Functional Activity of NMDA Receptors on Megakaryocytes

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Abstract

Cardiovascular disease is the most common cause of premature death in the developed world and is responsible for 39% of all mortalities in the UK. Three quarters of these deaths are a consequence coronary heart disease and stroke, conditions that often occur as a result of platelet aggregation and thrombus formation causing ischaemia-inducing vascular blockages. Currently the prevention of coronary occlusion in high-risk patients involves the inhibition of platelet aggregability, however, reducing the number of circulating platelets, which are produced by megakaryocytes, remains unexplored. Megakaryocytopoiesis is tightly regulated by a spectrum of cytokines ensuring differentiation and circulating platelet numbers remain within normal physiological restraints. Recently, it was identified that megakaryocytes express NMDA-type glutamate receptors and subsequent studies implied a role for the glutamate signalling in megakaryocytic cell line differentiation. This thesis characterises the expression and role of the megakaryocytic NMDA receptor in both cell lines and primary human cells. MEG-01 and HEL cell lines in addition to primary megakaryocytes express a range of regulatory NR2 and NR3 receptor subunits as well as the PSD proteins Yotiao and PSD-95. Blockade of the NMDA receptor ion channel with MK-801 reduced ERK1/2 activation and inhibited primary megakaryocyte differentiation. Formation of proplatelets *in vitro* was dramatically inhibited by MK-801 treatment, as were normal ultrastructural characteristics including α-granule formation and the expansion of demarcation membrane. It was also demonstrated that megakaryocytes express the vesicular glutamate transporter protein VGLUT2, whilst MEG-01 cells spontaneously release glutamate, providing a plausible glutamate source for NMDA receptor activation. It was also established that transgenic mice with significantly lowered levels of the NR1 subunit demonstrated a 5-fold increase in bleeding time compared to wildtype control. In the future, therapeutic manipulation of the megakaryocytic NMDA receptor may enable a greater level of control over platelet production by the modification of megakaryocyte differentiation.

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This is for all who believed in me.

In Memory

Kitty Watts

Les Hitchcock

Declaration

I performed all the work presented in this thesis, with exception to the generation of NR1^{neo} transgenic mice (Chapter 4; Dr. Bev Koller, University of North Carolina) and the mouse c-Mpl$^{+/+}$ and c-Mpl$^{-/-}$ tibial sections (Chapter 5; Dr. M. Perry, University of Bristol). This thesis has not been submitted for any other degrees. Some of the data has been presented at scientific conferences, invited talks and peer-reviewed journals (listed below). All the experiments were performed at the Department of Biology, University of York, with exception to some NR1^{neo} data (Chapter 4) that was carried out at the Department of Genetics, University of North Carolina, USA.

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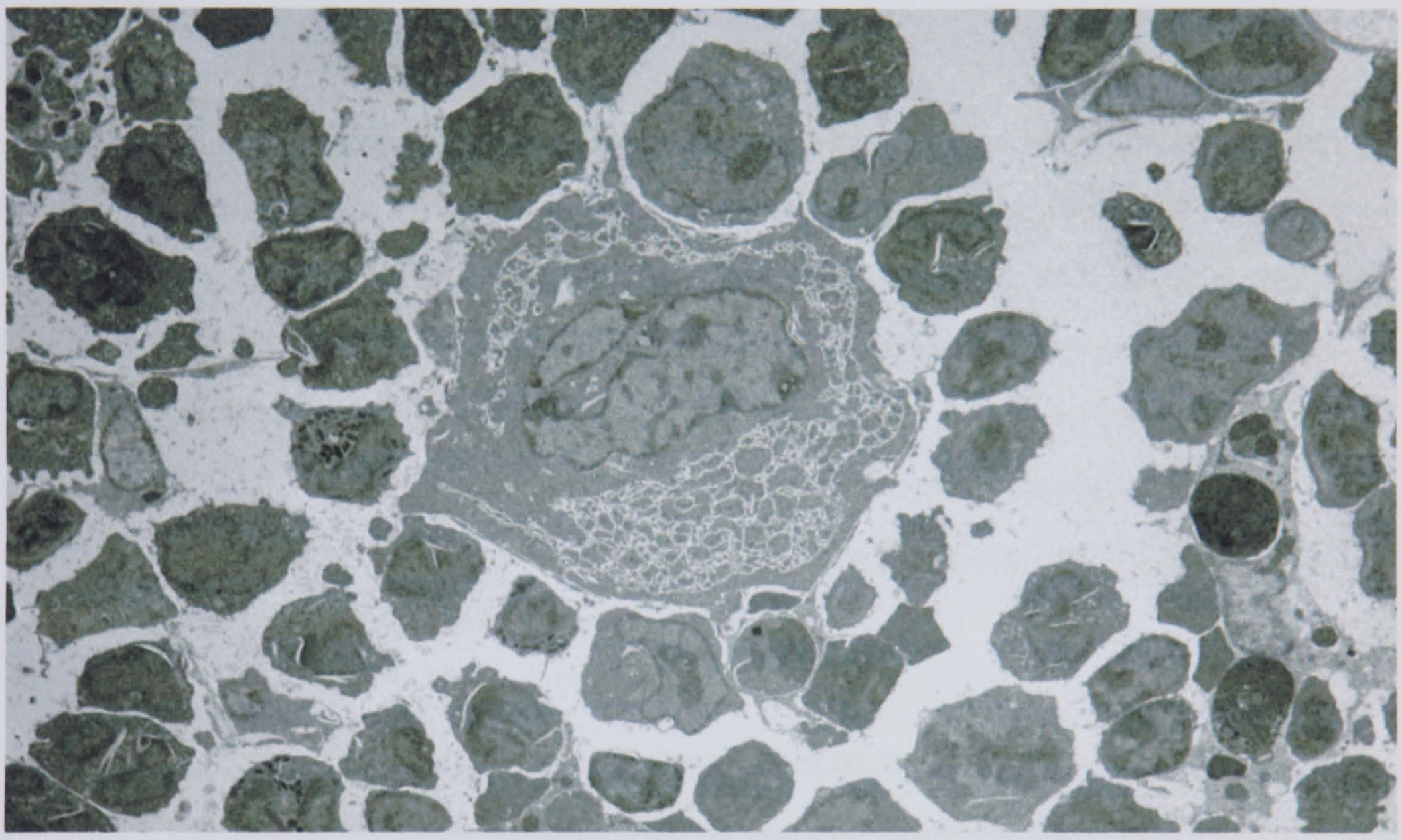
Chapter 1



Introduction

Transmission electron microscopy image of bone marrow demonstrating the size and unusual characteristics of the megakaryocyte (original magnification x 1250)

Chapter 1

Introduction

1.1 Cardiovascular disease

The human body relies on the circulatory system, driven by the heart, for the supply of oxygen and nutrients to its tissues. Breakdowns in this system, usually involving narrowing or blockages of the blood vessels or failure of the heart muscle, are often catastrophic.

The British Heart Foundation's 2002 statistics confirm that the main cause of death in the UK remains diseases of the heart and circulatory system (cardiovascular disease or CVD) (Fig. 1.1). CVD resulted in 235,000 UK deaths in 2000, 39% of all mortalities. Coronary heart disease (CHD; disease of the heart and blood vessels supplying the heart) and stroke (interruption of blood supply to areas of the brain) are the main forms of CVD, accounting for three quarters of mortalities. Although CVD-induced death increases dramatically with old age, it also causes one quarter (73,000) of premature mortalities (deaths before 75 years).

Worldwide, incidences of CHD are decreasing, a statistic mirrored in the UK (Fig. 1.2). However, the death rate from CHD in the UK remains amongst the highest in the world. Apart from Eastern and Central European countries, where CHD mortalities are increasing dramatically, only Ireland and Finland have a higher CHD death rate than the UK (Fig. 1.3). Although the death rates are decreasing in the UK, this is not at the same rate as other European counties, especially Scandinavia.

In the UK, CHD mortalities remain higher in males than females, although death rates in younger males (45-54) are decreasing more than in females of the same age. Incidences of CHD mortalities show a greater decrease in younger age groups. Death rates from stroke have also been falling throughout the last century and by 20% in the last 10 years (Fig. 1.4). As well as differences between sexes, there are also worrying variations between regions in the UK. Premature death rates in Scotland are 50% and

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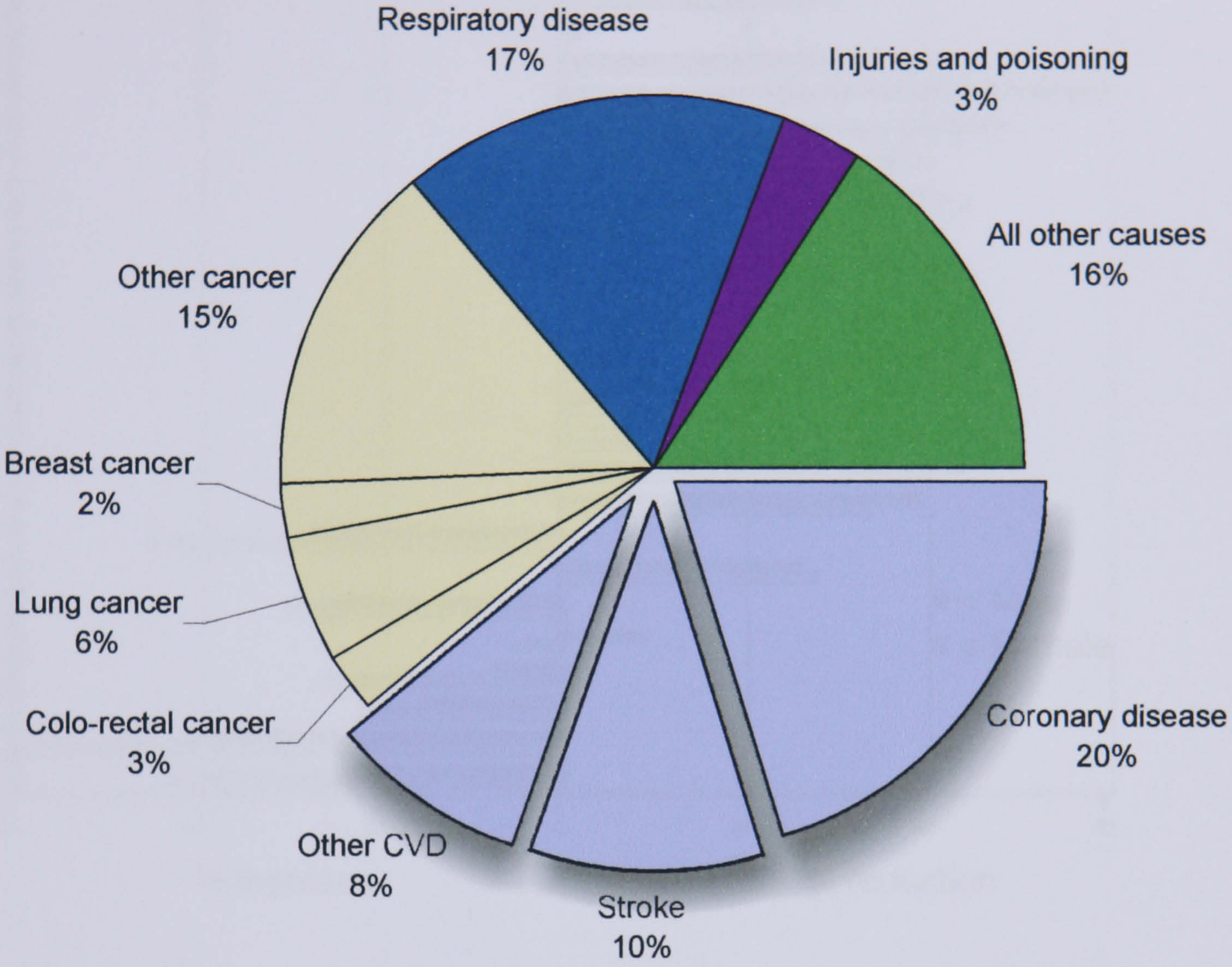


Figure 1.1. Causes of death in the UK in the year 2000. Cardiovascular disease (including CHD, stroke and other CVD) accounts for more deaths than any other cause, numbering 235,000, equivalent to 39% of all fatalities (taken from British Heart Foundation CHD statistics 2002; www.bhf.org.uk).

