). In the CNS the iGluRs are involved in mediating long-term potentiation (LTP), the mechanism responsible for the formation of memory and learning (Bliss and Collingridge, 1993a; Meyer and Shen, 2000; Liu and Cull-Candy, 2000).

1.3.2.1.1 AMPA receptors

AMPA receptors are thought to mediate the majority of rapid excitatory transmission in the CNS (Hollmann and Heinemann, 1994; Dingledine et al., 1999a). AMPA receptors have a rapid response to their agonists and desensitise quite rapidly. It has been proposed that AMPA receptors may play an important role in synaptic plasticity, and modulating membrane trafficking and synaptic targeting of receptors mediated by their direct phosphorylation by protein kinases and phosphatases (Lynch and Baudry, 1984; Raymond et al., 1993; Soderling and Derkach, 2000).

AMPA receptors are tetrameric heteromeric complexes of four homologous subunits GluR1, -2, -3 and -4, which combine to produce distinct receptor subtypes with different stoichiometries (Hollmann and Heinemann, 1994; Dingledine et al., 1999b). Multiple cloning of the AMPA receptor protein cDNA revealed that more than one isoform of these proteins was present in neuronal tissues. These alternative isoforms are formed by alternatively spliced exonic sequences of 38 amino acids near the M4

transmembrane domain of the GluR1-4 subunits in either a "flip" or "flop" configuration (Sommer et al., 1990a). AMPA receptors containing the flip isoform of the GluR1-4 subunits are found in prenatal and post-natal brain neurons during neuronal development and form non-desensitising receptors. whereas the flop isoform of the AMPA receptor subunits are only observed in postnatal brain maturation and receptors containing these subunits exhibit an increased desensitisation rate to glutamate-induced activation (Sommer et al., 1990b; Monyer et al., 1991).

AMPA and KA receptors are primarily permeable to Na^+ and K^+, and generally show relatively low unitary conductances (Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987; Trussell et al., 1988). The majority of the AMPA receptors are impermeable to Ca^{2+}, these receptors contain the GluR2 subunit which confers Ca^{2+} impermeability, however, a small population of neuronal cells in the retina, cerebellum, cerebral cortex and hippocampus contain AMPA receptors which form ion channels permeable to Ca^{2+} (Iino et al., 1990; Gilbertson et al., 1991; Burnashev et al., 1992a; Burnashev et al., 1992b; Jonas et al., 1994). The majority of AMPA receptors in the hippocampal pyramidal cells consist of GluR1 – GluR2 and GluR2 – GluR3 complexes, where as in Purkinje cells AMPA receptors mainly consist of just GluR2 – GluR3 heteromers.

1.3.2.1.2 Kainate receptors

KA receptors have many similar characteristics to the AMPA receptors, however unlike AMPA receptors, which are thought to play a role in glutamatergic synaptic transmission in the majority of excitatory synapses in the brain, the roles of KA receptors are still a matter of uncertainty. KA receptors are ligand-gated channels permeable to cations, activated by low concentrations of kainate and display rapid synaptic transmission and desensitization characteristics (Egebjerg et al., 1991; Kohler et al., 1993; for a review see Pinheiro and Mulle, 2006). KA receptors are thought to be involved in slow glutamatergic neurotransmission regulating presynaptic neurotransmitter release (Malva et al., 1995; Chittajallu et al., 1996; Vignes and Collingridge, 1997).

There are five KA receptor subunits GluR5, 6, 7, KA1 and KA2. which can be arranged in different ways to form a tetramer, a four subunit receptor. The mRNAs encoding the KA receptors are subject to post-translational modifications and/or alternative splicing as seen in the AMPA receptor subunits. All splice variants differ in their cytoplasmic C-domain apart from GluR5 which has an alternative 15-amino acid exon in the N-terminal domain. There are three main splice variants for GluR5, two main splice variants for the GluR6 subunit, and 2 additional splice variants for the GluR7 subunit, increasing the receptor diversity (for review see Hollmann and Heinemann, 1994).

1.3.2.1.3 NMDA receptors

NMDA receptors mediate excitatory neurotransmission in the CNS in different ways from AMPA and KA receptors. They are not believed to function as mediators of rapid synaptic transmission, unlike the AMPA and KA receptors, due to their slow response to glutamate (Lester et al., 1990). NMDA receptors also have high conductances, require glycine as a co-agonist and display a high permeability to Ca^{2+} (MacDermott et al., 1986; Mayer and Westbrook, 1987). The slow response of these receptors is thought to be due to the fact that they are inhibited by Mg^{2+} present in the extracellular environment of neuronal cells (Nowak et al., 1984; Mayer et al., 1984). NMDA receptors have a higher affinity for glutamate than the AMPA receptors, so a small population of these receptors are maintained in a partially activated state. However, full activation of the NMDA receptors requires the removal of Mg^{2+} from the glutamate binding site. The blockage of NMDA receptors by Mg^{2+} is voltage-dependent, therefore when glutamate is released into the synaptic cleft activating the KA and AMPA receptors, the subsequent depolarisation of the postsynaptic cell leads to the removal of Mg^{2+} and the activation of the NMDA receptors.

Seven NMDA receptor subunits have been identified; one NR1, four NR2 (A-D) and two NR3 (A, B) subunits. The majority of native NMDA receptor complexes are heterotetrameric, consisting of two glycine binding NR1 subunits and two glutamate binding NR2 subunits (Das et al., 1998; Rosenmund et al., 1998; Hawkins et al., 1999). However, expression studies in frog oocytes, where both NR1 and NR2 were co-expressed, indicated that it is the NR1/NR2 heterodimers that are the functional

unit in these receptors (Nakanishi et al., 1992c; Seeburg, 1993a; Mori and Mishina, 1995b; Furukawa et al., 2005; Chen and Wyllie, 2006). Eight isoforms of the NR1 subunit (NR1a – h) exist, derived from alternative splicing of the exonic sequences, however it is unknown whether more than one of these variants exist within one NMDA receptor complex (Anantharam et al., 1992; Sugihara et al., 1992; Nakanishi et al., 1992a; Durand et al., 1993; Hollmann et al., 1994; Koltchine et al., 1996). NR2 subunits are regarded as modulatory subunits, whereas NR1 acts as an essential subunit for the formation of the heteromeric NMDA receptors (For reviews see references Nakanishi et al., 1992b; Seeburg, 1993b; Mori and Mishina, 1995a).

1.3.2.2 Metabotropic Glutamate Receptors

The mGluRs (mGluR1-8) are G-protein-coupled receptors which can be divided into three classes, group I, II and III based on sequence similarity, pharmacology and intracellular signalling mechanisms (Figure 1.12). The members of the same subgroup share approximately 70% amino acid sequence identity, while the homology between different subgroups is approximately 45%. The different mGluR subtypes have similar structures, possessing a seven-transmembrane spanning domain with an extracellular N-terminus consisting of approximately 550 amino acid residues, and an intracellular C-terminal domain, the length of which depends on subtype (Hollmann and Heinemann, 1994; Dingledine et al., 1999c). The group I mGluRs (mGluR1 and -5) are positively coupled to phospholipase C, and are activated by (±) 1-amino-cyclopentane-*trans*-1, 3-dicarboxylic acid (*trans*-ACPD) leading to the activation of colocalised NMDA receptors via mechanisms involving protein kinase C-mediated tyrosine phosphorylation. In contrast group II (mGluR2 and -3) and group III (mGluR4, -6, -7 and -8) are negatively coupled to adenyl cyclase (Conn and Pin, 1997; Schoepp et al., 1999; Pin et al., 1999a; Bordi and Ugolini, 1999b; Brauner-Osborne et al., 2000; Schoepp, 2001a; Benquet et al., 2002). Group II receptors are also activated by *trans*-ACPD, while group III is activated by L (+)-2-amino-4-phosphonobutyric acid (L-AP4). All three classes of the mGluRs are enriched in the hippocampus, and the GluR2, -3 and -5 receptors can be found in the cortex, nucleus accumbens and amygdal (Fotuhi et al., 1994; Pin and Duvoisin, 1995; Blumcke et al., 1996; Shigemoto et al., 1997; Bordi and Ugolini, 1999a; Pin et al., 1999b; Spooren et al., 2001; Schoepp, 2001b).

1.3.3 Glutamate transporters

Specific, high affinity transport systems are required by all amino-acid neurotransmitters to complete the synaptic actions of the transmitter after receptor activation and to recycle the molecules involved. L-glutamate, as discussed previously, is the major excitatory transmitter of the mammalian CNS, and transportation of glutamate is vital as high glutamate concentrations in the synaptic milieu can cause excitotoxicity leading to neuronal injury. Glutamate transporters play an important role in maintaining the local integrity of excitatory synaptic transmission (Marcaggi and Attwell, 2004a).

Five high-affinity glutamate transporter subtypes have been identified in the CNS, EAAT-1 (GLAST), EAAT-2 (GLT-1), EAAT-3 (EAAC-1), EAAT-4 and EAAT-5 (Storck et al., 1992; Pines et al., 1992a; Kanai and Hediger, 1992b; Fairman et al., 1995; Arriza et al., 1997b). The different acronyms (EAAT, excitatory amino acid transporter; GLAST, glutamate-asparate transporter; GLT, glutamate transporter; EAAC, excitatory amino acid carrier) used to described the glutamate transporters are used to describe the glutamate transporters in different species, for example EAAT-1 is the name for human subtype whereas GLAST is the name for the equivalent subtype in the rat, they do not reflect the functional differences among the transporters. EAATs are members of a unique family of proteins, called the solute carrier family 1 (SLC1) (For reviews see Kanai and Hediger, 2003a; Kanai and Hediger, 2004a) and display considerable homology, sharing 50–60% sequence identity with each other at the amino-acid level, and 30-40% homology with ASCT1 and ASCT2 (alanine-serine-cysteine transporters) which are also members of the SLC1 family of proteins (Kanai and Hediger, 2004b).

All five of the glutamate transporter subtypes catalyse Na^+ and K^+ coupled transport of L-glutamate, maintaining a large concentration gradient across the cell membrane (Danbolt, 2001d; Kanai and Hediger, 2003b; Shigeri et al., 2004). It has been suggested by several studies that EAATs have a dual function as both a glutamate transporter (i.e. substrate recognition, binding, transport and ion coupling) and ion

channel (i.e. chloride flux) (Slotboom et al., 2001; Amara and Fontana, 2002; Kanner and Borre, 2002; Ryan et al., 2004).

EAATs express distinct molecular and pharmacological characteristics, and are observed in distinct localisations at both the cellular and regional level. EAAT1 is expressed by glial cells throughout the CNS and is found at high concentrations in Bergmann glia of the cerebellum (For reviews see Danbolt, 2001c; O'Shea, 2002b; Kanai and Hediger, 2004c; Beart and O'Shea, 2007a). EAAT2 is found predominantly in glial cells, and its expression can be observed abundantly throughout the CNS (For reviews see Danbolt, 2001b; O'Shea, 2002c; Kanai and Hediger, 2004d; Beart and O'Shea, 2007b). EAAT3 and EAAT4 are found almost exclusively in neurons; EAAT3 is widespread throughout the CNS, whereas EAAT4 is expressed mainly by cerebellar Purkinje cells (For reviews see Danbolt, 2001a; O'Shea, 2002a; Kanai and Hediger, 2004e; Beart and O'Shea, 2007c), but is also expressed at lower levels in the forebrain (Dehnes et al., 1998; Massie et al., 2001). EAAT5 is expressed predominantly in the retina where it is present in rod photoreceptor and bipolar cells (Arriza et al., 1997a; Pow and Barnett, 2000).

1.3.4 Vesicular Glutamate transporters

Specific transporters expressed on the vesicle membrane determine the transmitter cargo that will be packaged, carried and subsequently released following fusion with the plasma membrane.

The vesicular transport system for excitatory amino acids in the CNS has only recently been discovered. Research carried out by Ni et al. in 1994 (Ni et al., 1994c) discovered a novel gene which was upregulated in primary cultures of rat cerebellar granule neurons after exposure to subtoxic levels of the N-methyl-D-aspartate (NMDA) receptor agonist NMDA. Injection of this gene into Xenopus oocytes resulted in Na^+-dependent phosphate transport similar to that observed in native or recombinantly expressed kidney Na^+-dependent cotransporters. The gene was 560 amino acids (61kDa), and was predicted to have six to eight transmembrane domains, with postulated phosphorylation sites for protein kinase (PKC), tyrosine kinase and

calmodulin-dependent kinase II on the intracellular loops (Ni et al., 1994d). Analysis of the mRNA revealed its expression was restricted to the brain, and was shown to have 32% identity to a rabbit kidney type I Na^+/phosphate cotransporter, therefore it was named brain specific Na^+-dependent inorganic phosphate cotransporter (BNPI) (Ni et al., 1994c). The discovery that BNPI mRNA expression was restricted to neuronal cells which use glutamate as a neurotransmitter, including cortical cells, granule cells of the dendate gyrus, cerebellar granule cells, and hippocampal pyramidal cells (Ni et al., 1995) strongly suggested a specific function for BNPI in glutamatergic neurons (see Table 1.1). Further studies by Bellocchio et al. (1998, 2000), and Takamori et al. (2000) independently confirmed that BNPI functions to load L-glutamate into recycling intracellular vesicles, and due to this BNPI was renamed vesicular glutamate transporter 1 (VGLUT1) (Bellocchio et al., 1998e; Takamori et al., 2000c; Bellocchio et al., 2000c). Glutamate transport by VGLUT1 was inhibited by the H^+ ionophore, indicating that an electrochemical H^+ gradient is the driving force responsible for transport (Bellocchio et al., 2000b; Takamori et al., 2000b).

Evidence that VGLUT1 was absent from many excitatory synaptic pathways that were thought to use glutamate as a neurotransmitter, indicated that there may be more than one vesicular glutamate transporter (Bellocchio et al., 1998d). Recently a second brain-specific Na^+/phosphate cotransporter was identified, a 582 amino acid (64.4kDa) protein with 82% amino acid homology to VGLUT1 (Aihara et al., 2000c; Hisano et al., 2002e). This novel transporter known as differentiation-associated Na^+/phosphate cotransporter (DNPI) was cloned during a differential display technique to identify the genes expressed during rat pancreatic AR42J cell differentiation. The gene which was upregulated during differentiation was identified as a Na^+/phosphate cotransporter as expression of the cDNA in Xenopus oocytes, like VGLUT1, enhanced Na^+-dependent phosphate transport (Aihara et al., 2000b). DNPI has been predicted to have eight transmembrane domains from its amino acid sequence, with putative glycosylation sites between the first two putative transmembrane helices (Varoqui et al., 2002c). DNPI has been shown to be highly expressed in synaptic vesicles, where it has been shown to function as a vesicular glutamate transporter with properties similar to VGLUT1, and was therefore renamed vesicular glutamate transporter 2 (VGLUT2)

Region	VGLUT1		VGLUT2		VGLUT3	
	mRNA	Protein	mRNA	Protein	mRNA	Protein
Accumbens nuceus		**	*	**		
Amygdala	***	***	*	*	*	*
Cerebellar cortex	**	***		*		
Dorsal thalamic nuclei		*	***	**		
Habenula		***	***	**		
Hippocampus	*	*	**	**		*
Hypothalamus		*/**	**	**		*
Midbrain		*/**	***	**	***	*
Neocortex	***	***	*	*	*	*
Olfactory bulb	**	***	*	**		*
Piriform cortex	**	*	**	*		
Retina	**	*	*	*	*	*
Septum	*	***	*	*		
Striatum		***		***		*
Subthalamic nucleus		*	***	*		

Table 1.1: Regional distribution of VGLUT isoforms in rat brain. Expression levels: High (***), Medium (**) and Low (*) (Herzog et al., 2004; Hisano et al., 2002).

(Herzog et al., 2001a; Takamori et al., 2001b; Fremeau, Jr. et al., 2001f; Varoqui et al., 2002c). Glutamate transport by VGLUT2 was shown to be dependent on H^+ electrochemical gradient, like VGLUT1, as well as electric potential (Varoqui et al., 2002b).

Although these transporters share high sequence identity, and have highly similar substrate specificity, kinetics and pharmacology, these VGLUT proteins do differ in their expression profiles. VGLUT1 and VGLUT2 are predominantly expressed in the adult brain, expressing complementary expression patterns (Table 1.1). VGLUT1 mRNA has been shown to be predominately expressed in several discrete regions including the cerebral cortex, hippocampus, cerebellar cortex, and amygdale (Ni et al., 1994b), where as VGLUT2 mRNA is preferentially expressed in the spinal cord, brainstem, and the diencephalic structures, including the thalamus, of both human and rat brain (Aihara et al., 2000a; Herzog et al., 2001a; Fremeau, Jr. et al., 2001e; Varoqui et al., 2002a; Hisano et al., 2002d). In the cortex at both the mRNA and protein level, terminals in all layers label for VGLUT1, apart from fourth layer which principally labels for VGLUT2 (Takamori et al., 2001a; Fremeau, Jr. et al., 2001d). In the denate gyrus and the hippocampus, granule and pyramidal cell layers label for VGLUT2 (Fremeau, Jr. et al., 2001c), however all the terminals in the dendritic field stain for VGLUT1 (Bellocchio et al., 1998c). Although the majority of glutamatergic neurons in the brain express one of the VGLUT isoforms several studies have suggested that cerebellar mossy fibres, pinealocytes, primary sensory neurons, cerebellar unipolar brush cells, and certain pancreatic islet cells coexpress both VGLUT1 and VGLUT2 (Hisano et al., 2002c; Hioki et al., 2003; Hayashi et al., 2003; Li et al., 2003; Nunzi et al., 2003; Morimoto et al., 2003a; Bai et al., 2003d).

A third vesicular glutamate transporter, VGLUT3, has been discovered which has more than 70% structural identity to VGLUT1 and VGLUT2, and shares the glutamate transport characteristics similar to those of VGLUT1 and VGLUT2 (Reimer et al., 1998; Takamori et al., 2002; Hisano et al., 2002b; Fremeau, Jr. et al., 2002b; Gras et al., 2002b; Bai et al., 2003c). However, VGLUT3 has a very different distribution to either VGLUT1 or VGLUT2, since it is restricted to non-glutamatergic neurons and was often found to colocalise with other neurotransmitter transporters (Table 1.1). VGLUT3 mRNA and protein is expressed by a proportion of the GABAergic

interneurons in the cortex, hippocampus and interpeduncular nucleus, and cholinergic interneurons in the striatum and nucleus accumbens. This isoform has also been shown to be expressed in serotoninergic neurons of the raphe nuclei (Schafer et al., 2002a; Gras et al., 2002a; Somogyi et al., 2004; Herzog et al., 2004). These results suggest that VGLUT3 in conjunction with other neurotransmitters is involved in the co-release of two neurotransmitters by neurons in the adult brain. Recently experiments have also identified a role for VGLUT3 in glutamate release from astrocytes (Montana et al., 2004). The precise number of amino acids composing VGLUT3 is not definite, as studies by Gras et al. (2002) suggest that rat VGLUT3 is 589 amino acids (64.7 kDa) where as another study by Fremeau et al. (2002) have reported rat VGLUT3 be 530 amino acids (Fremeau, Jr. et al., 2002a; Gras et al., 2002c). However, a 601 amino acid (65kDa) secondary structure for mouse VGLUT3 has been reported which is predicted to have eight to ten transmembrane domains (Schafer et al., 2002b). As seen with VGLUT1 and VGLUT2 glutamate transport was shown to be driven by a H^+ electrochemical gradient (Varoqui et al., 2002d).

1.4 Non-neuronal glutamate signalling

Evidence is accumulating for the expression of components required for glutamatergic signalling in non-neuronal tissues. Functional glutamate receptors have been identified in several peripheral tissues and organs including the heart, intestine, kidney, pancreas, placenta, mammary gland and bone (for reviews see Skerry and Genever, 2001b; Hinoi et al., 2004a). Table 1.2 summaries the expression of the glutamatergic system in peripheral tissues.

1.4.1 Glutamate signalling in other tissues

The pancreas consists of several types of endocrine cells, predominantly found in the islets of Langerhans, including glucagon-secreting alpha (α) cells, and insulin-secreting beta (β) cells. The β-cells are the most abundant, accounting for two thirds of the islet cells, where as the α-cells are less abundant, accounting for only a fifth of the islet cells. Subunits of AMPA, KA, NMDA and metabotropic glutamate receptors

(mGluR2.-3,-4,-5,-8, GluR1. -2.-3.-4.-6.-7. KA-2. NR2A/C.D and NR1) have been shown to be expressed by the islets of Langerhans in the pancreas. modulating both insulin and glucagon secretion (Bertrand et al.. 1992; Bertrand et al.. 1993; Bertrand et al., 1995; Inagaki et al., 1995; Weaver et al.. 1996a; Liu et al.. 1997b; Brice et al.. 2002; Tong et al.. 2002; Bai et al.. 2003b). suggesting that glutamate may be an intracellular signal mediator in the islet. It has therefore been suggested that in β-cells glutamate transporters may be directly involved in controlling insulin release (Maechler and Wollheim. 1999). Glutamate transporters similar to EAAT-2 have been cloned from the pancreas, and immunocytochemical studies have shown that EAAT-2 is present in β-cells, but not by the α-cells (Manfras et al.. 1994; Gammelsæter et al.. 1999).

VGLUTs similar to VGLUT1 and -2 found in the CNS have also been identified in pancreatic α- and β-cells (Bai et al.. 2003a), however no other cloned glutamate transporters have been shown to be expressed by islet cells so far. The cells have been shown to express receptors for other neurotransmitters including acetylcholine and GABA (Molnar et al., 1995; Weaver et al.. 1996b; Liu et al.. 1997a). The expression of the inhibitory neurotransmitter GABA in pancreatic cells. suggests that a synapse-like organisation may be present in the pancreas.

In the gastrointestinal tract, glutamate has been shown to modulate histamine-induced acid secretion in the rat stomach (Tsai et al.. 1999). as well as contractility in the gastric fundus, jejunum, ileum and large intestine (Shannon and Sawyer. 1989; Sinsky and Donnerer, 1998; Li et al.. 2005). VGLUT2 has been shown to be abundant in the antrum and pylorus of the stomach, and in the intestine two types of glutamate transporters have been identified. VGLUT2 which is abundant in the ileum and EAAT-3 has been found in the intestine (Kanai and Hediger. 1992a).

Glutamate signalling has been shown to have an important. but poorly understood. function in the metabolism of heart myocytes (Dinkelborg et al.. 1996). EAAT-1 is expressed in myocardial mitochondria where it has been shown to function as a malate/aspartate shuttle. aiding metabolism (Ralphe et al.. 2004). Other glutamate

Tissue/cell	iGluRs			mGluRs			GluTs	VGluts
	NMDA	AMPA	KA	Group I	Group II	Group III		
Adrenal gland	+	+	+				+	
Bladder	+							
Gastrointestinal tract							+	+
Heart	+	+	+	+	+		+	
Hepatocytes				+				
Keratinocytes	+	+		+	+		+	
Kidney	+	+			+		+	
Lactating mammary glands							+	
Liver	+	+			+			
Lung	+	+			+			
Megakaryocytes	+							
Melanocytes				+				
Osteoblasts	+	+	+	+		+	+	+
Osteoclasts	+							
Osteocytes	+						+	
Pancreas	+	+	+	+	+	+		
Penile corpora cavernosa	+							
Pinealocytes	+	+	+	+	+			
Placenta							+	
Platelets							+	
Prostate	+	+						
Spleen	+	+			+			
T cells		+						
Taste buds						+		
Testis	+	+	+	+	+	+	+	+
Thymus				+	+			

Table 1.2: Expression of the glutamatergic system in peripheral tissues

transporters and GluRs have been identified in human and rat heart including GLAST, EAAT-3, GluR-1,-2, -3, -4, -5, -6 and -7, KA-1 and -2, and mGluR-1a, -2, -3 and -5, which are thought to improve the mechanical function of the ischaemic or hypoxic myocardium (Nakayama et al., 1996; Gill et al., 1998; Gill et al., 1999).

Glutamate signalling has also been documented in the skin, human keratinocytes have been shown to express functional NMDA receptors (NR1, NR-2A and -2B), AMPA receptors (GluR-2 and -3), mGluRs (-1α, -2 and -3) and glutamate transporters (EAAT-2 and -3) (Morhenn et al., 1994; Genever et al., 1999a; Nahm et al., 2004).

Expression/function profiling has identified some components of glutamate signalling in cells derived from haematopoietic stem cells (HSCs) including monocytes, macrophages, megakaryocytes (discussed in Chapter 1.5), platelets (discussed in Chapter 1.5), B and T lymphocytes, implicating glutamate-mediated intercellular signalling in the control of haemostasis and blood cell function (Kondo et al., 1997; Akashi et al., 2000; Hao et al., 2001; Manz et al., 2002).

Functional glutamate transport systems have been identified in macrophages derived from human blood monocytes including those mediated by high-affinity glutamate transporters similar to those found in embryonic neurons and neonatal astrocytes (EAATs), and sodium independent cysteine/ glutamate antiporters, which transport extracellular cysteine in exchange for intracellular glutamate (Bannai, 1986; Rimaniol et al., 2000b). Interestingly, these transporters are only functional in the presence of inflammatory factors such as TNFα and liposaccharide, which increase cysteine uptake suggesting that glutamate transport is regulated locally by inflammation and acts to increase antioxidant activity. Monocyte-macrophages have been reported to remove excess glutamate from the medium of neuronal cells cultured in the presence of high concentrations of this amino acid, leading to the suggestion that EAATs on macrophages protect neurons against the damaging effects of glutamate-mediated excitotoxicity during inflammation. It has been suggested that monocyte-macrophage cysteine/ glutamate transporters play important roles in the regulation of glutathione (GSH) synthesis (a ubiquitous antioxidant synthesised from cysteine, glutamate and glycine), increasing intracellular GSH levels and reducing oxidative stress in response to elevated extracellular glutamate (Rimaniol et al., 2000a; Rimaniol et al., 2001). In

HIV infection. neuronal damage is caused by micoglial activation. involving oxidative stress. glutamate-medicated excitotoxicity and apoptosis. In healthy individuals. astrocytes normally act to maintain low extracellular glutamate concentrations in the brain preventing neurotoxicity. This neuoroprotective function is impaired following HIV infection. Expression of EAAT1 on brain macrophages may compensate for impaired astrocyte function by restoring glutamate transport activity. removing excess extracellular glutamate and locally increasing GSH and antioxidants levels (Gras et al.. 2003; Vallat-Decouvelaere et al.. 2004). Other experiments have demonstrated that factors secreted from HIV-1-infected monocyte-macrophages have the ability to activate NMDA receptors comprised of NR1a and NR2B subunits in the brain. implicating a role for these cells and NMDA receptors in the neuronal degeneration observed during HIV associated dementia (Xiong et al., 2003a). On a similar vein. secretory factors derived from amyloid stimulated monocyte-macrophages found in patients with Alzheimer's disease have been shown to directly activate NMDA receptors which may contribute to the pathogenesis of the disease (Xiong et al., 2004). Together. these data indicate that monocyte-macrophages play an important role in modulating glutamate signalling in the CNS. and raise the possibility that they also regulate glutamatergic pathways within the bone marrow from where they are derived.

In recent studies it has been demonstrated that resting peripheral blood lymphocytes (PBL) and Jurkat T cells express genes for NMDA receptors including NR1 and NR2B. PBL induced to proliferate with phytohemagglutinin (PHA) were demonstrated to express elevated levels of NR1 in addition to NR2A and NR2D. suggesting T-cell activation causes the up-regulation of gene transcription for NR1 and these additional subunits (Miglio et al., 2005a). These data confirm previous observations that resting rat T-cells express the NR1 subunit (Boldyrev et al., 2004). Functional effects of NMDA receptor signalling in T-cells has been investigated using MK-801 and D-AP5. a competitive antagonist. Both compounds were shown to inhibit PHA-induced T-cell proliferation. but did not cause cell cycle arrest and had no effect on cell viability. suggesting NMDA receptor activation does not influence these factors. However inhibition of the expression of CD25. a cell–surface marker of T-cell activation by these antagonists suggested that NMDA receptor signalling may control the early phases of cell activation (Miglio et al., 2005b).

1.4.2 Glutamate signalling in bone

The cylindrical cavities of bones are filled with a network of thin, calcified trabeculae and bone marrow. Bone marrow contains a diverse population of cells including both haematopoietic and non-haematopoietic stem cells, which are the precursors to osteoclasts and osteoblasts respectively.

The identification of glutamate signalling in bone originated from attempts to identify potential targets for therapeutics to manipulate bone mass to treat bone disorders (Mason et al., 1997f). A technique known as differential RNA display was used to investigate the changes that occur in osteocyte gene expression after mechanical loading of rat bone in vivo. One of the genes found to be down regulated by this process was GLAST (EAAT-1 in humans). Immunohistochemical studies using neonatal rat tibiae, confirmed the expression of GLAST in old as well as newly incorporated osteoblasts and osteocytes (Mason et al., 1997e). A novel splice variant of EAAT-1, EAAT-1a, has also been cloned from bone which is identical to the neuronal EAAT-1 but lacks exon 3. The deletion of exon 3 is thought to alter the membrane topology of the transporter leading to the cellular efflux of glutamate instead of its accumulation (Huggett et al., 2000). More recent studies have identified that osteoblasts demonstrate functional glutamate uptake in vitro, and these findings have been confirmed by the identification of a number of glutamate transporters including EAAT-1, -2 and -3 (Takarada et al., 2004).

Molecular and cellular biological techniques have identified functional GluRs on bone forming osteoblasts (GluR3, KA-1 and -2, NR-1, -2A/B/D, mGluR-1, -4 and -8), and bone resorbing osteoclasts (NR-1, -2A/B/D) in vivo and in vitro, where they thought to modulate cellular function (Mason et al., 1997d; Chenu et al., 1998a; Gu and Publicover, 2000; Itzstein et al., 2001; Hinoi et al., 2001; Bhangu et al., 2001c; Hinoi et al., 2002; Kalariti and Koutsilieris, 2004). NMDA receptor antagonists, MK-801 and APV have been shown to inhibit osteoblast differentiation and bone formation (Birch et al., 1997; Skerry, 1999), as well as bone resorption in osteoclasts (Chenu et al., 1998b; Itzstein et al., 2000a). Sustained exposure to NMDA antagonists was shown to inhibit the expression of CBFA-1, a bone differentiation transcription factor,

at both the mRNA and protein level in osteoblasts (Hinoi et al., 2003), and reduced the number of resorption pits on cortical bone slices inhibiting the formation of the adhesion-induced sealing zone between osteoclasts and the bone required for bone resorption (Chenu et al., 1998c; Itzstein et al., 2000b). These reports strongly suggest that NMDA receptor antagonists affect osteoclastogenesis possibly through osteoblast-derived growth factors (Peet et al., 1999; Taylor, 2002).

Recent studies have identified components essential for regulated glutamate exocytosis in presynaptic neurons in osteoblasts (Bhangu et al., 2001b). Molecular techniques including RT-PCR, northern blot analysis and western blot analysis have shown expression of the target membrane-SNARE proteins SNAP-25 and syntaxin 4 and the vesicular-SNARE protein VAMP, the minimum molecular requirements for core exocytotic complex formation in osteoblasts. Immunofluorescent localisations revealed peripheral SNAP-25 expression on osteoblastic cells, particularly at intercellular contact sites, colocalising with immunoreactive glutamate and the synaptic vesicle-specific protein, synapsin I. Osteoblastic expression of SNARE accessory proteins, including munc18, rSec8, DOC2, syntaxin 6, and synaptophysin, required for glutamate exocytosis in the CNS have been identified. mRNA for the putative Ca^{2+}-dependent regulators of vesicle recycling activity, synaptotagmin I and the GTP-binding protein Rab3A are also identified by northern blot analysis. Evidence for vesicular glutamate exocytosis from osteoblasts has also been supported by the observation that osteoblasts express the vesicular transport protein VGLUT1, which is involved in the packaging of glutamate into synaptic vesicles with in the synapse (Bhangu et al., 2001a) and the identification of vesicles which are able to undergo repetitive recycling at peripheral sites, a process central to glutamate exocytosis in glutamatergic neurons, in osteoblasts by FM1-43 labelling, a fluorescent-based technique developed to study intracellular trafficking (P.G. Genever and P.S. Bhangu, personal communications). It has also been demonstrated that osteoblastic cells actively release glutamate spontaneously in a differentiation-dependent manner (Bhangu et al., 2001d; Genever and Skerry, 2001e). These data provide evidence that osteoblasts are able to direct glutamate release by regulated vesicular exocytosis, mimicking presynaptic glutamatergic neurons, indicating that a process with similarities to that observed in synaptic neurotransmission occurs in bone.

For a recent review of NMDA signalling in bone the reader is directed to a review by Spencer et al. (Spencer et al., 2007) and Figure 1.13 which summaries glutamate signalling with in the bone microenvironment.

1.5 Glutamate signalling in megakaryocytes and platelets

1.5.1 Glutamate release and uptake in platelets

Previous reports have identified functional NMDA-type glutamate receptors on human platelets. In neuronal cells NMDA receptors bind MK-801 at high glutamate or glycine concentrations (Ransom and Stec, 1988). However, in platelets MK-801 failed to bind to the NMDA receptors in the presence of either glutamate or glycine suggesting that in platelets these receptors are functionally distinct from those found in neuronal cells. NMDA receptors have an anti-aggregation activity within platelets. Activation of NMDA receptors causes glutamate to antagonise the aggregation properties of arachidonic acid (AA), platelet activating factor (PAF) and adenosine 5'-diphosphate (ADP) (Franconi et al., 1998a). Also, stimulation of the NMDA receptors has been shown to prevent platelet aggregation by inhibiting the synthesis of thromboxane A_2, a platelet aggregation promoter, from AA (Franconi et al., 1996b).

Molecular and morphological studies have shown the presence of three major glutamate transporters, EAAT1, EAAT2 and EAAT3, in human platelets similar to those found in the CNS (Zoia et al., 2004c; Rainesalo et al., 2005; Hoogland et al., 2005c). Zoia et al have shown that the EAAT1 and EAAT3 inhibitor SOS significantly reduced glutamate uptake by platelets, while the specific EAAT2 inhibitor DHK, did not significantly affect glutamate uptake, suggesting EAAT2 does not seem to play a major role in platelet glutamate uptake activity (Zoia et al., 2004b). However, studies by Hoodland et al concluded that EAAT2 was the predominant glutamate transporter in platelets, responsible for platelet glutamate uptake (Hoogland et al., 2005b). Their studies have shown that EAAT2 is responsible for the increase in sodium-dependent

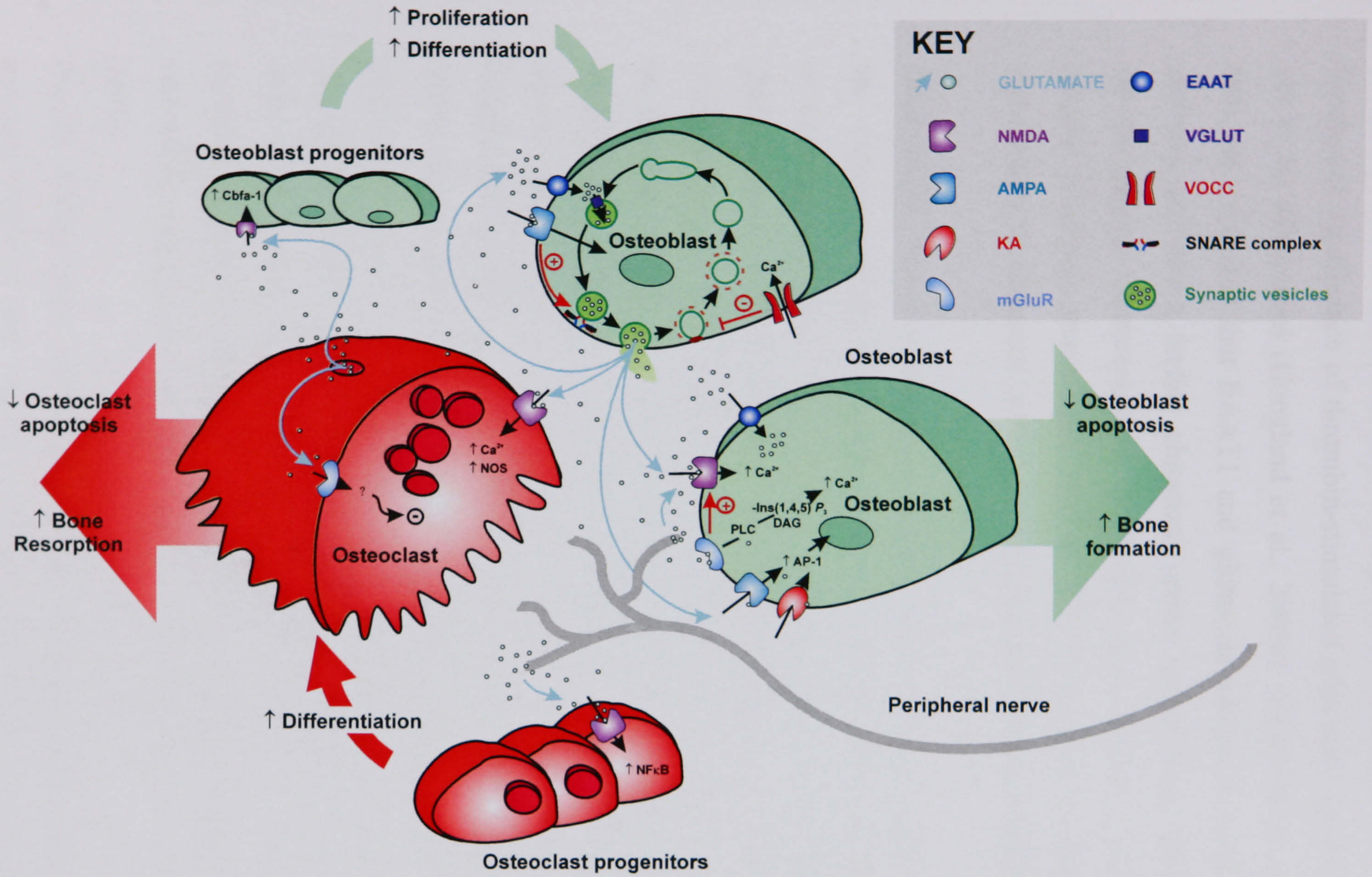


Figure 1.13: Glutamate and NMDA receptor signalling during bone remodelling. Glutamate, released into the bone microenvironment by mature osteoblasts, peripheral nerves and actively resorbing osteoclasts, activations NMDA receptors leading to increased proliferation of osteoblast progenitors and stimulates bone formation via Cbfa-1. NMDA receptor activation also stimulates NFκB-mediated osteoclast differentiation and inhibits mature osteoclast apoptosis, increasing osteoclast number and bone resorption. Changes in bone mass are likely to be governed by glutamate signaling from both cell types via autocrine and paracrine activity, regulating bone cell function. This dual mechanism of action may contribute to resorption/formation coupling during bone remodelling. Released glutamate acts via an autocrine loop and stimulates osteoclastic mGluR8 receptors, inhibiting bone resorption. Coupling is achieved by 'osteoclastic' glutamate acting in a paracrine signalling role, stimulating NMDA receptors on osteoblast progenitors, promoting their initial differentiation. Mature osteoblasts at the remodelling site subsequently release more glutamate and encourage neighbouring progenitor cells to differentiate, further stimulating bone formation. Other glutamate signalling pathways converging on NMDA receptor function are also shown (adapted from Spencer et al., 2006).

glutamate uptake in human platelets activated with thrombin, and EAAT2 immunoreactivity was found to be predominately in α-granules, suggesting thrombin stimulates glutamate transport from α-granule EAAT2 to the cell membrane. The functional significance of thrombin-stimulated glutamate uptake by blood platelets is yet to be determined (Hoogland et al., 2005a). Biochemical and molecular studies have also revealed that EAAT1 and glutamate uptake is down regulated in ageing patients, and that a further glutamate uptake reduction is observed in Alzheimer's disease patients (Ferrarese et al., 2000; Zoia et al., 2004a). These results indicate that platelet glutamate transporters may be able to be used as markers to identify the significance of glutamate in patients with neurophychiatric disorders.

Interestingly, recent studies have established that platelet exocytosis occurs through the formation of SNARE complexes involving t-SNAREs SNAP-23, and syntaxin 2 or 4 (Polgar and Reed, 1999; Reed et al., 2000; Chung et al., 2000; Shirakawa et al., 2000; Lemons et al., 2000; Chen et al., 2000b), and the v-SNARE cellubrevin/VAMP-3 (Bernstein and Whiteheart, 1999a). Cellubrevin/VAMP-3 is the only v-SNARE to be described in platelets (Bernstein and Whiteheart, 1999b), botulinum toxin–based studies indicate that one v-SNARE is required for α-granule exocytosis, however it is unclear whether it is VAMP-3/cellubrevin (Flaumenhaft et al., 1999). Platelets also contain the general accessory proteins α-SNAP, γ-SNAP, and NSF (Lemons et al., 1997). Dense core granule secretion is mediated by the SNARE complex accessory proteins such as α-SNAP and NSF, and in vitro exocytosis assays have determined that the t-SNAREs SNAP-23 and syntaxin 2, but not syntaxin 4 or 7, are involved in dense core granule exocytosis from platelets (Chen et al., 2000a). Sec/Munc (SM) proteins, such as Munc18, have a critical role in platelet granule secretion, selectively interacting with platelet syntaxins leading to platelet granule exocytosis (Houng et al., 2003).