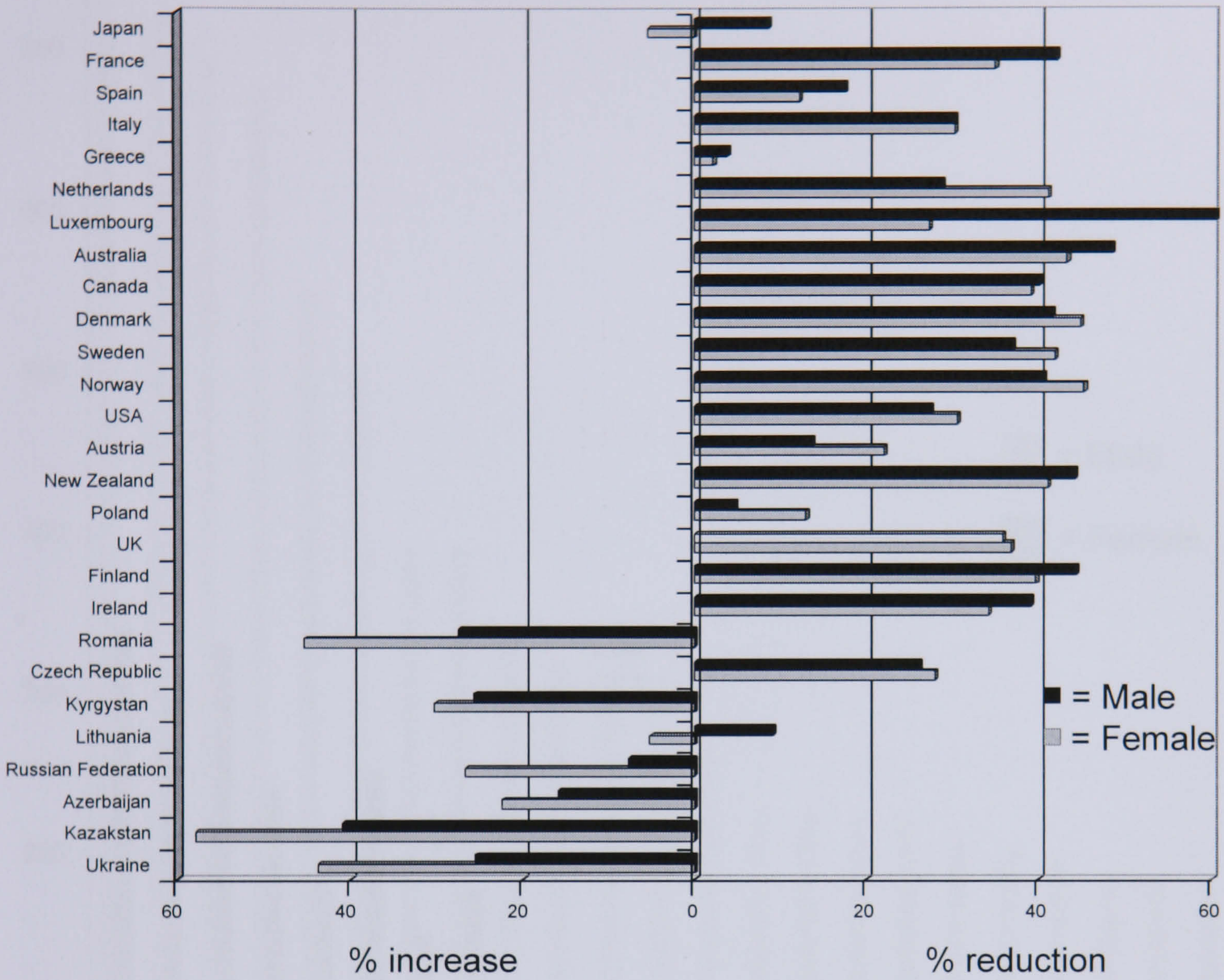


Figure 1.2. Percentage decrease in total incidences of CHD mortalities in selected countries worldwide from 1986 to 1996. Death rates have generally decreased, apart from many Eastern European countries which show worrying increases in the number of CHD deaths (taken from British Heart Foundation CHD statistics 2002; www.bhf.org.uk).

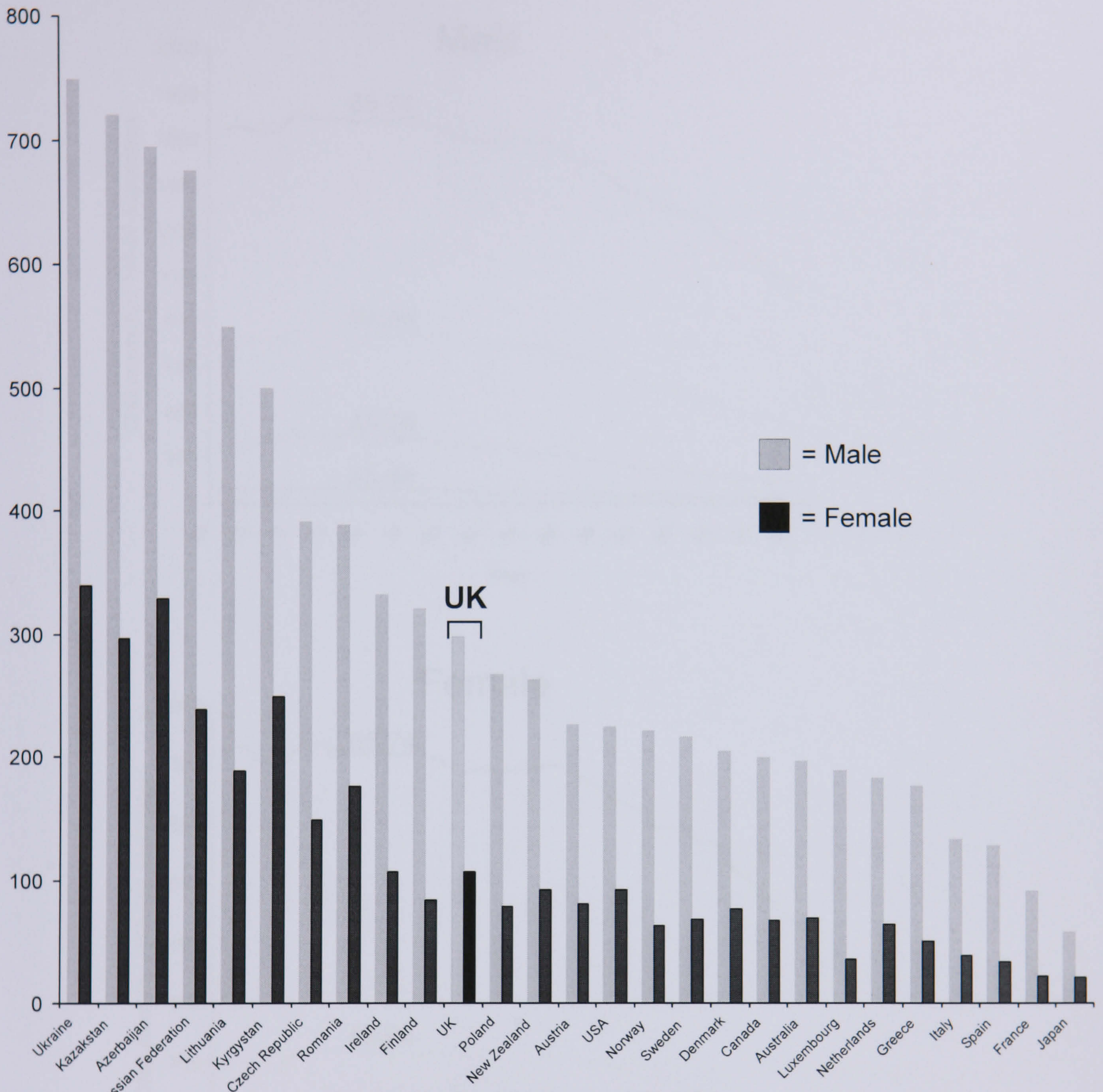
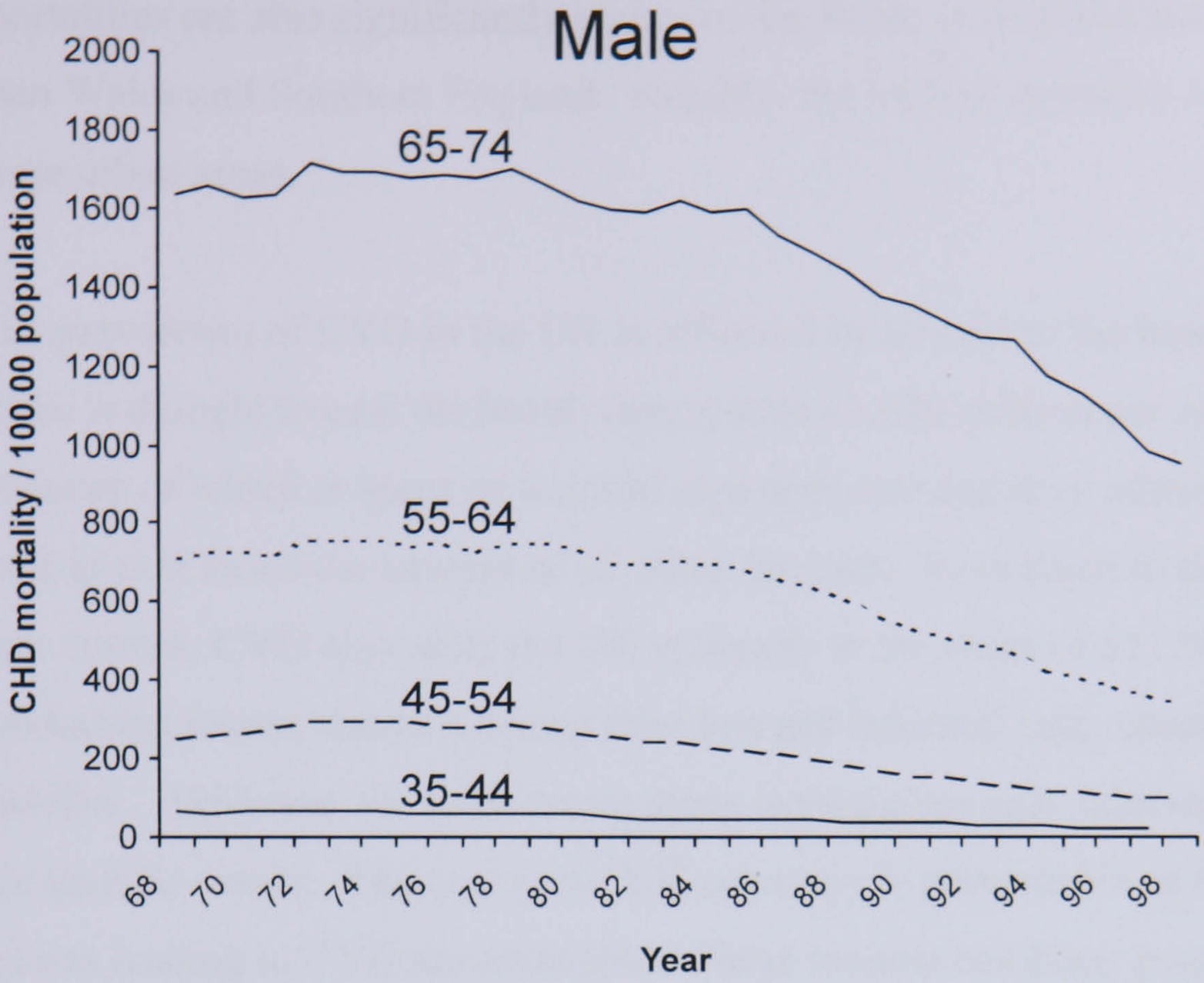


Figure 1.3. Death rates from CHD / 100,000 population in men and women aged between 35-74 years in 1996 in selected countries. CHD deaths in the UK are amongst the highest in the developed world. Death rates in the majority of Eastern European countries are far greater than the worldwide average (taken from British Heart Foundation CHD statistics 2002; www.bhf.org.uk).



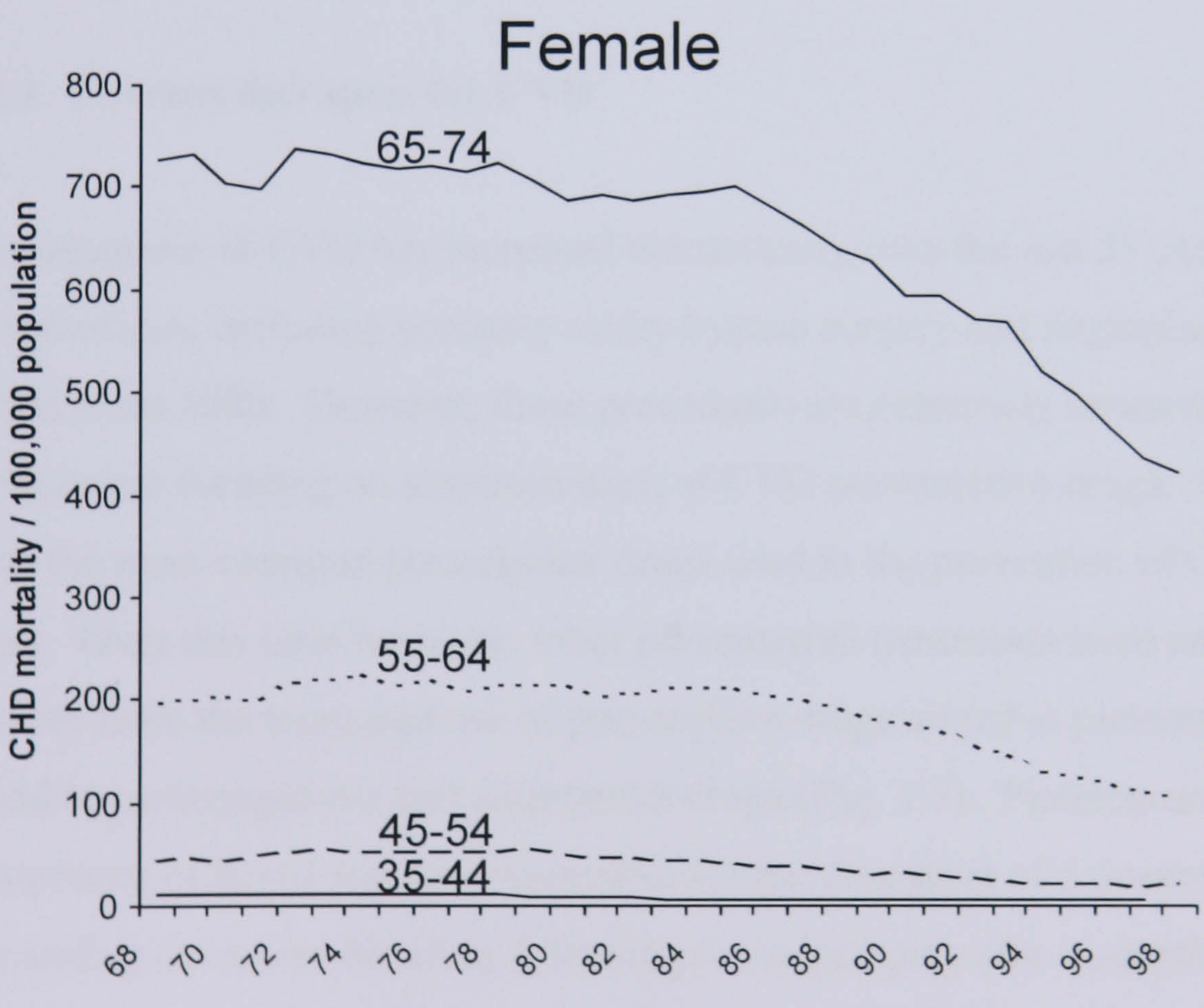


Figure 1.4. Age specific death rates in males and females / 100,000 population in the UK of CVD from 1968-1998. The death rates are higher in males and is significantly more prevalent in older age groups. Fatalities have decreased in all age groups, especially amongst the younger generations (taken from British Heart Foundation CHD statistics 2002; www.bhf.org.uk).

80% higher in men and women respectively than those living in East Anglia and CHD mortalities are also significantly higher in the North of England and Northern Ireland than Wales and Southern England. Notably, the highest mortality rates are localised to large urban areas.

The prevalence of CVD in the UK is reflected by its cost to the health services. CHD alone is thought to cost the health care system £1,600 million per annum (p.a.), the majority of which is spent on hospital inpatient care and drug administration. This total is over twice the amount of all other diseases. In addition to the costs to the health care system, CVD also costs the UK economy in the order of £11,000 million p.a. in production losses, due to working time lost and informal care, usually provided by families. However, the most recent study investigates only selected diseases and does not include cancer. The cost to the UK economy is preventable as the majority of risk factors leading to CVD are avoidable. These include smoking, poor diet, lack of physical activity, obesity, raised blood pressure and raised blood cholesterol.

1.1.1 Current therapies for CVD

The treatment of CVD has increased dramatically over the last 20 years. The number of operations, including coronary artery bypass surgery and angioplasty have increased 5-fold since 1980. However, these procedures are extremely invasive and health services are focusing on administration of CVD preventative drugs. Diuretics have been the most common prescription drugs used in the prevention of CVD in the last 20 years. Over this time however, other preventative treatments have emerged, most notably from the increased use of prescriptive drugs aimed at preventing clotting in the blood by anticoagulants and antiplatelet drugs (Fig. 1.5). Platelets are the smallest component of blood and are responsible for the formation of a thrombus (clot), preventing excessive bleeding following vascular injury (for in-depth platelet review, see Introduction 1.2). Platelets are activated at sites of injury, therefore any damaged vascular area, especially major and coronary arteries, is at risk of thrombus formation (Topol, 1998). Platelet activation and aggregation subsequently causes a positive feedback mechanism, resulting in the release of components promoting vasoconstriction and further platelet aggregation (Verheugt and Gersh, 2002). Blockages of blood vessels caused by the formation of clots, reduce the supply of

oxygen to the heart and the brain (ischaemia). often causing severe damage by ischaemic heart disease and stroke.

Aspirin is currently the most widely used anticoagulant and consistently reduces the incidence of ischaemic attack in high-risk patients (Theroux et al., 1988; The Risc Group, 1990) and is also beneficial when combined with low molecular weight heparin (The Frisc Study Group, 1996; Cohen et al., 1997; Harding et al.. 2002). Aspirin acts as an anticoagulant by inhibiting the production of the aggregation promoting thromboxane A2 by platelets (Fig. 1.6). Consequently, platelets exposed to aspirin do not respond to the pro-thrombotic agents collagen and adenosine diphosphate (ADP) (O'Brien, 1968; Zucker and Rothwell, 1978). Aspirin is cheap and as a preventative, greatly lowers the incidence of CVD in high-risk patients (Antithrombotic Trialists` Collaboration, 2002). However, recent evidence indicates an increase in aspirin resistance and fatal post-operative bleeding (Cambria-Kiely and Gandhi, 2002; Howard, 2002), leading to the development of improved therapies for modulating platelet function.

The use of antiplatelet therapies in the UK has increased nearly 60-fold in the last 20 years (Fig. 1.5). Antiplatelet drugs function by inhibiting the activation of the glycoprotein (GP) IIb/IIIa receptor on platelets, the role of which is to initiate the final stages of platelet aggregation (Harding et al., 2002) (Fig. 1.6). Intravenous administration of antibodies raised against GPIIb/IIIa, especially abciximab, has provided very positive results in numerous clinical trials (The CAPTURE Investigators, 1997; The PRISM Investigators, 1998; The PURSUIT Trial Investigators, 1998; The PARAGON Investigators, 1998; Chew and Moliterno, 2000; The GUSTO IV-ACS Investigators, 2001). Similar to aspirin, these antagonists create the potential to provoke major haemorrhage and are also extremely expensive (Verheugt and Gersh, 2002). Orally administered GPIIb/IIIa antagonists have proved significantly less successful. A recent report indicates that these drugs have no effect on ischaemic events and actually increase in the number of mortalities (Chew et al., 2001).

The control of ischaemic disease is currently managed by a combination of the previously mentioned therapeutics. However, the "all-or-nothing" irreversible effect