Recent studies have shown that human platelets express the vesicular transport proteins VGLUT1 and VGLUT2, and have the ability to release glutamate following aggregation (Tremolizzo et al., 2006). The role of glutamate in platelets is currently unknown but considering that human platelets express NMDA-like receptors, a role for glutamate in platelet activation and aggregation has been proposed (Franconi et al., 1996a; Franconi et al., 1998b).

1.5.2 Identification of functional NMDA receptors in megakaryocytes

As previously discussed megakaryocytes represent less than 0.5% of the rare myeloid cell population in the bone marrow and are responsible for the production and release of 1×10^{11} platelets into blood and the systemic circulation each day. Megakaryocytes are vital to the maintenance of haemostasis (blood clotting and wound healing) (Bruno and Hoffman, 1998; Italiano, Jr. et al., 1999e) although there is now accumulating evidence that megakaryocytes have additional roles within their bone marrow microenvironment including the regulation of haematopoietic and mesenchymal stem cell differentiation, through the local release of growth factors and cytokines. It has also been demonstrated that megakaryocytes express numerous factors involved in bone remodelling and bone cell function, including NFκB ligand (RANKL) (Kartsogiannis et al., 1999), osteonectin, osteocalcin (Kelm, Jr. et al., 1992; Thiede et al., 1994), TGF-β and their receptors, calcium-sensing receptors (House et al., 1997), oestrogen receptors (Bord et al., 2001) and NMDA-type glutamate receptors (Genever et al., 1999k). Functional roles for megakaryocytes in the regulation of bone remodelling have been suggested based on the phenotypic analysis of transgenic mice, which display skeletal abnormalities and from pharmacological studies. Mouse models over-expressing thrombopoietin (TPO), the major hormone controlling megakaryocytopoiesis display osteopetrosis (Yan et al., 1996), and TPO itself has been shown to inhibit osteoclastogenesis (Wakikawa et al., 1997). These data indicate that megakaryocytes have direct effects on remodelling, affecting the osteoblastic factors involved in both bone formation and resorption.

Using Northern blot analysis and reverse-transcriptase polymerase chain reaction (RT-PCR) it has been shown that rat bone marrow cells, human megakaryocytes and the megakaryoblastic cell line, MEG-01, expressed the mRNA for the NMDA receptor subunits, NMDAR1 (NR1) and NMDAR2 (NR2) (Genever et al., 1999j; Hitchcock et al., 2003k). In vivo radioligand binding of [3H]-MK-801, confirmed that the megakaryocytic NMDA subunits coordinated into a functional open-channel forming NMDA-type glutamate receptor, and that antagonism of these receptors with MK-801 had profound inhibitory effects on MEG-01 differentiation (Genever et al., 1999i). When treated with phorbol-myristate acetate (PMA) MEG-01 cells differentiate into a

megakaryocyte-like phenotype, increasing expression of CD41 (GPIIb'IIIa) and producing platelet-like particles (Takeuchi et al., 1991b). Treatment of cells with MK-801 (50-150μM) in the presence of PMA significantly inhibited CD41 expression by 30-55%. In more recent studies it has been demonstrated by RT-PCR that $CD34^+$-derived human megakaryocytes express mRNA for NR1A and NR2D receptor subunits, in addition to the NMDA receptor accessory proteins, Yotiao and post-synaptic density protein 95 (PSD-95). NMDA receptor antagonism has been demonstrated to inhibit TPO-induced megakaryocytic differentiation of $CD34^+$ HSCs, as determined by reduced expression of the megakaryocyte markers CD61, CD41a and CD42a. Treatment of mature $CD34^+$-derived megakaryocytes with MK-801 has also been shown to inhibit proplatelet formation, without affecting cell proliferation or apoptosis and when examined by transmission-electron microscopy (TEM) these cells lack α-granules, multi-lobed nuclei and demarcated membranes, which were abundant in untreated mature megakaryocyte controls (Hitchcock et al., 2003j). These findings support a functionally significant role for NMDA receptor signalling in megakaryocytopoiesis and platelet production.

Therefore the observations that bone cells express the glutamate/aspartate transporter, GLAST (GLT-1) (Mason et al. 1997), and that NMDA-type glutamate receptors are found on bone marrow megakaryocytes suggest a role for glutamate signalling in paracrine intracellular communications within the bone marrow microenvironment.

1.6 Primary aims

Work from our laboratory has demonstrated that megakaryocytes express functional neuroreceptors with similarities to the NMDA-type glutamate receptors in the central nervous system (CNS) and that antagonism of these receptors prevents normal megakaryocyte differentiation and platelet function (Genever et al., 1999h; Hitchcock et al., 2003i). This was the first evidence of glutamate signalling in megakaryocytes and identified a potentially novel mechanism for regulating megakaryocytopoiesis and platelet release. However the cellular source of glutamate within bone marrow has yet to be determined. Preliminary evidence from our laboratory suggests that

megakaryocytes are able to both send and receive glutamate signals (Genever et al., 1999g; Hitchcock et al., 2003h). Therefore the aims of this PhD project are to:

- determine the expression patterns of SNARE and related exocytotic proteins in megakaryocytes
- measure glutamate release from megakaryocytes in the presence and absence of appropriate stimuli
- identify the functional effects of activation and inhibition of glutamate release on megakaryocyte differentiation and platelet production in vitro.

Chapter 2

General Materials and Methods

Chapter 2: General Materials and Methods

2.1 Cell Culture

2.1.1 Plasticware and cell culture reagents

The plasticware and reagents used for cell culture were purchased from Life Technologies (Paisley, UK) unless otherwise stated. Cells were maintained in a humidified atmosphere at 37°C in 5% CO_2. All medium was supplemented with 100U/ml penicillin, 100μg/ml streptomycin and 2 mmol/l L-glutamine unless stated otherwise.

2.1.2 Cell line cultures

MEG-01 and human erythroleukaemia (HEL) megakaryoblastic cell lines were maintained as suspension cultures in RPMI 1640 medium (without L-glutamate) with 10% fetal bovine serum (FBS). For the experiments investigating cell viability, the cell lines were incubated in RPMI 1640 medium (without L-glutamate) without phenol red. Medium was changed every 3 days, passaged by centrifugation (440g for 5 minutes at room temperature) and re-plated at 1×10^5 cells/ml. Megakaryocytic differentiation was induced by treating both cell lines with 100nM phorbol myristate acetate (PMA; dissolved in ethanol) for 72 hours. For non-PMA treated samples, an equivalent volume of ethanol vehicle was added.

2.1.3 Extraction and expansion of mouse megakaryocytes

Primary mouse megakaryocytes were extracted and expanded using a technique, described by Prof. Kenneth Kaushansky, San Diego (personal communication).
The femora and tibiae were removed from five adult B6Cba mice, the ends of the bones removed and the bone marrow flushed out with Iscove's modified Dulbecco's medium (IMDM, Sigma) plus 2% FBS (3ml/ femur and 2ml/ tibia) in to a 50ml falcon

tube. The suspension was passed twice though a 22-gauge needle to break up cell clumps, and then passed through a 70 – 100 μm filter to remove debris. The cell suspension was centrifuged (240g for 5 minutes) and the supernatant removed carefully. The cell pellet was then resuspended in 95ml Optimem (Invitrogen) supplemented with 100U/ml penicillin, 100μg/ml streptomycin, 2 mmol/l L-glutamine, and 5ml BHK-TPO conditioned media (see section 2.1.3.1 for details). The cells were then cultured for 3 days in a T175 culture flask in a humidified atmosphere at 37°C in 5% CO_2. After 72 hours the cells were centrifuged at 160g for 5 minutes and resuspended in 8ml IMDM containing 2% FBS, the megakaryocytes were then purified on a BSA gradient (as described by Drachman et al., 1997) for 30 minutes to allow the larger megakaryocytes to settle to the bottom. The lower 7ml was siphoned off into a 15ml universal and centrifuged at 160g for 5 minutes and resuspended in 1ml Optimem supplemented with 100U/ml penicillin, 100μg/ml streptomycin and 2mol/l L-glutamine for use in other experiments.

2.1.3.1 TPO conditioned media

BHK-TPO cells (Baby Hamster Kidney cells engineered to constitutively release thrombopoietin (TPO), a kind gift from Prof. Kenneth Kaushansky, San Diego) were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) with 10% FCS in a humidified atmosphere at 37°C in 5% CO_2. The medium was changed every 72 hours until the cells were 70% confluent. The medium was removed from the adherent cells by gentle aspiration and they were washed with 5ml 1xPBS, and incubated for 5 minutes with 2ml trypsin/ EDTA at 37°C to remove the cells from the flask. DMEM with 10% FBS was added to the cells and they were centrifuged at 400g for 5 minutes. The cell pellet was re-suspended in DMEM with 10% FBS and grown until 70% confluent. The cells were then divided into 4 petri dishes and cultured until 70% confluent, the medium was then removed and the cells were washed twice with 1 × phosphate buffered saline (PBS). The cells were then cultured for another 24 hours in DMEM containing 1% FBS. After this incubation the conditioned medium was removed from the cells, filtered through a 0.2μm filter, split into 5ml aliquots and stored at -20°C.

2.1.4 Isolation of $CD34^+$ cells from umbilical cord blood

The purification and culture of CD34 positive ($CD34^+$) umbilical cord blood cells was carried out as previously described by Mathur et al. (Mathur et al., 2004). Umbilical cord blood was withdrawn from the umbilical cord and placenta from pre-term mothers giving birth by caesarean section. Informed consent was given by all the mothers at least 12 hours before donating cord blood. Approximately 50-100ml of blood was extracted from each placental preparation and collected into heparinised 50ml centrifuge tubes. The umbilical cord blood was diluted 1 volume to 3 volumes with Hanks buffered saline solution (HBSS) and overlaid on ficoll-paque (Fischer). The preparation was centrifuged at 440g for 35 minutes in a bench top centrifuge equipped with a swing-out rotor, and the mononuclear cells were removed from the ficoll interface. The cells were washed twice in 10ml cold "buffer A" (PBS containing 2% bovine serum albumin (BSA) (Sigma) and 5mM EDTA), centrifuging at 440g for 10 minutes at 4°C to pellet cells, the mononuclear cells were then re-suspended in 300µl buffer A. The $CD34^+$ cells were isolated using a magnetic labelling protocol (Miltenyi Biotec, Germany). To 1×10^8 mononuclear cells 100µl of blocking reagent and 100µl of magnetic anti- human $CD34^+$ fluorescein isothiocyanate I (FITC) conjugated antibody were added, incubated for 30 minutes at 4°C and washed in buffer A. The cells were resuspended in 500µl of buffer A and passed through a MiniMACs magnetic column attached to the magnetic base unit (Miltenyi Biotec). The magnetic column was washed three times with 500µl buffer A to remove the CD34-negative ($CD34^-$) populations. The column was then removed from the magnetic source, and the $CD34^+$ population collected by flushing 1ml of buffer A through the column. This process was then repeated with a second magnetic column. To determine the purity of the $CD34^+$ population, 25µl of cells were taken from both $CD34^+$ and $CD34^-$ cell populations for analysis by flow cytometry using the DakoCytomation CyAn analyser and the fluorescein isothiocyanate (FITC) filter. The average purity of the $CD34^+$ cells was approximately 86% (see Figure 2.1).

$CD34^+$ cells were cultured in MK basal culture medium (MKBM): IMDM (Sigma, 13390) supplemented with 0.2% (w/v) BSA (Sigma), 2 mM glutamine, 1mM sodium pyruvate, MEM non-essential amino acids (Gibco BRL, diluted 1:100), MEM vitamins (Gibco BRL, diluted 1:100), 0.1 mM 2-mercaptoethanol, 100U/ml penicillin

and 0.1 mg/ml streptomycin. The medium was also supplemented with 10% cord blood plasma and 40ng/ml thrombopoietin (TPO) (R & D systems, UK). To obtain cord blood plasma, undiluted umbilical cord blood was centrifuged at 1800g for 35 minutes at 4°C, and the plasma fraction removed.

$CD34^+$ cells were plated at 1.5×10^5 cells/ml in 24-well plates and incubated for 4 days in a humidified atmosphere at 37°C in 5% CO_2. After the incubation period the nonadherent cells were transferred to a 50 ml tube and the cells counted. The cell suspension was then adjusted to a density of 1.5×10^5 cells/ml by addition of fresh MKBM supplemented with 10% umbilical cord blood plasma and 40ng/ml TPO and incubated for 12 days for use in further experiments. After 14 days of culture, CD34 expression had reduced to 2.56% whereas CD61 and CD41 expression was approximately 90%, indicating megakaryocyte differentiation (see Figure 2.1).

2.2 Statistical analysis

Statistical significance in the various assays was initially assessed by determining whether the data was normally distributed using a Kolmogorov-Smirnov test (using Minitab statistical software). Data which followed a normal distribution pattern was further assessed by a Levene`s test (using SPSS statistical software) to determine the equality of variances. If the data was not normally distributed or the variances were not equal the data would have been analysed by a Mann-Whitney U test. However, the data in this thesis was found to be normally distributed with equal variances, therefore it was analysed by one-way ANOVA, a parametric test, and differences between test groups were determined using a Tukeys test (using Minitab). P values <0.05 were considered statistically significant.

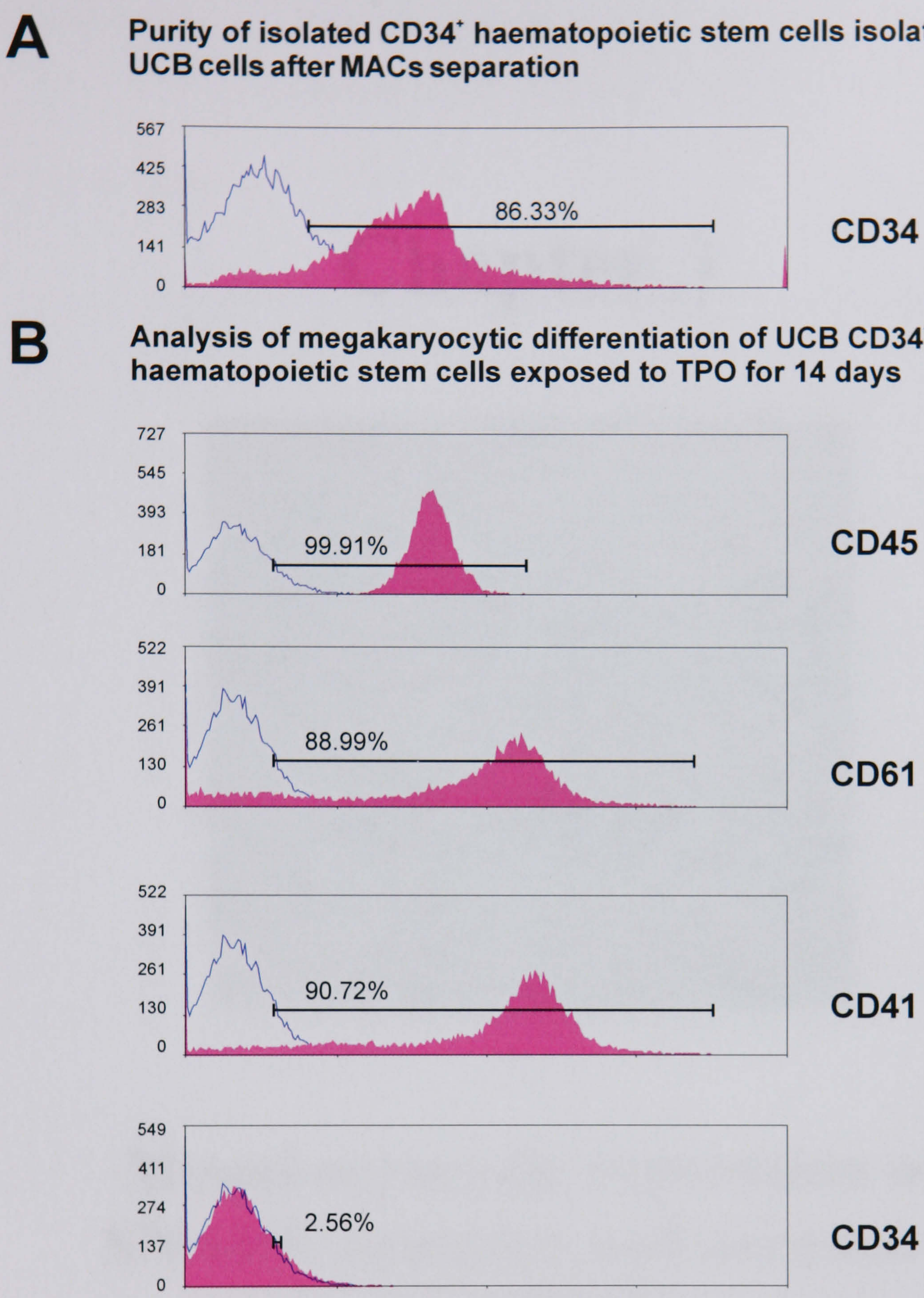
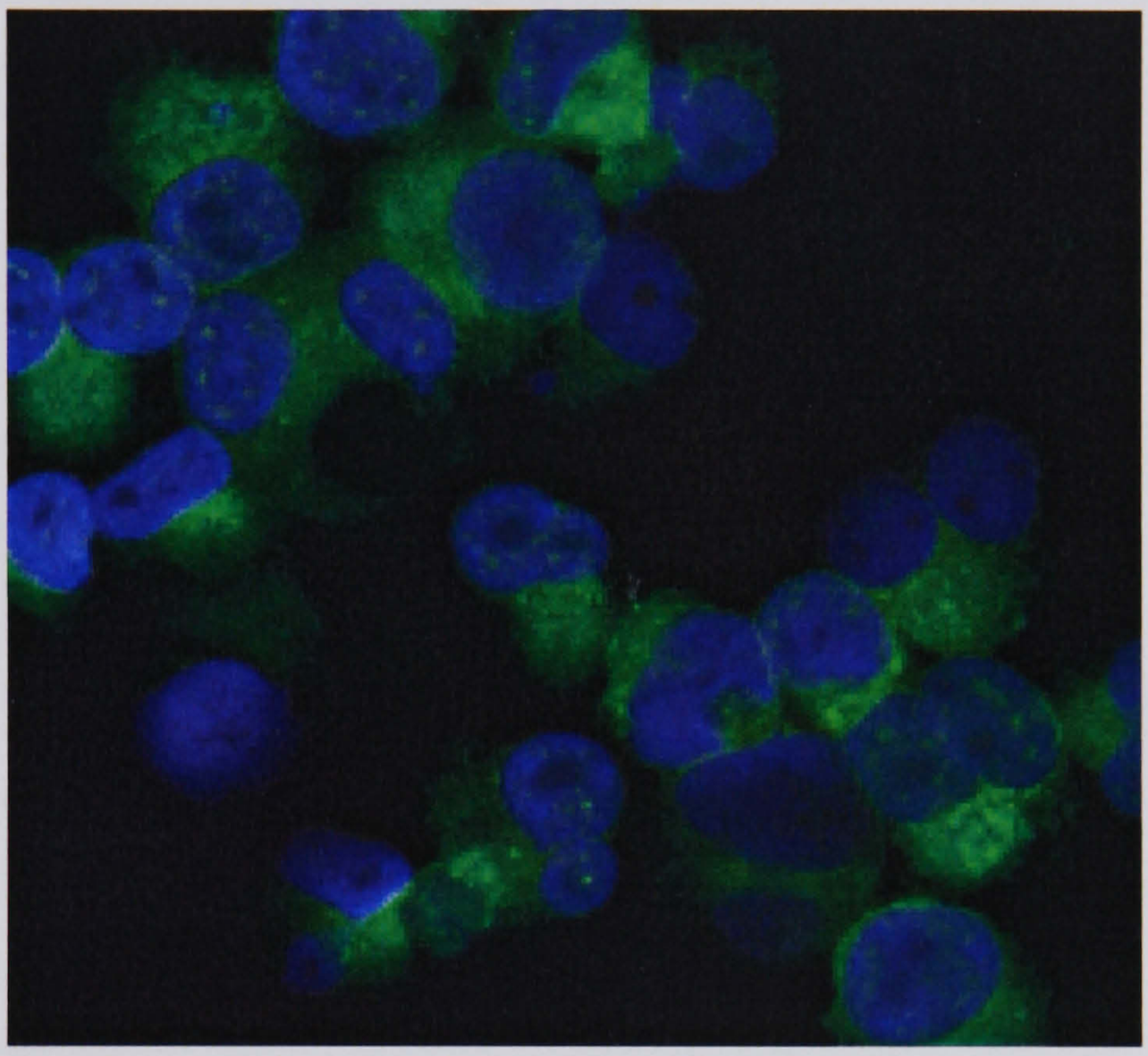


Figure 2.1: Flow cytometry data from differentiated CD34$^+$ umbilical cord blood (UCB) cells. (A) Purity analysis of isolated CD34$^+$ haematopoietic cells following MACS separation from UCB. CD34$^+$ cells were labelled with a mouse anti-human CD34-FITC antibody (Miltenyi Biotech) after MACS separation. During the separation process both the CD34$^-$ and CD34$^+$ flow-through were collected and tested for purity by flow cytometry. CD34-positivity typically ranged from 85-92% (Pink filled peak) compared to CD34$^-$ (blue peak). **(B) Analysis of megakaryocytic differentiation of CD34$^+$ UCB cells after 14 days exposure to 40ng/ml thrombopoietin (TPO).** The differentiated cells were labelled with mouse anti-human CD34-FITC, mouse anti-human CD41-FITC (Caltag laboratories), mouse anti-human CD45-FITC (Caltag laboratories), mouse anti-human CD61-FITC (Caltag laboratories). Results show increased expression of the megakaryocytic markers CD41, CD61, CD45 and a reduction in expression of CD34.

Chapter 3



Megakaryocytic expression of SNARE complex and accessory proteins

Expression of the SNARE complex protein SNAP-23 by differentiated MEG-01 cells after 72 hours incubation with 100nM PMA. Green fluorescence shows positivity for SNAP-23, blue fluorescence indicates the nuclei of the cell.

Chapter 3: Megakaryocytic expression of SNARE complex and accessory proteins

3.1 Introduction

In the CNS the release of a neurotransmitter from the pre-synaptic cell is controlled by several processes: docking of synaptic vesicles at the active site, priming of docked vesicles to make them fusion competent, and calcium-dependent fusion of vesicles with the plasma membrane (For reviews see Jahn and Sudhof, 1999; Murthy and De Camilli, 2003). The docking and fusion of synaptic vesicles at the plasma membrane is mediated by soluble *N*-ethylmaleimide-sensitive factor [NSF] attachment protein [SNAP] receptor (SNARE) proteins, which form a highly stable SNARE complex (Sollner et al., 1993a; Sollner et al., 1993g; Sutton et al., 1998b). SNARE proteins are divided into two categories defined by their localisation within the nerve terminal. SNAREs present on the vesicle membrane are known as v-SNAREs, and SNAREs present on the target plasma membrane are known as t-SNAREs. Synaptic vesicle exocytosis requires three neuronal SNAREs: vesicle associated membrane protein (VAMP) the main v-SNARE, and t-SNAREs syntaxin 1 and 25kDa synaptosomal-associated protein (SNAP-25) (Trimble et al., 1988a; Oyler et al., 1989d; Bennett et al., 1992c; Sollner et al., 1993f; Sutton et al., 1998a) (See section 1.3.1).

VAMPs are essential components of the exocytotic fusion machinery, and represent a family of small-type II integral membrane proteins of approximately 11-13 kDa. Structurally they consist of a variable N-terminal region which is exposed to the cytoplasm, followed by one extended or two short amphipathic α-helices predicted to from coiled-coil structures, and a C-terminal transmembrane domain. These α-helical regions are necessary for SNARE proteins to form the protein-protein interactions required for the formation of the SNARE complex (Grote et al., 1995; Regazzi et al., 1996; Gerst, 1997; Hao et al., 1997). There are at least four VAMP isoforms (excluding alternative splicing) which have been identified as functioning upon secretory granule, dense core vesicle and synaptic vesicle release in mammalian systems. The two isoforms which have been associated with trafficking events that involve regulated exocytosis in the mammalian CNS are VAMP1 and VAMP2 (for

review see Gerst, 1999d). These VAMP proteins are a major constituent of synaptic vesicles and peptidergic secretory granules in neurons (Elferink et al., 1989; Baumert et al., 1989c). VAMP2 is involved in stimulus-coupled secretion in a variety of cell types, including neurotransmitter release from neuronal cells (Trimble et al., 1988c; Baumert et al., 1989b). VAMP1 is widely expressed in nervous tissue and exists in two alternatively spliced forms VAMP1a and VAMP1b which may be tissue- and organelle- specific (Mandic et al., 1997; Isenmann et al., 1998). The VAMP1a splice variant is similar to VAMP2 and is involved in synaptic vesicle release. A third ubiquitously expressed isoform, Cellubrevin/VAMP3, is expressed by non-neuronal cells and localises to numerous types of secretory organelles, including secretory granules in pancreatic acinar cells and GLUT4 containing vesicles in adipocytes (McMahon et al., 1993; Aledo et al., 1996; Sengupta et al., 1996).

The syntaxin family of proteins are t-SNAREs which participate in membrane fusion (Sollner et al., 1993h). There are sixteen described members of the syntaxin family in mammalian cells, of which at least four are localised to the plasma membrane and participate in synaptic vesicle exocytosis. These include syntaxin1a, -1b and possibly -1c, syntaxin2, -3 and -4 (Inoue et al., 1992b; Bennett et al., 1992d; Bennett et al., 1993b; Jagadish et al., 1997a). Syntaxin 1 is found primarily at the plasma membrane of neuronal cells and at the synapse where pools of the protein are involved in the fusion of transport vesicles with the plasma membrane, whereas syntaxin 4 is localised to the plasma membrane of a wide variety of cells, but similarly to syntaxin 1 it appears to be involved in the fusion of transport vesicles with the plasma membrane. Syntaxins are small integral membrane proteins of approximately 300 amino acids which possess a single transmembrane domain (Bennett et al., 1992c; Inoue et al., 1992c), and have at least three putative coiled-coil forming regions (H1, H2 and H3) in the N-terminal and C-terminal domains. The C-terminal domain H3 coiled-coil region is involved in protein-protein interactions with VAMP (Calakos et al., 1994; Hayashi et al., 1994c; Hayashi et al., 1995; Kee et al., 1995b) and SNAP-25 (Hayashi et al., 1994b; Chapman et al., 1994d; Kee et al., 1995a).

The third of the SNARE complex protein families is the synaptosome-associated protein, SNAP-25 (Oyler et al., 1989c). SNAP-25 is a highly conserved protein of 25kDa which was originally identified in nervous tissue, and exists as two variants

SNAP-25A and SNAP-25B (Bark et al., 1995b). However, it has also been shown to be expressed in other tissues such as adipose and skeletal muscle (Roth and Burgoyne, 1994; Sadoul et al., 1995; Jagadish et al., 1996; Kannan et al., 1996). SNAP-25 is anchored to the cytosolic face of the plasma membrane via palmitolyated side chains in the middle of the molecule, and has a putative coiled-coil region located at the C-terminus which associates with VAMP and syntaxin leading to the formation of the SNARE complex (Hayashi et al., 1994a; Chapman et al., 1994c). SNAP-23 (synaptosome-associated protein of 23,000 Dalton) is a ubiquitously expressed isoform of SNAP-25, able to function in regulated exocytosis by associating with VAMP and syntaxin forming the SNARE complex (Ravichandran et al., 1996c).

The function of the SNARE proteins is controlled by numerous positive and/or negative regulators at different stages of assembly and disassembly of the trans-SNARE complex. Syntaphilin, an expression-limited pre-synaptic protein, has been shown to have a regulatory role in the assembly of the SNARE complex by competing with SNAP-25 for binding to syntaxin 1 preventing the formation of the SNARE complex by absorbing free syntaxin 1 (Lao et al., 2000a). Transient overexpression of syntaphilin in cultured hippocampal neurons significantly reduces neurotransmitter release, suggesting that syntaphilin is a molecular clamp which regulates synaptic vesicle exocytosis by controlling the availability of free syntaxin 1 for SNARE complex assembly (Lao et al., 2000b). Syntaphilin also has a function in endocytosis where it binds to dynamin 1 preventing dynamin-mediated endocytosis (Das et al., 2003).

The priming of synaptic vesicles is a critical step in the synaptic vesicle cycle, enabling docked vesicles to fuse to the plasma membrane and undergo rapid exocytosis upon Ca^{2+} influx (Klenchin and Martin, 2000). The Munc13 family of proteins, which are homologues of the *C. elegans* unc-13 family of proteins, have been shown to be involved in priming synaptic vesicles for exocytosis (Brenner, 1974a; Tokumaru and Augustine, 1999; Brose et al., 2000a; Martin, 2002). Munc13s, of which there are three mammalian isoforms: Munc 13-1, -13-2, and -13-3, are differentially expressed in the brain and localise to the synapse (Brose et al., 1995; Betz et al., 1998b; Augustin et al., 1999a; Brose et al., 2000b). Munc 13-1 is peripheral membrane localised to plasma membranes of synaptosomes, where it is

essential for the priming of synaptic vesicles at 90% of the glutamatergic synapses (Augustin et al., 1999b). Munc13 proteins contain multiple regulatory domains (C1 and C2 domains) that bind endogenous second-messenger diacylglycerol, phospholipid, and calcium (Maruyama and Brenner, 1991; Betz et al., 1998a). The C terminus of Munc 13-1 interacts with the N-terminal putative coiled-coil domain of syntaxin 1, and its interaction appears to aid the assembly of the SNARE complex, suggesting Munc13 is involved in priming synaptic vesicles (Betz et al., 1997b). These activated vesicles can be released immediately on Ca^{2+} influx.

Another component of the general fusion machinery involved in the priming of vesicular vesicles at the pre-synaptic membrane is Sec1/Munc-18 (SM). SM proteins are members of a family of conserved hydrophilic proteins with no recognisable domains originally identified in yeast and *C.elegans* (Brenner, 1974b; Novick and Schekman, 1979; Novick et al., 1980; Hosono et al., 1992; Halachmi and Lev, 1996; Jahn, 2000). There are at least seven mammalian homologues of the SM protein family: three of which, Munc18 (also known as Munc18a, Munc-18-1, nSec1, rbSec), Munc18b (also known as Munc18-2), and Munc18c (also known as Munc18-3), are involved in exocytosis (Hata et al., 1993a; Garcia et al., 1994; Pevsner et al., 1994b; Tellam et al., 1995; Gengyo-Ando et al., 1996; Bock et al., 2001). The mammalian SM protein involved in exocytosis of synaptic vesicles is Munc18, which was initially thought to bind selectively to the t-SNARE syntaxin 1 with nanomolar affinity, preventing the *in vitro* formation of the SNARE complex (Hata et al., 1993b; Pevsner et al., 1994a). However, more recent studies using *munc18-1* null mice have shown that in the absence of Munc18 the stability of syntaxin 1 is compromised, but targeting of syntaxin 1 to the presynaptic membrane and its assembly into the SNARE complex is Munc18 independent (Toonen et al., 2005). The lack of neurotransmitter release observed in the *munc18-1* null mice suggests that these SNARE complexes are non-functional or assembled in the wrong localisation (Verhage et al., 2000a), suggesting that Munc18 has an important role in vesicle docking and fusion, and does not act just as a chaperone protein.

The Rab family of small monomeric GTPases, which act upstream of SNARE complex assembly aiding intracellular vesicle transport, are potential SNARE regulators (Bean and Scheller, 1997; Novick and Zerial, 1997a). The Rab 3

superfamily, of which there are four related isoforms Rab-3a, -3b, -3c, and 3d, are involved in regulated exocytosis (Touchot et al., 1987; Matsui et al., 1988; Zahraoui et al., 1989; Baldini et al., 1992a). Rab -3a and -3c are predominantly expressed in neuronal cells in the brain, where they are localised to synaptic vesicles, and are involved in the regulated Ca^{2+} -dependent exocytosis (Fischer et al., 1990). Rab -3b and -3d are expressed in non-neuronal tissues such as adipocytes (Baldini et al., 1992b). The Rab 3 proteins cycle between a GDP-bound inactive and a GTP-bound active state, which is regulated by three types of regulator: Rab GDP dissociation inhibitor (GDI), Rab 3 GDP/GTP exchange protein (GEP), and Rab GAP (Takai et al., 1996; Geppert and Sudhof, 1998; Takai et al., 2001; Zerial and McBride, 2001). Rab proteins associate with the membranes of synaptic vesicles in their GTP-bound state, and upon hydrolysis of GTP they are removed from the membrane. This cycle of attachment and removal of Rab 3 from the synaptic vesicle membrane is synchronised with exocytosis, suggesting an important role for Rab 3 proteins in neurotransmitter release (Fischer et al., 1991; Fischer et al., 1994a; Fischer et al., 1994b). However, studies using Rab 3 knock out mice have shown that deletion of Rab 3 reduces the probability of synaptic vesicle exocytosis without changing the basic parameters, indicating that Rab 3 proteins are not involved in the docking and fusion of synaptic vesicles but regulate neurotransmitter release by acting as modulators of the release machinery (Schluter et al., 2004).

Several putative effector proteins have been identified for Rab 3, including rabphilin (Kishida et al., 1993; Shirataki et al., 1994; Li et al., 1994), RIM (Wang et al., 1997; Wang et al., 2000; Schoch et al., 2002; Calakos et al., 2004) and rabin (Brondyk et al., 1995). Rabphilin binds to Rab 3s in a GTP-dependent manner, it co-localises with Rab 3s on synaptic vesicles, but dissociates from Rab 3s after GTP hydrolysis during exocytosis (Stahl et al., 1996). Rabphilin is composed of an N-terminal Rab 3-interacting domain, a central phosphorylation domain, and two C-terminal C_2 domains that may bind Ca^{2+} (Fykse et al., 1995). In rabphilin knock out mice Rab 3A is targeted to synaptic vesicles normally with normal synaptic transmission and plasticity, suggesting that although rabphilin has the properties of a Rab 3 effector it is not essential for regulatory functions of Rab 3 in exocytosis (Schluter et al., 1999). Recent studies have suggested a role for rabphilin in regulating SNARE-dependent re-priming of synaptic vesicles for fusion at the plasma membrane. Rabphilin has been

shown to bind SNAP-25 via the C_2 domain regulating synaptic vesicle exocytosis after the readily releasable pool has been physiologically exhausted by use-dependent depression (Deak et al.. 2006).

Once exocytosis has taken place at the pre-synaptic membrane, the SNARE complex is disassembled allowing regeneration of the synaptic vesicles. The major regulator of this process is N-ethylmaleimide-sensitive factor (NSF). NSF is a hexameric protein that belongs to the AAA super-family of chaperone-like ATPases (Hanson et al., 1997c; Vale, 2000; May et al., 2001: Zinsmaier and Bronk, 2001b). It is made up of three distinct domains: an amino acid terminal (N) and two homologous ATP-binding domains (D1 and D2). The hexameric NSF binds to the SNARE complex through three molecules of α-SNAP (Soluble NSF attachment protein) to form a large 20S complex (Clary et al., 1990a). Hydrolysis of ATP by the D1 ATPase domain leads to the disassembly of the SNARE complex after vesicle exocytosis has occurred at the synapse (Hohl et al., 1998; Wimmer et al., 2001; Marz et al., 2003; Furst et al., 2003).

To investigate the expression of SNARE-complex and associated proteins in megakaryocytes we used megakaryocytic cell lines, primary megakaryocytes isolated from mouse bone marrow and primary megakaryocytes derived from $CD34^+$ haematopoietic stem cells from umbilical cord blood. Due to the rarity of megakaryocytes with in the bone marrow the majority of research in to megakaryocytic processes has been performed on megakaryocytic cell lines. The most commonly used are the MEG-01 and human erythroleukaemia (HEL) cells (Saito, 1997b). These cell lines are normally derived from the bone marrow of leukaemia patients and have been shown to express many of the characteristics of megakaryocytopoiesis and differentiated human primary megakaryocytic cells. The MEG-01 cell line was derived from a patient suffering with chronic myeloid leukaemia (CML), whereas the HEL cell line was developed from bone marrow cells from a patient with acute myelogenous leukaemia. Phorbol esters have been shown to induce terminal megakaryocytic differentiation of erythroid and megakaryoblastic leukaemic cell lines (Saito, 1997a).

The MEG-01 cell line exhibits megakaryoecytic characteristics (CD41, CD61, CD42a, and vWf) whilst lacking markers for other lineages (Ogura et al., 1985). MEG-01 cells

can be differentiated into mature megakaryocytic cells using the phorbol ester PMA which increases their CD41, CD42a and vWf expression (Ogura et al., 1988). PMA is thought to induce megakaryocytic differentiation through the activation of protein kinase C (PKC), translocating from the cytoplasm to the plasma membrane in MEG-01 cells stimulated by PMA (Long et al., 1990b). It has been reported that MEG-01 cells have the ability to produce platelet-like particles that are of similar size and morphology to circulating platelets (Takeuchi et al., 1991a). MEG-01 cells do not express the TPO receptor c-Mpl, therefore, PMA is the only known differentiation-promoting agent for MEG-01 cells (Matsumura et al., 1996b).

HEL cells in their undifferentiated state possess features common to macrophages, erythrocytes and megakaryocytes (Tabilio et al., 1984; Long et al., 1990a). Stimulation of HEL cells by low concentration doses of PMA, lead to an increased megakaryocytic phenotype recognised by the expression of the megakaryocyte-specific markers CD41 and CD42a, as well as morphological changes such as cytoplasmic maturation and the formation of a polyploid nucleus. These changes are believed to be due to PMA-mediated activation of PKC, which controls the switch between megakaryocyte and erythrocyte lineage (Hong et al., 1996), unlike MEG-01 cells, HEL cells express the TPO receptor c-Mpl and can therefore differentiation can also be stimulated by TPO (Matsumura et al., 1996a). These characteristics make the HEL cell line a useful model in lineage determination studies.

Recent developments in stem cell separation techniques and the increased commercial availability of human recombinant TPO has meant that $CD34^+$ haematopoietic cells can now be promoted to form large numbers of differentiated megakaryocytes in vitro. The three main sources of human $CD34^+$ cells are umbilical cord blood (UCB), bone marrow and peripheral blood (Majka et al., 2001). Due to its accessibility and availability UCB is the most widely used source for generating megakaryocytes following $CD34^+$ separation. $CD34^+$-derived megakaryocytes exhibit numerous characteristics of mature bone marrow megakaryocytes, including; expression of the megakaryocyte and platelet-specific markers CD41, CD61, CD42a and CD62P, TPO receptor c-Mpl and PDGF receptor (Gewirtz et al., 1995a; Majka et al., 2000). In this study we used $CD34^+$-derived megakaryocytes and primary murine megakaryocytes

isolated from the bone marrow of mice and expanded in a TPO-conditioned media (see Chapter 2 for details).

The aim of this chapter is to investigate the expression the SNARE complex proteins and regulatory proteins involved in SNARE-dependent glutamate release in the CNS by megakaryocytes using these well-established cell lines, primary murine megakaryocytes and primary human megakaryocytes (see Chapter 2 for details). This would provide a greater understanding of the glutamate signalling pathways in megakaryocytic cells.

3.2 Methods

3.2.1 RNA isolation

Total RNA was extracted from megakaryocytic cell lines, primary megakaryocytes and whole mouse brain using TRIzol® (GibcoBRL). Cells were grown to confluency in 25cm^2 culture flasks, washed twice with PBS and lysed using 1ml of the TRIzol® reagent. The whole mouse brain was snap frozen using liquid nitrogen and ground to a powder using a Braun dismembranator before 1ml of the TRIzol® reagent was added. The lysate was then transferred them to a 1.5ml eppendorf tube and incubated for 5 minutes at room temperature. 200µl chloroform (Sigma) was added to the TRIzol®-treated samples and vortexed before being centrifuged at 12,000g for 10 minutes at 4°C. 500µl of isopropanol was added to the aqueous phase and incubated at -20°C for 15 minutes before further centrifugation. The RNA pellet was then washed with 75% ethanol, centrifuged at 7,500g for 5 minutes at 4°C, and re-suspended in 11µl RNase free dH_2O (Life Technologies). The quality and quantity of the RNA was determined by calculating the ratio of A_{260} and A_{280} using a UV spectrometer. The samples were then stored at -80°C.