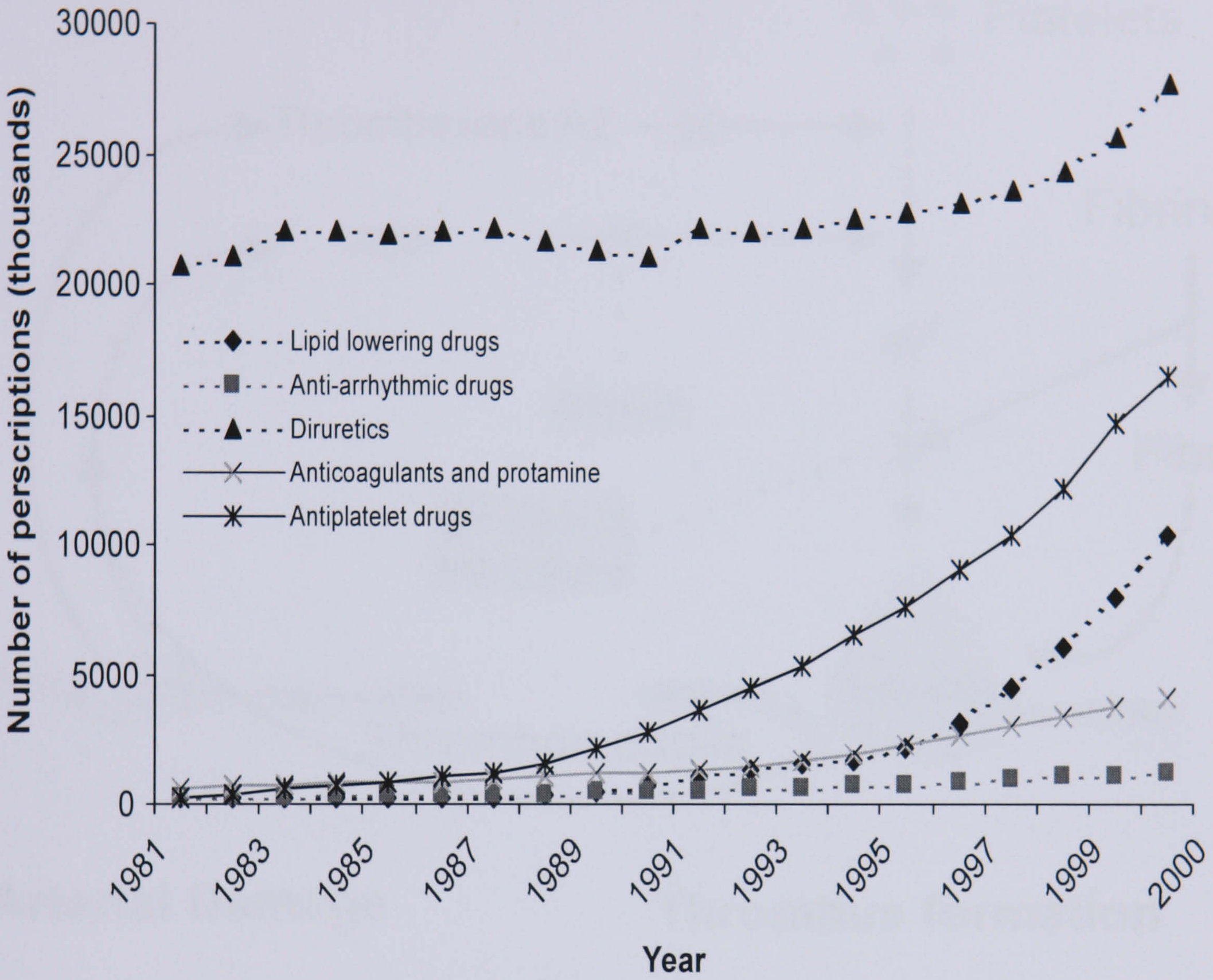


Figure 1.5. Selected prescriptions used in the prevention and treatment of CVD from 1981-2000 in England. The number of prescriptions for CVD have increased dramatically over this time, especially anti-platelet and lipid lowering drugs. The greater focus on CVD preventative drugs accounts for the decreasing death rates (taken from British Heart Foundation CHD statistics 2002; www.bhf.org.uk).

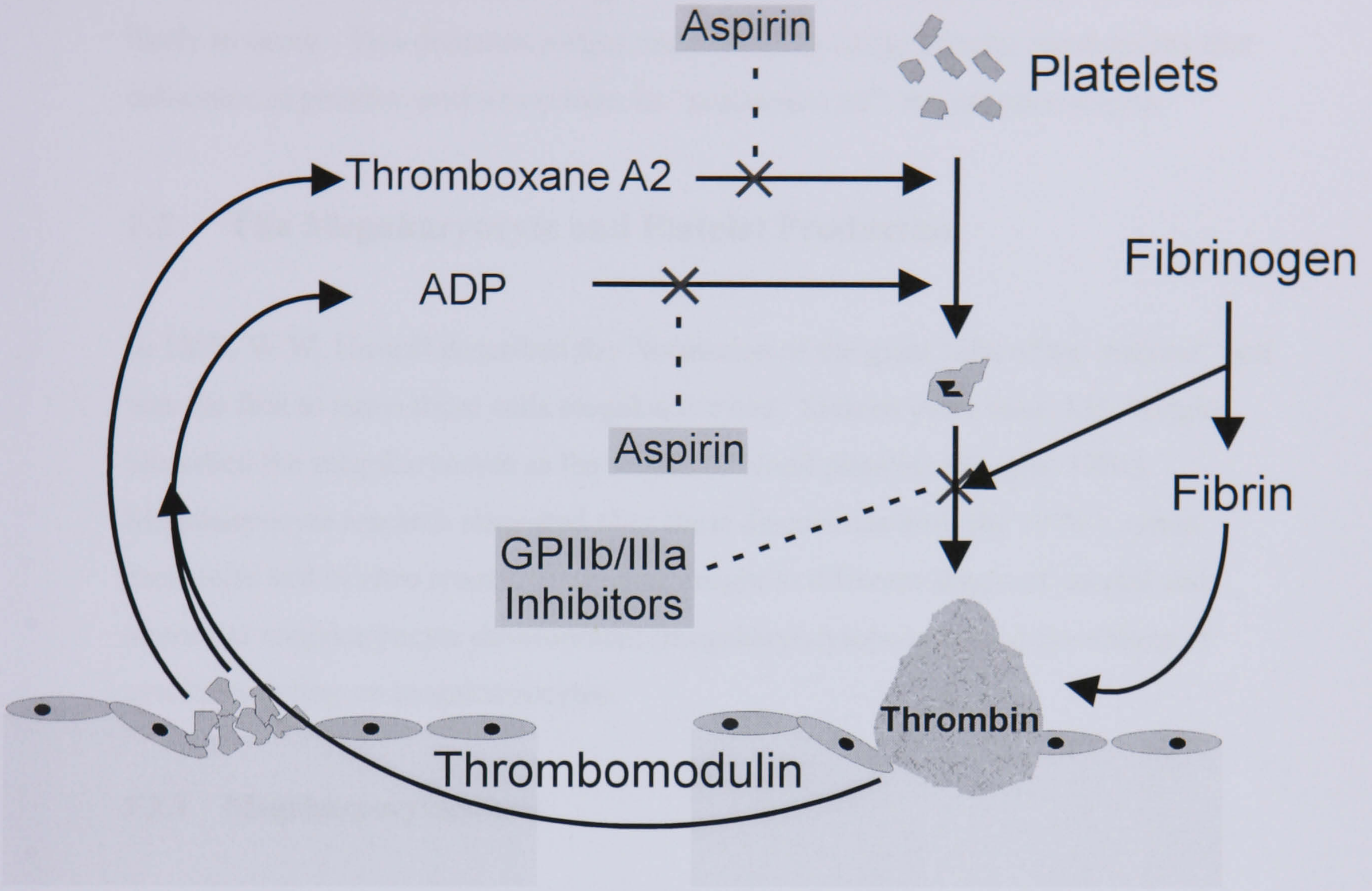


Figure 1.6. Diagram summarising the action of aspirin and GPIIb/IIIa inhibiting drugs on thrombus formation. Aspirin inhibits the actions of ADP and thromboxane A2, whilst the GPIIb/IIIa inhibitors prevent the binding of activated platelets to fibrinogen. The methods of both drugs successfully reduce the occurrence of CVD, but can complicate the process of coagulation, especially following surgery.

on all platelets causes subsequent detrimental effects. Rather than ensure that the circulating platelets do not function, a more effective mechanism of preventing ischaemia would be to tightly modulate the total number of circulating platelets. This would ensure that the risk of clotting would decrease, but haemorrhage would be less likely to occur. This demands a clear understanding of the cellular mechanisms that culminate in platelet production from its "precursor cell", the megakaryocyte.

1.2 The Megakaryocyte and Platelet Production

In 1890, W.W. Howell described the "formation of the giant cells of the marrow" and was the first to name these cells megakaryocytes. Sixteen years later, J.H. Wright identified the megakaryocyte as the source of blood platelets (Wright, 1906). Megakaryocyte research stagnated after these discoveries until the 1970's, when molecular and *in vitro* research provided insight to different stages of normal and abnormal megakaryocyte development (megakaryocytopoiesis) and the effects of cytokines acting on megakaryocytes.

1.2.1 Megakaryocytopoiesis

Megakaryocytes reside in a number of tissues. Predominantly these are the main locations of blood cell production, bone marrow and spleen. It is also notable that megakaryocytes have also been identified in the lung (Martin et al., 1983; Slater et al., 1983; Trowbridge et al., 1988) and whole cells passed from the bone marrow to the circulation (Tavassoli and Aoki, 1981). In the foetus, the megakaryocyte appears first in the yolk sac and then the liver before appearance in the spleen and marrow during embryogenesis (Gilman, 1942). The vast majority of megakaryocyte research focuses on cells within the bone marrow. Bone marrow fills the cylindrical cavities of long bones such as the femur and ulnae and is comprised of fatty "yellow" marrow and haematopoietic "red" marrow. Although only 0.5% of cells within the bone marrow are megakaryocytes, they are easily recognisable due to their size. A fully matured megakaryocyte is several-times the size of any other cell within the bone marrow, within the region of 30 μm in diameter.

Haematopoiesis is the process by which all mature blood cells are produced from multipotent haematopoietic stem cells (HSCs). HSCs are a small subset of blood cells with the potential to self-renew and in the relevant conditions, differentiate to all components of the haematopoietic system (Fig. 1.7). The HSC differentiates to the myeloid lineage, from which the megakaryocyte lineage-committed progenitor cell is derived (for review see Bruno and Hoffman, 1998). The earliest megakaryocyte-restricted progenitor cell to develop *in vitro* from the adult bone marrow is the burst forming unit megakaryocyte (BFU-MK) (Long et al., 1985; Brindell et al., 1989), followed by the colony forming unit megakaryocyte (CFU-MK) (Brindell et al., 1990), both of which contain only megakaryocytes. However, some mixed colonies do form, the most common is the BFU-erythroid/megakaryocyte (BFU-E / MK). The single BFU-E / MK cell has bi-potentiality and after 12 days in culture, mixed erythroblasts /megakaryocyte colonies form (Debili et al., 1996). This demonstrates just one of the associations between the differentiation of the megakaryocyte and the erythrocyte.

The CFU-MK is the most differentiated megakaryocyte progenitor cell. The later stages of differentiation are essentially characterised by morphological differences (Long, 1998). The immature megakaryocyte (or megakaryoblast) has a sparse cytoplasm and high nucleus to cytoplasm ratio, due to the large amount of protein synthesis at this stage. The nuclear and cytoplasm size both increase at the promegakaryocytic stage, in addition to the appearance of platelet-specific particles. Megakaryocyte development culminates in cytoplasmic maturation and nuclear endoreplication (see Chapter 1.2.2.). Platelets are then shed into the circulation, although the exact means of this remains unknown (see Chapter 1.2.3.).

During differentiation, megakaryocytes and their precursor cells express certain cell surface markers at stages of differentiation. All haematopoietic progenitor cells express CD34 during the early stages of differentiation. The expression of CD34 declines during megakaryocytopoiesis and is absent in the terminally differentiated cells. Cells of the megakaryocyte lineage express the specific lineage marker CD41 (also known as platelet glycoprotein or (GP)IIb), from the CFU-MK stage through to maturity. CD61 (GPIIIa) is expressed during early megakaryocyte