3.2.2 cDNA synthesis

Genomic DNA was eliminated using the DNA-free™ DNase treatment and removal reagents according to the manufacturer's instructions (Ambion).

cDNA was synthesised from 5µg DNase-treated total RNA using the Superscript II RT-PCR system (Life technologies). 1µl of oligo $(dT)_{12-18}$ and 1µl 10mM dNTP mix were added to the RNA and made up to 12µl with RNase-free dH_2O. The samples were incubated at 65°C for 5 minutes and then placed on ice. A master-mix of reagents was prepared containing, per reaction, 4µl 5x first strand buffer, 2µl 0.1M DTT and 1µl RNase OUT. To each reaction 7µl of the master-mix was added and incubated at 42°C for 2 minutes. 1µl SuperScript II (or the equivalent volume of RNase-free dH_2O for the no-RT control) was added to the reaction tubes and incubated for 42°C for 60 minutes. The reaction was terminated by heating to 70°C for 15 minutes and RNA digested by treating with RNase H (2units) for 20 minutes at 37°C. The cDNA

samples were diluted 1:10 in RNase-free dH_2O and stored at -20°C until use. cDNA synthesis was confirmed by PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primers (see table 3.1). The foetal human brain cDNA used as a positive control was obtained from Ambion (0.5ng/µl) and stored at -80°C.

3.2.3 Polymerase Chain reaction (PCR)

The PCR master-mix was prepared containing, per reaction, 5µl 10x PCR buffer, 1.5µl 50mM $MgCl_2$, 1µl 10mM dNTP mix, 0.5µl Platinum Taq polymerase (Life Technologies), 1µl forward primer, 1µl reverse primer (Sigma-Genosys, or Eurogentec) and 35µl dH_2O. 45µl of master-mix was then added to 5µl cDNA and run on a specific PCR program designed for the individual primer set (see table 3.1 for primer sequences). The PCR products (10µl) in bromophenol blue loading buffer were analysed by 1% Tris-Acetate-EDTA (TAE) agarose gel electrophoresis, using HyperLadder IV (Bioline) DNA quantification marker to determine the size of the PCR product.

3.2.4 Immunolocalisations

3.2.4.1 Cytospin preparations

A Cytospin 3 cytocentrifuge (Shandon Scientific Ltd, Runcorn, UK) was used for all cytospin preparations. The cell concentration was adjusted to 1×10^5 cells/ml and 200µl of cell suspension added to the sample chamber. Cells were spun for 5 minutes at 100g onto polylysine microscope slides (BDH) with low (LO) acceleration and slides removed and fixed in 4% paraformaldehyde and kept at -20°C until use.

3.2.4.2 Fluorescent immunocytochemistry

Cytospun slides were thawed and washed 3 times in 1×PBS. Cells were then incubated in 10% horse, sheep, or goat serum (Sigma), depending on the secondary antibody used, for 30 minutes at room temperature. Excess serum was removed and cytospun cells were incubated with primary antibody or control IgGs overnight at 4°C (see table

Protein	Species	Accession number	Forward Primer Reverse Primer	Actual Product Size
Alpha SNAP	Human/Mouse	BC091511	F-5'-AGGCTCATCCAAAATAGAGGAAGCA-3' R-5'-AGCCACCTTCAGCAGACACTTGTT-3'	409
Cellubrevin (VAMP 3)	Human	NM_004781	F-5'-TGGACAAGGTTCTGGAAAGAGACCA-3' R-5'-TGAAGAGACAACCCACACGATGATGATG-3'	203
Cellubrevin (VAMP3)	Mouse	NM_009498	F-5'-GTAATCGAAGACTCCAGCAGACACA-3' R-5'-ACACTGATCCCTATCGCCCACATCT-3'	223
Gamma SNAP	Human	NM_003826	F-5'-CACCTCGCCAAAGCAGAGAAATACC-3' R-5'-AGTGCCGCCTCATCAAACCTACG-3'	491
Gamma SNAP	Mouse	NM_028017	F-5'-GATGCTGAAGGAGATGCAGAAGCTG-3' R-5'-ACTGCCGTTAAAGCCTGGTATGCTG-3'	439
GAPDH	Human/Mouse	BT006893	F-5'-GGTGAAGGTCGGWGTCAACGG-3' R-5'-GGTCATGAGYCCTTCCACGAT-3'	520
Munc-13	Human	BC067084	F-5'-AAGGCAGAACAGCAGGAGGA-3' GGACACTACATCACCCACAACC-3'	486
Munc-13	Mouse	NM_021468	F-5'-GCCTAAGTGTGGAGGTGTGGAACAA-3' R-5'-GGCTGGGTAGTCGTGTGGTAAGGAG-3'	451
Munc-18-1	Human/Mouse	BC031728	F-5'-GACCTGTCCCAGATGCTGAAGAA-3' R-5'-GAGGTGAGCCATGTTGGTGATG-3'	363
NSF	Human/Mouse	BC013314	F-5'-GCTCAGTTGTGTGGTTGTGGATGAC-3' R-5'-GCTGCTCTCCTGTGGCAATGTT-3'	239
Rab 3A	Human	AF254795	F-5'-CGTCCTTCCTCTTCCGCTATGCT-3' R-5'-TGACACCACCCGCTCATCCTC-3'	332
Rab 3A	Mouse	NM_009001	F-5'-AAGGTCAAAACCATCTACCGCAACG-3' R-5'-CCTCAAAGAACTCAAAGCCCAGGTG-3'	313
Rab 3B	Human	BC005035	F-5'-GTTCACCCCCAGCCTTCGTTAGCAC-3' R-5'-GCCTGCCTTACACTGATGTTCTCCTTT-3'	387
Rab 3B	Mouse	BC051918	F-5'-ACCTCCTTCCTTTTCCGCTATGCTG-3' R-5'-TGCCTCACACTGATGTTCTCCTTGG-3'	416
Rabphillin 3A	Human/Mouse	BC017259	F-5'-GGAGCCAGCAAGTCCAACAAGC-3' R-5'-CCATGTCCGGTTTCAGCCAGAG-3'	481
SNAP-23	Human	NM_130798	F-5'-AGAAGATGAAATGGAAGAGAACCTG-3' R-5'-CTGTTGGTGTCAGCCTTGTC-3'	141
SNAP-23	Mouse	NM_009222	F-5'-GCAAGGGGAACAACTAAATCGCATA-3' R-5'-TCCATCTCATCTTCTCTGGCATCATT-3'	315
SNAP-25	Human	BC010647	F-5'-GGTATCAGGACTTTGGTTATGTTGGATG-3' R-5'-TGGTTTTGTTGGAATCAGCCTTCT-3'	445
SNAP-25	Mouse	NM_011428	F-5'-GTTATGTTGGATGAGCAAGGCGAAC-3' R-5'-ATCTGGCGATTCTGGGTGTCAAT-3'	392
Syntaphilin	Human/Mouse	BC035788	F-5'-GCGCACCTCTCCACCTGTGA-3' R-5'-GAGCCGGTCCTGTGTGTCCTT-3'	250
Syntaxin 1a	Human/Mouse	NM_004603	F-5'-GAGGAGGTGAAGCGGAAGCACAG-3' AGAGGCAAAGATGGCGGGGTTC-3'	384
Syntaxin 4	Human	AF026007	F-5'-CAGGAGTTGGAGAAACAGCAGGTCA-3' R-5'-ATCAGACACCATCCCAGCATTGG-3'	333
Syntaxin 4	Mouse	U76832	F-5'-AGTGGGCAGAGTGAGGTGTTTGTGT-3' R-5'-CCAATGATGACAGCCAAGATGAGAA-3'	341
VAMP	Human/Mouse	NM_014231	F-5'-ACCCAGGCACAAGTGGAGGAGGT-3' R-5'-CAATAACTACCACGATGATGGCACAGA-3'	229

Table 3.1: Primer sequences, and product sizes for RT-PCR reactions

3.2 for primary antibodies used and concentrations). Excess primary antibody or control IgGs were removed, washed 3 times for 5 minutes in 1×PBS and incubated with either FITC, or Cy3 conjugated secondary antibodies (Sigma, Chemicon, or Santa Cruz) for 1 hour in the dark at room temperature (see table 3.2 for secondary antibodies used and concentrations). Excess secondary antibody was removed and the cells were washed 3 times with 1×PBS for 10 minutes. The samples were mounted in Vectashield (Vector Laboratories) and viewed using a Leica DMLA microscope under UV illumination.

3.2.5 Western Blot analysis

3.2.5.1 Protein Extraction

Megakaryoblastic cell lines, MEG-01 and HEL, were plated at an initial concentration of 2×10^5 cells/ml and grown in the presence and absence of PMA for 72 hours. Cells were then removed and washed 3 times in 1×PBS. Cells were lysed with 250µl cold 1×lysis buffer (Cell Signalling: 2mM Tris (pH 7.5), 15mM NaCl, 0.1mM EDTA, 0.1mM EGTA, 0.1% Triton X-100, 0.25mM Sodium pyrophosphate, 0.1mM β-glycerolphosphate, 0.1mM Na_3VO_4, 0.1µg/ml Leupeptin) for 10 minutes on ice. Lysates were transferred into a 1.5ml eppendorf tube and centrifuged at 12,000g for 10 minutes. The supernatant was retained and the protein concentration was determined using the BCA protein assay (Pierce) according to the manufacturer`s instructions. The adult human brain lysate used as a positive control was obtained from abcam (5mg/ml).

3.2.5.2 Polyacrylamide gel electrophoresis and protein detection

40µg of total cell protein and 20µg of human brain lysate (control) was denatured at 95°C for 5 minutes in SDS loading buffer. Proteins were fractionated by SDS gel electrophoresis on a 15% polyacrylamide resolving gel using a 5% stacking gel in running buffer at 200V, 100mA for 1 hour. The electrophoresis gels were then equilibrated in transfer buffer and proteins transferred onto a nitrocellulose membrane (Amersham Biosciences) at 100V, 250mA for 1 hour.

Primary Antibody	Supplier	Dilution	Secondary Antibody	Supplier	Dilution
Mouse anti VAMP	Sigma	1:250	Goat anti mouse – FITC	Sigma	1:100
Mouse anti Syntaxin 4	BD Biosciences	1:250	Goat anti mouse – FITC	Sigma	1:100
Rabbit anti SNAP-23	Synaptic systems	1:50	Goat anti Rabbit – FITC	Sigma	1:100
Mouse anti Synaptotagmin	Synaptic systems	1:100	Goat anti mouse – FITC	Sigma	1:100

Table 3.2: Immunocytochemistry primary and secondary antibody suppliers and concentrations

3.2.5.3 Immunodetection

The membrane was blocked by gentle shaking for 15 minutes and incubation overnight in 4% marvel in TBS-T (20mM Tris, 137mM NaCl, 0.1% Tween 20) at 4°C. Following two rinses and a 15 minute incubation in TBS-T, membranes were incubated with primary antibody at room temperature for 1 hour, with extensive washes in TBS-T between incubations of primary and secondary antibodies. Membranes were then incubated for 1 hour at room temperature with the appropriate horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnologies).

Both primary and secondary antibodies were prepared in 4% marvel in TBS-T, see table 3.3 for details of primary and secondary antibodies used. Specific antibody binding was detected by enhanced chemilluminescence using ECL chemiluminescent solution, according to the manufacturer's instructions (Amersham Pharmacia Biotech) and exposed onto Hyperfilm (Amersham Pharmacia Biotech) at room temperature for up to 5 minutes. Films were developed using a xograph automated developer.

3.2.5.4 Membrane stripping

Antibodies were stripped from the western blot membrane by gentle agitation of the membrane in pre-warmed stripping buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris HCl; pH 6.7) at 60°C for 20 minutes. Membranes were then washed twice for 15 minutes in TBS-T before being blocked in 4% marvel TBS-T overnight prior to immunodetection as described in section 3.2.5.3.

3.2.6 Immunohistochemistry

3.2.6.1 Tissue collection and cryosectioning

The tibiae and femora were removed from adult rats and immediately frozen in liquid nitrogen and store at –80°C until use. Specimens were mounted in Cryo-m-bed embedding compound (Bright, UK) on brass chucks and frozen using dry ice and ethanol. Frozen sections were collected on polylysine slides using a Bright OTF500 cryostat (Bright) fitted with a tungsten carbide tipped knife and stored at -20°C until required.

Primary Antibody	Supplier	Dilution	Secondary Antibody	Supplier	Dilution
Mouse anti VAMP	Sigma	1:1000	Goat anti Mouse – HRP	Santa Cruz	1:1000
Rabbit anti VAMP1/2/3	Santa Cruz	1:1000	Goat anti Rabbit – HRP	Santa Cruz	1:1000
Rabbit anti SNAP-23	Synaptic systems	1:1000	Goat anti Rabbit – HRP	Santa Cruz	1:1000
Mouse anti Syntaxin 4	BD Biosciences	1:5000	Goat anti Mouse – HRP	Santa Cruz	1:1000
Mouse anti GAPDH	Advanced Immunoche-mical Inc	1:800	Goat anti mouse – HRP	Santa Cruz	1:1000

Table 3.3: Western blot analysis primary and secondary antibody suppliers and concentrations

3.2.6.2 Peroxidase immunolocalisations

Sections were fixed in 4% paraformaldehyde for 5 minutes before blocking with 3% hydrogen peroxide (Sigma) for 30 minutes to deplete endogenous peroxide activity. The Vector Avidin / Biotin Blocking Kit (Vector Laboratories) was used according to the manufacturer's protocol to prevent any non-specific avidin binding to endogenous biotin. Sections were blocked using 10% serum, chosen to match the species in which the secondary antibody was raised. Sections were incubated with primary antibody for 30 minutes (see table 3.4 for concentrations of primary antibody used), followed by exposure to biotinylated IgG secondary antibodies (1:200; Vector Laboratories). Avidin-biotinylated-peroxidase reagent (Vectastain Elite ABC reagent, Vector Laboratories, 1:50 dilution) was then applied to the specimens and incubated for 20 minutes followed by incubation with DAB tablets (0.5mg/ml 3,3'-diaminobenzidine and 0.3% hydrogen peroxide; Sigma) dissolved in dH_20, for 2 to 8 minutes. The sections were counterstained with haematoxylin before mounting in 90% v/v glycerol/PBS and viewed using a Leica DMRB microscope. Antibody negative controls received identical treatment apart from the application of either mouse or rabbit IgGs (Vector Laboratories; 1μg/ml) instead of the primary antibody, depending on what species the primary antibody was raised in. Incubations were carried out at room temperature with 3 × 5 minute PBS washes between incubations.

Primary Antibody	Supplier	Dilution	Secondary Antibody	Supplier	Dilution
Rabbit anti SNAP-23	Synaptic systems	1:500	Goat anti Rabbit – Biotinylated	Vector laboratories	1:200
Mouse anti syntaxin 4	BD Biosciences	1:250	Horse anti Mouse – Biotinylated	Vector laboratories	1:200
Mouse anti VAMP	Sigma	1:250	Horse anti Mouse – Biotinylated	Vector laboratories	1:200
Mouse anti Munc-18	BD Biosciences	1:500	Horse anti Mouse – Biotinylated	Vector laboratories	1:200
Mouse anti Rat CD61	BD Pharmingen	1:250	Horse anti Mouse – Biotinylated	Vector laboratories	1:200

Table 3.4: Immunohistochemistry primary and secondary antibody suppliers and concentrations

3.3 Results

3.3.1 Expression of SNARE complex and accessory proteins by megakaryocytic cell lines

3.3.1.1 mRNA expression of SNARE complex and accessory protein genes by megakaryocytic cells

Identification of a single band using RT-PCR confirmed the expression of mRNA for the core SNARE complex proteins, the v-SNAREs VAMP2, VAMP3 (cellubrevin), and t-SNAREs syntaxin 1a and syntaxin 4 by the megakaryocytic cell line MEG-01 in the presence and absence of 100nM PMA, a reagent which leads to the differentiation of MEG-01 cells (Figure 3.1). The expression of mRNA for the t-SNARE SNAP-25 which is normally present in the SNARE complex in neuronal cells was not detected absent from megakaryocytic cells, however its ubiquitously expressed homologue SNAP-23 was found to be expressed.

RT-PCR was also used to identify the mRNA expression of accessory proteins required for SNARE-dependent glutamate release in the CNS. In MEG-01 cells, mRNA expression of NSF, Rab 3a, Rab 3b, Rabphilin 3a, syntaphilin, Munc-13, Munc-18-1, α-SNAP, and γ-SNAP were identified in MEG-01 cells treated with and without PMA (Figure 3.2).

The mRNA for the SNARE complex and accessory proteins appeared to be more abundantly expressed by PMA-treated MEG-01 cells compared to control untreated cells. Products from MEG-01 cells were identical in size to those found in the human foetal forebrain positive controls and sequencing identified the products as the genes of interest.

3.3.1.2 Protein expression of SNARE complex and accessory proteins in megakaryocytic cells

SNARE complex proteins, SNAP-23, VAMP and syntaxin 4, were shown to be expressed at the protein level in MEG-01 cells and a similar megakaryocytic cell line HEL, in the presence and absence of PMA, by western blot analysis (Figure 3.3). VAMP expression in MEG-01 cells and HEL cells identified a molecular species at approximately 36kDa, instead of the expected size of approximately 18kDa.

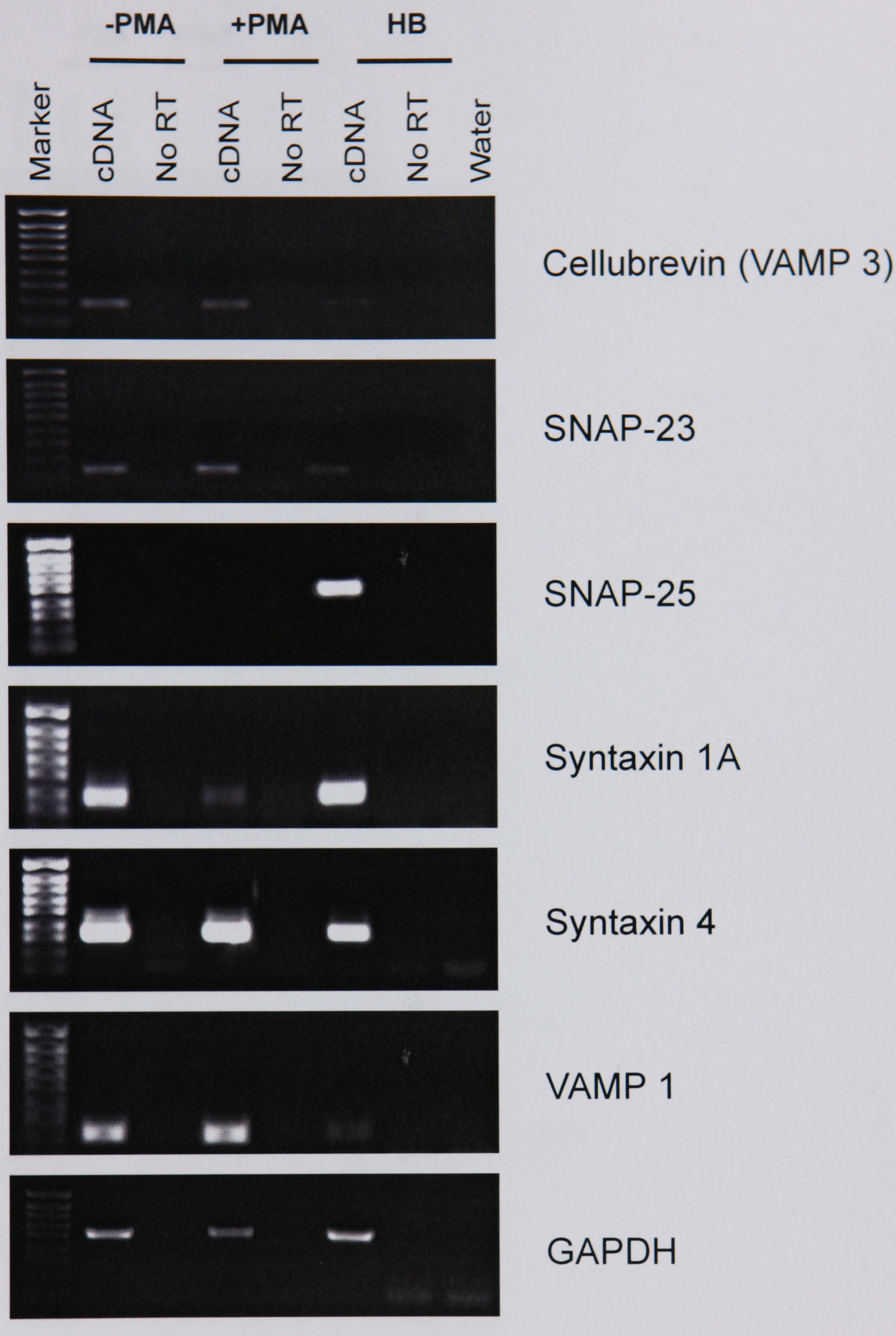


Figure 3.1: mRNA expression of SNARE complex protein genes by megakaryoblastic cell line, MEG-01, in the presence and absence of PMA. RT-PCR was used to demonstrate the mRNA expression of SNARE complex genes Cellubrevin, SNAP-23, Syntaxin 1a, Syntaxin 4 and VAMP by the megakaryoblastic cell line MEG-01 in the presence and absence of PMA. Human foetal forebrain (HB) cDNA was used as a positive control and expressed all SNARE complex components. Control reactions (no RT) were performed in parallel, in the absence of cDNA.

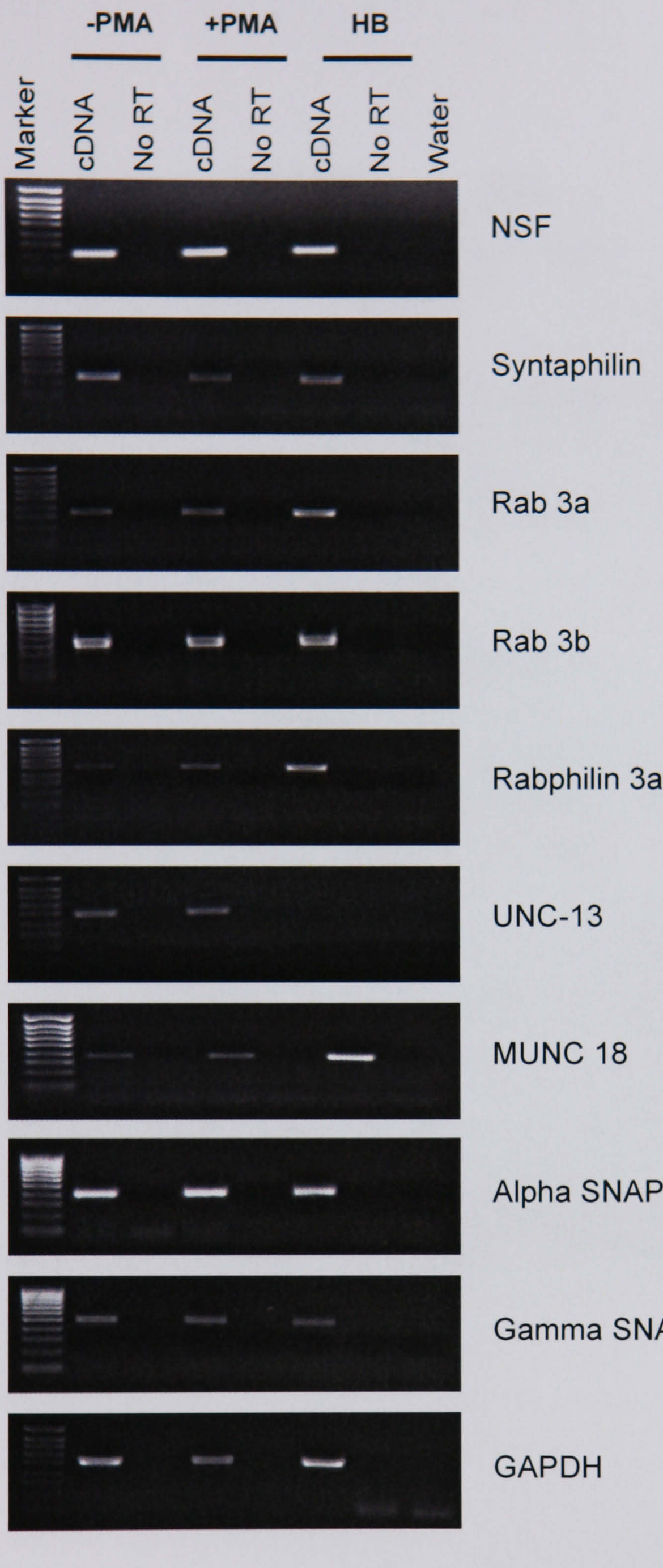


Figure 3.2: mRNA expression of SNARE complex accessory protein genes by megakaryoblastic cell line, MEG-01, in the presence and absence of PMA. RT-PCR was used to demonstrate the mRNA expression of SNARE complex accessory proteins NSF, syntaphilin, Rab 3a, Rab 3b, Rabphilin 3a, UNC-13, MUNC-18, α-SNAP and γ-SNAP by the megakaryoblastic cell line MEG-01 in the presence and absence of PMA. Human foetal forebrain (HB) was used as a positive control and expressed all SNARE complex accessory proteins. Control reactions (no RT) were performed in parallel, in the absence of cDNA.

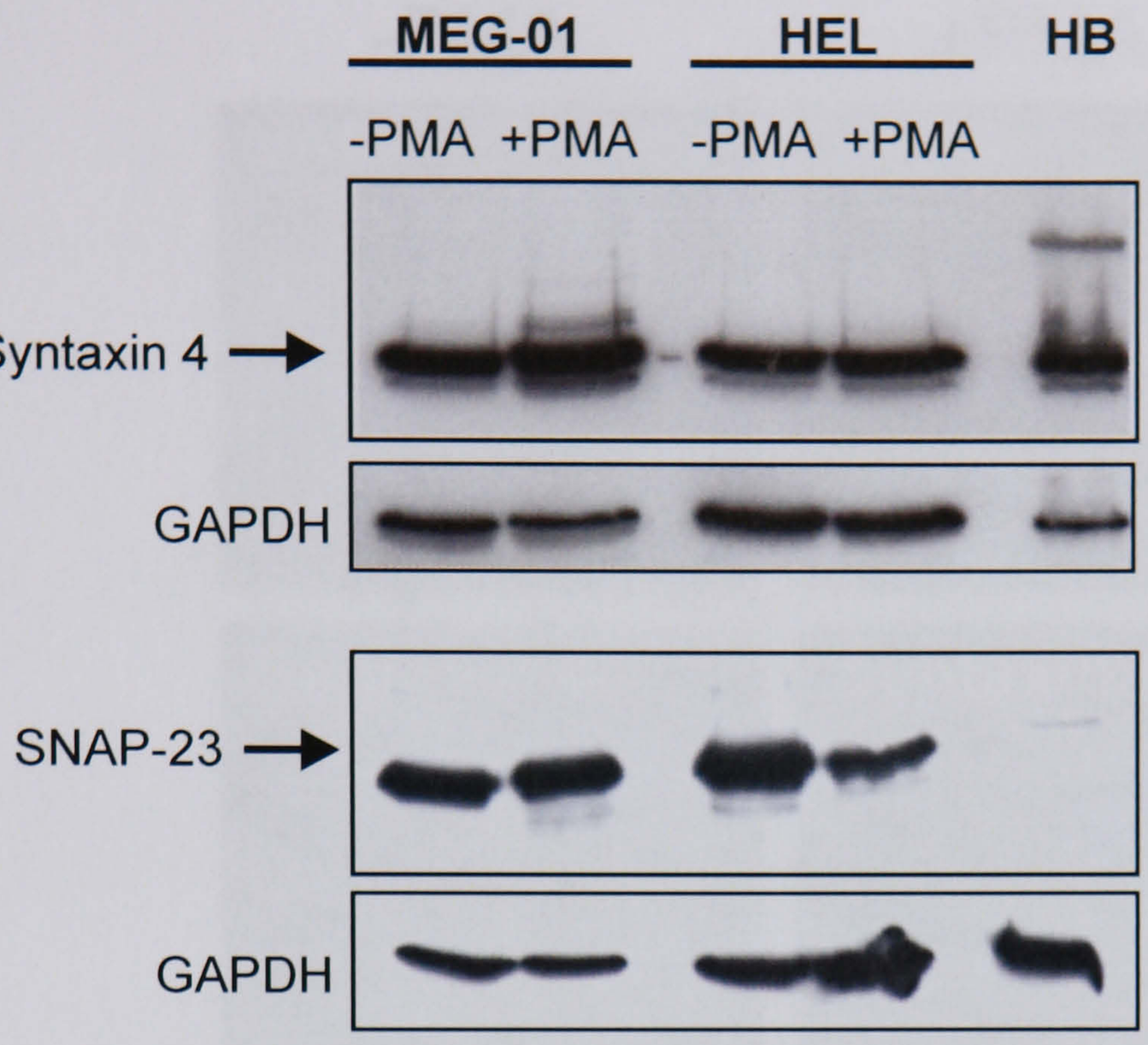


Figure 3.3: Western blot analysis of SNARE complex proteins in megakaryoblastic cell lines, MEG-01 and HEL, in the presence and absence of PMA. Protein expression of SNARE complex proteins SNAP-23, Syntaxin 4 and VAMP was determined by western blot analysis in megakaryoblastic cell lines, MEG-01 and HEL, in the presence and absence of PMA. Human brain lysate was used as a positive control and expressed all SNARE complex components.

Original in colour

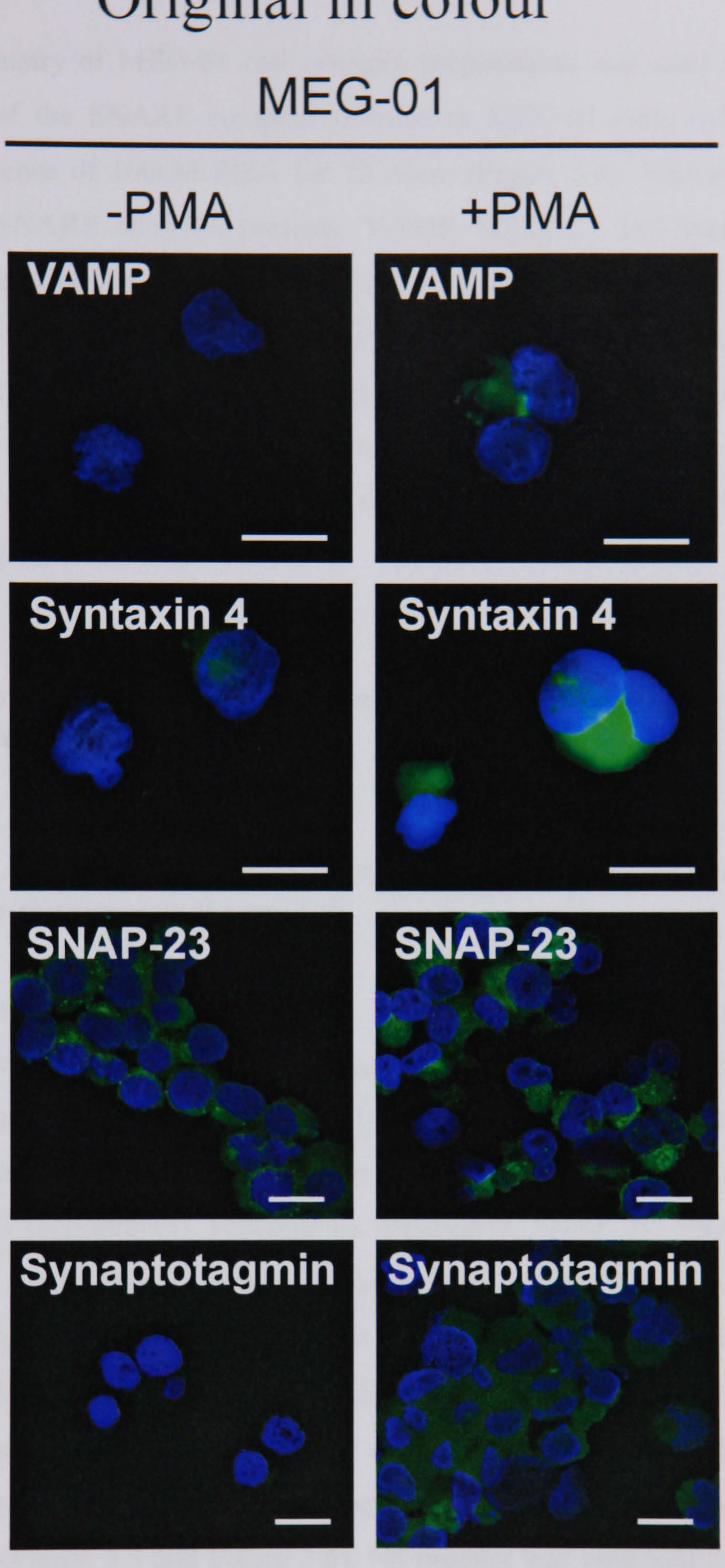


Figure 3.4: Immunofluorescent localistaion of SNARE complex and accessory proteins in the megakaryoblastic cell line, MEG-01 in the presence and absence of PMA. Protein expression of SNARE complex proteins SNAP-23, Syntaxin 4 and VAMP, and accessory proteins VGLUT1, VGLUT2 and synaptotagmin were determined by immunocytochemistry in the megakaryoblastic cell line, MEG-01 in the presence and absence of PMA. The protein of interest is indicated by green fluorescence (FITC) and the blue fluorescence (DAPI) shows the nucleus of the cell. Bar indicates 30μm.

Immunocytochemistry of MEG-01 cell cytospin preparations was used to determine the localisation of the SNARE complex proteins in MEG-01 cells cultured in the absence and presence of 100nM PMA for 72 hours (Figure 3.4). Primary antibodies recognising the SNARE complex proteins, VAMP, SNAP-23 and syntaxin 4, and accessory protein synaptotagmin were used in conjunction with a FITC conjugated secondary antibody to localise the expression of the SNARE complex proteins in MEG-01 cells (green positive staining). The location of the nucleus was identified by counterstaining with the nuclei dye DAPI (blue). Control samples, in which non-specific mouse and rabbit IgGs were used as opposed to the primary antibody exhibits the level of background staining.

3.3.2 Expression of SNARE complex and associated proteins by primary murine megakaryocytes

3.3.2.1 mRNA expression of SNARE complex and accessory protein genes by primary murine megakaryocytes

RT-PCR analysis was used to determine expression of SNARE complex and associated proteins by primary murine megakaryocytes, derived from the bone marrow from the femora and tibiae of mice, followed by exposure to TPO conditioned media for 3 days and separation on a BSA gradient. Gene-specific primers to mouse brain VAMP2, VAMP3 (cellubrevin), syntaxin 1a, syntaxin 4, SNAP-25, SNAP-23, NSF, Rab 3a, Rab 3b, Rabphillin 3a, Munc-13, Munc-18-1, α-SNAP and γ-SNAP were designed and specific PCR programmes were used (Primer details are listed in Table 3.1). The RT-PCR amplification products for SNARE complex and associated proteins from primary murine megakaryocytes were identical in size to those found in the whole mouse brain positive control and sequencing identified the products as the genes of interest (Figure 3.5 and Figure 3.6). No product was observed for SNAP-25, as observed in the MEG-01 cell line, nor for syntaxin 1a.

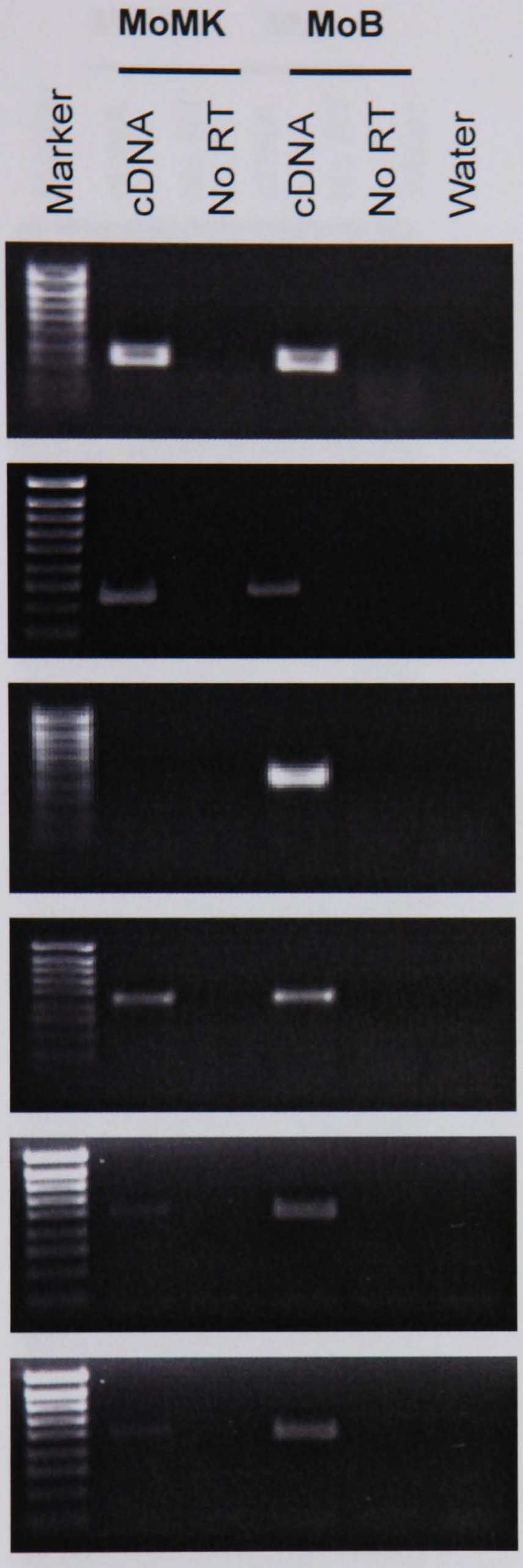


Figure 3.5: mRNA expression of SNARE complex protein genes by primary murine megakaryocytes. RT-PCR was used to demonstrate the mRNA expression of SNARE complex proteins Cellubrevin, SNAP-23, Syntaxin 4 and VAMP by primary murine megakaryocytes (MoMKs). Whole mouse brain (MoB) was used as a positive control and expressed all SNARE complex components. Control reactions (no RT) were performed in parallel, in the absence of cDNA.

Figure 3.6: mRNA expression of SNARE complex accessory protein genes by primary murine megakaryocytes. RT-PCR was used to demonstrate the mRNA expression of SNARE complex accessory proteins NSF, Rab 3a, Rab 3b, Rabphilin 3a, UNC-13, MUNC-18, α-SNAP and γ-SNAP by primary murine megakaryocytes (MoMKs). Whole mouse brain (MoB) was used as a positive control and expressed all SNARE complex accessory proteins. Control reactions (no RT) were performed in parallel, in the absence of cDNA.

3.3.3 Expression of SNARE complex and associated proteins by primary human megakaryocytes from CD34$^+$ umbilical cord blood (UCB) cells

3.3.3.1 mRNA expression of SNARE complex and accessory protein genes by primary human megakaryocytes from CD34$^+$ umbilical cord blood cells

RT-PCR analysis was used to determine expression of SNARE complex and associated proteins by primary human megakaryocytes, derived from UCB CD34$^+$ cells, following exposure to 25ng/ml TPO for 14 days. Gene-specific primers to human brain VAMP2, VAMP3 (cellubrevin), syntaxin 1a, syntaxin 4, SNAP-25, SNAP-23, NSF, Rab 3a, Rab 3b, Rabphillin 3a, syntaphilin, Munc-13, Munc-18-1, α-SNAP, and γ-SNAP and RT-PCR programmes used in these experiments were identical to those previously described in section 3.3.1.1 and listed in Table 3.1. The RT-PCR amplification products for the SNARE complex and associated proteins from primary human megakaryocytes were identical in size to those found in human foetal cerebellums (Figure 3.7 and Figure 3.8). No product was observed for SNAP-25 as seen previously with MEG-01 cells and primary murine megakaryocytes. No products were observed for Rab3b or Rabphillin 3a in primary human megakaryocytes. Human foetal cerebellum was used as a positive control for all RT-PCR reactions.

3.3.3.2 Protein expression of SNARE complex and accessory proteins in primary human megakaryocytes derived from CD34$^+$ umbilical cord blood cells

SNARE complex proteins, SNAP-23, VAMP and syntaxin 4, were shown to be expressed at the protein level in primary human megakaryocytes by western blot analysis (Figure 3.9). VAMP expression in primary human megakaryocytes identified a molecular species at approximately 68kDa, instead of the expected size of approximately 18kDa. As stated in section 3.3.1.2, previous experiments have identified a 68 kDa band, probably corresponding to a VAMP/synaptophysin heterodimer, suggesting this isoform is present in primary human megakaryocytes.

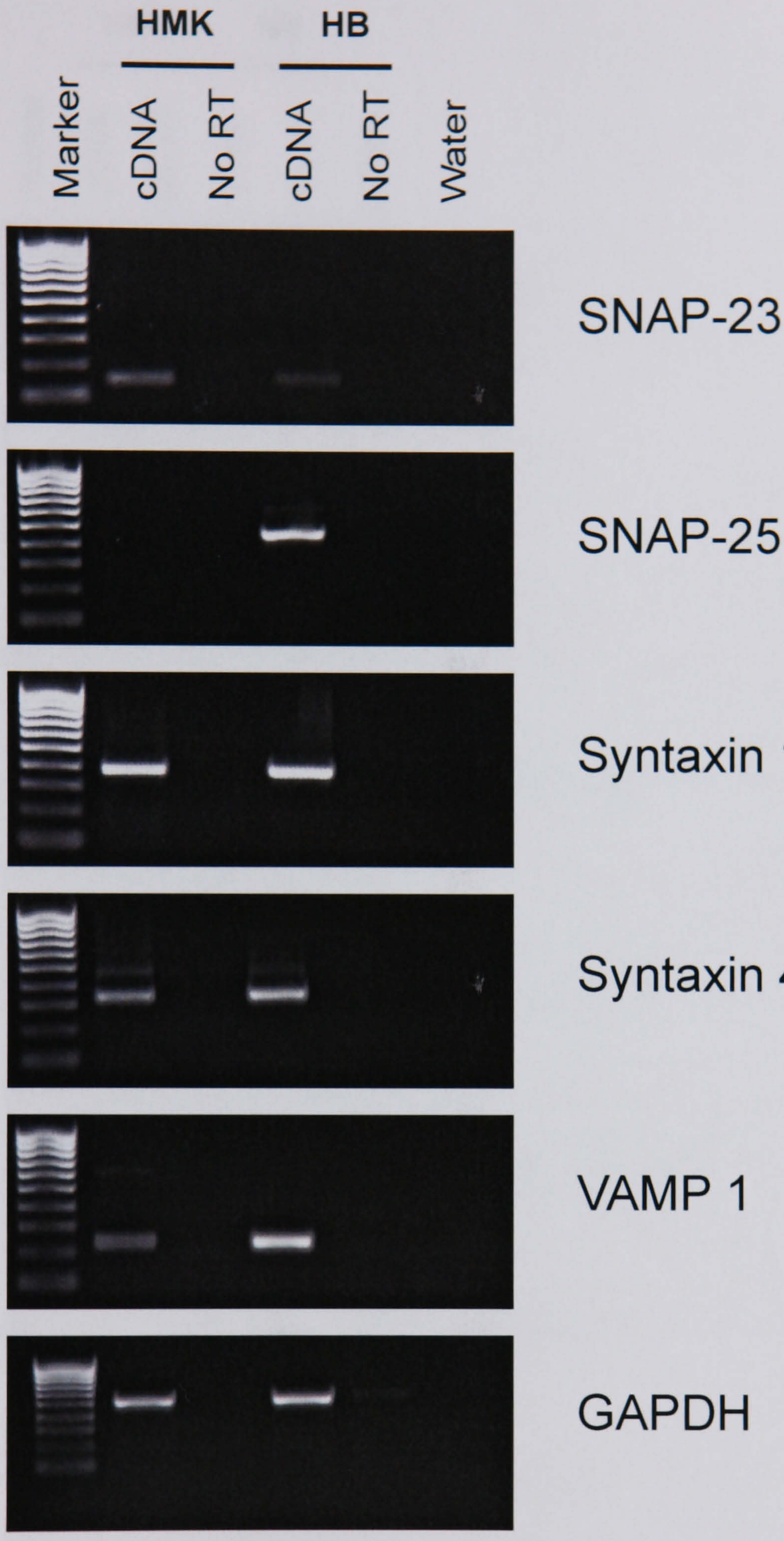


Figure 3.7: mRNA expression of SNARE complex protein genes by primary human megakaryocytes derived from UCB CD34+ cells. RT-PCR was used to demonstrate the mRNA expression of SNARE complex proteins SNAP-23, Syntaxin 1a, Syntaxin 4 and VAMP by primary human megakaryocytes (hMKs). Human foetal forebrain (HB) was used as a positive control and expressed all SNARE complex components. Control reactions (no RT) were performed in parallel, in the absence of cDNA.

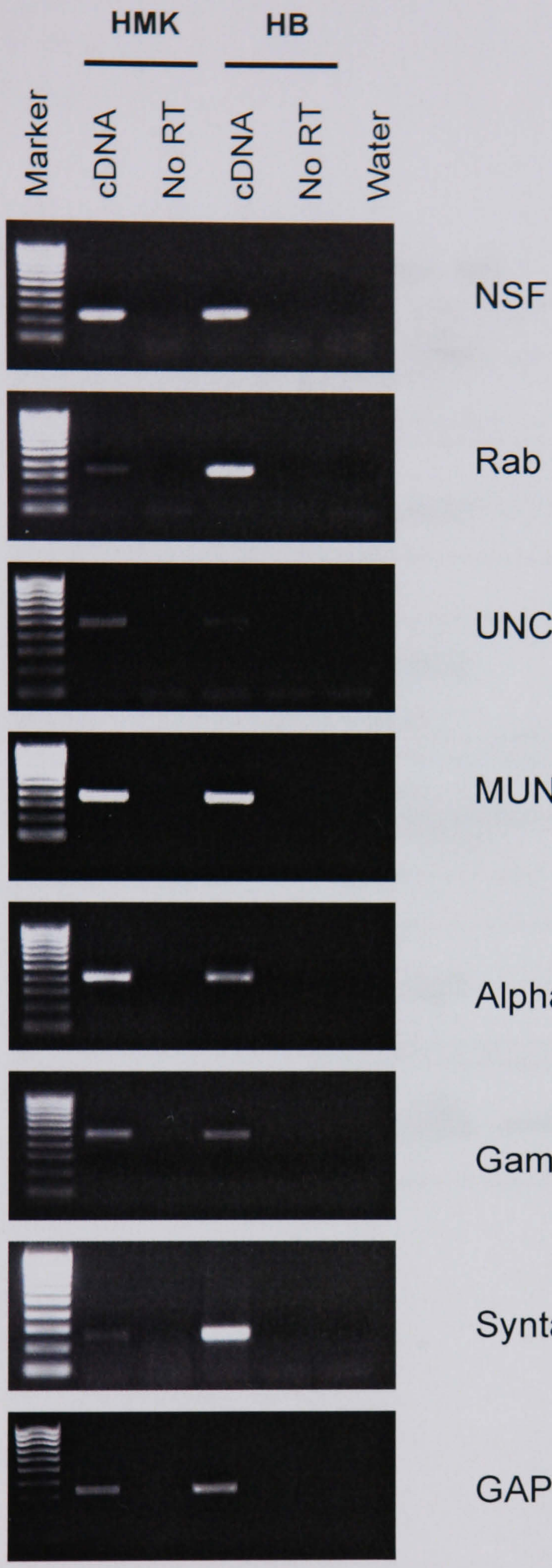


Figure 3.8: mRNA expression of SNARE complex accessory protein genes by primary human megakaryocytes derived from UCB CD34+ cells. RT-PCR was used to demonstrate the mRNA expression of SNARE complex accessory proteins NSF, Rab 3a, UNC-13, MUNC-18, α-SNAP, γ-SNAP and syntaphilin by primary human megakaryocytes (hMKs). Human foetal forebrain (HB) was used as a positive control and expressed all SNARE complex accessory proteins. Control reactions (no RT) were performed in parallel, in the absence of cDNA.

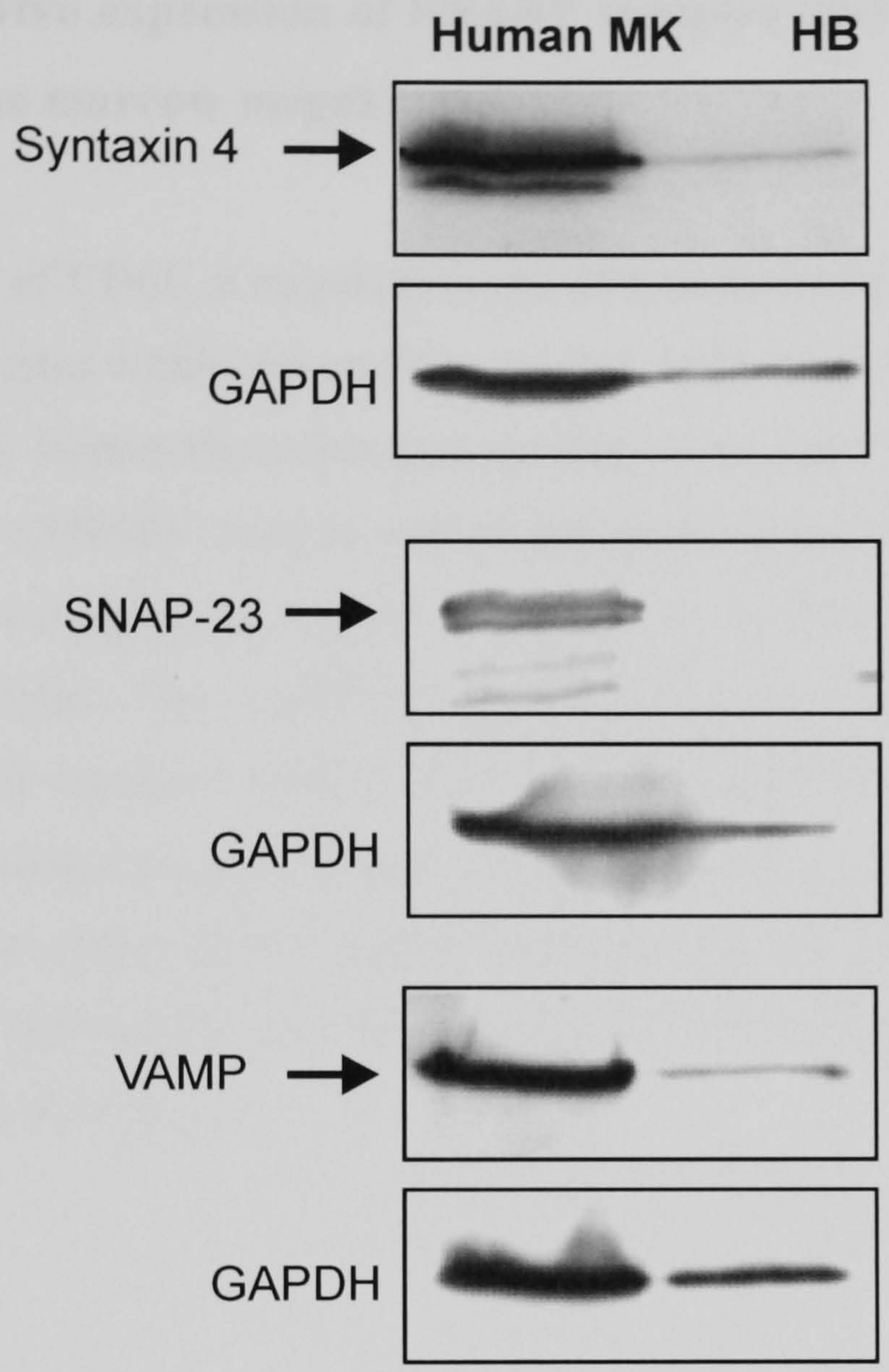


Figure 3.9: Western blot analysis of SNARE complex proteins in primary human megakaryocytes (MK). Protein expression of SNARE complex proteins SNAP-23, Syntaxin 4 and VAMP was determined by western blot analysis in primary human megakaryocytes. Human brain lysate (HB) was used as a positive control and expressed all SNARE complex components. GAPDH loading controls were carried out.

The 68kda band was also identified in the human brain lysate control (Calakos and Scheller. 1994a; Washbourne et al., 1995a; Edelmann et al., 1995b; Roy et al., 2004a).

3.3.4 In vivo expression of SNARE complex and accessory proteins in rat bone marrow megakaryocytes

Expression of CD61, a megakaryocytic cell surface marker, was used to identify the megakaryocytes within the rat bone marrow sections, shown by the brown staining in Figure 3.10. Immunohistochemical staining of rat bone marrow sections was used to identify core SNARE complex and accessory proteins in bone marrow. The t-SNAREs SNAP-23 and syntaxin 4 were identified in a cytoplasmic location with in the megakaryocytes. The v-SNARE VAMP however was not identified in rat bone marrow. The accessory protein Munc-18-1 was also identified in the in vivo rat bone marrow megakaryocytes (Figure 3.10). Munc-18-1 was observed throughout the cytoplasm as well as at the plasma membrane. Control samples, in which non-specific mouse and rabbit IgGs were used as opposed to the primary antibody exhibits the level of background staining.

Original in colour

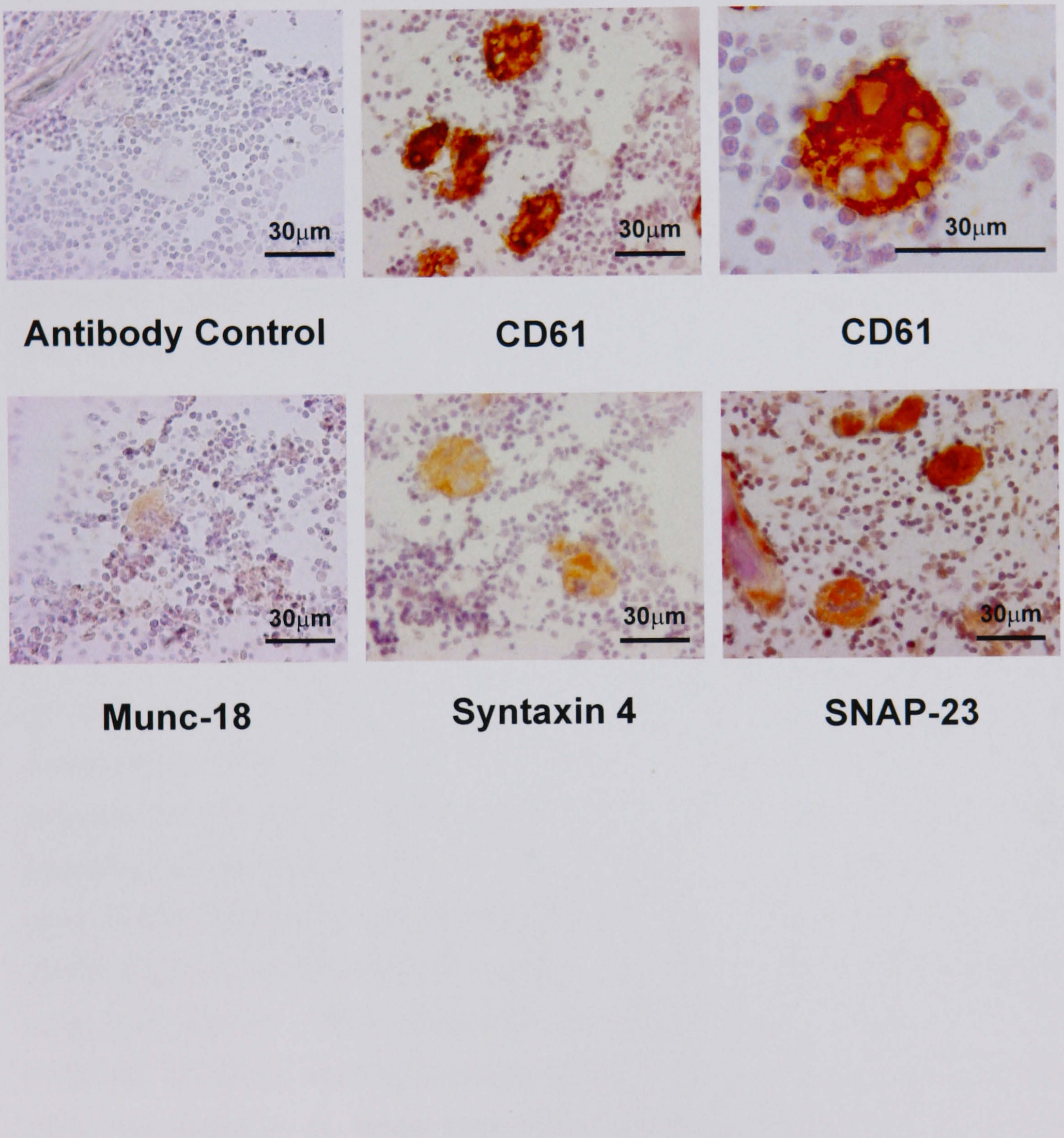


Figure 3.10: Rat megakaryocyte expression of SNARE complex proteins in vivo. Protein expression of SNARE complex proteins SNAP-23 and Syntaxin 4, and associated protein Munc-18 were determined by immunohistochemistry in ex vivo rat bone marrow sections. The megakaryocytic cell surface marker CD61 was used to identify megakaryocytes within the bone marrow. Brown DAB staining indicates the proteins of interest.

3.4 Discussion

Glutamate signalling was once thought to have been restricted to the mammalian CNS, where it is involved in aspects of normal brain function including learning, cognition and memory (Ottersen and Storm-Mathisen, 1984b; Fonnum, 1984b; Collingridge and Lester, 1989b; Headley and Grillner, 1990b). However, the binding of glutamate to glutamate receptors has been identified in non-neuronal tissues such as bone, spleen, lung, liver pancreas, heart, stomach, testis and intestine (Hosli and Hosli, 1993b; Vernadakis, 1996b; Steinhauser and Gallo, 1996b; Shelton and McCarthy, 1999a; Conti and Weinberg, 1999b; Bergles et al., 2000a; Skerry and Genever, 2001c; Hinoi et al., 2004b). The finding that bone marrow megakaryocytes express open channel forming N-methyl-D-aspartate (NMDA) – type glutamate receptors suggests that glutamate signalling mechanisms are important in these cells (Genever et al., 1999f; Hitchcock et al., 2003g). This was the first evidence of glutamate signalling in megakaryocytes and identified a potentially novel mechanism for regulating megakaryocytopoiesis and platelet release. To further investigate the role of glutamate signalling in megakaryocytopoiesis, this chapter addresses the megakaryocytic expression of the SNARE complex and key accessory proteins required for SNARE-dependent glutamate release in the CNS, for the first time.

RT-PCR, western blot analysis and immunolocalisations have identified of several components of the core exocytotic complex in megakaryocytic cells, including syntaxin 1a, syntaxin 4, VAMP2, cellubevin/VAMP3, which are the same as those identified and characterised in the CNS as mediators for glutamate exocytosis (Sollner et al., 1993e; Chapman et al., 1994b; Bennett, 1995b; Hanson et al., 1997a; Betz et al., 1997c; Robinson and Martin, 1998b; Sassa et al., 1999a; Zinsmaier and Bronk, 2001a; Chen and Scheller, 2001a; Richmond and Broadie, 2002b). Expression of the t-SNARE SNAP-25, which is normally present in the SNARE complex in neuronal cells, was found to be absent from both megakaryocytic cell lines and primary megakaryocytes at the mRNA level, however its homologue SNAP-23 was shown to be expressed. Despite amino acid sequence similarities, SNAP-23 and SNAP-25 have been shown to be expressed in different tissues. SNAP-25 is expressed predominantly in the brain, whereas SNAP-23 is ubiquitously expressed in other human tissues such

as heart, placenta. kidney. muscle. lung. liver and pancreas where it has been shown to be vital for membrane fusion and vesicle docking (Ravichandran et al., 1996b).

Western blot analysis of VAMP expression in MEG-01 cells and HEL cells identified a molecular species at approximately 36kDa, instead of the expected size of approximately 18kDa. In previous experiments monomeric VAMP (18kDa), VAMP homodimers (36kDa), and 68 kDa bands probably corresponding to a VAMP/synaptophysin heterodimers have been observed (Calakos and Scheller, 1994b; Edelmann et al., 1995a; Washbourne et al., 1995b; Roy et al., 2004b). Therefore it is likely that the molecular species identified in this study are VAMP homodimers. The monomer, homodimer and heterodimer can all be observed in the human brain lysate control.

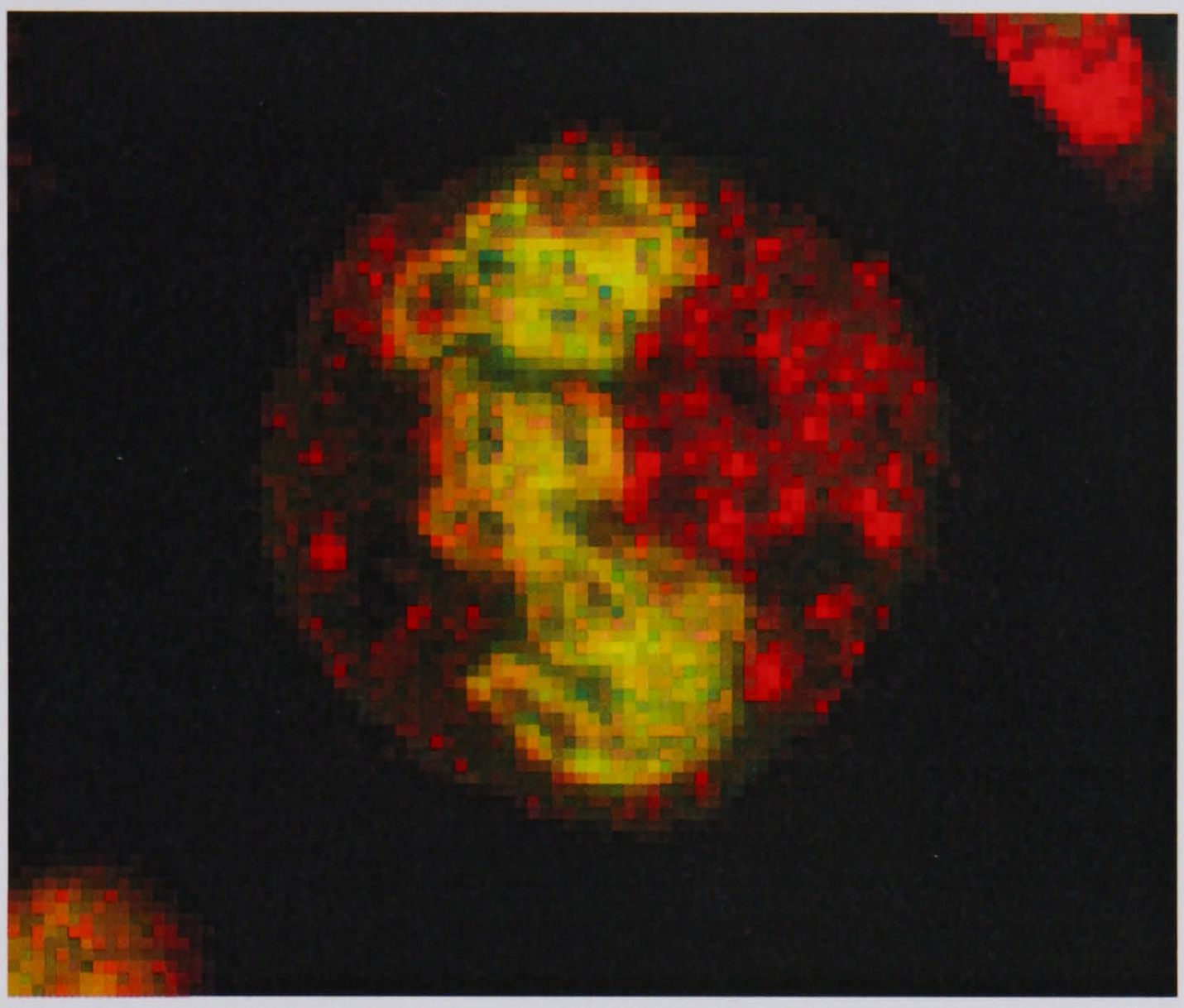
The identification of genes encoding multiple accessory proteins involved in targeted vesicular glutamate release, NSF, α-SNAP, γ-SNAP, Munc18-1, Munc13, and syntaphilin, by megakaryocytic cells, indicates that the essential components required for neurotransmitter release in the CNS were present in these cells. For example in *munc18-1* null mice there is a lack of neurotransmitter release, suggesting Munc18 has an important role in vesicle docking and fusion (Verhage et al., 2000b), therefore it is significant that megakaryocytic cells express this protein and other regulatory proteins such as Munc13 which aids the disassembly of the SNARE complex after exocytosis has occurred.

Megakaryocytic cells also expressed genes encoding key regulatory proteins, synaptotagmin, Rab3a, Rab3b, and the Rab3a binding protein rabphilin 3a, providing evidence for Ca^{2+}-dependent glutamate exocytosis from megakaryocytes. In megakaryocytes Rab3a, a neuronal rab protein, and Rab3b, a non-neuronal rab, were identified in both the MEG-01 cell line and the primary murine megakaryocytes, however only Rab3a was identified in primary human megakaryocytes. The expression of these Rab proteins and the Rab3 binding protein rabphilin3a, provide evidence that the SNARE-dependent glutamate release pathway in megakaryocytes is regulated in a similar fashion to that observed in the CNS.

In neuronal cells, voltage-gated Ca^{2-} channels are required for the rapid influx of Ca^{2+}, to mediate a fast response to an incoming stimulus, leading to neurotransmitter release. Voltage-gated Ca^{2+} channels contain a long intracellular linker which associates with synaptic proteins such as synaptotagmin, a tight interaction between presynaptic Ca^{2+} channels and the SNARE complex is essential for stabilising neurotransmitter release (Zamponi, 2003). The identification of synaptotagmin I, the putative Ca^{2+} sensor that drives SNARE complex formation in neuronal cells in the megakaryocytic cell line, MEG-01, further supports the evidence for Ca^{2+}-dependent glutamate exocytosis by megakaryocytes. However, megakaryocytes are classified as non-excitable cells and are not believed to express voltage-gated Ca^{2+} channels (Mahaut-Smith et al., 1999; Thomas et al., 2001; Mason and Mahaut-Smith, 2004). Therefore, these observations suggest that glutamate release mechanisms in megakaryocytes although similar, are different to those described for neurons, or that alternative Ca^{2+} trafficking pathways are involved.

This chapter provides the first evidence that megakaryocytes possess the relevant machinery to carry out SNARE-dependent glutamate release mechanisms, similar to those observed in the CNS at glutamatergic synapses. These data support the previous findings that glutamate signalling occurs in megakaryocytic cells (Genever et al., 1999e; Hitchcock et al., 2003f), and suggests a regulatory role for glutamate in autocrine signaling and signaling within the bone marrow microenvironment.

Chapter 4



Evidence of vesicular glutamate signalling and release in megakaryocytic cells

Identification of acidic vesicles using acridine orange staining in primary human megakaryocytes, green fluorescence and red fluorescence co-localised in a nuclear region within the cell, and red fluorescence observed in the cytoplasm and periphery of the cells.

Chapter 4: Evidence of vesicular glutamate signalling and release in megakaryocytic cells

4.1 Introduction

The identification of a megakaryocytic SNARE-complex and other vesicular proteins associated with glutamate release in neuronal cells, described in Chapter 3, provides the first evidence for SNARE-dependent glutamate release from megakaryocytic cells. These findings and the characterisation of the megakaryocytic NMDA receptor suggest an important role for glutamate signalling in megakaryocyte maturation and platelet production and paracrine signalling within the bone marrow (Genever et al., 1999d; Hitchcock et al., 2003e). Previous studies have also identified glutamate signalling in other cells of the bone marrow including osteoblasts and osteoclasts (see section 1.4). Osteoblasts have been shown to express SNARE and other vesicular proteins associated with glutamate release in the CNS (Bhangu et al., 2001a; Bhangu et al., 2001e), and the functional glutamate transporter molecule EAAT1 (GLAST), which has been implicated in glutamate uptake in neuronal cells, has been identified in osteoblasts and osteoclasts (Mason et al., 1997c). From these findings it is possible to hypothesise that megakaryocytes, osteoblasts and osteoclasts may be responsible for glutamate release and recycling within the bone marrow aiding bone resorption and remodelling, and regulating megakaryocytopoiesis.

Vesicular exocytosis is a fundamental process leading to the release of neurotransmitter by the fusion of the vesicle and plasma membrane (as discussed in section 1.3.1 and section 3.1). Exocytosis occurs through distinct mechanisms, two of which have been investigated are full fusion of the vesicular and plasma membranes leading to the collapse of the vesicle releasing the neurotransmitter (Heuser and Reese, 1973; Sudhof, 1995), and "kiss and run" exocytosis where the a transient fusion pore temporarily connects the lumen of the vesicle and the extracellular space releasing the neurotransmitter without the collapse of the vesicle which is then recycled (Ceccarelli et al., 1973; Pyle et al., 2000; Stevens and Williams, 2000; Sudhof, 2000).

Previously an enzyme-linked fluorimetric glutamate release assay has been employed in the study of neuronal glutamate release (Nicholls et al., 1987a; Bezzi et al., 2004) and has been modified for the study of glutamate release from osteoblastic cells (Genever and Skerry, 2001d). The assay is based on the principle that addition of glutamate dehydrogenase (GDH) and β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) will result in released glutamate being oxidised to α-ketoglutarate, resulting in the formation NADPH that can subsequently be determined fluorimetrically. This assay was used in this chapter to determine the glutamate release capacity of megakaryocytic cells.

Until recently, technical limitations prevented the investigation of exocytotic events in small synaptic vesicles, however the development of optical techniques to track vesicle recycling has allowed more extensive studies of small synaptic vesicle exocytosis and endocytosis broadening our knowledge of this subject (Betz et al., 1992; Ryan et al., 1993; Sankaranarayanan and Ryan, 2000; Gandhi and Stevens, 2003; Aravanis et al., 2003c). In particular, the development of styryl dyes as fluorescent molecules that label recycling vesicles have revolutionised the way in which synaptic vesicle recycling can be investigated. FM1-43 is one of the styryl dyes which have been used to investigate exocytosis and endocytosis from individual vesicles (Aravanis et al., 2003a; Aravanis et al., 2003b). Investigations carried out by Richards et al. (Richards et al., 2005a) using this technique suggest that two modes of exocytosis occur at the hippocampal synapses. The first a slow event, where a population of vesicles lose their dye with slow kinetics, mediated by "kiss and run" exocytosis, and the second a larger event, where a population of vesicles lose their dye with faster kinetics, mediated by the full collapse of the vesicle into the plasma membrane (Richards et al., 2005b). These styryl dyes can therefore give a vital insight into exocytosis kinetics at the synapse.

During normal synaptic transmission, the excitatory action of released glutamate is rapidly terminated due to its efficient removal from the synapse by glutamate uptake systems in glia and nerve terminals (Nicholls and Attwell, 1990; Clements et al., 1992). Neuronal cells contain approximately 5mM glutamate in their cytoplasm, with extracellular glutamate levels of approximately 1 to 2μM being reported (Benveniste

et al., 1984; Anderson and Swanson, 2000). It is crucial that extracellular glutamate concentrations within the CNS are kept low, as excess glutamate is highly neurotoxic causing neuronal damage (Olney, 1971; McCall et al., 1979; Choi, 1988a). To reduce the risks of neurotoxicity within the CNS the metabolism of glutamate is compartmented by the blood-brain barrier. This essentially prevents glutamate from the blood plasma (where it is present in a higher concentration) entering the CNS, where is at a lower concentration, therefore the majority of all glutamate within the CNS is synthesised within its own boarders (Gruetter et al., 1994). Glial cells play an important role in the correct functioning of glutamatergic neurotransmission at the synapse (Hamberger et al., 1981; Erecinska and Silver, 1990). In particular, astrocytes are responsible for maintaining extracellular concentrations of glutamate at a low level. Astrocytes are non-excitable cells that are involved in the recycling and synthesis of glutamate with in the CNS. The levels of glutamate found in cultured astrocytes are much lower than those found in neuronal cells, due to conversion of glutamate to glutamine during recycling of the synaptically released glutamate by glutamine synthase during the glutamate-glutamine cycle (for reviews see Hertz et al., 1999; Nedergaard et al., 2002). The synthesised glutamine is released back into the extracellular space from the astrocyte into the synaptic cleft, where it is then taken up by nerve terminals and reconverted into glutamate by the enzyme glutaminase. This extracellular glutamine has no neurotransmitter action and cannot therefore activate excitatory amino acid receptors. The newly resynthesised glutamate is then ready to be released in a new round of synaptic transmission.

Astrocytes and neuronal cells express high affinity, sodium-dependent excitatory-amino-acid transporters (EAATs), which as discussed in section 1.3.3 enable excess extracellular glutamate to be retrieved from the synaptic cleft after synaptic transmission has taken place leading to the termination of synaptic transmission. The removal of glutamate by these transporters prevents neurotoxicity and is vital in maintaining the integrity of the synapse and neurotransmission (Marcaggi and Attwell, 2004b). The identification of EAATs within the bone marrow suggests that glutamate recycling may occur in a similar manner to that observed in the CNS. Here we investigate the expression of these transporters in megakaryocytic cells, hypothesising that megakaryocytic cells may have a functional role with in the bone marrow recycling and releasing glutamate maintaining the bone microenvironment.

An important stage of the synaptic vesicle cycle is the regeneration of the synaptic vesicles with in the nerve terminal, where vesicles are reloaded with neurotransmitter via specific vesicle transporters. for glutamate these are the VGLUTs (for a review of vesicular glutamate transporters please see section 1.3.4) (Maycox et al., 1990). These transporters require a proton electrochemical gradient, $\Delta\mu H^+$ for active transport of the neurotransmitter into the vesicle. This gradient is generated by proton ATPases of the vacuolar type that electrogenically pump protons into the vesicle interior (Moriyama et al., 1992; Nelson, 1992; Schuldiner et al., 1995). In the absence of membrane-permeable anions. v-ATPases are able to generate an inside-positive membrane potential ($\Delta\Psi$), which leads the accumulation of Cl^- ions and an increase of anions entering the vesicle via chloride channels, decreasing the $\Delta\Psi$, and increasing ΔpH due to the net accumulation of HCl. The ΔpH component of the $\Delta\mu H^+$ can drive the uptake of most neurotransmitters apart from glutamate, which shows little or no sensitivity to ΔpH. A common property of all synaptic vesicles appears to be their acidification coincides with the loading of the neurotransmitter.

The pH-sensitive dye acridine orange was used by Zoccarato et al. (Zoccarato et al., 1999h) to monitor exocytosis and endocytosis in synaptosomes (Tabb et al., 1992a; Zoccarato et al., 1999g). It had been used in previous studies to detect acidification of synaptic vesicles, lysosomes, endocytotic granules and whole cells (Gluck et al., 1982; Bowman et al., 1988; Tabb et al., 1992b; Hartinger and Jahn, 1993; Dell'Antone et al., 1995). Acridine orange is a weak metachromatic base, which is freely permeable due to unprotonated amine however once it has entered an acidic synaptic vesicle it becomes protonated and trapped with in the acidic compartments where its accumulation and dimerisation leads to a change in its optical parameters (Zoccarato et al., 1999f). Exocytosis of the neurotransmitter leads to vesicular alkalisation and the neutralisation of acridine orange allowing it to cross the vesicle membrane, and a decrease in acridine orange fluorescence. The study by Zoccarato et al. (Zoccarato et al., 1999e) indicated that the rates of synaptic retrieval measured using acridine orange were slightly faster than those observed using FM1-43 dye (Ryan, 1996; Ryan et al., 1996), although acridine orange does monitor postendocytotic acidification of the vesicle during neurotransmitter loading, indicating that the response to acridine orange is as fast as that of the FM1-43 and/ or that neurotransmitter loading takes places

immediately following synaptic vesicle retrieval (Zoccarato et al., 1999d). In this chapter acridine orange was used to visualise acidic vesicles within megakaryocytic cells supporting our evidence for SNARE-dependent glutamate release in these cells similar to that observed in neuronal cells. We also investigated the expression of VGLUT proteins with in megakaryocytic cells, these transporters as discussed previously were once considered brain-specific markers of glutamatergic neurons, their expression by these cells is important in investigating their glutamatergic phenotype.

The aim of this chapter is to investigate whether functional glutamate signalling occurs with in megakaryocytic cells, focusing on release and recycling mechanisms within both megakaryocytic cell lines and primary megakaryocytes using the well-established methods discussed above.

4.2 Methods

4.2.1 Acridine Orange Staining

The acidic vesicles were stained with acridine orange (Sigma) at a final concentration of 5µg/ml in culture medium. After 15 min at 37°C, the stain was removed by centrifugation at 1500rpm for 5 minutes and the cell pellet washed three times with 1×PBS, and the cells were imaged by confocal microscopy using a Zeiss LSM 510 meta on an Axiovert 200M. This experiment was repeated 3 times to ensure consistency of results (n=3).

4.2.2 FM1-43 staining

4.2.2.1 Preparation of fibronectin coated coverslips

13mm glass coverslips were first sterilised by immersion in 100% ethanol for 30seconds, washed in sterile distilled water (dH_2O) and allowed to air dry. The coverslips were then placed in 24-well culture plates and coated with 200µl of 25ng/ml fibronectin (Sigma Aldrich), diluted in dH_2O overnight at 4°C. Fibronectin solution was then removed and the coverslips washed 3 times in dH_2O.

4.2.2.2 FM1-43 labelling of megakaryocytic cells

MEG-01 cells in the presence of 100nM PMA and primary murine megakaryocytes were plated at 5×10^4 cells/cm^2 onto sterile fibronectin-coated coverslips and maintained in culture for 72 and 24 hours respectively. Medium was removed and the cells were incubated for 5 minutes in Hank's buffered saline solution (HBSS) containing 1mM $CaCl_2$. Recycling vesicles were labelled, in live cells, using FM1-43 (Molecular Probes) at a final concentration of 10µM and incubated for various periods up to 20 minutes. After these incubation periods cells were washed in HBSS, fixed in 4% paraformaldehyde for 5 minutes, mounted and viewed under UV illumination. For destaining experiments, FM1-43 was added to MEG-01 cells, incubated for 20 minutes, washed in HBSS and then incubated for a further 30 to 60 minutes in FM1-43-free HBSS. The cells were then fixed, mounted and viewed under UV

illumination. This experiment was repeated 3 times to ensure consistency of results (n=3) and a stop watch was used to ensure timings were accurate.

4.2.3 RNA extraction, cDNA synthesis and PCR

RNA was extracted and cDNA synthesised from MEG-01 cells and primary human megakaryocytes derived from $CD34^+$ umbilical cord blood cells using the methods described in section 3.2.1 and 3.2.2 respectively, and run on a specific PCR program designed for the individual primer set (see table 4.1 for primer sequences). The PCR products were then analysed by 1% TAE agarose gel electrophoresis, as described in section 3.2.3.

4.2.4 Immunolocalisations

Cytospin preparations and immunocytochemistry were carried out as described in section 3.2.4.1 and 3.2.4.2 respectively, using the MEG-01 cell line cultured in the presence and absence of 100nM PMA, and primary human megakaryocytes derived from $CD34^+$ umbilical cord blood cells. See table 4.2 for primary and secondary antibodies used and concentrations.

4.2.5 Western blot analysis

Protein extraction, polyacrylamide gel electrophoresis and immunodetection were carried out as described in section 3.2.5, using the megakaryocytic cell line MEG-01 cultured in the presence and absence of 100nM PMA. See table 4.3 for primary and secondary antibodies used and concentrations.

4.2.6 Immunohistochemistry

Tissue collection, cryosectioning and peroxidase immunolocalisations were carried out as described in section 3.2.6 using the tibiae and femora from adult rats. Table 4.4

Protein	Species	Accession number	Forward Primer Reverse Primer	Product Size
VGLUT1	Human	NM_020309	F-5'-GCCTCCCTCGCCGCTACATTATCG-3' R-5'-CGCCGCCAGGGAGTGCTA-3'	718
VGLUT2	Human/Mouse	AB032435	F-5'-TGGACATGGTCAACAACAGCA-3' R-5'-GCATAGGAACCACAAAAGGAGGT-3'	385
EAAT1	Human	U03504	F-5'-GGAAGGGCACAAAGGAAAACA-3' R-5'-CCACGGGGCATACCACATTA-3'	431
EAAT2	Human	U03505	F-5'-AGGGCTTCTTCCGCTTGGCATCTC-3' R-5'-CTCCGGCACCTCAGTCAGTCACAGTCCA-3'	489

Table 4.1: Primer sequences, and product sizes for RT-PCR reactions

Primary Antibody	Supplier	Dilution	Secondary Antibody	Supplier	Dilution
Rabbit anti VGLUT1	Synaptic systems	1:1000	Donkey anti rabbit – FITC	Santa Cruz	1:200
Rabbit anti VGLUT2	Synaptic systems	1:500	Donkey anti rabbit – FITC	Santa Cruz	1:200

Table 4.2: Immunocytochemistry primary and secondary antibody suppliers and concentrations

Primary Antibody	Supplier	Dilution	Secondary Antibody	Supplier	Dilution
Rabbit anti GLT-1	abcam	1:500	Goat anti Rabbit – HRP	Vector laboratories	1:1000
Mouse anti GAPDH	Advanced Immunoche-mical Inc	1:800	Goat anti mouse – HRP	Santa Cruz	1:1000

Table 4.3: Western blot analysis primary and secondary antibody suppliers and concentrations

Primary Antibody	Supplier	Dilution	Secondary Antibody	Supplier	Dilution
Rabbit anti VGLUT1	Synaptic systems	1:1000	Goat anti Rabbit – Biotinylated	Vector laboratories	1:200
Rabbit anti VGLUT2	Synaptic systems	1:500	Goat anti Rabbit – Biotinylated	Vector laboratories	1:200
Goat anti EAAT-1	Santa Cruz	1:250	Horse anti Goat – Biotinylated	Vector laboratories	1:200
Rabbit anti EAAT-2	Santa Cruz	1:200	Goat anti Rabbit – Biotinylated	Vector laboratories	1:200

Table 4.4: Immunohistochemistry primary and secondary antibody suppliers and concentrations

provides information on the primary and secondary antibodies used and concentrations.