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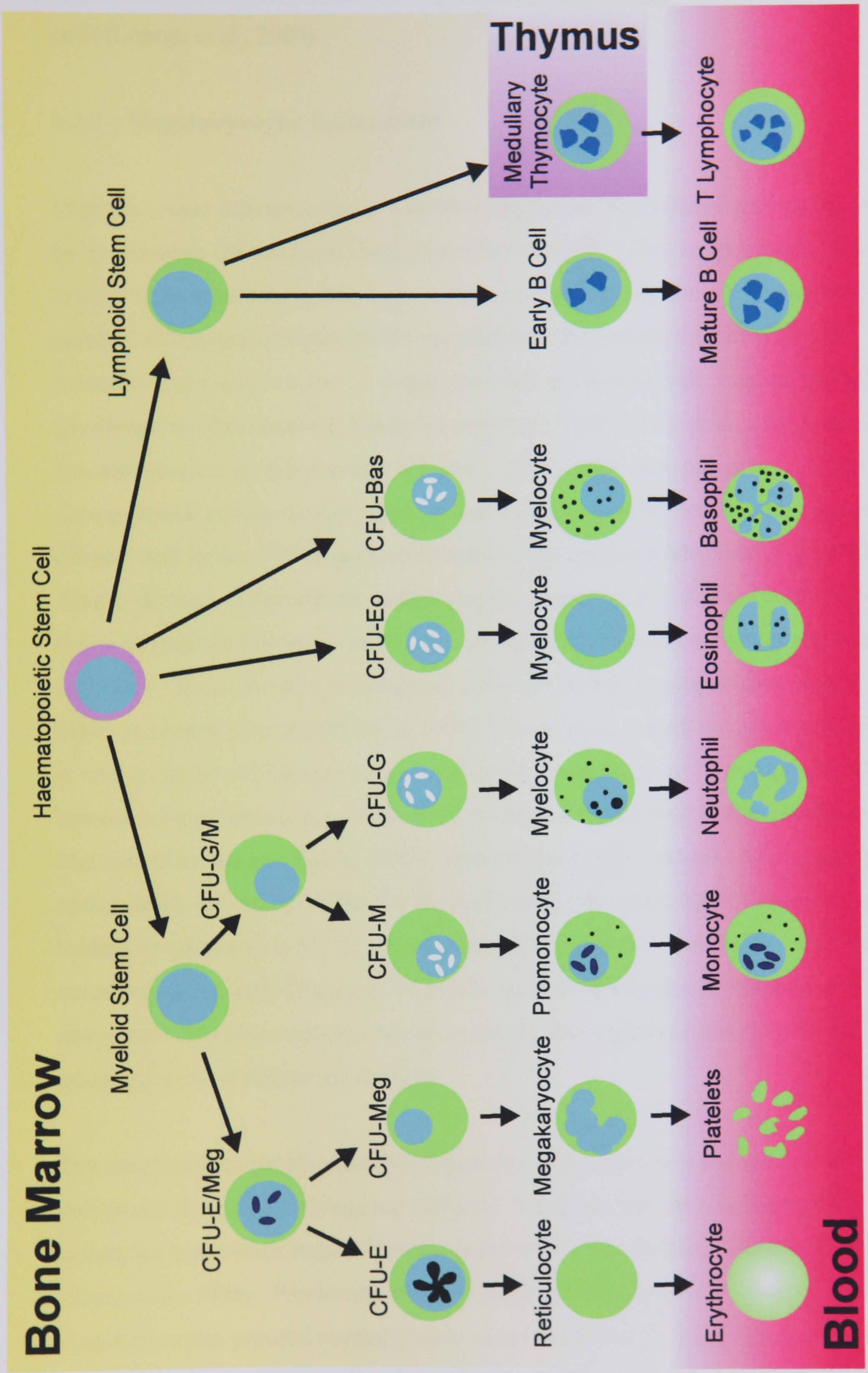

Figure 1.7. Diagram summarising the process of haematopoiesis. The haematopoietic stem cell (HSC) is able to self replicate and differentiate into all components of blood. The majority of haematopoiesis occurs in the red bone marrow, prior to release into the circulation. Megakaryocytopoiesis, the process of megakaryocyte and platelet formation, originates from a common erythroid / megakaryocyte progenitor cell in the myeloid lineage

differentiation, and CD42a (GPIX) expression is limited to terminally differentiated cells (Lepage et al., 2000).

1.2.2 Megakaryocytic Endomitosis

Megakaryocyte differentiation is characterised by the formation of a polyploid nucleus by endomitosis (reviewed in Zimmet and Ravid, 2000). This is a process in which nuclear replication takes place in the absence of cellular replication, leading to a large number of repeated chromosomes compared with the normal diploid (2N) cell. Endomitosis is not exclusive to megakaryocytes in the mammal. Cardiac myocytes (Sandritter and Scomazzoni, 1964), hepatocytes (Kudryavtsev et al., 1993) and arterial smooth muscle cells (Owens and Schwartz, 1983) all become polyploid at some point during normal cellular development as well as smooth muscle cells during pregnancy (Heiden and James, 1975) and thyroid cells in hyperthyroidism (Auer et al., 1985). Abnormalities in chromosome number are also common in malignant cells. However this is a condition known as aneuploidy, in which specific chromosomes are deleted or replicated. Quantification of aneuploid cells is now used in prognosis of prostate, colon and breast cancers (Barlogie, 1984). However, megakaryocyte polyploidisation is unique, as the cell becomes highly polyploid during normal differentiation. The immature megakaryocyte is a diploid (2N) cell and in this state is able proliferate at a high rate (Zimmer and Ravid, 2000). When the cell differentiates and undergoes endomitosis, the cell no longer divides and undergoes further endomitotic cycles, leading to cells of 4, 8, 16, 32, 64 and even 128N, with the mode being 16N. Polyploid megakaryocytes would therefore be able to produce much greater amounts of mRNA and protein than a normal diploid cell, enabling the megakaryocytes to perform the unique process of platelet production.

The exact mechanism by which the megakaryocyte achieves such high levels of polyploidy is a subject of ongoing research. It appears that the megakaryocyte undergoes a quiescent stage following cycles of nuclear division (Odell et al., 1968; Zhang et al., 1996). Previously, it was thought that during mitosis, polyploid megakaryocytes proceed normally up to anaphase A, but do not proceed with anaphase B, telophase or cytokinesis (Fig. 1.8) (Nagata, 1997). A more recent publication did however identify the characteristics of anaphase (DNA condensation, nuclear

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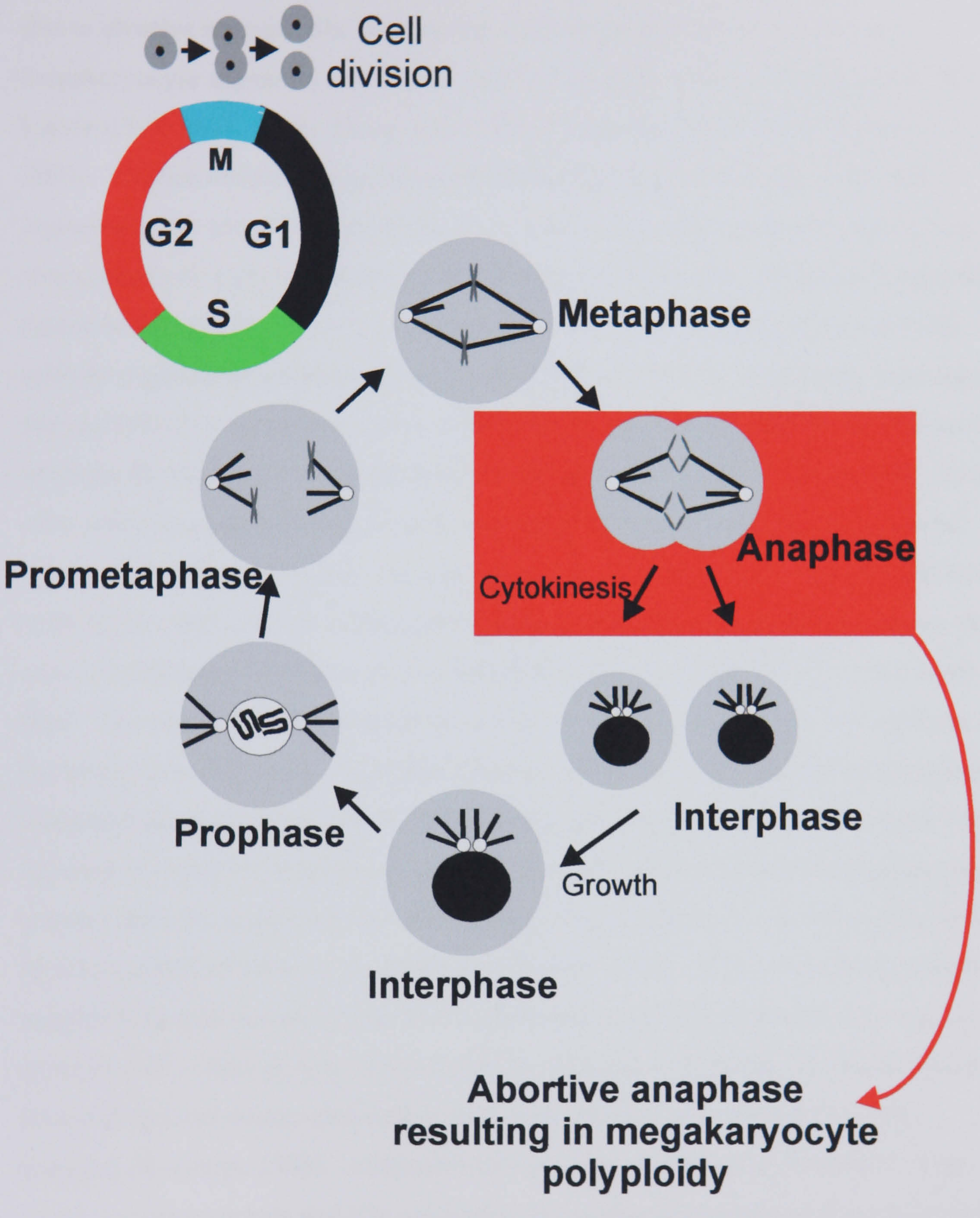

Figure 1.8. Schematic diagram of normal mitosis, showing the proposed mechanism for megakaryocyte polyploidy. The formation of a polyploid nucleus relies upon abortive mitosis, specifically cytokinesis in which the two daughter nuclei separate following chromosomal replication. Consequently, the megakaryocyte develops a nucleus with numerous copies of all chromosomes, up to 128N.

membrane breakdown and formation of a mitotic spindle), suggesting that only cytokinesis was absent in megakaryocytes compared to normal nuclear replication (Vitrat, 1998). It would therefore appear that the formation of a polyploid nucleus is due to abortive mitosis. The mechanisms underlying abortive mitosis in the megakaryocyte appears to involve the cell cycle regulator cyclin B-dependent cdc2 kinase (Datta et al., 1996; Zhang et al., 1996; Garcia and Cales, 1996, Carow et al., 2001). Cyclins are proteins synthesised during interphase of the cell cycle and degraded at the end of mitosis (Patel et al., 1999). Lowered levels of cyclin B1 causes megakaryocytic cells to undergo polyploidisation (Zhang et al., 1996) and ubiquitin-mediated degradation of cyclin B1 allows re-entry to the S-phase of the cell cycle without completing anaphase (Zhang et al., 1998). Datta and co-workers demonstrated that polyploidy in megakaryocytic cells is actually associated with increases in levels of cyclin B and cdc2, but it is the down regulation of the phosphatase cdc25-C (cdc2 is only active in a phosphorylated state) that causes the subsequent inactivity of cdc2 (Datta et al., 1996). However, considering the rarity of the megakaryocyte, combined with the fact that only 1% of megakaryocytes at anytime are undergoing mitosis, the exact mechanism of megakaryocytic polyploidisation may remain elusive for some time. The reason for megakaryocytes becoming polyploid appears to be important in the production of platelets. Idiopathic thrombocytopenic purpura (ITP) is an inducible immunologic condition in which platelet number is greatly reduced. This causes an increase in levels of megakaryocyte polyploidy as well as increases in megakaryocyte number and size, suggesting that the megakaryocyte endomitosis is responding to low platelet numbers (Mazur et al., 1988; Tomer et al., 1989). When circulating platelet number becomes normal, the level of megakaryocyte ploidy reverts to its normal modal score of 16N. Other reports also suggest that high levels of ploidy in megakaryocytes have a direct non-linear relationship with mean platelet mass (platelet volume x number) (Bessman, 1984). Atherosclerotic patients often display abnormally high ploidy megakaryocytes and it is postulated that as platelets produced from these cells are larger and more easily activated, they may have a causative effect on this condition (Brown et al., 1997). Work by Kuter and Rosenberg conclusively demonstrated the existence of a "feedback loop" between the number of circulating platelets and megakaryocyte ploidy (Kuter and Rosenberg, 1990). Thrombocytopenia induced in mice by application of anti-platelet serum correlates with increases in megakaryocyte ploidy, which is reduced by platelet transfusion. Therefore, in response to changes in

platelet number, megakaryocyte ploidy varies inversely with and proportionally to platelet change. The mediators of this feedback loop remain unclear. However, following the finding that circulating platelets actively bind TPO (Fielder et al, 1996), it seems likely that platelets can regulate the plasma TPO levels directly by its binding.