4.2.7 Glutamate Release Assay

Glutamate release was determined using an enzyme-linked fluorimetric assay based on previously published protocols (Nicholls and Sihra, 1986: Nicholls et al., 1987b) with some modifications (Genever and Skerry, 2001c). Cells were cultured prior to the experiment as described in section 2.1.1. Before the assay MEG-01 cells, primary human megakaryocytes or primary murine megakaryocytes were plated at 5×10^5 cells/ml (100µl per well) in blackened 96-well plates (µclear, Greiner Labortechnik Ltd., Stonehouse, U.K.) in release buffer pre-warmed to 37°C, containing NaCl (120 mM), KCl (3 mM), NaH_2PO_4 (1.25 mM), HEPES-Na (25 mM), Glucose (4 mM), $MgCl_2$ (1 mM), $CaCl_2$ (2mM), pH 7.4. The reaction was initiated by the addition of release buffer containing GDH (40 U/ml, Sigma) and NADP+ (1mM), and the plate was transferred to a Dynex MFX fluorescent plate reader equipped with a thermostated incubation chamber (37°C). Fluorescence was monitored using excitation and emission wavelengths of 355 nm and 460 nm, respectively. For each assay, a standard curve was produced using known concentrations of exogenous glutamate. Test compounds were added to cell suspension 30 min before the addition of GDH and were maintained throughout the assay period. Glutamate release was normalised to cell number.

4.3 Results

4.3.1 Identification of acidic vesicles in megakaryocytic cells

The pH sensitive dye acridine orange was used to determine whether synaptic vesicles were present in megakaryocytic cells. Acridine orange is an optical probe which has been shown to be trapped in acidic vesicles (Barasch et al., 1991) where it exhibits different absorption and fluorescence properties depending on the form present. Green fluorescence is observed when the dye is in its monomeric form in solution and when it is bound to double-stranded nucleic acids, and a red emission can be observed when acridine orange molecules accumulate with in the vesicles, either in the presence of single-stranded nucleic acids, or as crystals (Kapuscinski et al., 1982). Acidic vesicles were identified in the MEG-01 cell line, with red fluorescence observed around the periphery of the cells, and green fluorescence observed with in the nucleus of the cell (Figure 4.1A). Acidic vesicles were also observed in primary human megakaryocytes, where green fluorescence and red fluorescence co-localised in a nuclear region within the cell, and red fluorescence observed in the cytoplasm and periphery of the cells (Figure 4.1B).

4.3.2 Identification of vesicular recycling in megakaryocytic cells

FM1-43 staining and destaining has become an important method for studying vesicular turnover in neuronal cells. To determine whether vesicle recycling could take place in megakaryocytic cells, FM1-43 staining was carried out as described in section 4.2.2 using the MEG-01 cell line treated with 100nM PMA and primary murine megakaryocytes. The FM1-43 dye is incorporated into the vesicle membrane and acts as a fluorescent reporter of vesicle recycling. The results show an accumulative increase in the amount of fluorescent dye within the cells over a 20 minute incubation period. Following removal of FM1-43 dye from the culture media cells showed destaining within 60 minutes (Figure 4.2).

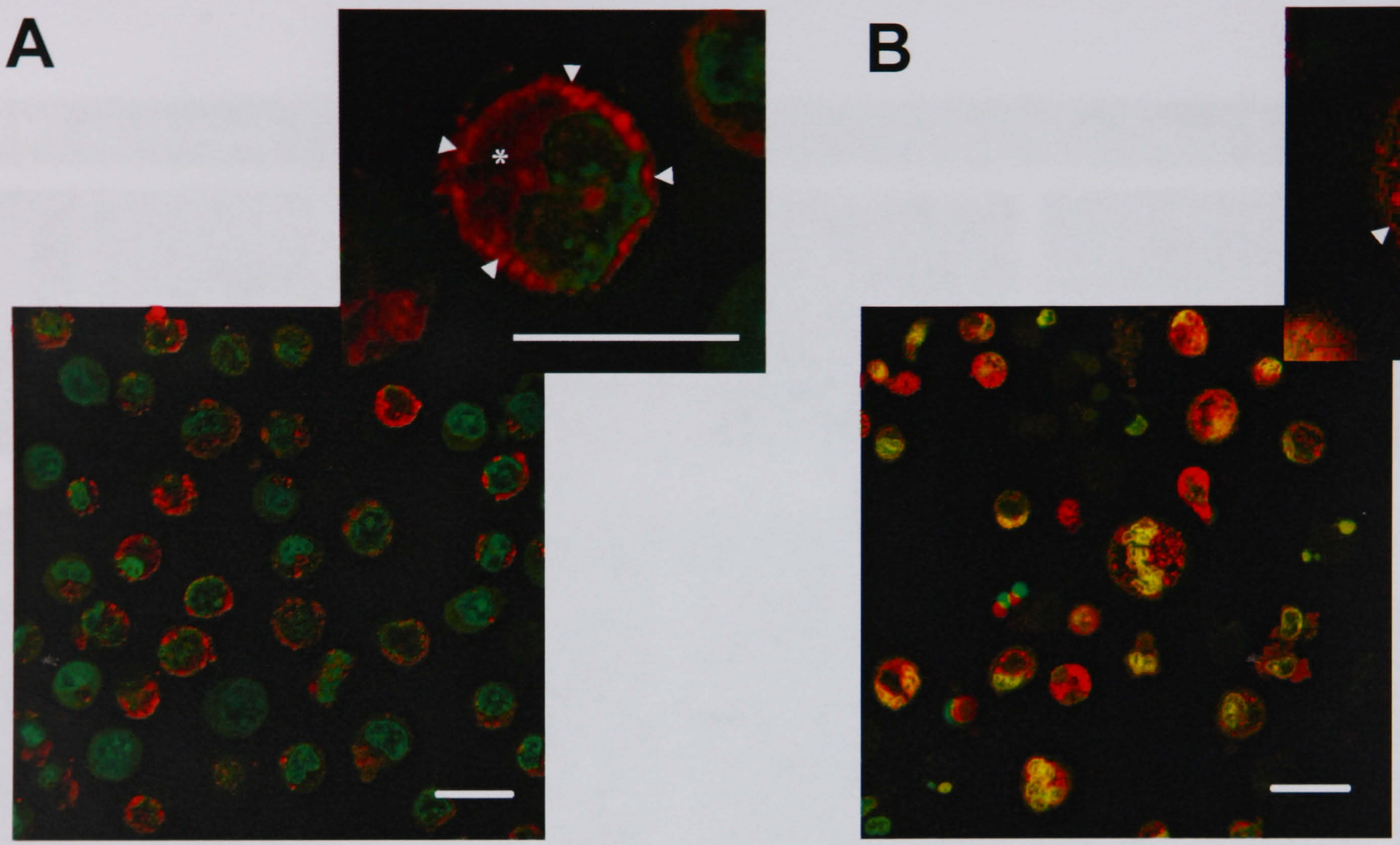


Figure 4.1: Identification of acidic vesicles in MEG-01 cells and primary human megakaryocytes using acridine orange. (A) Acidic vesicles were identified in the MEG-01 cell line, with red fluorescence observed around the periphery (arrow heads) and cytoplasm (asterisks) of the cells, and green fluorescence observed with in the cytoplasm and nuclear region of the cell. **(B)** Acidic vesicles were also observed in primary human megakaryocytes, where green fluorescence and red fluorescence co-localised in a cytoplasmic and nuclear region within the cell, and red fluorescence observed in the cytoplasm (asterisks) and periphery (arrow heads) of the cells. Bar region indicates 20μm.

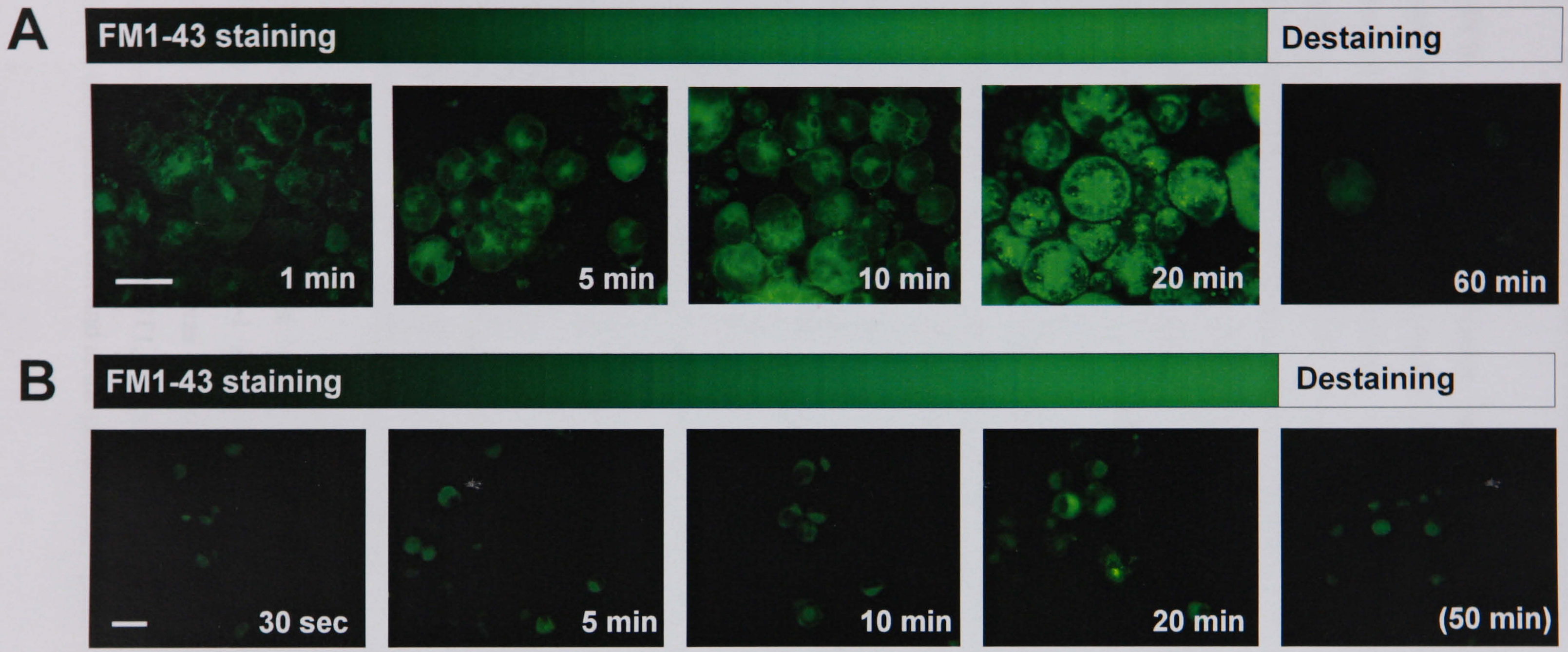


Figure 4.2: (A) and (B) Identification vesicular recycling in primary murine megakaryocytes and MEG-01 cell line respectively using FM1-43 dye . The incorporation of the FM1-43 dye into the vesicle membrane, shown by green fluorescence, increased over the time course and was reduced in destaining experiments. Bar regions indicate 20μm.

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4.3.3 Identification of vesicular glutamate transporters (VGLUTs) and glutamate transporters (EAATs) in megakaryocytic cells

RT-PCR was used to determine mRNA transcription of vesicular glutamate transporters (VGLUTs), responsible for packaging glutamate into vesicles, and glutamate transporters responsible for recycling glutamate (EAATs) in the megakaryocytic cell line MEG-01 and primary human megakaryocytes derived from $CD34^+$ umbilical cord blood cells. VGLUT1 was shown to be expressed at the mRNA level by MEG-01 cells, treated in the presence or absence of 100nM PMA for 72 hours, and primary human megakaryocytes (Figure 4.3), however no transcription at the mRNA level was observed for VGLUT2 in these cells.

Immunocytochemistry of MEG-01 cell cytospin preparations was used to determine the localisation of the VGLUT proteins in MEG-01 cells cultured in the presence or absence of 100nM PMA for 72 hours (Figure 4.4A). Primary antibodies recognising VGLUT1 and VGLUT2 were used in conjunction with a FITC conjugated secondary antibody to localise the expression of these proteins in MEG-01 cells (green positive staining). The location of the nucleus was identified by counterstaining with the nuclei dye DAPI (blue). Control samples, in which non-specific mouse IgGs were used in place of the primary antibody exhibits the level of background staining.

The glutamate transporter protein EAAT-2 (GLT-1) was shown to be expressed in MEG-01 cells and HEL cells cultured in the presence or absence of 100nM PMA for 72 hours by western blot analysis, the anti-EAAT-2 antibody recognised a protein with the molecular weight of approximately 70 kDa (Figure 4.4B), consistent with the known molecular weight of neuronal EAAT-2 (Pines et al., 1992b).

Immunohistochemical staining of rat bone marrow sections was used to identify VGLUT1, VGLUT2, EAAT-1 and EAAT-2 proteins in megakaryocytes in vivo. VGLUT2 immunoreactivity was observed at the periphery of the cell, at the plasma membrane, where as VGLUT1, EAAT-1 (GLAST) and EAAT-2 (GLT-1) were observed throughout the cell and at the periphery (Figure 4.5). Control samples, in

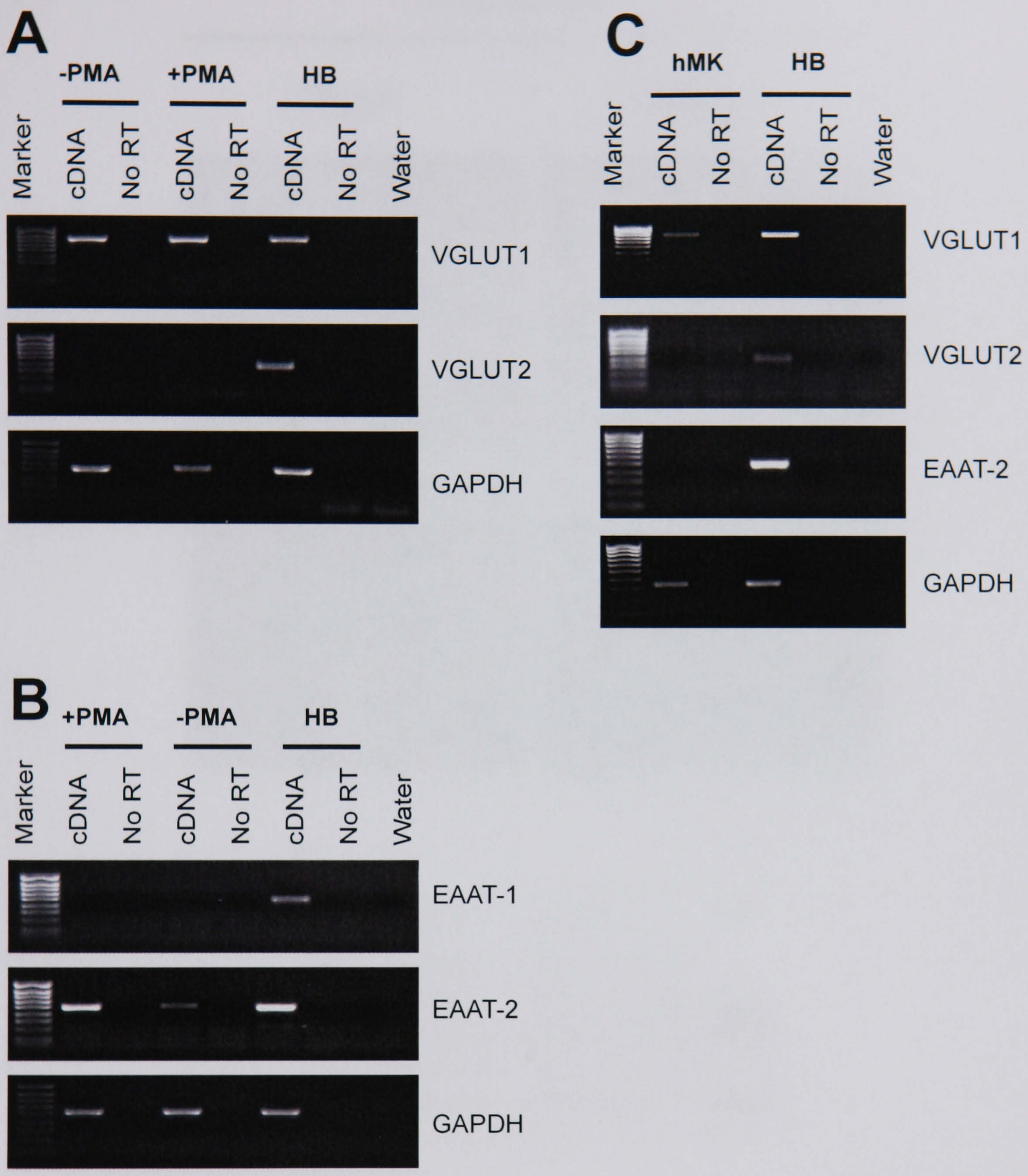

Figure 4.3: mRNA expression of vesicular glutamate transporter (VGLUT) and glutamate transporter (EAAT) protein genes by megakaryocytic cell line, MEG-01, in the presence and absence of PMA (A,B) and primary human megakaryocytes (hMKs) derived from CD34+ cord blood (C). RT-PCR was used to demonstrate mRNA expression of VGLUTs and EAATs in megakaryocytic cells. Human foetal forebrain (HB) was used as a positive control and control reactions (no RT) were performed in parallel.

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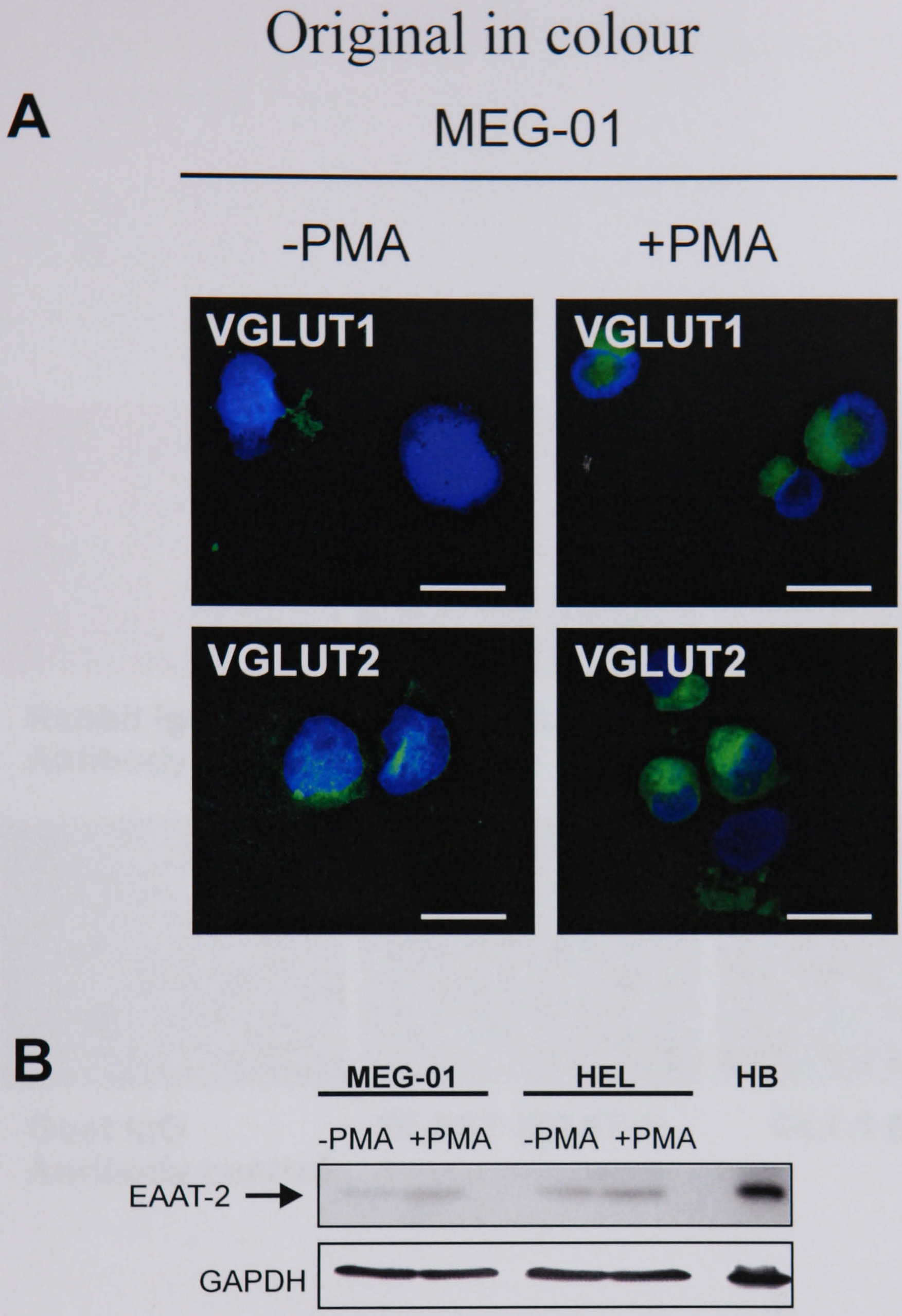

Figure 4.4: Protein expression of vesicular glutamate transporter (VGLUT) and glutamate transporter (EAAT) proteins by megakaryocytic cell lines, MEG-01 and another megakaryocytic cell line, HEL, in the presence and absence of PMA. (A) Protein expression of VGLUT1 and VGLUT2 were determined by immunocytochemistry in the megakaryoblastic cell line MEG-01, in the presence and abscence of PMA (green fluorescence (FITC) indicates the protein of interest, blue fluorescence is a DAPI nuclear stain). Bar indicates 30μm **(B)** Western blot analysis was used to determine the expression of EAAT-2 in MEG-01 and HEL cells, in the presence and absence of PMA. Human brain lysate was used as a positive control and GAPDH loading controls are shown.

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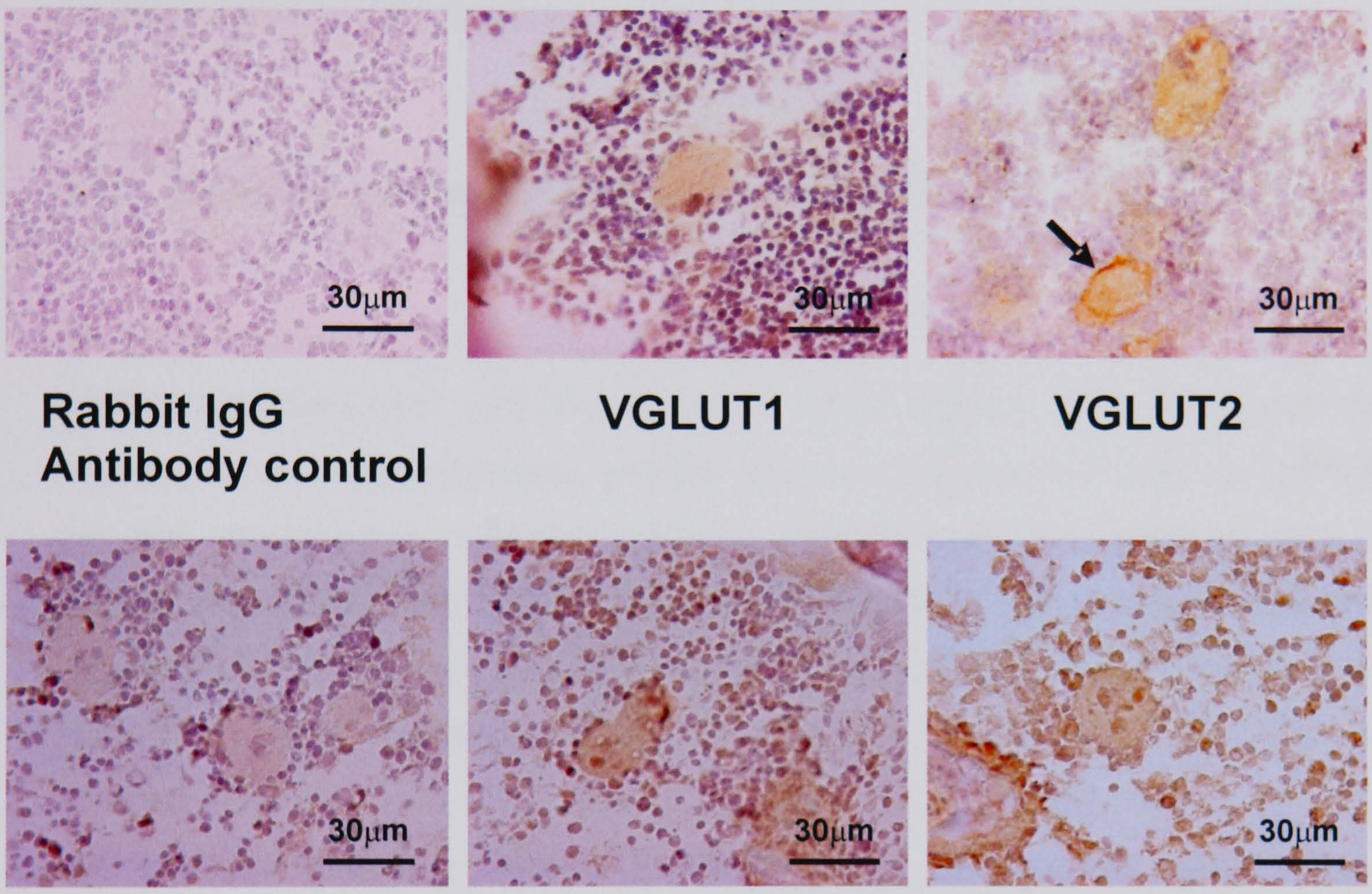

Figure 4.5: Rat megakaryocyte expression of vesicular glutamate transporter (VGLUT) and glutamate transporter (EAAT) proteins in vivo. Protein expression of VGLUT1, VGLUT2, GLAST (EAAT-1) and GLT-1 (EAAT-2) were determined by immunohistochemistry in rat bone marrow sections. Rabbit and goat IgG controls show back ground levels of staining.

which non-specific goat and rabbit IgGs were used in place of the primary antibody exhibit the level of background staining.

The identification of the vesicular glutamate transporters (VGLUTs) and the glutamate transporter proteins EAAT-1 and EAAT-2 in megakaryocytic cells indicates that megakaryocytes may participate in a release and recycle mechanisms with similarities to those observed in the CNS, this data is supports our observations that glutamate recycling occurs in megakaryocytic cells.

4.3.4 Glutamate release by megakaryocytic cells

An enzyme-linked fluorometric glutamate release assay was used to determine whether megakaryocytic cells had glutamate release activity. We demonstrated that primary murine megakaryocytes, primary human megakaryocytes and the MEG-01 cell line spontaneously released glutamate. MEG-01 cells, differentiated with the phorbol ester PMA, were shown to release significantly more glutamate than cells without PMA ($P<0.001$) (Figure 4.6). Primary murine megakaryocytes released 23.4% significantly more glutamate than MEG-01 cells differentiated with 100nM PMA for 72 hours ($P<0.001$), and 13.5% more compared to human megakaryocytes ($P<0.05$). The levels of glutamate released by the MEG-01 cells and the primary human megakaryocytes were similar and differences in release were not significant ($P>0.05$) (Figure 4.7). To determine the effects of calcium and potassium on glutamate release from megakaryocytic cells, MEG-01 cells cultured in the presence and absence of 100nM PMA were treated with or without 2mM $CaCl_2$ and/ or 60mM KCl (Figure 4.8). There was shown to be no significant difference in the amount of glutamate released by MEG-01 cells, which had not been exposed to PMA, when treated or untreated with 2mM $CaCl_2$ and/ or 60mM KCl. MEG-01 cells treated with 100nM PMA for 72 hours released 29% significantly less glutamate in the presence of 2mM $CaCl_2$ than untreated controls ($P<0.001$). Exposure to a depolarizing concentration of KCl (60mM) in the absence of 2mM $CaCl_2$ lead to a 13% reduction in the amount of glutamate released by PMA treated MEG-01 cells, however there was no significant difference in the glutamate released from cells exposed to 60mM KCl in the presence

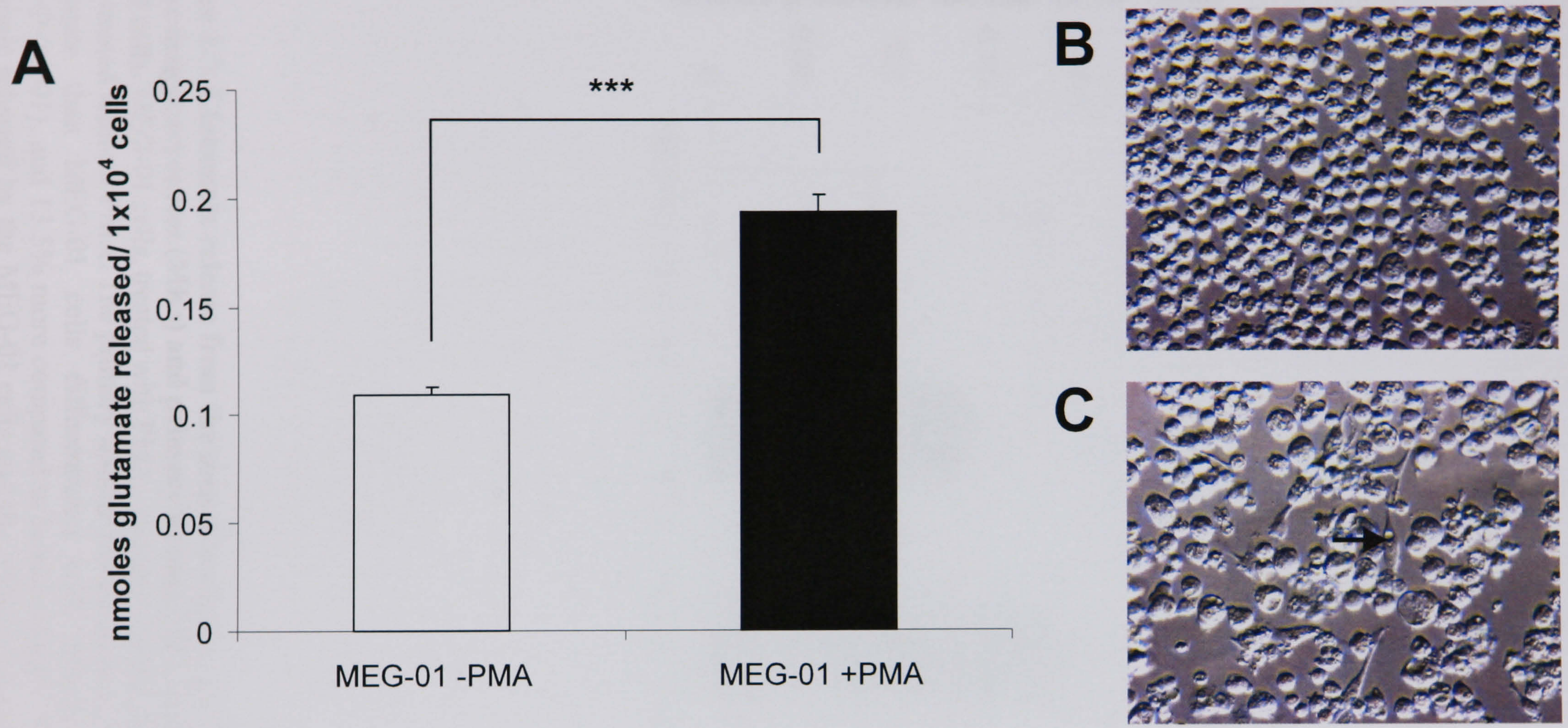

Figure 4.6: Glutamate release from the megakaryocytic cell line, MEG-01. (A) MEG-01 cells, treated with PMA, were shown to release significantly more glutamate than cells treated without PMA (***=P<0.001 using a one-way ANOVA, n=3, error bars indicate standard deviation). **(B)** Light microscopy image of MEG-01 cells treated without PMA (×20 magnification). **(C)** Light microscopy image of MEG-01 cells treated with 100nM PMA for 72 hours, cells are enlarged indicating advanced maturity and adhere to the tissue culture plastic (arrow) (×20 magnification).

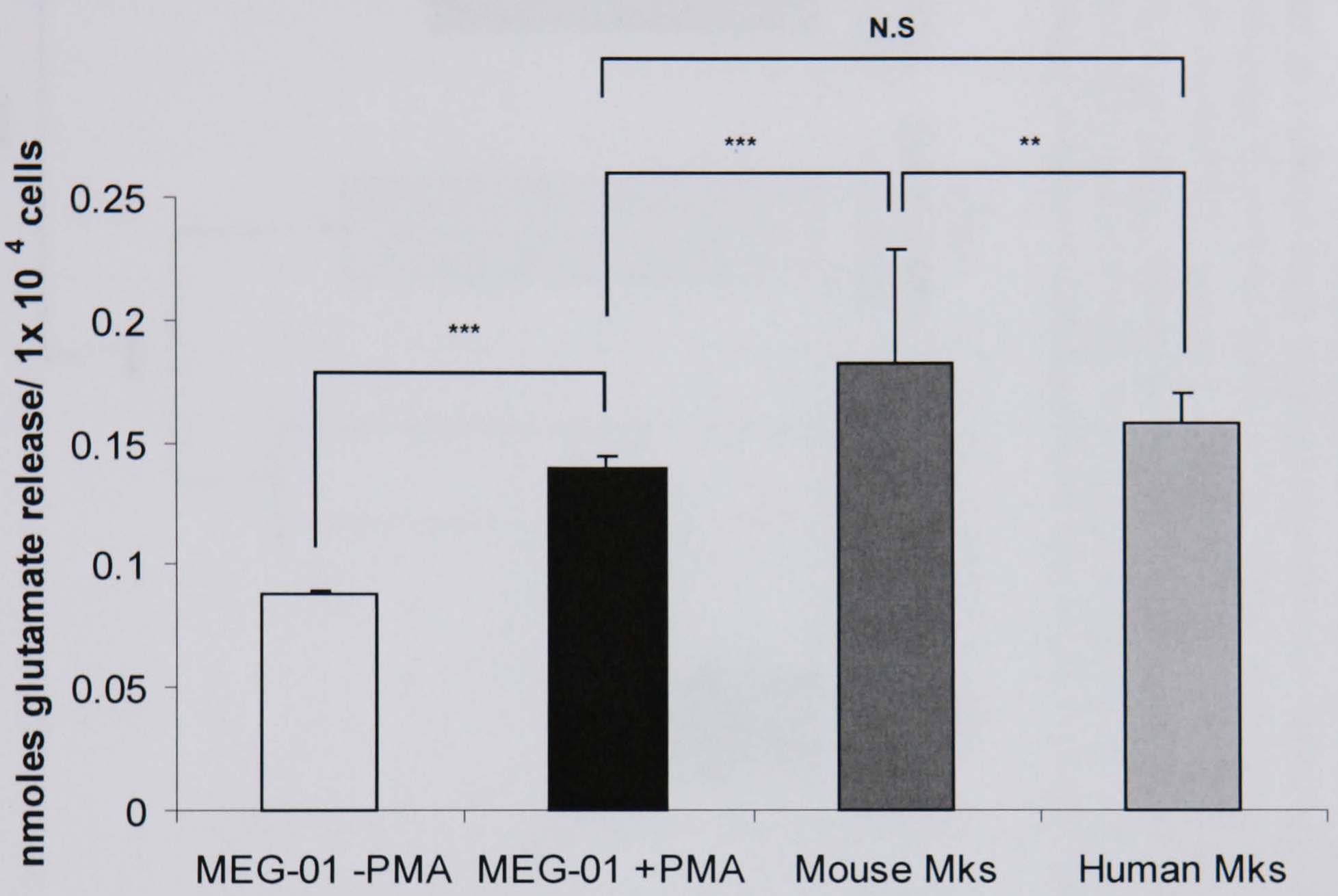

Figure 4.7: Glutamate release from the megakaryocytic cell line, MEG-01, primary mouse megakaryocytes (MKs) and primary human MKs derived from CD34+ cord blood cells. MEG-01 cells, treated with PMA, released significantly more glutamate than cells treated without PMA. The primary murine MKs released 23.4% significantly more glutamate than MEG-01 cells differentiated with 100nM PMA for 72 hours (***=$P<0.001$), and 13.5% more compared to human MKs (**=$P<0.05$). The levels of glutamate released by the MEG-01 cells and the primary human Mks were similar and differences in release were non significant (N.S at $P>0.05$). The statistical test used was a one-way ANOVA, n=3, error bars indicate standard deviation.

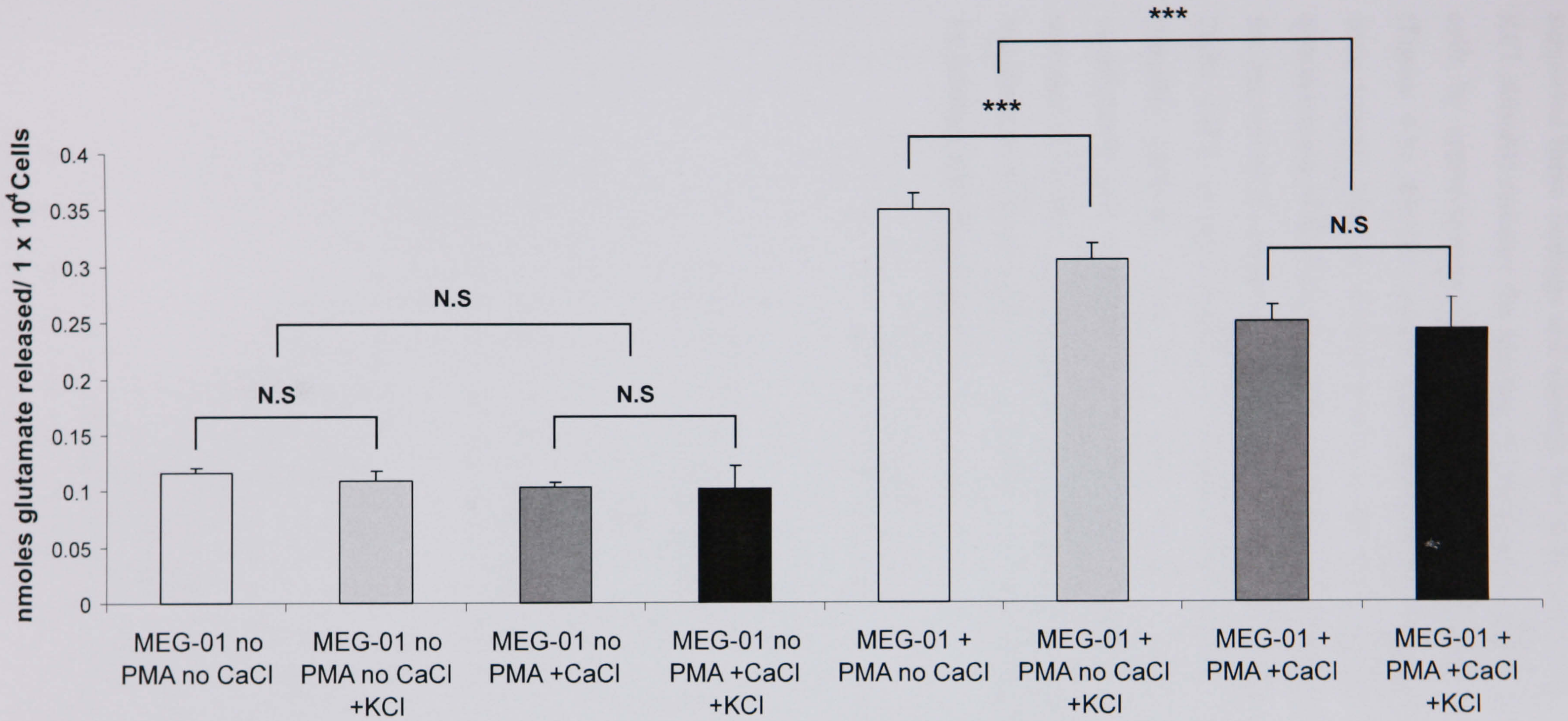

Figure 4.8: Glutamate release from the megakaryocytic cell line, MEG-01 treated with and without 100nM PMA for 72 hours in the presence of 2mM $CaCl_2$ and 60mM KCl. There was shown to be no significant difference in the amount of glutamate released by MEG-01 cells, which had not been treated with PMA, when treated or untreated with 2mM $CaCl_2$ and/ or 60mM KCl. MEG-01 cells treated with 100nM PMA for 72 hours released 29% significantly less glutamate in the presence of 2mM $CaCl_2$ (***=P<0.001). Exposure to a depolarising concentration of KCl (60mM) in the absence of 2mM $CaCl_2$ lead to a 13% reduction in the amount of glutamate released by PMA treated MEG-01 cells , however there was no significant difference in the glutamate released from cells exposed to 60mM KCl in the presence of 2mM $CaCl_2$ (***=P<0.001, N.S at P>0.05). The statistical test used was a one-way ANOVA, n=3, error bars indicate standard deviation).

of 2mM $CaCl_2$ ($P<0.001$ and $P<0.05$ respectively). Real-time glutamate release assays, supported these findings and showed that exposure to depolarising concentrations of KCl (60mM) reduced the amount of glutamate released from PMA treated MEG-01 cells by approximately 20% compared to controls in the absence of 2mM $CaCl_2$ (Figure 4.9). Similar results were observed when primary human megakaryocytes were treated with or without $CaCl_2$ in the presence or absence of a depolarising concentration of KCl (Figure 4.10). There was shown to be no significant difference in the amount of glutamate released by primary human megakaryocytes when exposed to 2mM $CaCl_2$ in the presence or absence of a depolarising concentration of KCl (60mM) ($P<0.05$). However, primary human megakaryocytes released 23% significantly less glutamate in the presence of 2mM $CaCl_2$ compared to untreated samples ($P<0.001$), and exposure to a depolarising concentration of KCl (60mM) in the absence of 2mM $CaCl_2$ lead to a 9% reduction in the amount of glutamate released by primary human megakaryocytes ($P<0.05$).

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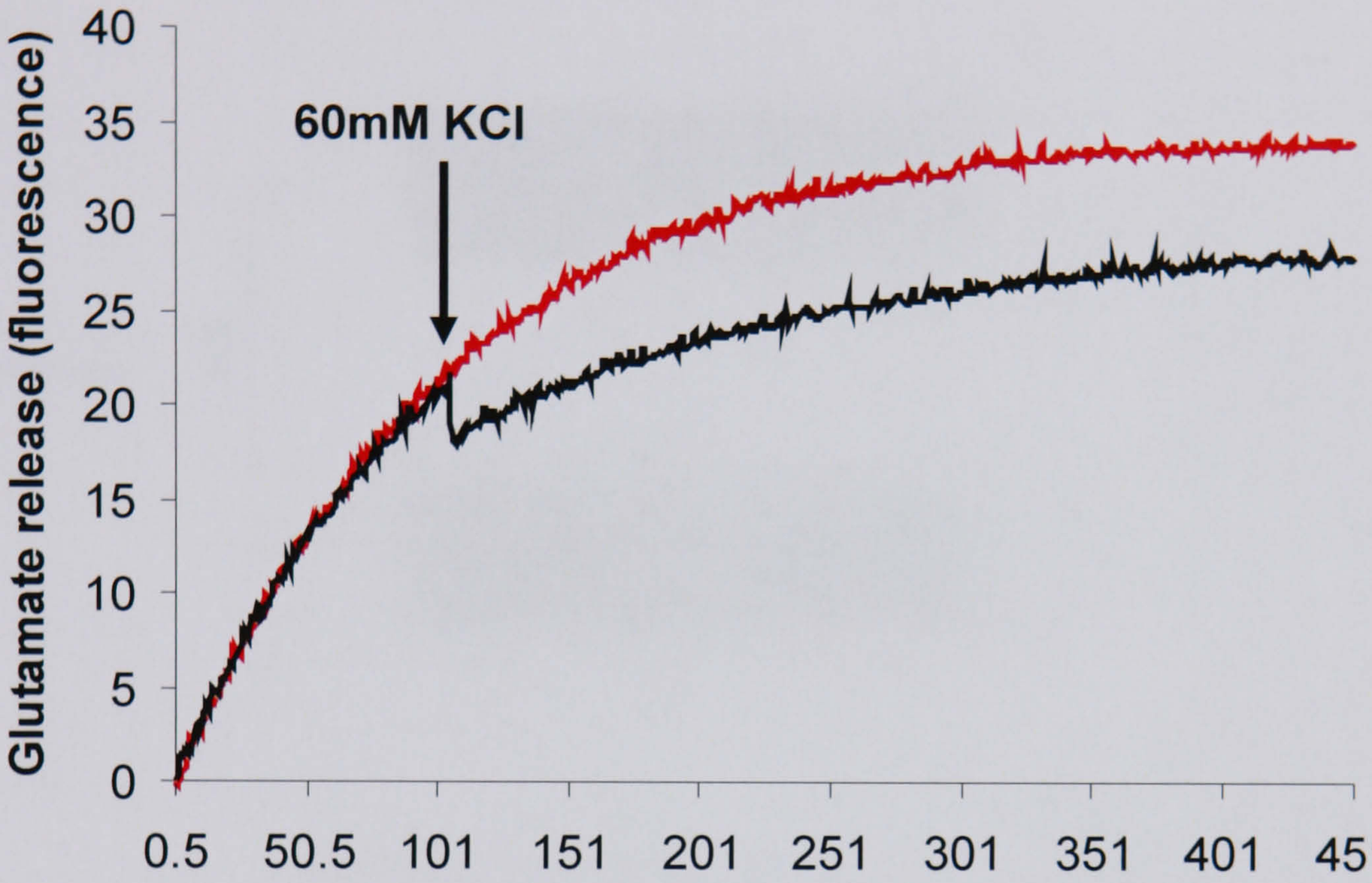


Figure 4.9: Real-time glutamate release from the megakaryocytic cell line, MEG-01 in the presence of a depolarising concentration of KCl. Real-time glutamate release assays indicated a 20% reduction in glutamate release after exposure to potassium chloride (60mM) compared to controls (n=3).

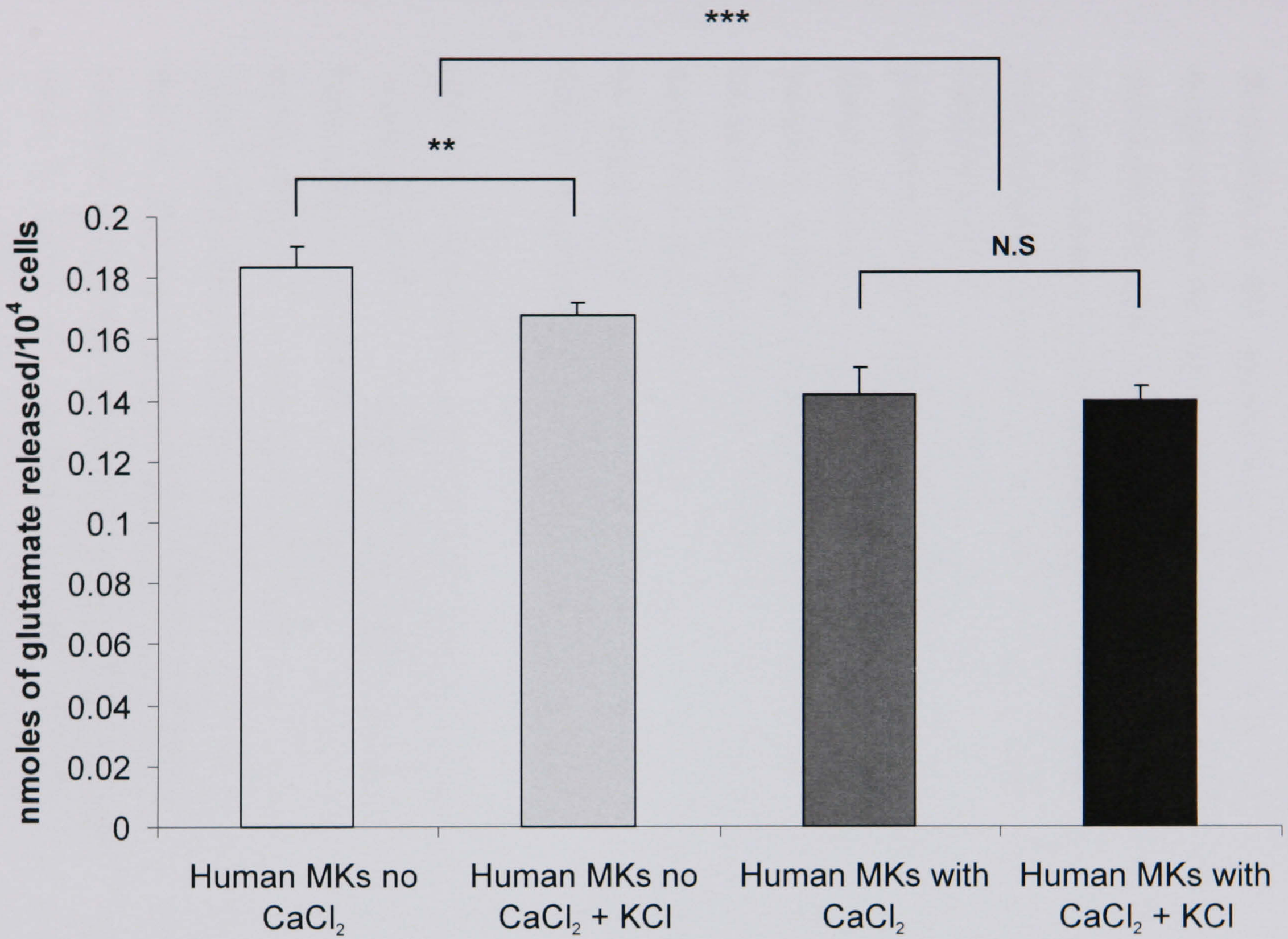


Figure 4.10: Glutamate release from primary human megakaryocytes (MKs) derived from CD34+ umbilical cord blood cells exposed to 2mM $CaCl_2$ and 60mM KCl. There was shown to be no significant difference in the amount of glutamate released by primary human MKs when exposed to 2mM $CaCl_2$ in the presence or absence of a depolarising concentration of KCl (60mM) (N.S at $P>0.05$). Primary human MKs released 23% significantly less glutamate in the presence of 2mM $CaCl_2$ compared to untreated samples (***=$P<0.001$). Exposure to a depolarising concentration of KCl (60mM) in the absence of 2mM $CaCl_2$ lead to a 9% reduction in the amount of glutamate released by primary human Mks (**=$P<0.05$). The statistical test used was a one-way ANOVA, n=3, error bars indicate standard deviation).

4.4 Discussion

The pH-sensitive dye acridine orange has previously been used by Zoccarato et al. (1999) to monitor exocytosis and endocytosis of acidic neurotransmitter-containing vesicles in synaptosomes (Zoccarato et al., 1999c), as synaptic vesicles appear to become acidified in response to neurotransmitter loading. After exocytosis, it has been demonstrated that accumulated acridine orange (red emission) is released from synaptosomes, leading to a prompt decrease in acridine orange fluorescence (green emission) (Zoccarato et al., 1999b). Then in response to neurotransmitter loading there is a large uptake of acridine orange as the recycling synaptic vesicles become acidified (red emission) (Zoccarato et al., 1999a). In both MEG-01 cells and primary human megakaryocytes we identified abundant cytoplasmic and peripheral vesicle populations, both green and red emissions of acridine orange were observed simultaneously in acidic vesicles, with red emissions predominantly at the cell periphery, indicating that both monomeric (green emission) and accumulated acridine orange (red emission) coexist with in megakaryocytic cells. These results indicate that acidic neurotransmitter-containing vesicles are present in megakaryocytic cells, and the presence of both red and green acridine orange fluorescence strongly suggest that vesicle recycling is occurring in megakaryocytic cells.

FM1-43, a styrylpyridinium dye, was first employed by Betz et al. (1997) (Betz and Angleson, 1997b) to observe vesicle recycling in living nerve terminals, and has also been used to investigate synaptosomal recycling in a range of species including mouse, frog and rat (Betz and Angleson, 1997a; Betz et al., 1997d; Kavalali et al., 1999; Fergestad and Broadie, 2001; Rheuben et al., 2004). The incorporation of the FM1-43 dye into megakaryocytic cells over the 20 minute time-course indicates that vesicular endocytosis takes place within megakaryocytes. In destaining experiments when the dye is removed from the culture medium, the reduction of fluorescence provides evidence for vesicular exocytosis. These data strongly indicate vesicular recycling in megakaryocytic cells.

Vesicular glutamate transporters (VGLUTs) are required for the storage of glutamate into synaptic vesicles in glutamate releasing neurons. In MEG-01 cells and human

megakaryocytes the expression of VGLUT1 was observed at the mRNA level by RT-PCR, whereas VGLUT2 expression appeared to be absent. However, both VGLUT1 and VGLUT 2 were shown to be expressed at the protein level by immunocytochemistry in MEG-01 cells. VGLUT1 and VGLUT2 were also observed in megakaryocytes in rat bone marrow sections. These results suggest that VGLUT1 and VGLUT2 may play a similar role in megakaryocytes to that seen in the CNS, facilitating glutamate packaging into vesicles prior to SNARE-dependent exocytosis (Morimoto et al., 2003b). Expression of the VGLUT1 and VGLUT2 proteins was more prominent in differentiated MEG-01 cells, undifferentiated cells (cells not treated with PMA) showed weaker expression of the VGLUT proteins. This suggests a differentiation dependent VGLUT expression in these cells. It was also noticeable that the expression of the VGLUT proteins in both the primary and MEG-01 cells was prominent at the plasma membrane where the red acridine orange staining was observed, consistent with vesicular release activity identified in specialised zones in neuronal cells (Bellocchio et al., 1998b; Hisano et al., 2002a). The identification of 'brain-specific' vesicular glutamate transporters (VGLUTs) in megakaryocytes highlights the glutamatergic phenotype of megakaryocytic cells, as it has been shown in the CNS that no other component is required by neuronal cells to store and release of glutamate by exocytosis (Takamori et al., 2001b; Herzog et al., 2001b; Fremeau, Jr. et al., 2001b; Varoqui et al., 2002e).

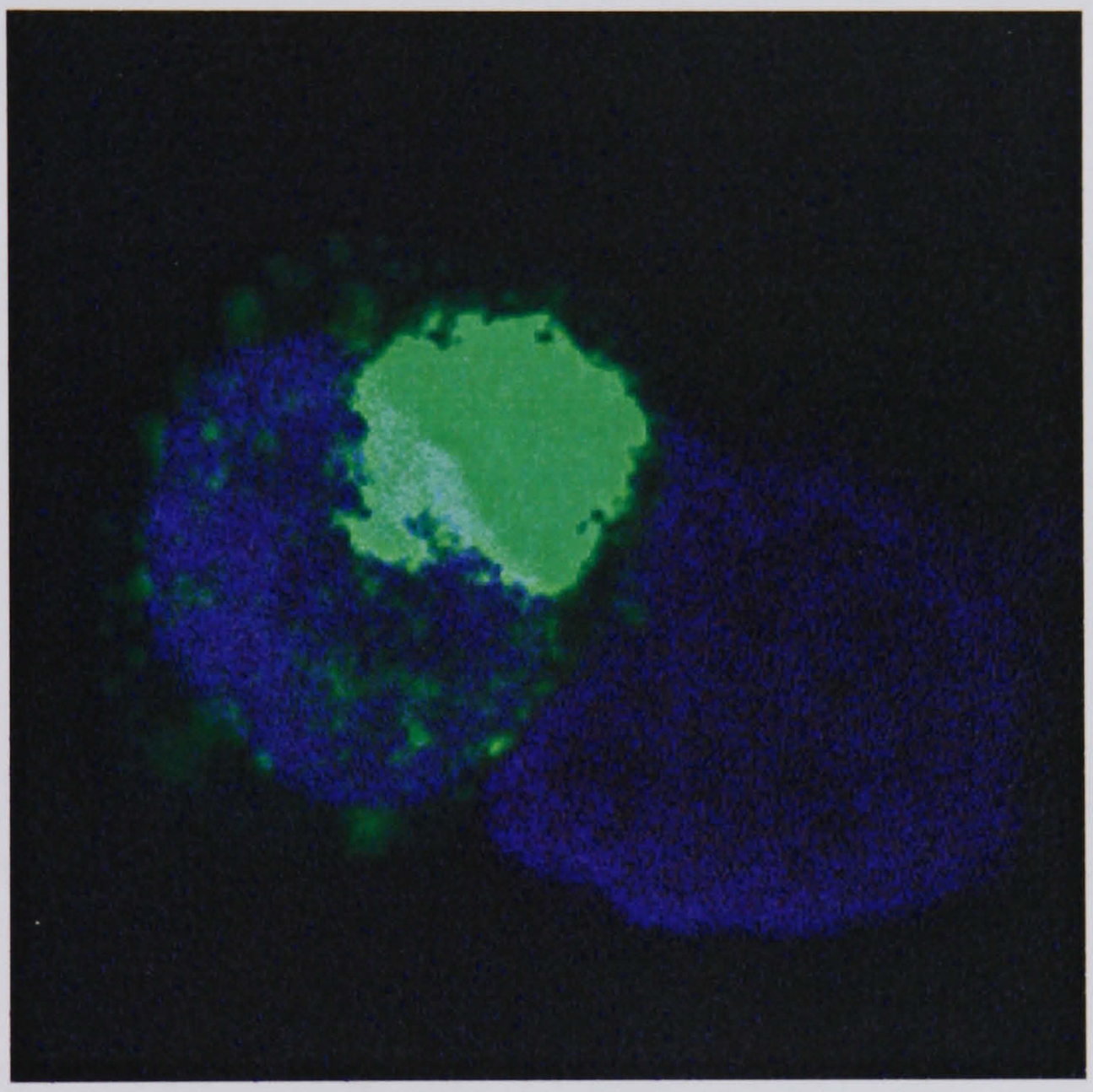
The identification of the glutamate/aspartate transporter, EAAT-1 (GLAST) and EAAT-2 (GLT-1) (Mason et al. 1997) within the bone marrow suggest a role for glutamate signalling in paracrine intracellular communications within the bone marrow microenvironment. The identification of EAAT-1 and EAAT-2 by RT-RCR, western blot analysis and immunohistochemistry in megakaryocytic cells strongly suggests that these cells may also play an important role in glutamate recycling with in the bone marrow microenvironment, and further supports the evidence for glutamate signalling from megakaryocytes similar to that observed in neuronal cells.

Studies in cortical neurons and chromaffin cells (neurosecretory cells) have shown that these cells release glutamate when stimulated by KCl, a depolarising agent (Romero et al., 2003). To determine glutamate release activity in megakaryocytes a real-time glutamate release assay was carried out. The results demonstrate that MEG-01 cells

released glutamate in the presence of Ca^{2+}, but that unlike neuronal cells, there was a decrease in glutamate release when the cells were depolarised with 60mM KCl. These results suggest that in comparison to neuronal cells, megakaryocytes spontaneously released glutamate and that glutamate release is negatively regulated by depolarisation with 60mM KCl. The results from glutamate release assays indicate that MEG-01 cells differentiated with PMA release significantly more glutamate than untreated cells, suggesting that more mature megakaryocytes have the ability of releasing more glutamate. These findings are supported by the higher expression patterns of exocytotic proteins in PMA treated MEG-01 cells. The results also indicated that release of glutamate from megakaryocytic cells can occur in a calcium-independent manner, unlike neuronal cells where calcium influx in response to pre-synaptic cell depolarisation leads to the release of the neurotransmitter. Spontaneous release of glutamate was also identified in primary murine megakaryocytes and human megakaryocytes. Similar observations have been seen in osteoblastic cells which were also shown to spontaneously release glutamate at concentrations similar to those observed from neuronal cells (Genever and Skerry, 2001b). During differentiation, the concentration of glutamate released by osteoblastic cells was shown to be significantly increased, and unlike neuronal cells, osteoblastic K^+-mediated depolarisation was also shown to inhibit glutamate release, as seen in the megakaryocytic cells suggesting that voltage-mediated megakaryocytic and osteoblastic calcium entry reduced the level of glutamate exocytosis. These findings suggest that glutamate signalling within the bone marrow may be similar yet distinct from that observed in neuronal cells.

This chapter provides the evidence for functional glutamate recycling in megakaryocytic cells. Expression of glutamate transporters EAATs and VGLUTs strongly suggest that glutamate signalling pathways similar to those identified in the CNS occur in megakaryocytes. The release of glutamate by these cells also supports evidence for vesicular glutamate release mechanisms in megakaryocytic cells, and the findings that other bone marrow cells release glutamate indicates that glutamate may have an important role within the bone marrow microenvironment for megakaryocytopoiesis and bone remodelling via autocrine/paracrine interactions.

Chapter 5



Manipulation of glutamate release from megakaryocytic cells

Expression of the pEGFP-VGLUT1 by MEG-01 cells 48 hours after transformation. Green fluorescence indicates EGFP fluorescence of VGLUT1 and blue fluorescence indicates the nuclei of the cell.

Chapter 5: Manipulation of glutamate release from megakaryocytic cells

5.1 Introduction

The characterisation of the megakaryocytic SNARE complex (described in chapter 3) and the identification of vesicle recycling and glutamate release (described in chapter 4) confirms the existence of components required for SNARE-dependent glutamate release in megakaryocytes. These findings provoke intriguing questions regarding the function of glutamate release in megakaryocytes. Previous studies, as discussed in section 1.5.2, have shown that antagonism of megakaryocytic NMDA-type glutamate receptors leads to impaired megakaryocyte maturation and platelet production indicating an important role for glutamate signalling in megakaryocytopoiesis. In this chapter we used the glutamate release inhibitor, riluzole (discussed below), to determine the functional role of glutamate release in megakaryocyte proliferation and differentiation. To further investigate the molecular mechanisms involved in megakaryocytic glutamate release and establish that megakaryocytic glutamate release occurs via a SNARE-dependent mechanism we transiently overexpressed the vesicular glutamate transport protein (VGLUT1) (discussed previously in section 1.3.4 and 4.1) and clostridrial tetanus and botulinum neurotoxins (discussed below) to determine their effects on glutamate release from these cells.

Glutamate, as discussed in section 1.3 is the major excitatory neurotransmitter in the CNS, existing at very high concentrations in the brain. Normal glutamate release is physiologically important for maintaining neuronal functions including learning and memory formation or plasticity of the brain (Malgaroli and Tsien, 1992; Bliss and Collingridge, 1993b). A number of neuropathological conditions including strokes and chronic neurodegenerative disorders such as Huntington's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS) have been linked to abnormal glutamate release in the CNS therefore maintaining low levels of presynaptically released glutamate is an important neuroprotective mechanism (Choi, 1988b; Meldrum and Garthwaite, 1990; Choi and Rothman, 1990; Lipton and Rosenberg, 1994).

Riluzole (2 - amino - 6 - [trifluoromethoxy] - benzothiazole) is a neuroprotective agent with anticonvulsant, sedative and anti-ischaemic properties (Malgouris et al., 1989b; Bensimon et al., 1994b; Doble, 1996; Wokke, 1996). Clinical trials have shown that riluzole prolongs the life of patients with ALS, a rapidly progressive and fatal disease characterised by the degeneration of both upper and lower motoneurons (Bensimon et al., 1994a; Lacomblez et al., 1996; Louvel et al., 1997; Dengler, 1999). As a result of these studies riluzole has been approved and is marketed for the treatment for patients with ALS. In addition, it has also proved effective in animal models of Parkinson's disease, Chorea Huntington and cerebral and retinal ischaemia (Malgouris et al., 1989a; Pratt et al., 1992; Mary et al., 1995; Barneoud et al., 1996; Lagreze et al., 1999).

The precise mechanisms by which riluzole produces its neuroprotective effects in ALS are unknown, however it is hypothesised that riluzole acts to limit glutamate excitotoxicity by inhibiting the release of glutamate from presynaptic terminals (Malgouris et al., 1989c; Martin et al., 1993a; Shaw and Ince, 1997; Centonze et al., 1998). Several studies have shown that riluzole inhibits the release of glutamate from cultured cerebellar granule and hippocampal cells (Doble et al., 1992; Prakriya and Mennerick, 2000), and from slices of hippocampal area CA1 (Martin et al., 1993b), and from cat caudate nucleus *in vivo* (Cheramy et al., 1992). Riluzole has also been shown to reduce currents through a wide variety of ion channels, including voltage dependent sodium channels (Stefani et al., 1997b; Zona et al., 1998a; Urbani and Belluzzi, 2000), high-voltage-activated calcium channels (Siniscalchi et al., 1997; Huang et al., 1997; Stefani et al., 1997a), potassium channels (Zona et al., 1998b; Duprat et al., 2000; Xu et al., 2001), and NMDA receptor-gated channels (Hubert et al., 1994). In this chapter we used riluzole to inhibit glutamate release from megakaryocytic cells to determine the functional effects of glutamate release on megakaryocyte proliferation and differentiation.

As discussed previously in sections 1.3.1 and 1.3, regulated synaptic vesicle exocytosis at mature synapses involves the interaction of v-SNAREs VAMP-1 and -2 (Trimble et al., 1988b; Baumert et al., 1989a) and t-SNAREs SNAP-25 (Oyler et al., 1989b) and syntaxin (Bennett et al., 1992b), forming the SNARE-complex. Direct evidence for the involvement of the SNARE-complex proteins syntaxin and VAMP in

neurotransmission came from genetic studies carried out in *Drosophila* and C.*elegans* (Broadie et al., 1995; Schulze et al., 1995; Deitcher et al., 1998; Ogawa et al., 1998; Saifee et al., 1998), which identified SNARE-complex proteins as targets for the proteolytic action of clostridial tetanus and botulinum neurotoxins, blocking neurotransmitter release in the nerve terminal (Schiavo et al., 1992b; Montecucco and Schiavo, 1994a; Niemann et al., 1994d).

The clostridial neurotoxins are a family of structurally and functionally related toxins. To date seven distinct botulinum serotypes have been identified BoNT/A, B, /C, /D, /E, /F and /G. The various BoNTs are produced by distinct strains of *Clostridium botulinum*, where as the tetanus neurotoxin (TeNT) is produced by a uniform group of *C.tetani* (for reviews see Schantz and Johnson, 1992; Humeau et al., 2000). These neurotoxins are composed of two disulphide-linked polypeptide chains (Helting et al., 1979; Weller et al., 1989; Weller et al., 1991; Montecucco and Schiavo, 1994b; Niemann et al., 1994b). The heavy chain (100kDa) mediates the specific binding of the toxin to the neuronal presynaptic membrane and the internalisation of the light chain (LC; 50kDa), which is responsible for the intracellular proteolytic activity and subsequent blockage of neurotransmitter release (Niemann et al., 1994a).

Only three of the SNARE-complex proteins have been identified as targets of the closridial neurotoxins (for reviews see Niemann et al., 1994c; Montecucco and Schiavo, 1995). In mammals VAMP-1 and VAMP-2 are cleaved by TeNT, BoNT/B, /D, /F and /G, SNAP-25 by BoNT/A, /C and /E and syntaxin only by BoNT/C (Blasi et al., 1993a; Rossetto et al., 1994; Schiavo et al., 1995; Pellizzari et al., 1996; Foran et al., 1996c; Pellizzari et al., 1997; Cornille et al., 1997; Washbourne et al., 1997b; Vaidyanathan et al., 1999b). Previous studies have used targeted expression of TeNT and BoNT neurotoxins to investigate synaptic function and exocytosis in neuronal cells, providing useful tools for investigating SNARE-dependent neurotransmission in vitro and in vivo. Presynaptic expression of TeNT-LC in *Drosophila* was shown to abolish evoked neuromuscular synaptic transmission, indicating that *Drosophila* VAMP (n-syb) is susceptible to cleavage by the TeNT-LC, however spontaneous release still occurred in these TeNT-LC expressing synapses, although at a reduced rate, indicating that VAMP is not essential for spontaneous release (Sweeney et al., 1995). Another study examined the effect on exocytosis in PC12 endocrine cells

transiently transfected with the BoNT/C1 LC. which cleaves syntaxin and SNAP-25 (Blasi et al.. 1993b: Williamson et al.. 1996a: Foran et al.. 1996b: Fisher and Burgoyne, 1999c). The expression of the BoNT C1 LC in PC12 cells resulted in a marked inhibition of both basal and evoked exocytosis providing a useful method for analysing the function of components of the exocytotic machinery (Fisher and Burgoyne. 1999b). These methods are used in this chapter to investigate the role of the SNARE-complex in megakaryocytic glutamate release.

Megakaryocytopoiesis, as discussed previously in section 1.2.3. is regulated by numerous cytokines. (Kaushansky et al.. 1986: Ikebuchi et al.. 1987: Bruno and Hoffman, 1989; Bruno et al., 1989: Avraham et al.. 1992; Burstein et al., 1992; Kaushansky, 1995b), chemokines (Keller et al.. 1994), growth factors (Han et al., 1992) and extra cellular matrix molecules (Mossuz et al., 1997). many of which are produced by the stromal cells within the bone marrow microenvironment (Dorshkind. 1990; Eaves et al., 1991; Deryugina and Muller-Sieburg. 1993). The primary regulator controlling megakaryocytic differentiation and platelet production. as discussed in section 1.2.3, is thrombopoietin (TPO) (Kaushansky. 1995a). However. studies have shown that TPO knockout mice produce normal megakaryocytes and platelets indicating that other cytokines have an important role and are sufficient to support megakaryocytopoiesis in the absence of TPO (Bunting et al.. 1997b).

The establishment of co-culture systems with defined stromal cells and haematopoietic progenitor cells has been vital in broadening our understanding of the role of stromal cells in the regulation of megakaryocytopoiesis. Haematopoietic progenitor cells have been characterised and stromal cells have been shown to be derived from mesenchymal stem cells (MSCs). MSCs are pluripotent and have the ability to self-renew and differentiate into a range of mesenchymal tissues. including bone. adipose. cartilage. muscle. marrow stroma. tendon. and ligament. Isolation and culture of MSC's from human bone marrow aspirates have demonstrated that these cells display a fibroblastic morphology and retain their pluripotent potential following extensive culture expansion (Haynesworth et al.. 1996b: Majumdar et al.. 1998a: Pittenger et al., 1999b).

Studies by Pittenger et al. (Pittenger et al.. 1999a) established the multilineage potential of human MSC's (hMSC's) isolated from adult marrow in vitro. and it has

been further demonstrated that these cells express various haematopoietic cytokines including IL-6, IL-11, LIF, SCF, and Flt3/Flk2 ligand (FL) (Haynesworth et al., 1996a; Majumdar et al., 1998b; Mbalaviele et al., 1999); cell adhesion molecules such as VCAM-1, E-selectin, and activated leukocyte cell adhesion molecule (ALCAM); and ECM proteins such as collagen I and fibronectin (Haynesworth et al., 1992; Mosca et al., 1996; Bruder et al., 1997). These hMSCs also have the ability to maintain long-term culture-initiating cells (LTC-IC) in long-term bone marrow culture with $CD34^+$ haematopoietic progenitor cells and support both myeloid and erythroid differentiation (Majumdar et al., 1998c). In the absence of exogenous cytokines, hMSCs have been shown to support megakaryocyte and pro-platelet formation (Cheng et al., 2000a), suggesting that the cytokines and other molecules produced by hMSCs are sufficient for sustaining megakaryocytopoiesis and platelet formation, or alternatively MSCs may produce small quantities of TPO, which work with the MSC-produced cytokines to enable thrombopoiesis to occur (Cheng et al., 2000b). However the latter hypothesis is based on the idea that TPO is essential for this process to take place, a notion that is inconsistent with the observations that TPO knockout mice produce normal megakaryocytes and platelets (Bunting et al., 1997a; Cheng et al., 2000c).

Studies from our laboratory (personal communications, Dr Gary Spencer) provide evidence for glutamate signalling in MSCs. MSCs have been shown to release glutamate into their extracellular environment and express the necessary molecular machinery required for vesicular glutamate exocytosis, including v-SNARE VAMP, t-SNARE SNAP-23, key accessory proteins Munc-18, Rab3A, Rab 3B and NSF, and vesicular glutamate transporters VGLUT1 and VGLUT2. They have also been shown to express functional glutamate receptors, AMPA and NMDA-type glutamate receptors (NMDAR1, 2B, 2C and 2D), and antagonism of these receptors inhibits osteogenic differentiation of MSCs. These findings suggest that glutamate signalling has an important role in MSCs and that the release of glutamate by these cells with in the bone marrow provides a source of agonist (glutamate) for receptor activation and paracrine communications within the bone marrow. In this chapter we use MSC-megakaryocytic cell co-cultures to investigate the role of glutamate signalling with in the bone marrow microenvironment, and to determine the effects of glutamate on osteoblast differentiation in vitro.

5.2 Methods

5.2.1 MTT viability assay

To determine the effects of a glutamate release inhibitor, riluzole (Tocris), on megakaryocyte viability, MEG-01 cells (2 x 10^5 cells/ml) were cultured in 96 well plates with and without PMA for 72 hours in the presence of varying concentrations (0 to 100μM) of the glutamate release inhibitor riluzole (dissolved in DMSO (Sigma)). For non-riluzole treated samples, an equivalent volume of DMSO was added. The methylthiotetrazole (MTT) assay was then used to determine cell viability. After 72 hours, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide: Thiazol blue; Sigma) solution was added directly to the wells to give a final concentration of 1 mg/ml and the plates were incubated for a further 3 hours. The cells were lysed with acidic isopropanol (0.1M HCl) and absorbances read at 570 nm.

5.2.2 Annexin V-FITC Apoptosis detection kit (Sigma)

To determine the effects of riluzole, on megakaryocyte apoptosis, MEG-01 cells (1 x 10^6 cells/ml) were cultured in 96 well plates with and without PMA for 72 hours in the presence of varying concentrations (0 to 100μM) of riluzole (dissolved in DMSO (Sigma)). For non-riluzole treated samples an equivalent volume of DMSO was added. After 72 hours incubation, the cell suspension was washed twice with 1×PBS. Cells were then resuspended in 1×binding buffer at a concentration of approximately 1×10^6 cells/ ml. 500μl of the cell suspension was transferred to a 1.5ml microcentrifuge tube, and 500μl of the non-treated cell suspension to a second 1.5ml microcentrifuge tube (control). To each cell suspension 5μl of annexin V-FITC and 10μl of propidium iodide was added. The tubes were incubated at room temperature for exactly 10 minutes in the dark. Fluorescence of the cells was determined immediately with a flow cytometer. Cells in early apoptosis stain with the annexin V-FITC alone, live cells

show no staining by either propidium iodide or annexin V-FITC, and necrotic cells are stained by both propidium iodide and annexin V-FITC.

5.2.3 Transmission electron microscopy

MEG-01 cells were treated with and without 100nM PMA, in the presence of 25μM Riluzole for 72 hours. Cells were washed twice in 1xPBS before being fixed in 4% paraformaldehyde/ 2.5% glutaraldehyde in 100mM phosphate buffer (pH7.0) at room temperature for 90 minutes. Cells were then washed 3 times for 10 minutes in 1xPBS and fixed for a second time in 1% osmium tetroxide for 1 hour on ice and washed twice for 10 minutes in 1xPBS. The cells were dehydrated through graded ethanols, dried over a molecular sieve and washed twice in epoxypropane for 5 minutes. The cells were embedded in 60% epoxypropane/ 40% epon araldite for 30 minutes and were desiccated overnight in silica gel. Fresh epon araldite was added the next day, and was allowed to polymerise for 48 hours at 60°C. Sections were cut and stained with saturated uranyl acetate in 50% ethanol with Reynolds lead citrate and viewed using a transmission electron microscope (TEM; Tecnai 12 BioTwin, FEI). Samples were prepared by the Technology facility, University of York, for use on the TEM.

5.2.4 Plasmid constructs and transfections

5.2.4.1 Plasmid constructs

pcDNA3.1- and pcDNA3.1/V5-HIS B were obtained from Invitrogen, and pEGFP-N2 was obtained from Clontech. The expression construct pTNT, carrying active tetanus toxin light chain (TeNTLC) gene, was a kind gift from Dr S. Sweeney, University of York, UK. This gene was directionally cloned into pcDNA3.1/V5-HIS B using *EcoR*I and *Xho*I restriction sites for use in mammalian cells. VGLUT1 was directionally cloned into pEGFP-N2 using the *Bgl*II and *Sal*I restriction sites. Primers used to generate the VGLUT1 constructs were: 5`-ttagcagatctcaggagccgccaccat-3` and 5`-gattacgtcgacgggaggcacatggtctgtag-3` and the reverse primer contained a one base pair mismatch to mutate the translational stop codon and created an open reading frame in which EGFP protein is fused to the C-terminus of VGLUT1 to allow expression of EGFP within the transfected cells. VGLUT1 was also directionally

cloned into pcDNA3.1- using the *Bgl*II and *Kpn*I restriction sites. In this case the primers used to generate the VGLUT1 constructs were: 5'-ttagcagatctacaggagccgccaccat-3' and 5'-gattacggtaccgggaggcacatggtcagtag-3'. The botulinum toxin construct pBoNT/C1 was a kind gift from R.D. Burgoyne and was constructed as described previously by Fisher and Burgoyne, 1999 (Fisher and Burgoyne, 1999a). In brief, DNA encoding the botulinum toxin light chain was amplified by PCR using the following forward (5') and reverse (3') primers d(ataggtaccatgccaataacaattaacaac) and d(tgcagggcccagtgatggtgatggtgatgcc) respectively. These primers incorporated a *Kpn*I restriction site in the forward primer and an *Apa*I site in the reverse primer to facilitate subcloning in to the pcDNA3 vector (Invitrogen), producing the pBoNT/C1 construct.

5.2.4.2 Transforming Chemically One Shot® TOP10 Competent Cells (Invitrogen)

Vials containing the ligation reactions were centrifuged briefly and placed on ice. One 50µl vial of One Shot® cells for each ligation/transformation was thawed on ice. Each ligation reaction (1 to 5µl) was pipetted directly into the vial of competent cells and mixed by tapping gently. The remaining ligation mixtures were stored at -20°C. The vials were incubated on ice for 30 minutes before heat shocking cells at 37°C in a water bath for exactly 45 seconds. The vials were then immediately placed on ice and 250µl of pre-warmed S.O.C medium (Invitrogen) was added to each vial. The vials were then incubated at 37°C for exactly 1 hour with shaking at 225 rpm in a shaking incubator. From each transformation vial 50µl and 100µl was plated on separate, labeled LB-agar plates containing the appropriate antibiotic 100µg/ ml ampicillin or 50µg/ml kanamycin. The remaining transformation were stored at +4°C. Plates were incubated at 37°C overnight.

5.2.4.3 Growth of bacterial cultures in tubes

Single colonies were picked from freshly streaked selective plates and were used to inoculate 5 ml of LB medium containing the appropriate selective antibiotic. These cultures were then incubated overnight at 37°C with vigorous shaking at 225rpm.

5.2.4.4 QIAprep® Miniprep (Qiagen)

The kit was used according to the manufacturer's instructions. In brief. bacterial cells were harvested by centrifugation at > 8000 rpm (6800 x *g*) in a table-top microcentrifuge for 3 min at room temperature. The bacterial cell pellet was resuspend 250µl of Buffer P1 and transferred to a microcentrifuge tube. 250µl of Buffer P2 was added and mixed thoroughly by inverting the tube 4–6 times. 350µl of Buffer N3 was then added and mixed immediately and thoroughly by inverting the tube 4–6 times. The microcentrifuge tube(s) were centrifuged at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge for 10 minutes. The supernatants were then added to a QIAprep spin column by decanting or pipetting, before centrifugation at top speed for 60 seconds and the flow-through discarded. The QIAprep spin column was then washed by addition of 0.75 ml of Buffer PE and centrifuging for 60 seconds. The flow-through was discarded, and the tube centrifuged for an additional 1 minute to remove residual wash buffer. The QIAprep column was then placed into a clean 1.5 ml microcentrifuge tube and the DNA eluted by the addition of 50µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, letting it stand for 1 minute before centrifugation for 1 minute.