1.2.3 Platelet Production

Once the megakaryocyte develops a highly polyploid nucleus, the cytoplasm also undergoes a unique maturation process. Firstly, the cytoplasm develops a demarcation membrane system (DMS), the origin of which remains unknown. In immature megakaryocytes, the DMS is concentrated to local areas of the cell, but as the cell matures, the DMS is present in the vast majority of the cytoplasm. At this stage, primitive platelets begin to form within the cytoplasm of the megakaryocyte (known as platelet territories) and the DMS dilates to eventually fragment the cytoplasm and release the matured platelets (Nurden et al., 1997). During this process, platelet-specific proteins are expressed and small dense bodies known as α-granules, form. These contain substances vital for the function of the mature platelet, such as von-Willebrand factor (vWF), ADP, GPIIb/IIIa, serotonin and β-thromboglobulin (Long, 1998). α-Granule disorders, such as von Willebrand disease type III, Glazmann thrombasthenia and Gray platelet syndrome, although quite rare, often result in decreased platelet number and aggregation causing clotting abnormalities (Smith et al., 1997).

As previously eluded, the major function of the megakaryocyte is in the formation and release of mature, functional platelets. The various stages of differentiation ensure that the mature megakaryocyte cytoplasm is weak and easily ruptured and the platelets which are already formed can be released into the circulation. The process by which platelets are released from the mature megakaryocyte is termed thrombocytopoiesis. Each megakaryocyte can form between 1,000 and 5,000 platelets, which are subsequently released into the circulation, but the process by which this is achieved is poorly understood. The widely accepted process, hypothesised by Wright in 1910, is that megakaryocytes produce platelets from the bone marrow by forming long thin cytoplasmic structures known as proplatelets. As mature megakaryocytes in the bone

marrow are often observed in close proximity to blood-filled sinusoids (Tavassoli, 1980), it has been suggested that the proplatelet structures are able to invade the sinusoid and the circulating blood pressure will cause the proplatelet to disintegrate, resulting in platelet release in the sinusoid lumen. Scanning electron microscope (SEM) pictures have supported this idea (Ihzumi et al., 1977; Radley and Scurfield, 1980; Handagama et al., 1984), with the identification of such cytoplasmic extensions observed in the canine marrow sinusoid. It is also possible that entire intact megakaryocytes can enter circulation via the sinusoid, entailing transendothelial migration through parajunctional areas of the sinusoid, approximately 6μm in diameter (Tavassoli and Aoki, 1981). The other plausible method of platelet release is by passage of whole megakaryocytes from the marrow through the microcirculation of the lungs causing cytoplasmic disintegration with pulmonary macrophages destroying the released nuclei (Martin et al., 1993; Zucker-Franklin and Philipps, 2000). It is believed that splenic megakaryocytes only contribute to thrombocytopoiesis following complete bone marrow ablation (Layendecker and McDonald, 1982). The contribution of each site to circulating platelet number is far from clear, owing to major experimental problems with these investigations.

Apoptosis is the process of programmed cell death, often induced by extracellular signals such as a death signal or withdrawal of factors vital for cell survival. This process takes place in virtually all cells during development, to control cell number and eradicate senescent or unhealthy cells. The differentiated megakaryocyte however, is able to undergo apoptosis completely of its own accord, in the absence of extracellular stimuli (for review see Li and Kuter, 2001). Bcl-x is a member of the Bcl-2 family of potent antiapoptotic proteins and is expressed at very high levels almost throughout megakaryocytopoiesis. Bcl-x expression has however been shown to decrease dramatically in large terminally differentiated megakaryocytes on the verge of producing platelets (Sanz et al., 2001). Transgenic mice overexpressing Bcl-x in megakaryocytes showed, as expected, a reduction in the number of megakaryocytes undergoing apoptosis, although the majority of these cells were unable to form proplatelets in culture and lacked normal DMS morphology (Kaluzhny et al., 2002). Overexpression of Bcl-2 results in a 2-fold reduction in the number of circulating platelets, although megakaryocyte number remains unchanged (Ogilvy et al., 1999).

More recently a family of proteases, known as caspases, which are activated during apoptosis have also been implicated in the formation of proplatelets from bone marrow-derived megakaryocytes (De Botton et al., 2002). Cleavage and subsequent activation of procaspase-3 and –9 occurs in megakaryocytes undergoing proplatelet formation and caspase inhibition with the specific inhibitor z-VAD.fmk, inhibited proplatelet formation from mature megakaryocytes. When z-VAD.fmk is added to cells already undergoing platelet formation, it has no effects. These results are consistent with the theory that platelet formation occurs through a process similar to that of apoptosis.

1.3 Regulation of Megakaryocytopoiesis

A wide range of different cytokines regulates the proliferation. survival and differentiation of progenitor cells to mature platelet-producing megakaryocytes (summarised in Fig. 1.9). The majority of the cytokines identified so far are positive regulators, promoting the differentiation of the haematopoietic stem cells down the megakaryocyte lineage and a few have been identified that inhibit megakaryocyte differentiation (reviewed in Wendling and Han, 1997). This ensures that the number of circulating platelets is tightly regulated. Although effects of several different classes of cytokines are important in megakaryocytopoiesis. most of which will be mentioned here, the most potent effects appear to be mediated by thrombopoietin (TPO).

1.3.1 Thrombopoietin

The exact nature of TPO remained a mystery from when it was first named as the humoral substance responsible for platelet production in 1958 (Kelemen et al., 1958) until it was characterised and cloned in 1994 (de Sauvage et al., 1994; Lok et al., 1994; Kaushansky et al.; 1994; Wendling et al., 1994). TPO was identified as a polypeptide of 353 amino acids, presumed to consist of two domains due to its homology with the major regulator of erythrocyte development, erythropoietin (EPO). The 155 residues at the amino-terminal have 46 percent sequence similarity to EPO and binds to the TPO receptor, c-Mpl (Bartley et al., 1994). The carboxy-terminal has no homology to any haematopoietic cytokines and the deletion of this domain has no overall effect on thrombopoietin activity (Hokom et al., 1995). It is clear that TPO affects nearly every aspect of megakaryocyte development. TPO, in conjunction with other cytokines involved in cell proliferation. supports HSC survival and promotes entry to the megakaryocyte lineage (Kaushansky. 1995a; Ku et al., 1996; Sitnicka et al., 1996). TPO then stimulates expression of platelet-specific markers associated with megakaryocyte differentiation, such as CD61 (Kaushansky et al., 1994), causes the development of a polyploid nucleus by endomitosis (Debili et al., 1995) and triggers the maturation of the cytoplasm (Choi et al., 1995: Cramer et al., 1997). The profound effects TPO has on megakaryocytes *in vitro*, lead to the development of transgenic mice lacking the genes

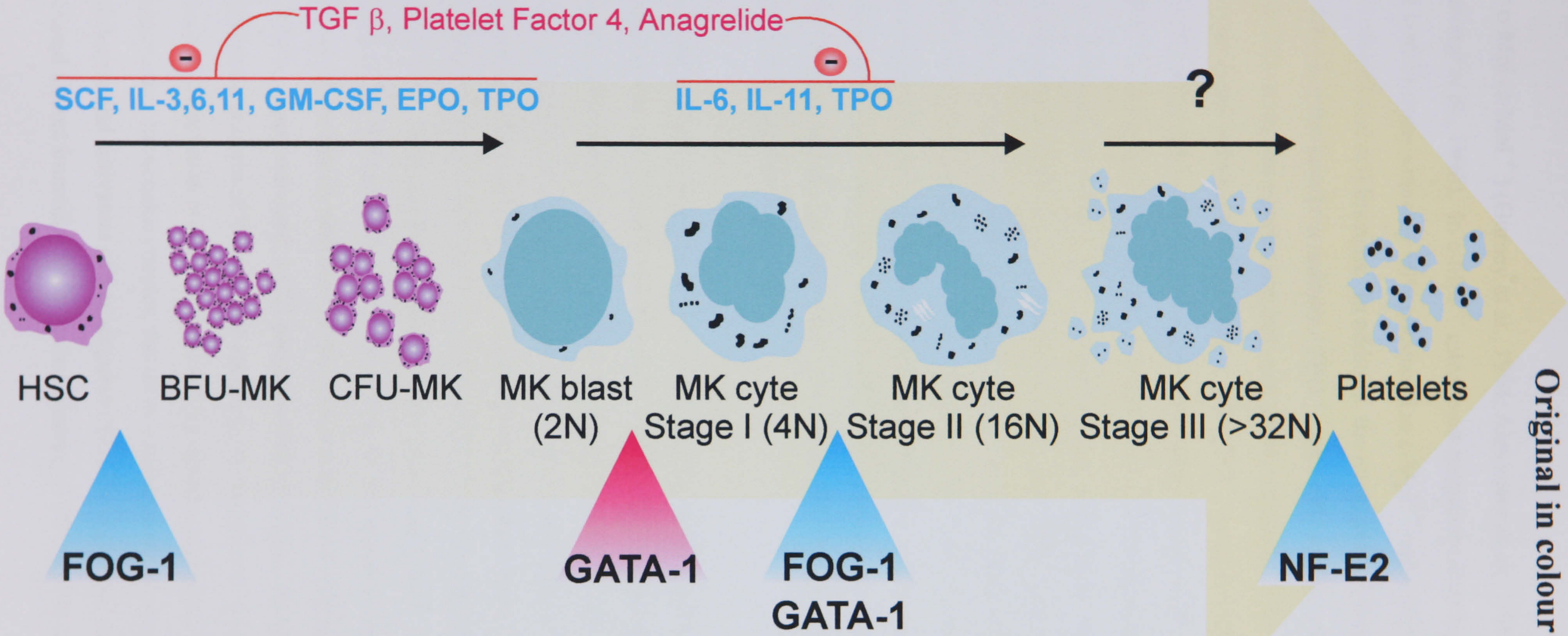

Figure 1.9. Diagram summarising the process of megakaryocytopoiesis and the regulation by cytokines and transcription factors. Megakaryocytes originate from the HSC and form colonies of megakaryocyte progenitors. Further differentiation gives rise to polyploid nuclei prior to cytoplasmic maturation and platelet release. Numerous cytokines positively regulate megakaryocytopoiesis (blue) whilst some appear to have a negative effect (red). Positive (blue) and negative (red) transcriptional regulation of megakaryocyte development centres around FOG-1, GATA-1 and NF-E2, each of which regulates a particular stage of differentiation and platelet release.

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for c-Mpl (c-Mpl$^{-/-}$) (Gurney et al., 1994; Alexander et al., 1996) or TPO (TPO$^{-/-}$) (de Sauvage et al., 1996). In c-Mpl$^{-/-}$ mice, both megakaryocyte and platelet numbers are reduced by approximately 90% compared to c-Mpl$^{+/+}$ and c-Mpl$^{+/-}$ without affecting any other blood cell lineages. In addition, the mean platelet volume is significantly increased in the knockout animals. TPO$^{-/-}$ mice also display a 90% reduction in megakaryocyte and platelet numbers and show a decrease in the levels of polyploidy in the knockouts compared to wild-types. The TPO$^{-/-}$ mice differ from the c-Mpl$^{-/-}$, as the TPO gene appears to work in a dose-dependant manner with heterozygous mice (TPO$^{+/-}$) with megakaryocyte and platelet counts intermediate of knockout and wild-type. However, in c-Mpl$^{+/-}$ mice, megakaryocyte and platelet counts are not significantly to wild-types. These data identify that TPO is the major regulator of megakaryocytopoiesis, acting through c-Mpl. The actions of TPO although most profound in megakaryocytes, are not restricted only to this lineage. TPO also enhances the proliferation and expansion of erythroid progenitors (Kaushansky et al., 1995b), CFU-granulocyte-macrophage (CFU-GM) lineage in normal mice (Kaushansky et al., 1996) and the proliferation of human and murine HSCs (Young et al., 1996; Kobayashi et al., 1996; Sitnicka et al., 1996).