5.2.4.5 Plasmid DNA Transfection using Lipofectamine 2000 reagents (Invitrogen)

MEG-01 cells were transiently transfected in 6-well plates using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The pGL3-luciferase plasmid (Promega) was used in transfection reactions to determine transfection efficiency. Prior to preparing DNA: Lipofectamine complexes, suspension cells were plated at 1.5×10^6 cells in 500µl of growth medium without antibiotics. For each transfection reaction, complexes were prepared as as follows: 4.7µg of sample DNA and 0.3µg of pGL3-luciferase plasmid were diluted in a total volume of 250µl of Opti-MEM® I Medium (Invitrogen) without serum and mixed gently; 20µl of Lipofectamine™ 2000 were then added to 230µl of Opti-MEM® I Medium and incubated for 5 minutes at room temperature. After the 5 minute incubation, diluted

DNA was added to the diluted Lipofectamine™ 2000 (total volume = 500 µl). The complexes were mixed gently and incubated for 20 minutes at room temperature. After 20 minutes incubation 500 µl of complexes were added to each well containing cells and medium and mix gently. Cells were incubated at 37°C in a 5% CO_2 incubator for 48 hours prior to testing for transgene expression.

5.2.5 Glutamate release assay for transiently transfected MEG-01 cells

Glutamate release was determined using the enzyme-linked fluorimetric assay previously described in section 4.2.7. Before the assay MEG-01 cells transiently transfected with VGLUT1, botulinum C or the tetanus toxin light chain were plated at 5×10^5 cells/ml (100µl per well) in blackened 96-well plates (µclear, Greiner Labortechnik Ltd., Stonehouse, U.K.) in release buffer pre-warmed to 37°C, containing NaCl (120 mM), KCl (3 mM), NaH_2PO_4 (1.25 mM), HEPES-Na (25 mM), Glucose (4 mM), $MgCl_2$ (1 mM), $CaCl_2$ (2mM), pH 7.4. The reaction was initiated by the addition of release buffer containing GDH (40 U/ml, Sigma) and NADP+ (1mM), and the plate was transferred to a Dynex MFX fluorescent plate reader equipped with a thermostated incubation chamber (37°C). Fluorescence was monitored using excitation and emission wavelengths of 355 nm and 460 nm, respectively. For each assay, a standard curve was produced using known concentrations of exogenous glutamate. Intracellular glutamate concentrations were determined by the addition of 0.1% triton-X (Sigma), incubating the plate at room temperature for 20 minutes and monitoring fluorescence as previously in a Dynex MFX fluorescent plate reader. Glutamate release was normalised luciferase activity.

5.2.5.1 Bright-Glo. Luciferase Assay System (Promega)

The Bright-Glo luciferase assay was carried out according to the manufacturer's instructions. In white 96-well plates 100µl of reagent was added to 100µl of the glutamate release assay cell culture and incubated for 2 minutes at room temperature to allow complete cell lysis and measured in a luminometer.

5.2.6 MEG-01 cell – Mesenchymal stem cell (MSC) co-cultures

5.2.6.1 Isolation and Culture of Primary BMSCs

Tissue culture plasticware and reagents were purchased from Invitrogen (Paisley, U.K., http://www.invitrogen.com) unless otherwise stated. Primary human bone marrow stromal cells (BMSCs) were isolated from femoral heads following informed consent (with kind authority from Harrogate District Hospital, Harrogate, U.K.), removed during hip-replacement operations. The marrow surrounding the trabecular bone fragments was collected and grown in control medium (alpha minimum essential medium (α- MEM) containing 2 mM glutamax, 100 U/ml penicillin, 100 µg/ml streptomycin, and 15% fetal bovine serum (FBS)) in 75-cm^2 flasks at 37°C in 5% CO_2 at 95% air atmosphere. After 7 days of incubation, the nonadherent cells were removed and medium was replaced every 3 days. On reaching confluency, the cells were subcultivated at a 1:3 ratio and then cultured up to passage 15. For co-culture experiments MSCs were plated at 1 x 10^5 cells per cm^2 in 24 well plates, and left 24 hours to adhere to the cell culture plastic forming a monolayer.

5.2.6.2 VG1MK-MSC Co-cultures

To determine the influence of glutamate on the osteogenic differentiation of human mesenchymal stem cells (MSCs) VGLUT1 over-expressing MEG-01 cells (VG1MK) and empty vector controls were parachuted onto MSC monolayers and allowed to form heterotypic contacts. MEG-01 cells were cultured and transiently transfected with the pEGFP-N2/VGLUT1 and pEGFP-N2 vector (empty vector control) as previously described in section 5.2.4. After 48 hours the transiently transfected MEG-01 cells were adjusted to a cell concentration of 5 x 10^4 cells/ml in control medium or in osteogenic medium (control medium, supplemented with 10 nM dexamethasone, 50 µg/ml L-ascorbic acid phosphate, and 5 mM ß-glycerophosphate (Sigma-Aldrich)) to induce osteogenic differentiation of the MSCs. 1ml of the cell suspension was parachuted onto the MSC monolayers and were incubated at 37°C in 5% CO_2 at 95% air atmosphere for 4 days.

5.2.6.3 Quantitative pNPP Alkaline Phosphatase Assay

After 4 days of incubation the media was removed from the monolayer of MSCs and MEG-01 cells was washed with 0.2M carbonate buffer (2:1 vol:vol of 0.2M Na_2CO_3 and 0.2M $NaHCO_3$, pH 10.2). Cells were then lysed in 150µl of 0.1% triton in 0.2M carbonate buffer and cells subjected to 3 cycles of freeze/ thawing (-80°C/ 37°C). 50µl of the cell lysates were transferred to a clear 96-well plate to measure alkaline phosphatase activity and 50µl were transferred to white-bottomed 96-well plates to calculate DNA content by pico green assay.

5.2.6.3.1 Alkaline Phosphatase activity

The cell lysates in each well were incubated with 50µl of 3mM p-nitrophenol (pNP) substrate (Sigma) in 3mM $MgCl_2$ in 60mM carbonate buffer at 37°C for up to 15 minutes. The pNP standards were prepared as described in Table 5.1, and 100µl added in triplicate to wells of the clear 96-well plate. The absorbance of the samples and the standard curve was measured at 405nm on a Dynex MFX plate reader.

5.2.6.3.2 Quant-iT™ Pico Green (Invitrogen) Assay

DNA standards were prepared as described in Table 5.2 using 100µg/ml Salmon sperm DNA (10mg/ml; Sigma) diluted in 0.1% Triton-X in 0.2M carbonate buffer and 50µl added in triplicate to wells of the white-bottomed 96-well plate. The Quant-iT™ Pico Green dsDNA reagent (Invitrogen) was diluted 1:50 in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) and 50µl added to each sample/ standard. The plate was then incubated in the dark at room temperature with gentle agitation for 2 minutes before fluorescence was measured at 485nm excitation and 583nm emission on a Dynex MFX fluorescent plate reader. The DNA content of the samples was determined using the DNA standard curve and alkaline phosphatase activity normalised against DNA content.

Concentration pNP (µmol/ml)	pNP (µl)	0.2M carbonate buffer (µl)
0.5	20	380
0.3	12	388
0.2	8	392
0.1	4	396
0.05	2	398
0.025	1	399
0.0125	0.5	399.5
0	0	400

Table 5.1: Alkaline phosphatase assay pNP standard curve

DNA conc (µg/ml)	Volume of DNA (µl)	Volume of 0.1% Triton (µl)	Total (µl)
8	80 (µl of 100µg/ml stock)	920	1000 (A)
4	500 of A	500	1000 (B)
2	500 of B	500	1000 (C)
1	500 of C	500	1000 (D)
0.5	500 of D	500	1000 (E)
0.25	500 of E	500	1000
0	0	500	500

Table 5.2: Pico green assay DNA standard curve

5.3 Results

5.3.1 Functional effects of inhibiting glutamate release in megakaryocytes

To determine the functional effects of inhibiting glutamate release on megakaryocytic proliferation and differentiation primary murine megakaryocytes and MEG-01 cells (treated with and without 100nM PMA) were cultured in the presence of riluzole (glutamate release inhibitor) for 48 and 72 hours respectively. Figure 5.1 shows the morphological changes in the cells, and Figure 5.2 shows the results of an MTT assay used to determine the viable number of MEG-01 cells following riluzole exposure. A dose-dependent decrease in the numbers of viable cells was observed following riluzole exposure ($P<0.001$). As expected there were fewer cells in the cultures treated with PMA due to the inhibition of cell proliferation and promotion of cell differentiation (at the beginning of the experiment 2×10^5 cells/ml were added to each well for both PMA treated and untreated samples). These results were supported by the results of the Annexin V apoptosis assay, which indicated that there was an increase in apoptosis and necrosis on exposure to increasing concentrations of riluzole (Figure 5.3).

To further investigate the effects of inhibiting glutamate release on the morphology of the MEG-01 cell line, transmission electron microscopy (TEM) was carried out on cells treated with 25μM riluzole for 72 hours (Figure 5.4). The results from TEM show an increased number of cell vacuoles and fewer, smaller mitochondria in the cells treated with riluzole compared to the un-treated control cells (Figure 5.4). There was also evidence of platelet shedding in the control cells, which was not observed in riluzole-treated MEG-01 cells (Figure 5.4).

To determine the inhibition of glutamate release from megakaryocytic cells on exposure to riluzole, glutamate release assays were performed (as described previously in section 4.2.7) on MEG-01 cells (treated with and without 100nM PMA) cultured in the presence of a range of riluzole concentrations (0μM to 100μM) for 72 hours. The results from glutamate release assays showed a dose-dependent increase in the amount of glutamate released from MEG-01 cells treated with and without PMA on exposure to riluzole (Figure 5.5).

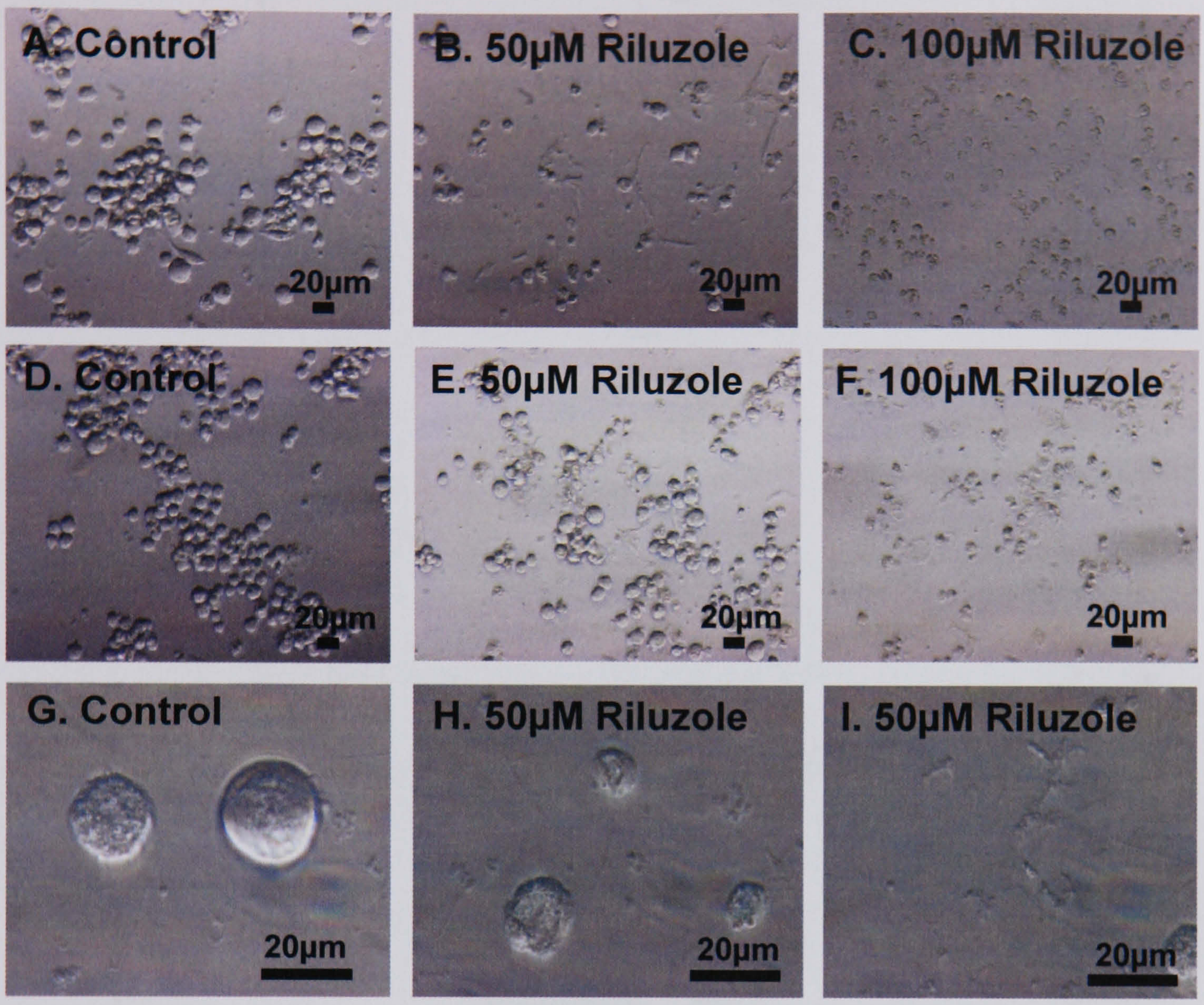


Figure 5.1: Morphological changes in MEG-01 cells observed using light microscopy after exposure to riluzole. (A - C) cells treated with 100nM PMA in the presence of riluzole (0, 50 and 100µM) after 72 hours incubation. **(D - F)** MEG-01 cells treated without PMA in the presence riluzole (0, 50 and 100µM) after 72 hours incubation; and **(G-I)** primary murine megakaryocytes treated with 0 and 50µM riluzole after 24 and 48 hours shown in H and I respectively.

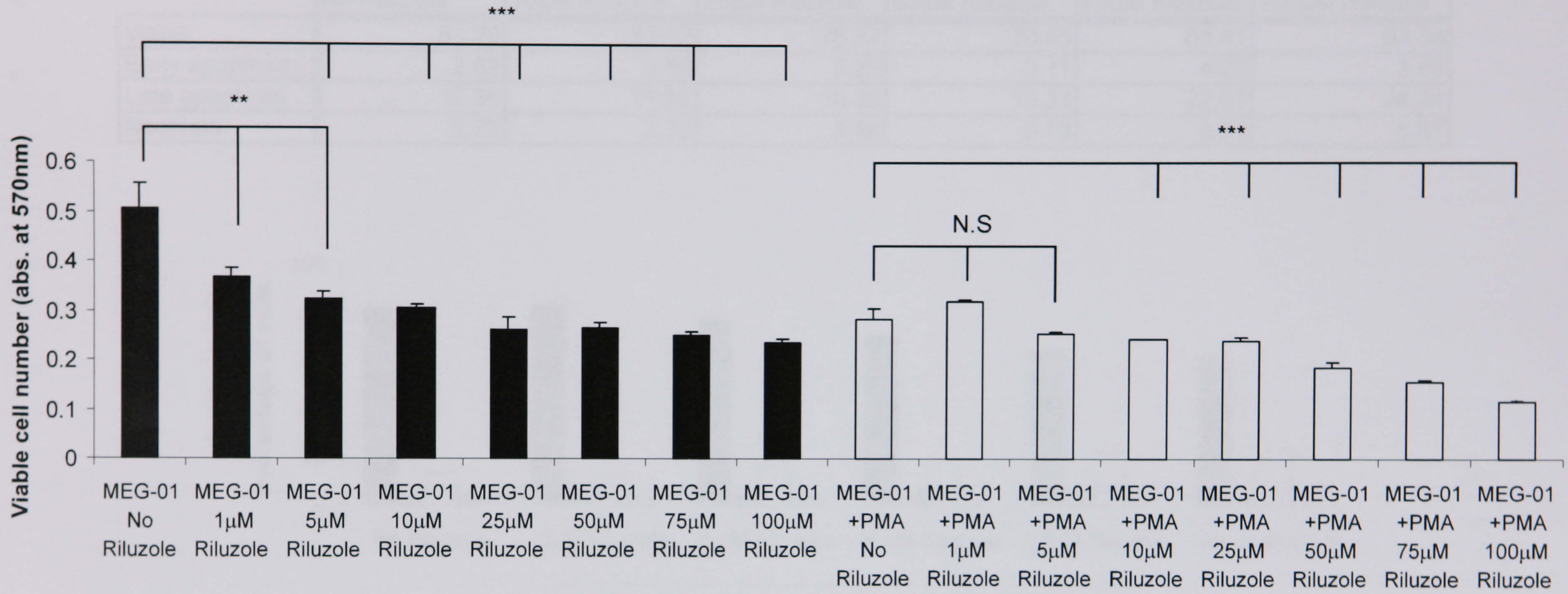


Figure 5.2: Viable MEG-01 cell numbers after exposure to the glutamate release inhibitor riluzole (0-100μM) in the presence and absence of 100nM PMA for 72 hours. Observations from MTT viability assays showed that there was a dose dependent decrease in viable MEG-01 cell number on exposure to riluzole (** $P< 0.05$, *** $P<0.001$, N.S = non significant at $P>0.05$). The statistical test used was a one-way ANOVA, n=3, error bars indicate standard deviation.

	No Riluzole	10μM Riluzole	25μM Riluzole	50μM Riluzole	75μM Riluzole	100μM Riluzole
Viable	81.29	83.32	76.87	70.45	64.41	63.04
Early apoptosis	2.62	2.59	3.22	3.11	4.29	3.86
Late apoptosis	13.36	11.83	16.35	18.31	22.91	24.24
Necrosis	2.73	2.26	3.56	8.13	8.39	8.87

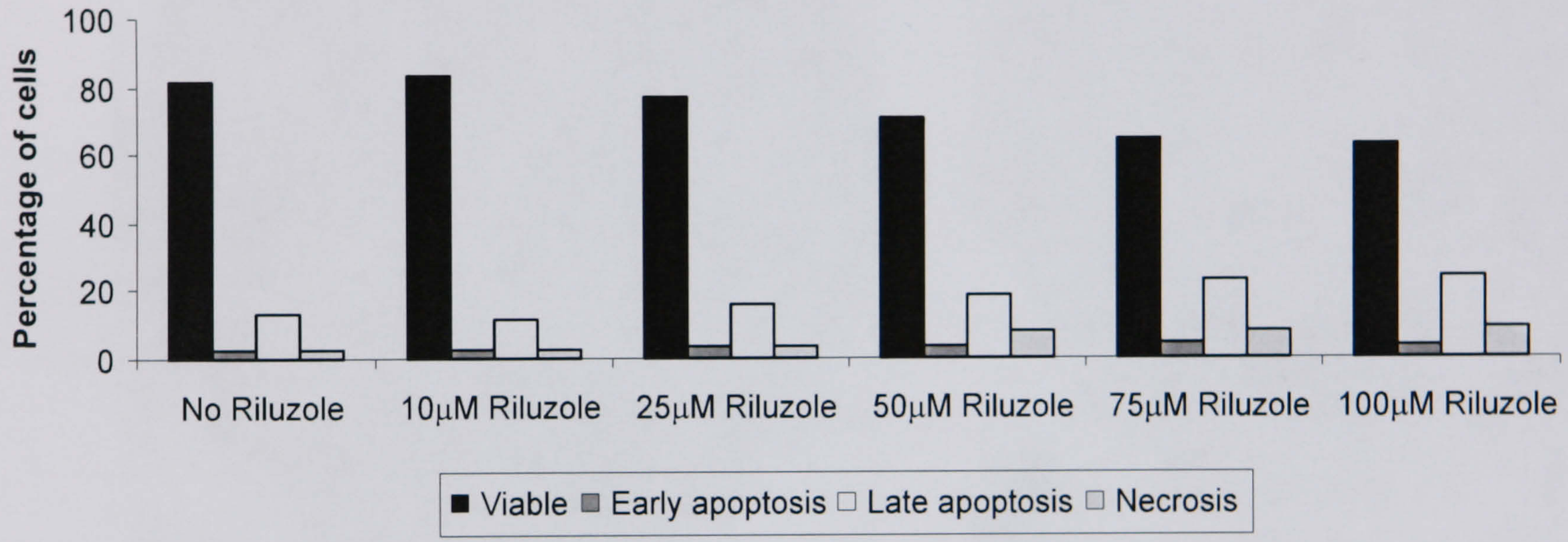


Figure 5.3: Detection of apoptotic cells after exposure to riluzole (0 - 100μM). Results from Annexin V apoptosis assays indicate a dose dependent decrease in MEG-01 cell viability that coincides with an increase in early and late apoptotic cells and necrosis (n=3).

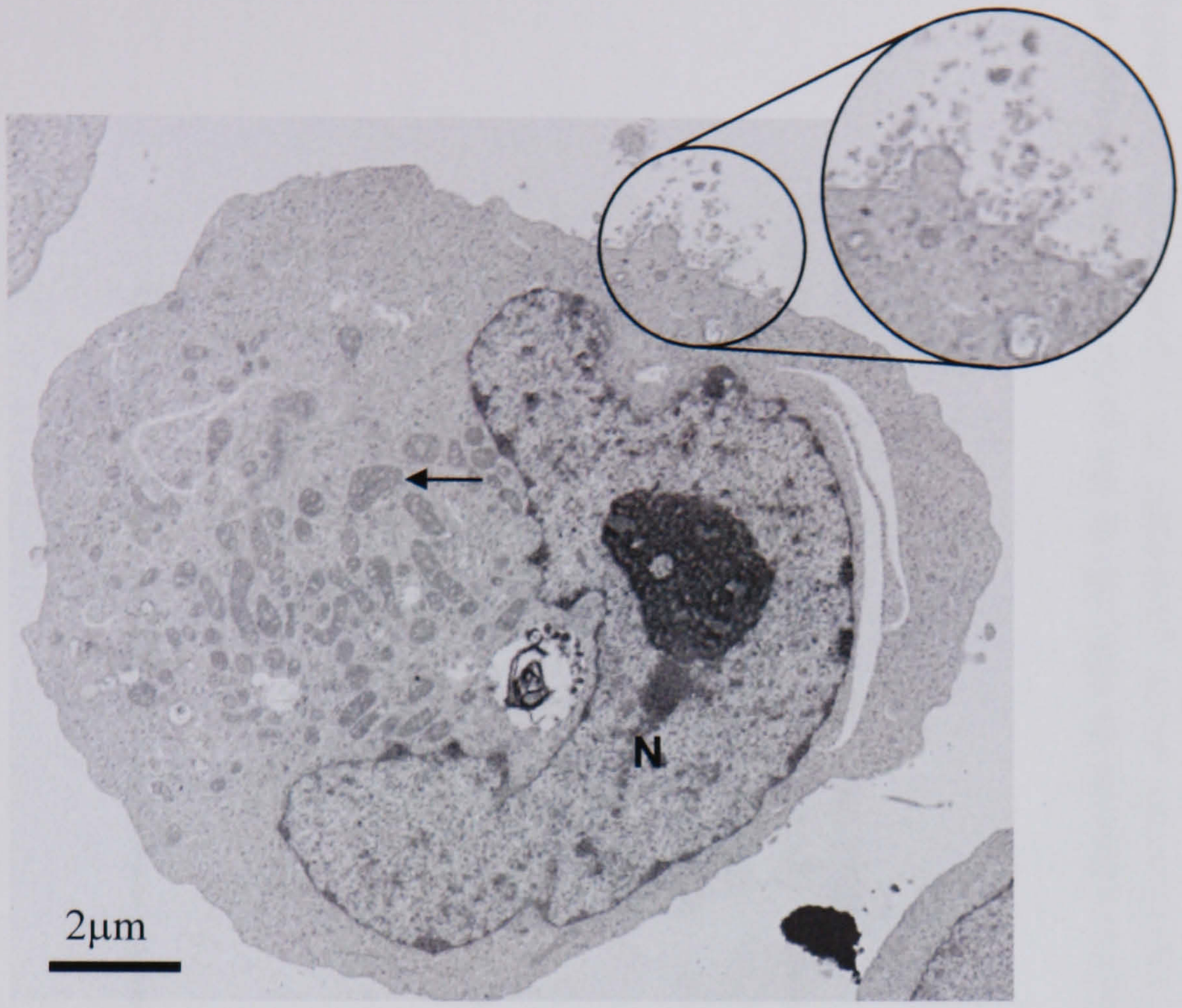


Figure 5.4: Morphological changes in MEG-01 cells visualised by TEM after exposure to riluzole. (A) Control MEG-01 treated with PMA for 72 hours, showing distinct nuclear lobes (N), large mitochondria (arrows) and possible platelet formation (magnified area). **(B)** MEG-01 cell treated with PMA in the presence of 25μM riluzole for 72 hours, the cell appears to have a more distorted periphery than the control, contains many large vacuoles (astricks), and smaller mitochondria (arrow head).

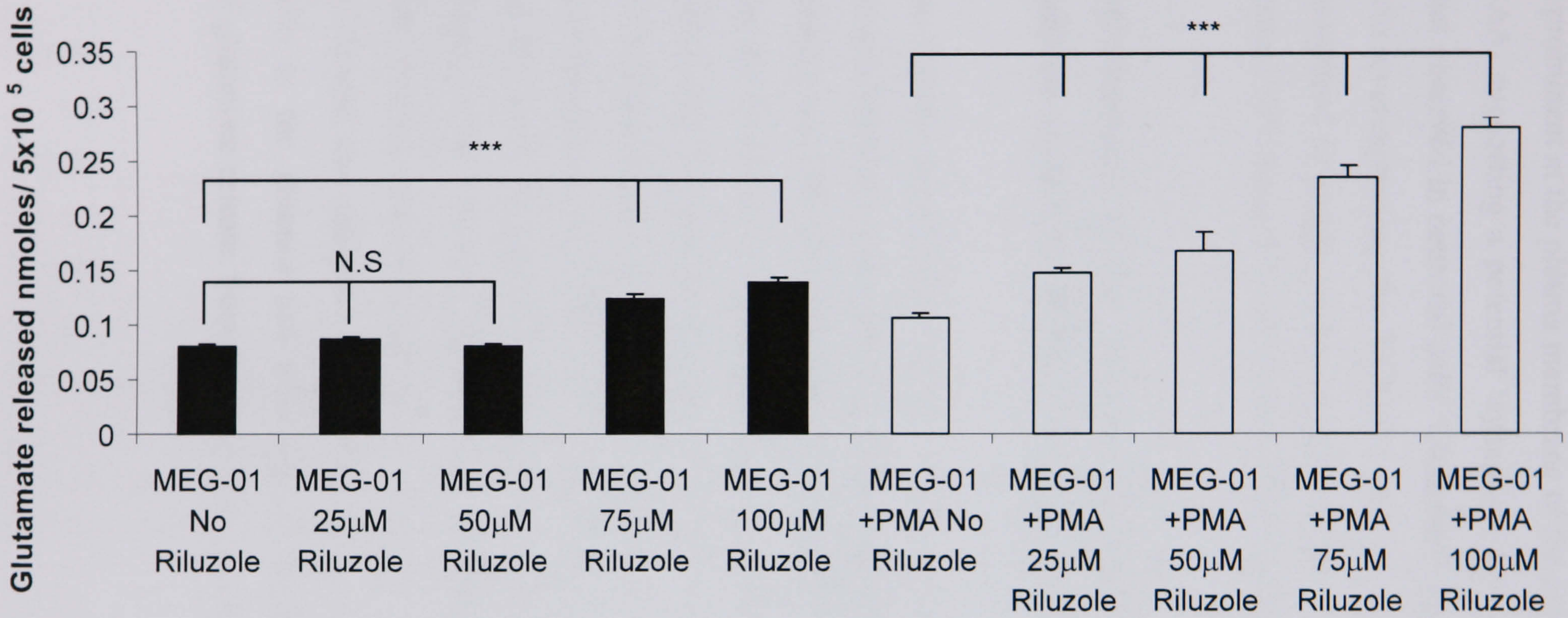


Figure 5.5: Glutamate release from the megakaryocytic cell line, MEG-01 exposed to riluzole (0-100μM) in the presence and absence of PMA. MEG-01 cells, treated with and with out PMA, were shown to release significantly more glutamate on exposure to increasing concentrations of riluzole (***=P<0.001) compared to untreated controls. There was however no significant difference in the glutamate released by MEG-01 cells in the absence of PMA on exposure to 25 and 50μM riluzole concentrations (N.S at P>0.05). The statistical test used was a one-way ANOVA, n=3, error bars indicate standard deviation.

5.3.2 Overexpression of VGLUT1 in megakaryocytic cells

In the MEG-01 cell line overexpressing EGFP tagged VGLUT1, the localisation of the protein was prominent at the plasma membrane of the cells. in a specific area as shown in Figure 5.6A, suggesting a potential 'synaptic zone' with in megakaryocytic cells similar to that observed in neuronal cells. Glutamate release assays demonstrated that MEG-01 cells overexpressing the VGLUT1 protein released 41% more ($P<0.001$) glutamate compared to empty vector controls (Figure 5.6B). These cells were also shown to contain 36% more ($P<0.001$) intracellular glutamate.

5.3.3 Overexpression of the tetanus toxin light chain (TeNTLC) and botulinum toxin C (BoNT/C1) in megakaryocytic cells

To determine whether the SNARE complex is responsible for glutamate release from megakaryocytes, MEG-01 cells were transiently transfected with the TeNTLC and BoNT/C1 which cleave VAMP and SNAP-25 respectively to disable vesicle recycling by preventing the formation of the SNARE complex. The results of glutamate release assays indicated that the overexpression of the TeNTLC induced a 30% decrease ($P<0.001$) in released glutamate compared to empty vector controls (Figure 5.7A), whereas overexpression of BoNT/C1 in MEG-01 cells showed no significant difference in the levels of glutamate released ($P<0.001$) compared to empty vector controls (Figure 5.7B). Western blot analysis of MEG-01 cells treated with and without 10nM tetanus toxin (Sigma) in the presence or absence of 100nM PMA (Figure 5.8) showed that tetanus toxin significantly decreases VAMP expression in MEG-01 cells in the absence and presence of PMA ($P<0.001$), suggesting the reduction of glutamate release from these cells is due to the proteolytic cleavage of VAMP.

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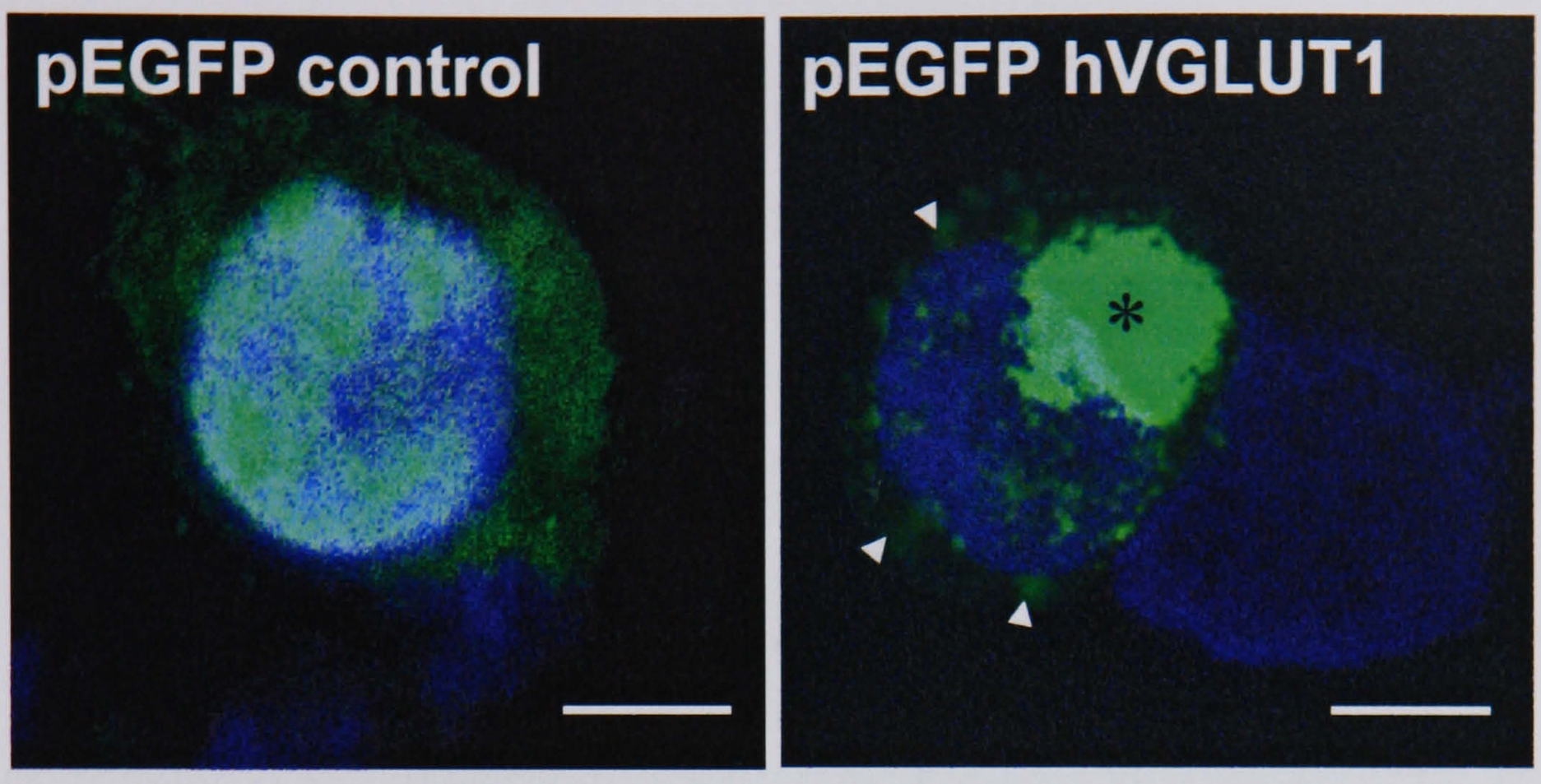
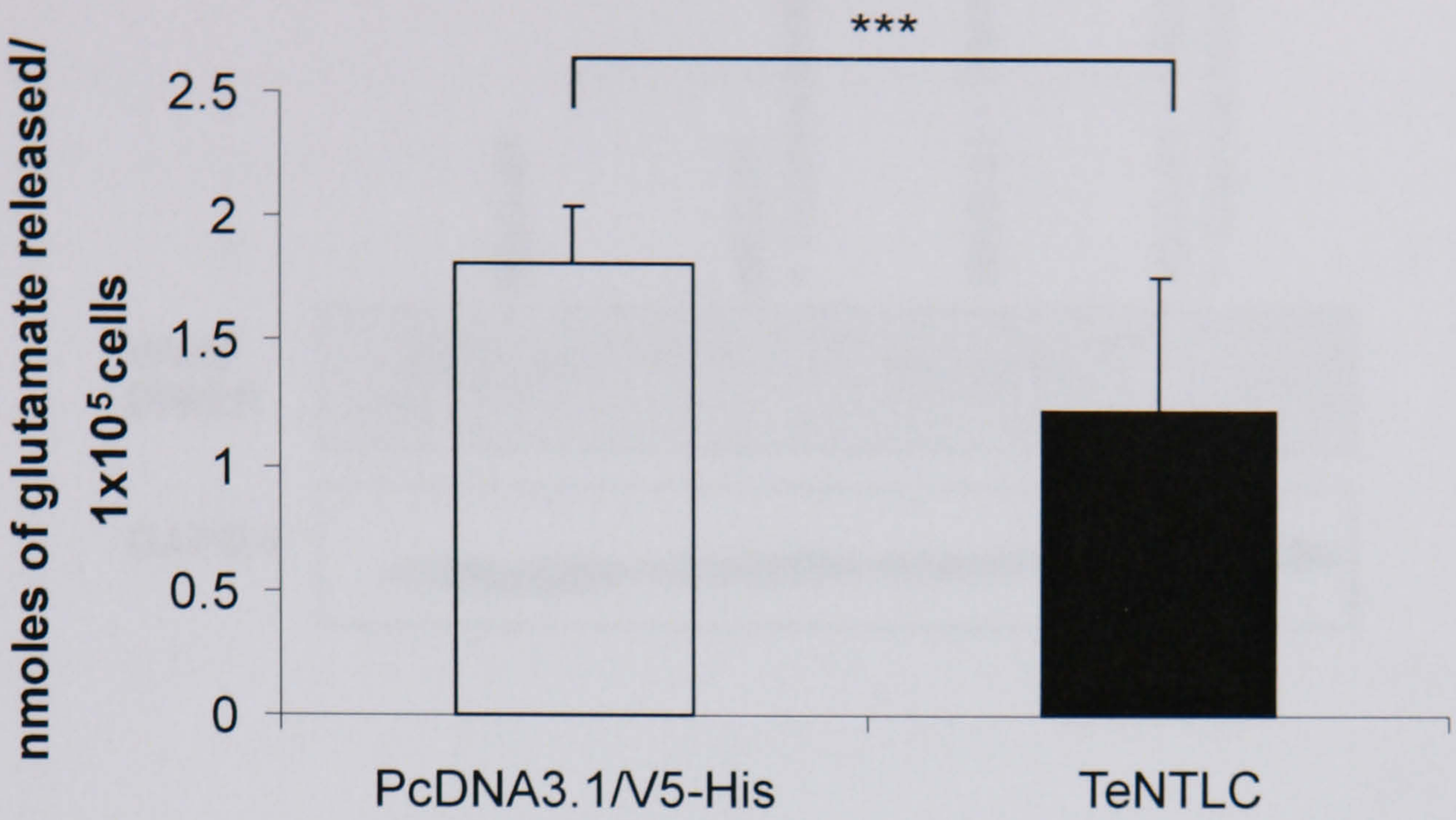


Figure 5.6: Overexpression of VGLUT1 in the MEG-01 cell line. (A) Expression of EGFP-VGLUT1 fusion construct in MEG-01 cells. EGFP-VGLUT1 accumulated at the cell periphery (arrow head) and in discrete zones (asterisk) compared to empty vector controls. Bar indicates 10μm.
(B) Using glutamate release assays , a significant increase in the amount of glutamate released by MEG-01 cells overexpressing VGLUT1 was observed compared to vector controls (***$P<0.001$). The statistical test used was a one-way ANOVA, n=3, error bars indicate standard deviation.

A



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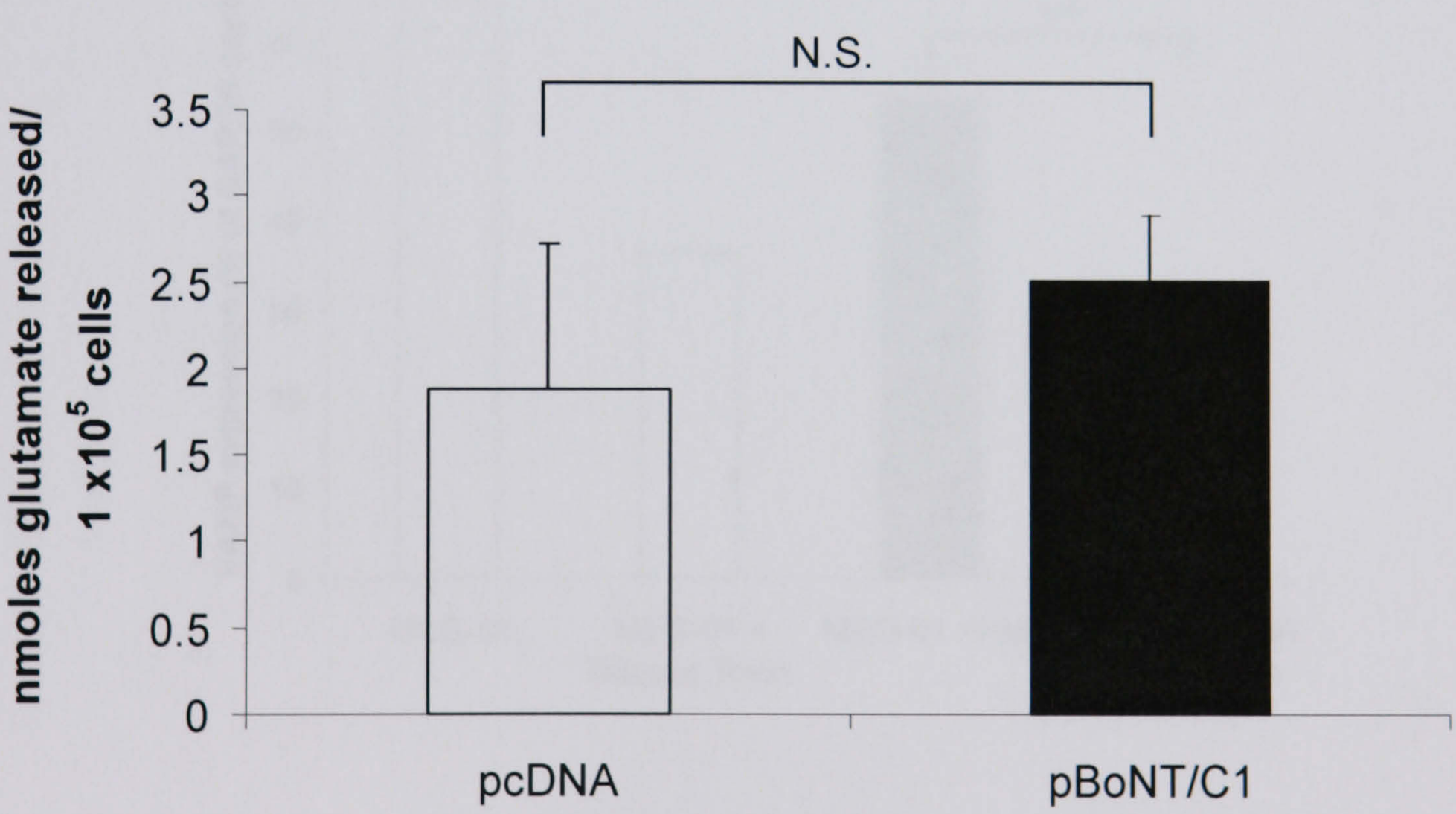

Figure 5.7: Glutamate release from MEG-01 cells overexpressing the tetanus toxin light chain (TeNTLC) and botulium toxin C (pBoNT/C1). **(A)** Overexpression of the TeNTLC in megakaryocytic cells significantly reduced glutamate release by 30% compared to empty vector controls (***$P<0.001$). **(B)** Megakaryocytic cells overexpressing pBoNT/C1 showed no significant difference in glutamate released compared to empty vector controls (N.S at $P>0.001$). The statistical test used was a one-way ANOVA, n=3, error bars indicate standard deviation.

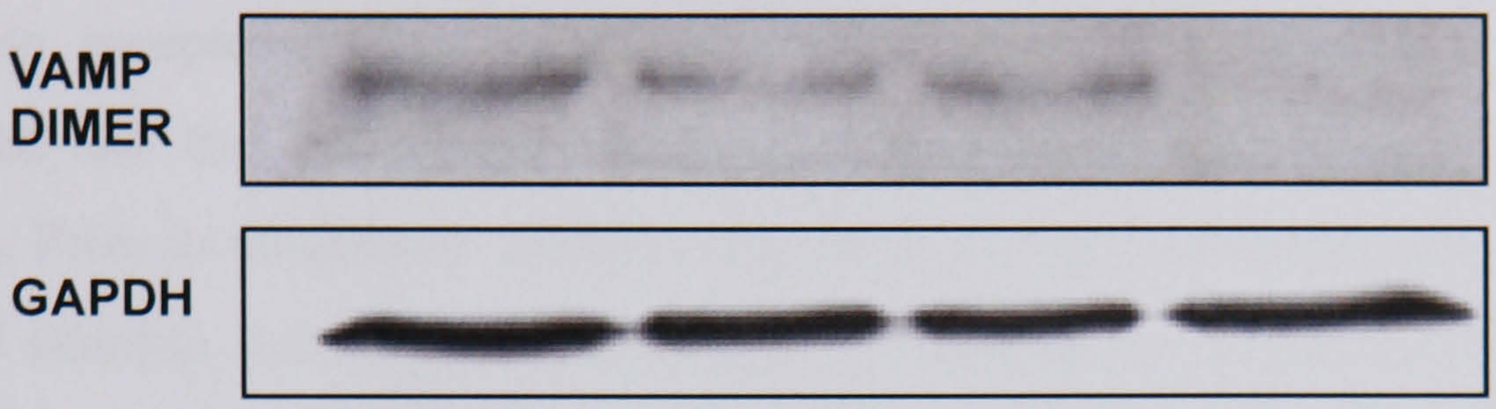

Figure 5.8: Inhibition of SNARE-complex formation in megakaryocytic cells using tetanus toxin. **(A)** Western blot analysis of VAMP expression in the megakaryocytic cell line, MEG-01, in the presence and absence of 100nM PMA following exposure to tetanus toxin (10nM).
(B) Densitometry was used to quantify VAMP expression against GAPDH controls (***$P<0.001$). The statistical test used was a one-way ANOVA.

5.3.4 Effect of MEG-01 cells overexpressing VGLUT1 on the osteogenic differentiation of MSCs in co-cultures

Previous studies in our laboratory (personal communication. Dr Gary Spencer) have shown, as discussed in section 5.1. that MSCs express components involved in regulated glutamate exocytosis and glutamate receptors AMPA and NMDA-type glutamate receptors. Here, co-cultures were used to investigate the influence of glutamate on the osteogenic differentiation of human mesenchymal stem cells (MSCs). Prior to co-culture, synaptic vesicle recycling was determined in MSCs using FM1-43 staining, carried out as described previously in section 4.2.2. The results show an accumulative increase in the amount of fluorescent dye within the cells over a 20 minute incubation period. Following removal of FM1-43 dye from the culture media cells showed destaining within 30 minutes (Figure 5.9). In co-culture experiments VGLUT1 overexpressing MEG-01 cells (VG1MK) and empty vector controls were parachuted onto MSC monolayers and allowed to form heterotypic contacts. After fours days of culture under osteogenic conditions, alkaline phosphatase activities were shown to be 26% greater ($P<0.05$) in VG1MK-MSC co-cultures compared to MSCs co-cultured with empty vector MEG-01 controls (Figure 5.10).

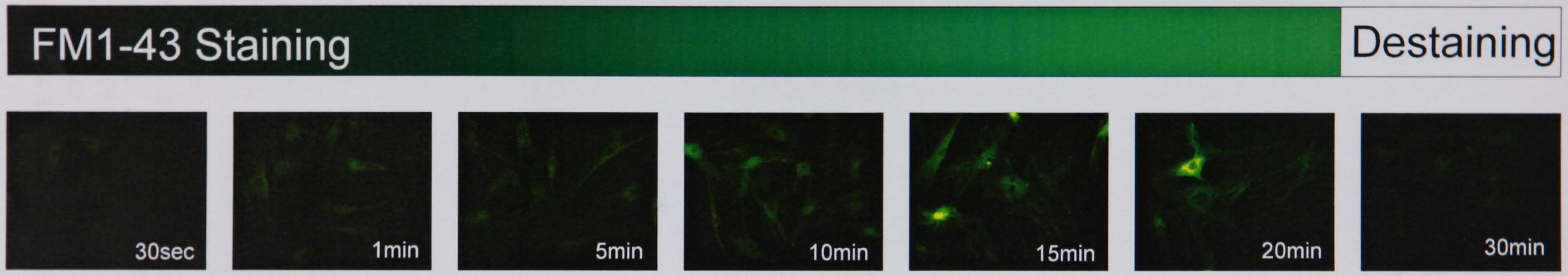

Figure 5.9: Identification vesicular recycling in primary mesenchymal stem cells (MSCs) using FM1-43 dye . The incorporation of the FM-43 dye into the vesicle membrane, shown by green fluorescence, increased over the time course and was reduced in destaining experiments.

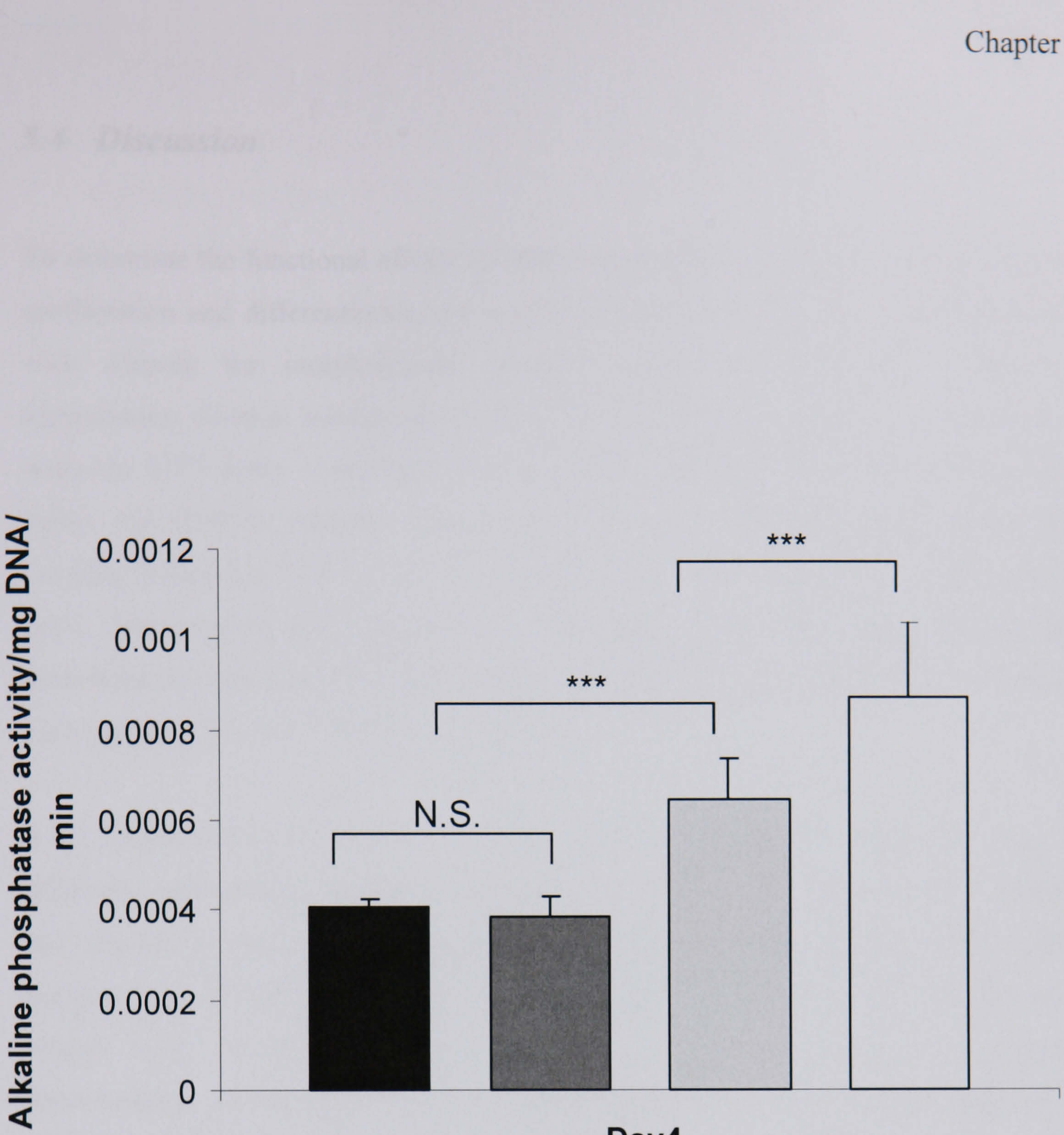

Figure 5.10: Osteogenic differentiation of human MSCs co-cultured with VGLUT1 overexpressing MEG-01 cells. MSCs cultured in osteogenic medium showed a significant increase (***P<0.001) in alkaline phosphatase activity after 4 days incubation in co-culture with VGLUT1 over expressing MEG-01 cells (VG1MK) compared to empty vector controls (EGFPMK). There was a significant difference in alkaline phosphatase activity observed between the osteogenic MSC-EGFPMK cocultures and the non-osteogenic controls (***P<0.001). However, no significant difference in the amount of alkaline phosphatase activity observed in MSCs cultured in non-osteogenic conditions (N.S. at P>0.005). The statistical test used was a one-way ANOVA, n=3, error bars indicate standard deviation.

5.4 Discussion

To determine the functional effects of inhibiting glutamate release on megakaryocytic proliferation and differentiation, the cells were observed after 72 hours of treatment with riluzole for morphological changes using light microscopy (LM) and transmission electron microscopy (TEM), and viable cell numbers were determined using the MTT assay. In neurons riluzole inhibits glutamate release by blocking ionic influx via NMDA channels, voltage-sensitive Na^{2+} channels, calcium influx and possibly interaction with G-proteins (Estevez et al., 1995d; Wang et al., 2004). It has been demonstrated that glutamate has a direct neurotoxic effect on isolated motorneurons, and that low concentrations of riluzole prevent cell damage induced by such glutamate toxicity (Estevez et al., 1995c).

After treatment with riluzole it was observed by LM that the number and size of MEG-01s and primary murine megakaryocytes was reduced, these findings suggest that riluzole is toxic to megakaryocytes. The appearance of the cells from TEM suggests that riluzole also alters the morphology of the cells. The MEG-01 cells treated with 25μM riluzole for 72 hours displayed significant cytoplasmic abnormalities, in the form of large open vacuoles and smaller mitochondria. The presence of an abnormal cytoplasm and numerous large vacuoles is also seen in Bernard-Soulier Syndrome, a severe bleeding disorder, where the lack of GPIb-IX complexes results in thrombocytopenia and giant platelets (Nurden et al., 1997b). GPIb-IX complexes therefore have an important role in normal megakaryocyte maturation and DMS production. The data presented here suggests that glutamate may also play an important role in cell development and differentiation, and indicates that there is a possibility that the inhibition of glutamate release in megakaryocytes, may have an effect on megakaryocyte differentiation and platelet size and function. However this has yet to be determined in future studies.

The observations made from TEM and LM are supported by the results from the MTT assay and the Annexin-V apoptosis assay, which suggest that glutamate release may be involved in megakaryocyte proliferation and/ or viability, as inhibition of glutamate release with riluzole leads to a significant reduction in viable cell numbers with an

increase in programmed cell death. However, this hypothesis presumes that the dose-dependent increase in glutamate release observed from megakaryocytic cells exposed to riluzole was due to leakage of intracellular glutamate from cells undergoing apoptosis and the breakdown of the cell membrane, not that riluzole exerts its effects on megakaryocytes via another mechanism. Further investigations are required to determine whether riluzole inhibits glutamate release from megakaryocytes as seen in neuronal cells or acts via alternative pathways to affect cell viability. These results are interesting as the effect of riluzole on megakaryocytes appears to be toxic, the opposite effect to what is observed in neuronal cells, where it is neuroprotective (Estevez et al., 1995b; Wang et al., 2004). Similar observations were made when osteoblastic cells were treated with riluzole. Exposure to low concentrations of riluzole (1 - 10μM) lead to a reduction in osteogenic differentiation without affecting viability, however, the addition of higher concentrations (≥25μM) of riluzole, which usually have no effect on neuronal viability, resulted in high levels of osteoblastic cell death and apoptotic characteristics, including increased Bax/Bcl-2 ratio, membrane blebbing, chromatin condensation and DNA fragmentation (Genever and Skerry, 2001a). Together these findings suggest that although glutamate is released by megakaryocytes and osteoblasts that the mechanisms of glutamate release in the bone marrow may be different to those seen in the CNS. Further work is required to investigate the role of glutamate on megakaryocyte survival.

The localisation of EGFP-tagged VGLUT1 in MEG-01 cells mirrored the immunolocalisation of the endogenous protein and the acridine orange staining of acidic vesicles (see section 4.3) again suggesting that there may be a 'synaptic zone' within megakaryocytes similar to that observed in pre-synaptic neurons.

Overexpression of VGLUT1 in the megakaryocytic cell line MEG-01 showed a significant increase in the amount of glutamate released compared to empty vector controls. These findings are supported by previous studies which have shown that over-expression of VGLUT1 in cultured glutamatergic neurons increases the amount of glutamate released at synapses (Wojcik et al., 2004). Also, overexpression of the *Drosophila* VGLUT, DVGLUT, in motoneurons was shown to increase vesicular glutamate release, leading to an increase in synaptic vesicle volume and a decrease in the number of synaptic vesicles released, to maintain normal levels of excitation at the

synapses (Daniels et al., 2004b). These data confirm the specificity and importance of VGLUT-dependent glutamate signalling in megakaryocytes.

The light chain of the tetanus toxin (TeNT) neurotoxin, an exotoxin of *Clostridium tetuni*, inhibits neurotransmitter release by proteolytically cleaving VAMP2 between Gln-76 and Phe-77, impairing synaptic vesicle exocytosis from neuronal cells (Link et al., 1992; Schiavo et al., 1992a). Treatment of the megakaryocytic cell line MEG-01 with TeNT lead to a marked reduction in the expression of VAMP2, indicating that in megakaryocytic cells TeNT is also able to cleave VAMP2, therefore preventing the formation of the SNARE complex. Transient transfection of the TeNT light chain in the megakaryocytic cell line MEG-01 significantly reduced the amount of glutamate released from these cells compared to empty vector controls, confirming that proteolyic cleavage of VAMP2 in megakaryocytic cells inhibits glutamate exocytosis through SNARE-dependent mechanisms.

The botulinum neurotoxin serotype C (BoNT/C) cleaves both syntaxin 1 and SNAP-25 (Blasi et al., 1993c; Foran et al., 1996a; Williamson et al., 1996b). In this study we transiently transfected MEG-01 cells with the BotNT/C1 light chain to determine its effects on exocytosis. The results indicated that unlike cells treated with the TeNTLC there was no significant reduction in glutamate released from cells overexpressing the BoNT/C1 compared to empty vector controls. These results support the findings from RT-PCR that megakaryocytic cells do not express one of the main targets of BoNT/C SNAP-25, expressing only its homologue SNAP-23. Previous studies have shown that SNAP-23 is either resistant to cleavage or is cleaved to a reduced extent by the different BoNT serotypes (Washbourne et al., 1997a; Vaidyanathan et al., 1999a), suggesting the megakaryocytic SNARE-complex is composed of SNAP-23 and the other exocytotic proteins, not SNAP-25. Megakaryocytic cells were also shown to express the other BoNT/C target syntaxin 1 by RT-PCR and its homologue syntaxin 4, however studies have shown that syntaxin 4 can not be cleaved by BoNT/C (Hansen et al., 1999). As glutamate exocytosis was not diminished in megakaryocytic cells using BoNT/C1 it suggests that megakaryocytic syntaxin 1 can not be cleaved or is cleaved to a reduced extent by BoNT/C, or that syntaxin 4 which is also expressed by megakaryocytes and is resistant to cleavage by BoNT/C replaces syntaxin 1 in the complex.

Co-culture studies using MSCs and MEG-01 cells overexpressing VGLUT1 have implicated megakaryocytic glutamate release in the regulation of bone marrow stem cell activity. The increase of glutamate released from the VGLUT1 overexpressing MEG-01 cells was shown to increase the osteogenic differentiation of MSCs compared to the empty vector controls. These findings support observations from our laboratory (personal communication. Dr Gary Spencer) that osteogenic differentiation of MSCs is significantly inhibited by exposure to glutamate receptor antagonists CFM-2 and MK-801, which inhibit AMPA and NMDA receptors respectively. Also prolonged exposure of MSCs to AMPA agonists, glutamate or NMDA, has been shown to lead to an increase in viable cell numbers, indicating that glutamate signalling is vital to the differentiation and viability of MSCs. As well as expressing glutamate receptors, MSCs express the necessary machinery required for vesicular glutamate exocytosis and have also been shown to release glutamate into their extracellular environment, these data are supported by findings in this study that functional synaptic vesicle recycling occurs in MSCs and suggest communication between the cells occurs via paracrine glutamate signalling. These results collectively suggest that glutamate signalling and communication between stem cells, bone cells and megakaryocytes in a paracrine and autocrine manner has a vital role within the bone marrow microenvironment in cell differentiation and cell viability.

This chapter confirms the importance of glutamate release in megakaryocytic differentiation and cell viability, and confirms via the use of tetanus toxin that SNARE-dependent glutamate release takes place in megakaryocytic cells. Also the observations that modified megakaryocytic glutamate release activity is sufficient to influence MSC differentiation through heterotypic cell interactions, indicates an important role for glutamate signalling from these cells in paracrine interactions within the bone marrow microenvironment.

Chapter 6

General discussion

Chapter 6: General discussion

Megakaryocytes, as discussed previously in section 1.2.3, are derived from HSCs via a tightly regulated process involving numerous cytokines and haematopoietic growth factors, ensuring the number of platelets released in to the circulation is adequate to meet physiological needs. Since the identification of TPO as a major regulator of megakaryocytopoiesis, the majority of research in this field has focused on this hormone (Wendling et al., 1994a; Lok et al., 1994a; Kaushansky et al., 1994a; de Sauvage et al., 1994a). However, studies by Bunting and colleagues have shown that normal platelets and megakaryocytes are produced *in vivo* in the absence of TPO (Bunting et al., 1997c), and other studies using transgenic mice deficient in the TPO gene and the c-mpl receptor indicated although these mice showed an 85% reduction in peripheral platelet, marrow and spleen megakaryocytes, the megakaryocytes and platelets that were present remained functionally normal (Gurney et al., 1994b; Kaushansky, 1995d; de Sauvage et al., 1996a), strongly suggesting that although TPO is important in the development of an adequate number of megakaryocytes and platelets, other factors must also be involved in platelet production. The data presented in this thesis presents additional evidence for a novel glutamate signalling system that regulates megakaryocyte differentiation and platelet production, enhancing our knowledge of peripheral glutamatergic signalling.

The main aim of this work was to further investigate and broaden our knowledge regarding glutamate signalling in megakaryocytes as a potential novel regulator of megakaryocytopoiesis and platelet production. Previous studies have shown that megakaryocytes express functional NMDA-type receptors, and antagonism of these receptors leads to impaired megakaryocytopoiesis and platelet production *in vivo* indicating an important role for glutamate signalling in these cells (Genever et al., 1999c; Hitchcock et al., 2003d). The findings described in this thesis support this evidence for neuronal-like glutamate signalling in megakaryocytic cells by identifying components required for regulated vesicular exocytosis, vesicle recycling and endocytosis.

6.1 Evidence for Pre-synaptic glutamate release mechanisms in megakaryocytes

The data presented in this thesis has shown that megakaryocytes express core exocytotic SNARE proteins identical to those identified and characterised in neuronal cells as effectors of regulated vesicular glutamate exocytosis. Identification of the v-SNARE isoforms VAMP-1 and Cellubrevin (VAMP-3) and the t-SNAREs SNAP-23 and syntaxins 1 and 4 in megakaryocytes confirmed the components required to form the ternary core exocytotic complex exist in megakaryocytic cells.

In the CNS, out of the eight identified VAMP isoforms (VAMP 1-8) it is only VAMP-1 and VAMP-2 which are thought to be exclusively associated with trafficking events that involve regulated exocytosis (for review see Gerst, 1999a), whereas the other VAMP isoforms such as cellubrevin (VAMP3) appear to function in both constitutive and regulated trafficking pathways in a variety of systems. Neuronal cells also express numerous t-SNAREs, to date, 16 syntaxin isoforms have been identified (excluding alternative spliceoforms) in mammalian cells, however only syntaxins 1A, B and, possibly, C, as well as syntaxins 2 through 4 have been shown to localise to the plasma membrane and are thought to participate in exocytosis (Inoue et al., 1992a; Bennett et al., 1992a; Bennett et al., 1993a; Jagadish et al., 1997b). In the CNS, syntaxin 1 is most commonly found to be associated within the SNARE complex involved in regulated glutamate release (Gerst, 1999b). SNAP-25 which exists in two variants (SNAP-25 A and B) is the only synaptosomal protein to be associated with VAMP and syntaxin in the mammalian CNS (Oyler et al., 1989a; Chapman et al., 1994a; Bark et al., 1995a), however, a constitutively expressed form of SNAP-25, SNAP-23 (Ravichandran et al., 1996a), which is present in nonneuronal tissues is predicted to participate in both constitutive and stimulus-coupled exocytic events (Ravichandran et al., 1996d; Chen et al., 1997; Sadoul et al., 1997; Leung et al., 1998; Guo et al., 1998; Rea et al., 1998).

The reason for the difference between the number of syntaxin and VAMP or SNAP-25 isoforms is unclear, but suggests some promiscuity in the v-SNARE-t-SNARE interactions of the latter two families. Differential expression of these v-SNAREs and

t-SNAREs in megakaryocytes suggests that v-SNARE-t-SNARE promiscuity or functional redundancy may also function in this system and indicates that the trafficking and formation of the exocytotic SNARE complex relies on other mechanisms to generate specificity as seen in neuronal cells within the CNS (Yang et al., 1999; Brunger, 2001b).

Evidence for SNARE-dependent glutamate release mechanisms in megakaryocytes, similar to those observed in the CNS, is further supported by findings in this study that cells transiently overexpressing the tetanus toxin light chain (TeNTLC) (discussed in Chapter 5) release significantly less glutamate compared to empty vector controls, confirming that proteolyic cleavage of VAMP2 in megakaryocytic cells inhibits glutamate exocytosis through SNARE-dependent mechanisms.

Furthermore, the identification of numerous accessory proteins involved in targeted vesicular glutamate exocytosis and endocytosis, NSF, α-SNAP, γ-SNAP, Munc18-1, Munc13, and syntaphilin, by megakaryocytic cells, indicates that the essential components required for neurotransmitter release in the CNS were present in these cells. These proteins are essential for neurotransmitter release throughout the CNS, for example in *munc18-1* null mice there is a lack of neurotransmitter release resulting in complete paralysis at birth, suggesting Munc18-1 has an important role in vesicle docking and fusion (Verhage et al., 2000c). The expression of Munc-18-1 expression is considered to be neuron- specific and it is therefore significant that megakaryocytes express not only this protein but also other regulatory proteins such as Munc13 which aid the disassembly of the SNARE complex after exocytosis has occurred (Gerst, 1999c; Verhage et al., 2000d). In addition, this work has also shown that megakaryocytic cells express key regulatory proteins, synaptotagmin, Rab3a, Rab3b, and the Rab3a binding protein rabphilin 3a, providing evidence for Ca^{2+}-dependent glutamate exocytosis from megakaryocytes similar to that observed in the CNS.

Identification of the 'brain-specific' vesicular glutamate transport proteins (VGLUT-1 and -2) in megakaryocytic cells, has provided evidence for uptake and packaging of glutamate into synaptic vesicles by megakaryocytes. These data have emphasised the 'glutamatergic phenotype' of megakaryocytic cells highlighting the similarities in

exocytotic mechanisms for both intracellular vesicular packaging and glutamatergic signalling between the CNS and megakaryocytes. The significance of these findings are supported by recent studies in the CNS which have shown that no other component is required to make a neuron store or release glutamate by exocytosis upon stimulation (Brunger, 2001a; Varoqui et al., 2002f), a selectivity setting glutamate-releasing neurons apart from other neurotransmitting neurons and provides compelling evidence for a similar mode in megakaryocytes (Ni et al., 1994a; Bellocchio et al., 1998a; Takamori et al., 2000a; Bellocchio et al., 2000a; Fremeau, Jr. et al., 2001a). Modulation of vesicular glutamate uptake in megakaryocytes by transiently overexpressing VGLUT-1 was shown to have a profound effect on the magnitude of glutamate signalling by regulating the quantity of glutamate available for release. As observed previously in neuronal cells (Daniels et al., 2004a), overexpression of the VGLUT1 protein lead to a significant increase in both packaged (intracellular) and released glutamate. This manipulation of glutamate signalling represents a possible target for drug development via the specific control of the megakaryocytic glutamate signalling pathway.

The work presented above, provides compelling evidence for possible 'pre-synaptic' glutamate release mechanisms in megakaryocytes, these observations were supported by findings in this study that megakaryocytes release glutamate at similar or even greater levels than those released by depolarised neurons at glutamatergic synapses. Elevated levels of glutamate release were observed in the megakaryoblastic cell line MEG-01 with a more differentiated megakaryocytic phenotype, after exposure to PMA, than undifferentiated cells. These results are supported by findings from RT-PCR and immunolocalisation experiments (see Chapter 3 and Chapter 4) which showed an increased expression of the SNARE complex and accessory proteins including the VGLUTs in differentiated cells compared to undifferentiated cells, and suggest that the glutamatergic phenotype is more prevalent in differentiated cells. However, unlike neuronal cells, glutamate release from megakaryocytes is spontaneous and is negatively regulated by depolarisation with 60mM KCl. Furthermore, the treatment of megakaryocytic cells with the glutamate release inhibitor riluzole lead to a significant reduction in viable cell numbers and an increase in apoptosis, suggest that glutamate release may be involved in megakaryocyte proliferation and/ or viability. These results are interesting as the effect of riluzole on

megakaryocytes appears to be toxic, the opposite effect to what is observed in neuronal cells, where it is neuroprotective (Estevez et al., 1995a; Wang et al., 2004). The reason for the differences in the release mechanisms between megakaryocytes and CNS system is unclear at this time.

In addition to this evidence for vesicular exocytosis, it has also been observed that megakaryocytes participate in vesicular glutamate recycling mechanisms similar to those described in the CNS. Expression of excitatory amino acid transporters (EAATs) by megakaryocytes and observations of megakaryocytic vesicular recycling by FM1-43 staining strongly suggests that these cells may also play an important role in glutamate recycling with in the bone marrow microenvironment, and further supports the evidence for glutamate release from megakaryocytes similar to that observed in neuronal cells.

6.2 Implications of megakaryocytic glutamate signalling mechanisms

Glutamatergic signalling, as discussed previously in section 1.4, has been identified in a variety of non-neuronal tissues including heart, intestine, kidney, pancreas, placenta, and mammary gland and bone (for reviews see Skerry and Genever, 2001a; Hinoi et al., 2004c). Unlike the majority of these other pheripheral tissues, which consist of a homogenous population of cells, the bone marrow where megakaryocytes are located consists of a heterogeneous cell population of both stem cells and mature cells. Therefore it is difficult to predict the precise impact that the data presented in this thesis and in previous studies (Genever et al., 1999b; Hitchcock et al., 2003c) will have on the current understanding of peripheral glutamatergic signalling, megakaryocytopoiesis and platelet production with in the bone marrow microenvironment. However, the fact that glutamate release and active vesicle recycling has been observed in megakaryocytes, as discussed above and in chapter 4, suggests that glutamate signalling from these cells could have significant implications on the modulation of NMDA receptor-mediated megakaryocytopoiesis (Hitchcock et al., 2003b) and in establishing interactions between the megakaryocytes and EAAT-2 (GLT-1) positive cells in the bone marrow microenvironment including osteoblasts

(Mason et al., 1997b). Due to the rarity of megakaryocytes within the bone marrow, it is possible with this current data to hypothesise that released glutamate would act predominantly on the cell from which it was released, in an autocrine fashion, helping to regulate megakaryocytopoiesis via NMDA-type glutamate receptor activation, and recycling the released glutamate through the megakaryocytic EAAT-1 (GLAST) and EAAT-2 (GLT-1) receptors. These findings and the fact that bone marrow cells also release and recycle glutamate (Mason et al., 1997a; Bhangu et al., 2001a; Bhangu et al., 2001f) suggests that there is always a constant source of glutamate within the vicinity of the megakaryocytes for NMDA receptor activation and paracrine interactions with other bone marrow cells (Figure 6.1). Observations during this study also suggest that glutamate signalling from megakaryocytes is sufficient to influence MSC differentiation through heterotypic cell interactions, providing further evidence for the importance of glutamate signalling from these cells in paracrine interactions within the bone marrow microenvironment.

The hypothesised autocrine glutamate signalling pathway in megakaryocytes suggest that glutamate signalling may be a potential target for anti-platelet therapies for treatment of CVD as manipulation of megakaryocytic glutamate release preventing autocrine glutamate receptor activation and/or blockage of the megakaryocyte NMDA-type would lead to impaired megakaryocytopoiesis and platelet production, as seen in previous studies (Hitchcock et al., 2003a). However further work is required in this area, including the use of siRNAs and viral transfection to knock down components of the SNARE apparatus and accessory proteins to determine the functional effects of glutamate release on megakaryocytopoiesis and platelet production, and the megakaryocyte NMDA-type glutamate receptor signalling.

6.3 Megakaryocytic 'presynaptic' glutamate release: Concluding remarks

Since the acceptance of the amino acid L-glutamate as the major excitatory neurotransmitter in the CNS just over 20 years ago after by a review by Fonnum

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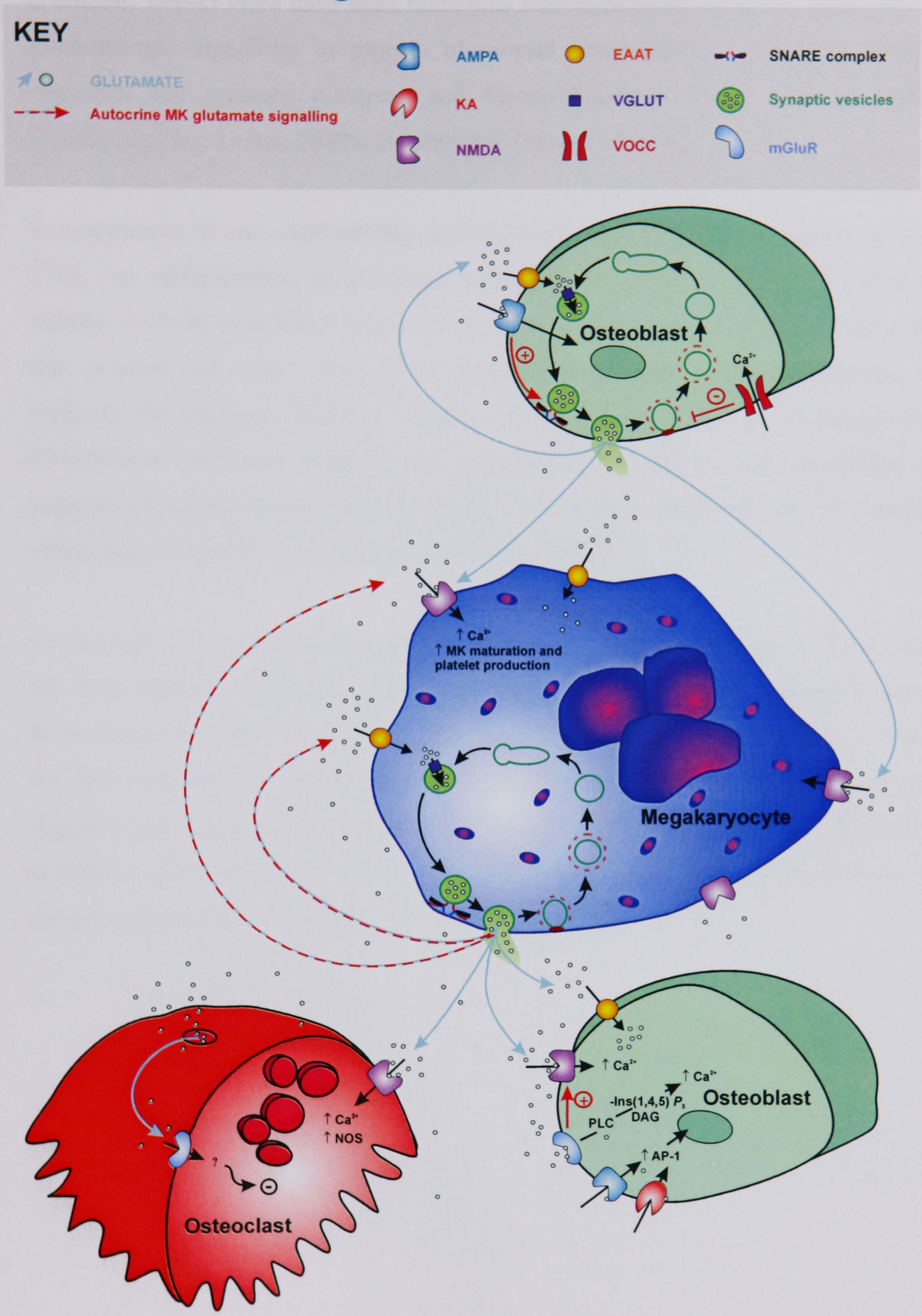

Figure 6.1: Hypothesised role of megakaryocytic glutamate release in the bone marrow microenvironment. Hypothesised autocrine glutamate signalling in megakaryocytic cells (MKs) and the possible regulation of megakaryocyte differentiation via interactions between glutamate-releasing megakaryoctyes and glutamate-releasing bone marrow cells expressing the glutamate transporter EAAT-2 (GLT-1) and glutamate receptors (Modified from Spencer et al., 2006).

(Fonnum, 1984c) there have been numerous studies demonstrating the importance of glutamatergic signalling in aspects of normal brain function including learning, cognition and memory (Ottersen and Storm-Mathisen, 1984a; Fonnum, 1984d; Collingridge and Lester, 1989a; Headley and Grillner, 1990a).

In comparison to our understanding and knowledge about glutamate signalling in the CNS, our understanding of glutamate signalling in peripheral tissues is still in its infancy, with the signalling mechanism in megakaryocytes less well characterised than that in other cell types. This thesis significantly advances our understanding and supports the previous findings, namely the identification of the megakaryocytic NMDA-type glutamate receptor, for megakaryocyte glutamatergic signalling and suggests glutamate release from these cells may have an important role in paracrine interactions within the bone marrow microenvironment.

To conclude, it is clear that megakaryocytes are an important source of glutamate in the bone marrow microenvironment and operate similar molecular mechanisms to those observed in the CNS to ensure regulated vesicular glutamate exocytosis. This is the first evidence for glutamatergic exocytotic mechanisms in megakaryocytes and suggests an autocrine activation mechanism for the megakaryocytic NMDA-type glutamate receptors influencing megakaryocytopoiesis and platelet production; and paracrine interactions with other cells within the bone marrow.

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Sigma-Genosys. Cambridge. UK

http://www.genosys.co.uk

Stratagene. California. USA

http://www.stratagene.com

Tocris Cookson Ltd, Bristol, UK

http://www.tocris.com

Vector Laboratories Ltd, Peterborough. UK

http://www.vectorlabs.com

Abbreviations

AA	arachidonic acid (not the first time this is used)
ADP	adenosine diphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ANOVA	analysis of variance
BFU-MK	burst forming unit megakaryocyte
BFU-E/MK	burst forming unit-erythroid / megakaryocyte
BMSC	bone marrow stromal cells
BNPI	brain-specific Na^+-dependent inorganic phosphate (Pi) transporter I
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CFU-GM	CFU-granulocyte-macrophage
CFU-MK	Colony forming unit megakaryocyte
CHD	coronary heart disease
CHO	Chinese hamster ovary
CML	chronic myeloid leukaemia
CNS	central nervous system
CVD	cardiovascular disease
DAB	3, 3`-diaminobenzidine
DAG	diacylglycerol
D-AP5	D-(-)-2-amino-5-phosphonopentanoic acid
dH_2O	distilled H_2O
DMS	demarcation membrane system
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNPI	differentiation-associated BNPI
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EAAT	excitatory amino acid transporter

EDTA	ethylenediaminetetraacetic acid
ETB	Ets binding sequence
FBS	foetal bovine serum
FGFβ	fibroblastic growth factor β
FITC	fluorescein isothiocyanate I
FN	fibronectin
FOG	friend of GATA
GAP	GTPase activating protein
G-CSF	granulocyte colony stimulating factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDH	glutamate dehydrogenase
GLAST	glutamate/aspartate transporter
GLT-1	glutamate transporter-1
GM-CSF	granulocyte macrophage colony-stimulating factor
GP	glycoprotein
GSH	glutathione synthase
GTPase	guanosine triphosphatase
HBSS	Hank`s buffered salt solution
HEL	human erythroleukaemia
HIV	human immunodeficiency virus
HSC	haematopoietic stem cell
IgG	immunoglobulins G
IL	interleukin
IMDM	Iscove`s Modified Dulbecco`s Medium
IP_3	inositol triphosphate
KA	kainate
kainate	[2S-(2a, 3b, 4b)]-2-carboxyl-4-(1-methylethenyl)-3-pyrrolidinaecetic acid
kb	kilobases
kDa	kilodalton
LPS	liposaccharide
LIF	leukaemia inhibitory factor
LTC-IC	long-term culture-initiating cells

LTP	long-term potentiation
m	meter
μ	micro
M	molar
MACS	magnetic activated cell sorting
mGluR	metabotropic glutamate receptors
min	minute
MK-801	5-Methyl-10, 11-dihydro-5H-dibenzo-[α,δ] cyclohepten-5, 10-imine
ml	millilitre
mRNA	messenger-ribonucleic acid
MTT	methylthiotetrazole
n	nano
$NADP^+$	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide hydrogen phosphate
NF-E2	nuclear factor-erythrocyte 2
NMDA	N-methyl-D-aspartate
NR	NMDA receptor
PAF	platelet-activating factor
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PF4	platelet factor 4
PI	propidium iodide
PI3-K	phosphatidylinositol-3-kinase
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristate acetate
PSD-95	postsynaptic density-95
RANK ligand	receptor activator of NF-kB
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minutes
RT-PCR	reverse transcriptase polymerase chain reaction

SCF	stem cell factor
SDS	sodium dodecyl sulphate
siRNAs	small interfering RNAs
SNARE	soluble N-ethyl maleimide-sensitive factor attachment protein receptors
v-SNARE	vesicle-SNARE protein
t-SNARE	target-SNARE protein
TBE	Tris-borate EDTA
TEM	transmission electron microscopy
TGFβ1	transforming growth factor β 1
TPO	thrombopoietin
TRIS	tri (hydroxy) methyl aminomethane
UCB	umbilical cord blood
U.V.	Ultraviolet
VGLUT1	vesicular glutamate transporter 1
VGLUT2	vesicular glutamate transporter 2
vWf	von-Willebrand factor

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