The commercial availability of recombinant TPO has enabled the characterisation of signalling events induced by its interaction with c-Mpl. On binding TPO, c-Mpl becomes a homodimer resulting in the activation of numerous downstream signalling events (reviewed in Fig.1.10). Janus kinases (JAK) are already bound to the cytoplasmic domain of the receptor and following dimerisation, the increase in local JAK concentration causes cross phosphorylation and subsequent activation (Miyazaki et al., 1994). There are four members of the JAK family, JAK 1, 2, 3 and TYK2. Of these JAK2 is the most important in c-Mpl mediated signal transduction as it is the only one of the family activated in the presence of TPO (Tortolani et al., 1995; Mu et al., 1995). Once activated, JAK2 phosphorylates tyrosine residues located on the cytoplasmic domain of the receptor enabling the receptor to activate independent pathways (Drachman et al., 1999), as well as directly activating other signalling mechanisms. In megakaryocytes, the most studied substrate of JAK is the signal transducers and activators of transcription (STATs) family, of which STAT3 and 5 are activated almost immediately following binding of TPO to c-Mpl. STATs bind to

Figure 1.10 TPO-mediated signalling events in megakaryocytes. TPO has been shown to be the major regulator of megakaryocyte differentiation by activation of the JAK/STAT pathway (1), MAPK (2) and PI3-K (3) via adaptor molecules. Activation of these signalling pathways in megakaryocytes initiates changes in gene transcription, regulating megakaryocyte survival, proliferation and differentiation. For review of TPO signal transduction see Drachman et al., 1999.

phosphotyrosines situated on the receptor and are themselves then activated by JAK. The phosphorylated STATs form homodimers and translocate to the nucleus causing transcriptional activation (Dorsch et al., 1997). The adaptor proteins Grb2, SOS and Shc, the GTP exchange protein Vav and the phosphatase SHP-2 are also activated by JAK2 on c-Mpl almost immediately on binding TPO (Miyakawa et al., 2001). These proteins activate the mitogen activated protein kinase (MAPK) pathway, also culminating in the activation of transcription factors (Rojnuckarin et al., 1999).

Another c-Mpl–activated pathway of great importance to megakaryocyte development is phosphoinositide-3-kinase (PI3-K). Activation of c-Mpl induces the regulatory domain of PI3-K to undergo a conformational change and recruits the PI3-K kinase domain. Activated PI3-K can then activate the MAPK pathway, members of the protein kinase C (PKC) family and also AKT (also known as protein kinase B (PKB)) (Sattler et al., 1997; Geddis et al., 2001; Rojnuckarin et al., 2001; Majka et al., 2002). The c-Mpl-mediated activation of MAPK is thought to promote megakaryocyte proliferation and differentiation, whilst AKT, via its downstream targets NFκB, β-catenin, forkhead transcription factor and Bad, influences cell survival in addition to differentiation (Rojnuckarin et al., 2001; Majka et al., 2002).

TPO is clearly essential for correct megakaryocyte development. However its influence appears to be restricted to megakaryocyte progenitor cell proliferation and ploidy. C-Mpl$^{-/-}$ and TPO$^{-/-}$ mice although thrombocytopenic, do not bleed spontaneously as would be expected if TPO signalling were the single most important cytokine in platelet production. Bunting and co-workers (1997) showed that the remaining megakaryocytes and platelets from c-Mpl$^{-/-}$ and TPO$^{-/-}$ mice have normal ultrastructural morphology compared to wild-type. Functional studies demonstrated that platelets from these mice respond as normal to agonists such as ADP and fibrinogen. This suggests that the c-Mpl / TPO system is not solely responsible for the production of normal megakaryocytes and platelets. *In vitro*, mature megakaryocytes isolated from mouse bone marrow, do not disintegrate into platelets following administration of TPO, but instead require the presence of serum (Kaushansky, personal communication). This observation confirms in vivo studies using baboons injected for a 28-day period with human recombinant TPO, in which a 7-day delay in

increased platelet numbers was observed after the first administration of TPO (Harker et al., 1996). This work also suggests that the role of TPO is predominantly megakaryocyte proliferation rather than megakaryocyte differentiation and platelet production.

1.3.2 Other Positive Regulators of Megakaryocytopoiesis

As previously mentioned a wide range of cytokines, in addition to TPO, have positive effects on megakaryocyte differentiation. These remaining cytokines are however much less megakaryocyte-specific than TPO and influence the production of nearly all cells during haematopoiesis. Stem cell factor (SCF; also known as steel factor and C-kit) in conjunction with interleukin (IL)-3 and granulocyte macrophage colony-stimulating factor (GM-CSF) has profound effects on megakaryocyte progenitor proliferation, greatly increasing colony size (Brindell et al., 1991). Transgenic mice lacking either the gene for SCF or its receptor show a decrease in total megakaryocyte number, although platelet numbers do not decrease due to a concomitant increase in megakaryocyte size and consequently, the ability to produce more platelets (Hunt et al., 1992). These findings suggest that SCF primarily regulates progenitor cell proliferation. IL-3 appears to have a greater effect on megakaryocytopoiesis than GM-CSF, with IL-3 treatment increasing platelet numbers in both primates and humans (Farese et al., 1993) whilst GM-CSF has no significant effect on platelet number, but instead increases the number of CFU-MK colonies (Vannucchi et al., 1990; Stahl et al., 1991). A greater increase in platelet number has been demonstrated when IL-3 and GM-CSF were used in conjunction, compared to independent application (O'Shaughnessy et al., 1995).

The role of IL-6 family of cytokines, comprising of IL-6, Leukaemia inhibitory factor (LIF), IL-11, oncostatin M, ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1) has been the subject of extensive research in megakaryocytopoiesis. The majority of these studies have focused on IL-6, which promotes megakaryocyte development, increasing the number and size of cells, as well as endomitosis and proplatelet formation (Ishibashi et al., 1989; Debili et al., 1993). Increased serum IL-6 concentrations in rheumatoid arthritis usually results in reactive thrombocytosis (over-active megakaryocytopoiesis), indicating that IL-6 may be a cause of pathological

megakaryocytopoiesis (de Benedetti et al., 1991; Hollen et al., 1991). Doubt was cast on these findings however, when the IL-6 knockout mouse was shown to have no platelet number abnormalities (Kishimoto et al., 1994). IL-11 and LIF have similar effects to each other, both inducing the maturation of immature megakaryocytes *in vitro*. Mice treated with IL-11 or LIF show a 1-2 fold increase in the circulating platelet number, without increasing the overall number of megakaryocytes (Burstein et al., 1992; Teramura et al., 1992). This is believed to be due to IL-11 and LIF increasing the megakaryocyte ploidy levels. Phase I clinical trials using IL-11 have proved disappointing, having no effect on patients suffering from chemotherapy-induced thrombocytopenia (Gordon et al., 1996).

Other factors promoting megakaryocytopoiesis include EPO, which has a proliferative action on the bi-potential BFU-E/M cells. Mice treated with low levels of EPO show a small increase in platelet number, although chronic EPO treatment causes thrombocytopenia (Macdonald et al., 1987), presumably due to the majority of bi-potential cells being forced down the erythrocyte lineage (Broudy et al., 1995). Granulocyte colony stimulating factor (G-CSF) and fibroblastic growth factor β (FGFβ) also stimulate megakaryocyte development, possibly by working in conjunction with IL-3 (Glapsy and Golde, 1992).

1.3.3 Negative Regulators of Megakaryocytopoiesis

The majority of megakaryocytopoiesis research has focused on positive regulation. However, it is becoming increasingly apparent that negative regulation is a vital control mechanism for platelet release. The majority of these factors are actually released by megakaryocytes and platelets, inducing a negative feedback mechanism, inhibiting differentiation (Caen and Han, 1995; Gewirtz et al., 1995). Transforming growth factor β 1 (TGFβ1) has the greatest inhibitory effect on megakaryocyte development and is extremely potent, with picogram amounts irreversibly affecting both human and mouse megakaryocyte progenitors *in vitro* (Keller et al., 1991; Kuter et al., 1992; Xi et al., 1996) (Fig. 1.9). Subcutaneous injection of TGFβ1 for a 14-day period decreased platelet number (Carlino et al., 1990) but also increased the number of macrophages and granulocytes (Bursuker et al., 1992), indicating that TGFβ1 may have a bi-

directional effect on haematopoiesis. Platelet factor 4 (PF4), released from platelet α-granules upon activation (Mantovani et al., 1994), has an inhibitory effect on the proliferation of megakaryocyte progenitors *in vitro* and in vivo (Gerwitz. 1989; Han et al.. 1990). Heparin-like molecules expressed on the cell surface of megakaryocytes and platelets are essential for the binding of growth factors involved in the positive regulation of megakaryocytopoiesis such as IL-3. IL-6, GM-CSF, bFGF (Rider, 1993) and possibly TPO. Many of these growth factors need to bind to heparin before they can exert any effect on the cell. PF4 has a very high affinity for heparin-like molecules and therefore may block the binding site for positive regulators (Sato et al.. 1993).

Anagrelide is the most common treatment for thrombocythaemic (characterised by increased megakaryocyte and platelet number) conditions such as essential thrombocythaemia (ET). Anagrelide reduces megakaryocyte and platelet number and lowers the level of ploidy from 32N to a modal of 16N in ET-derived megakaryocytes (Lane et al., 2001; Tomer, 2002). Anagrelide therefore has the ability to inhibit megakaryocyte proliferation and differentiation. The mechanism by which anagrelide achieves this is unknown, although it has been shown to affect megakaryocyte post-mitosis (Mazur et al., 1992), identifying the possible means by which ploidy levels are decreased.

1.3.4 Transcriptional Regulation of Megakaryocytopoiesis

The molecular control of megakaryocytopoiesis still in part remains obscure, possibly due to the fact that no megakaryocyte "master gene" has been identified. However, three transcription factors, GATA-1, FOG-1 and NF-E2, have been implicated as chief regulators of megakaryocytopoiesis at distinct stages during differentiation (for review see Shivdasani, 2001).

GATA-1 and GATA-2 have been identified as a key erythro-megakaryocyte transcription factors with the majority of megakaryocyte-expressed gene containing GATA *cis*-elements (Lepage et al.. 1999). It is possible to identify the effects of GATA-1 on megakaryocytes and platelet production by using transgenic mice lacking the gene for GATA-1 (Shivdasani et al.. 1997) and also human subjects with critical

GATA-1 point mutations (Nichols et al., 2000; Freson et al., 2001). An 85% reduction in platelet number, increased bleeding time, increased platelet size and erroneous platelet shapes are some of the identifiable differences between knockout and wild-type mice and human patients compared to normal (Vyas et al., 1999). Interestingly, megakaryocytes derived from GATA-1-null mice show massively increased levels of proliferation, although the resulting cells are small and do not display any mature morphology. It could therefore be hypothesised that GATA-1 inhibits proliferation and initiates aspects of megakaryocyte differentiation.

FOG (Friend Of GATA)-1 is a co-factor of GATA-1. The megakaryocyte phenotypes of mice completely lacking GATA-1 are very similar to humans with point mutations preventing interaction between GATA-1 and FOG-1. It could therefore be a distinct possibility that the characteristics of GATA-1 ablation could be due to the lack of FOG-1 function (Nichols et al., 2000). However, mice deficient in only FOG-1 show a complete lack of megakaryocyte progenitors, implicating a role in earlier megakaryocyte development (Tsang et al., 1998). Consequently, FOG-1 may be a positive regulator of megakaryocyte progenitor proliferation and differentiation. So it would appear that FOG-1 acts independently of GATA-1 during early megakaryocyte differentiation and again in conjunction with GATA-1 in later megakaryocytopoiesis.

The NF-E2 transcription factor is comprised of two domains. The 45kDa domain is expressed exclusively in haematopoietic cells whilst the other 18kDa domain is expressed in a wide range of cells. The role of the NF-E2 was initially investigated in erythrocyte differentiation, when its role in platelet production was identified. NF-E2 gene ablation results in mild erythrocyte phenotype (Shivdasani et al., 1995a), whilst platelet number was profoundly reduced (Shivdasani et al., 1995b). Apart from the great reduction in platelet number, the megakaryocytes differentiate normally, forming cells with a mature cytoplasm and high levels of polyploidy. The terminally mature megakaryocytes appear not to be able to form and release platelets in vivo and NF-E2 mutated mice completely lack circulating platelets. The transcriptional targets of NF-E2 would therefore appear to be involved in the final stages of platelet release. A likely target is β1 tubulin, which is restricted to haematopoietic cells and limited to only mature cells in the megakaryocyte lineage. Expression of β1 tubulin is reliant on

NF-E2 and localises to the proplatelet shafts during *in vitro* thrombopoiesis (Lecine et al., 2000) and mice lacking β1 tubulin do not produce platelets (Schwer et al., 2001). This certainly provides a feasible answer as to why NF-E2-null mice are so severely thrombocytopenic, although other genes involved in the extracellular matrix reorganisation of platelet production may be under the control of NF-E2.

The effective control of normal and pathological megakaryocyte development relies upon a greater understanding of cellular and molecular events during differentiation. The identification of TPO as a principle regulator of megakaryocytopoiesis has certainly been the most promising advance in the understanding of megakaryocyte biology since the discovery of their function. However, TPO fails to complete the story of megakaryocytopoiesis by leaving out the final chapter. Cytokines regulating platelet release remain a mystery.

1.4 Megakaryocytes and the Bone Marrow Microenvironment

The bone marrow is tightly packed with numerous cell types, most of which will constantly interact in the regulation of differentiation and function. The megakaryocyte is no exception and has been shown to modulate and be modulated by numerous different neighbouring cell types within the bone marrow microenvironment. One such interaction currently the subject of intensive study is the role of the megakaryocyte in bone remodelling (for review see Compston, 2002).

Bone and bone marrow can be considered as one concurrent tissue. Not only are they adjacent, they share blood supply, as marrow sinuses receive blood from arteries supplying nutrients to bone. The process of bone remodelling is effectively a balance between osteoblasts which form the calcified bone and the osteoclast, which resorbs bone (for review see Seeman, 2002). A disruption in this balance results in pathological conditions, the most common of which is osteoporosis. Osteoclasts are derived from marrow HSCs (Teitelbaum, 2000), whilst osteoblasts differentiate from stromal stem cells, also present in the marrow (Owen, 1988). Megakaryocytes express TGFβ and TGFβ receptors (Bord et al., 2001), osteonectin, osteocalcin (Kelm et al., 1992; Thiede et al., 1994), oestrogen receptors (Bord et al., 2001) and RANK ligand

(Kartsogiannis et al., 1999), all of which regulate bone remodelling, implying a role for megakaryocytes in bone remodelling. TPO has profound effects on normal bone remodelling by inhibiting osteoclastogenesis (Wakikawa et al., 1997) and causing osteoporosis in mouse models in which TPO is over-expressed (Yan et al., 1996). Postmenopausal women undergoing high-dose oestrogen replacement therapy to prevent excessive bone-loss by osteoporosis, display a significant increase in megakaryocyte number (Bord et al., 2000), presumably due to activation of the megakaryocytic oestrogen receptor. Osteopetrosis, a condition characterised by excessive bone formation, has been induced in transgenic rats. These animals known as "toothless" (tl/tl) are thrombocytopenic, but do however show an increase in megakaryocyte number and size (Thiede et al., 1996). In addition, hyperproliferative megakaryocyte conditions such as myelofibrosis often occur in conjunction with the bone hardening disease, osteosclerosis (Poulsen et al., 1998). Only speculative conclusions can be drawn on the role of the megakaryocyte in bone remodelling, but it certainly appears that dysfunctional bone conditions nearly always coincide with changes in megakaryocyte number and function.

Megakaryocyte differentiation can also be modulated via interactions with both endothelial and stromal cells present in the bone marrow. HSCs cocultured either in direct contact with or in the presence of bone marrow endothelial cells (BMEC) tend to differentiate to the myeloid lineage, especially megakaryocytic (Rafii et al., 1995). After 14 days of coculture, in the absence of any exogenous cytokines, 80% of HSCs were myeloid (CD15 or CD14 positive) and 20% were megakaryocytes (CD61 or CD41 positive). BMECs are able to produce IL-6, SCF, G-CSF and GM-CSF without stimulation, all of which are able to initiate and support megakaryocytopoiesis. Conversely it appears that bone marrow stromal cells (BMSC) can regulate megakaryocyte differentiation (Zweegman et al., 2000). *In vitro* coculture of HSCs in direct contact with BMSCs differentiated to BFU-MK and CFU-MK, but did not differentiate past that point. However, HSCs grown in the presence of, but not in direct contact with BMSCs did become large, polyploid, mature megakaryocytes. It would therefore appear that the interactions between megakaryocytes and numerous different cell types could affect cellular differentiation and function.

1.5 Megakaryocytic Disorders

The majority of megakaryocytic diseases come under the heading of the myeloproliferative disorders (MPD), in which excessive proliferation of abnormally developed megakaryocytes results in very high platelet numbers and myelofibrosis (for reviews see Briere et al., 1997; Cripe and Hromas, 1998). Three of the most common malignant megakaryocytic diseases are essential thrombocythaemia (ET), polycythaemia vera (PV) and agnogenic myeloid metaplasia (AMM).

ET is usually diagnosed incidentally, by the presentation of high platelet number following blood sample examination. ET bone marrow trephines are characterised by profoundly elevated in numbers of enlarged megakaryocytes that usually cluster in small groups (Georgii et al., 1996). Although platelet number is increased, the platelets can either be over-reactive causing thrombosis, or under-reactive resulting in haemorrhage. At first it was thought that the discovery of TPO function and the cloning of c-Mpl, would lead to a greater understanding of all MPDs and especially ET. However, there is no evidence of c-Mpl mutations, a reduction in level of c-Mpl expression, differences in serum TPO levels or c-Mpl-mediated signalling in ET patients (Kiladjian et al., 1997; Horikawa et al., 1997). This suggests strongly that the c-Mpl/TPO signalling pathway is not altered in ET. The prognosis of ET patients is good. There is no significant decrease in lifespan (Fenaux et al., 1990) and only 10% of patients suffer from haematological events (Cortelazzo et al., 1990), 13% of which are haemorrhage (Hehlmann et al., 1988), the remaining cases being vascular thrombotic disorders. However, 4.5% of patients suffering from ET do develop acute leukaemia as a direct cause, possibly due to hydroxyurea treatment (Sterkers et al., 1998).

ET can also develop into another megakaryocytic MPD, PV (Murphy et al., 1997). PV is similar in many aspects to ET. Megakaryocyte number and size are both increased as well as platelet number although not to the same extent as ET. The difference to ET is the increase in erythrocyte number and morphology and the development of myelofibrosis. Myelofibrosis results from dramatic increases in the levels of platelet-

derived growth factor (PDGF) and TGF-β from the malignant megakaryocytes. This stimulates the bone marrow fibroblasts and causes the production of fibronectin and collagen, resulting in fibrous outgrowths into the marrow (Ross et al., 1986). Myelofibrosis in ET is extremely rare, but by approximately 4 years after presentation, 20% of PV patients develop bone marrow fibrosis (Georgii et al., 1996). Myelofibrosis is the prominent abnormality in AMM, with over 50% of bone marrow being fibrotic 4 years after presentation. The large amount of fibrotic tissue in the marrow cavity and subsequent loss of progenitor cells results in splenomegaly, enlargement of the spleen due to increase blood cell production and extramedullary haematopoiesis. Extramedullary haematopoiesis can also occur in the central nervous system, such as the brain or spinal column or in the pleural and peritoneal cavities. This results in the enlargement of these tissues causing haemorrhage or possible seizures.

Thrombocytopenic conditions caused by dysfunctional megakaryocytopoiesis are less common than the previously mentioned malignant conditions. X-linked thrombocytopenia is believed to be due to a 2-base mutation on the X-chromosome leading to the substitution of a single amino acid. This substitution inhibits the interaction between GATA-1 and FOG-1, resulting in severe thrombocytopenia and large platelets (macrothrombocytes) (Mehaffey et al., 2001). Amegakaryocytic thrombocytopenia is present at birth and develops into other haematopoietic cell cytopenias during childhood. 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., 1999; Ballmaier et al., 2001). Patients' megakaryocyte colony formation does not respond to TPO treatment and point mutations have since been discovered in the c-Mpl gene in all patients in the studies. It has therefore been concluded that this condition is caused by the absence of response to TPO by c-Mpl. The finding that defective TPO signalling is the single cause only of this very rare disease suggests that other factors are involved in the pathogenesis of other megakaryocyte disorders. One such factor recently implicated in megakaryocyte differentiation is glutamate signalling (Genever et al., 1999a).

1.6 Glutamate Signalling in the Central Nervous System

The central nervous system (CNS) is subtly regulated by excitatory and inhibitory neurotransmission with almost the entirety of the former being mediated by the amino acid glutamate. Glutamatergic synaptic activity can essentially be divided into four stages; 1. The passage of an action potential along the axon of the pre-synaptic cell causes the vesicular release of glutamate into the synaptic cleft. 2. Released glutamate diffuses across the synaptic cleft where it binds to glutamate receptors located in the extracellular membrane of the post-synaptic cell. 3. Binding of the glutamate agonist to the receptor results in receptor activation, leading to an influx of charged ions such as Ca^{2+} and Na^{+} or activation of coupled G-proteins in to the post-synaptic cell, causing depolarisation, continuing the action potential and the activation of a wide range of downstream signalling events. 4. The termination of synaptic transmission by the removal of glutamate from the synaptic cleft, via glutamate transporter expressing neighbouring neuronal and glial cells (Fig.1.11). Abnormal glutamate signalling has been implicated in a range of neurological conditions, including epilepsy, ischaemic brain damage, Huntingdon's chorea, Alzheimer's, Parkinson's disease and amyotrophic lateral sclerosis (reviewed in Choi and Rothman, 1990; Lee et al., 1996). Glutamate-containing vesicles translocate to the area of the pre-synaptic neuron termed the active zones. Following stimulation, the vesicles dock with the plasma membrane and the vesicular membrane fuses with the pre-synaptic plasma membrane, releasing the vesicular glutamate into the synaptic space. The process of tethering the vesicle to the plasma membrane requires the interaction of specific proteins, known as the SNARE (soluble N-ethyl maleimide-sensitive factor attachment protein receptors) hypothesis, present on both the vesicle and the plasma membrane. The vesicle (v)-SNARE proteins bind with the target (t)-SNARE proteins on the plasma membrane resulting in the fusion of the membranes and subsequent glutamate release.

1.6.1 Metabotropic Glutamate Receptors

Glutamate receptors can be classified into two main groups: ionotropic, those with an integral ion channel and metabotropic, which are coupled to G-proteins. The finding

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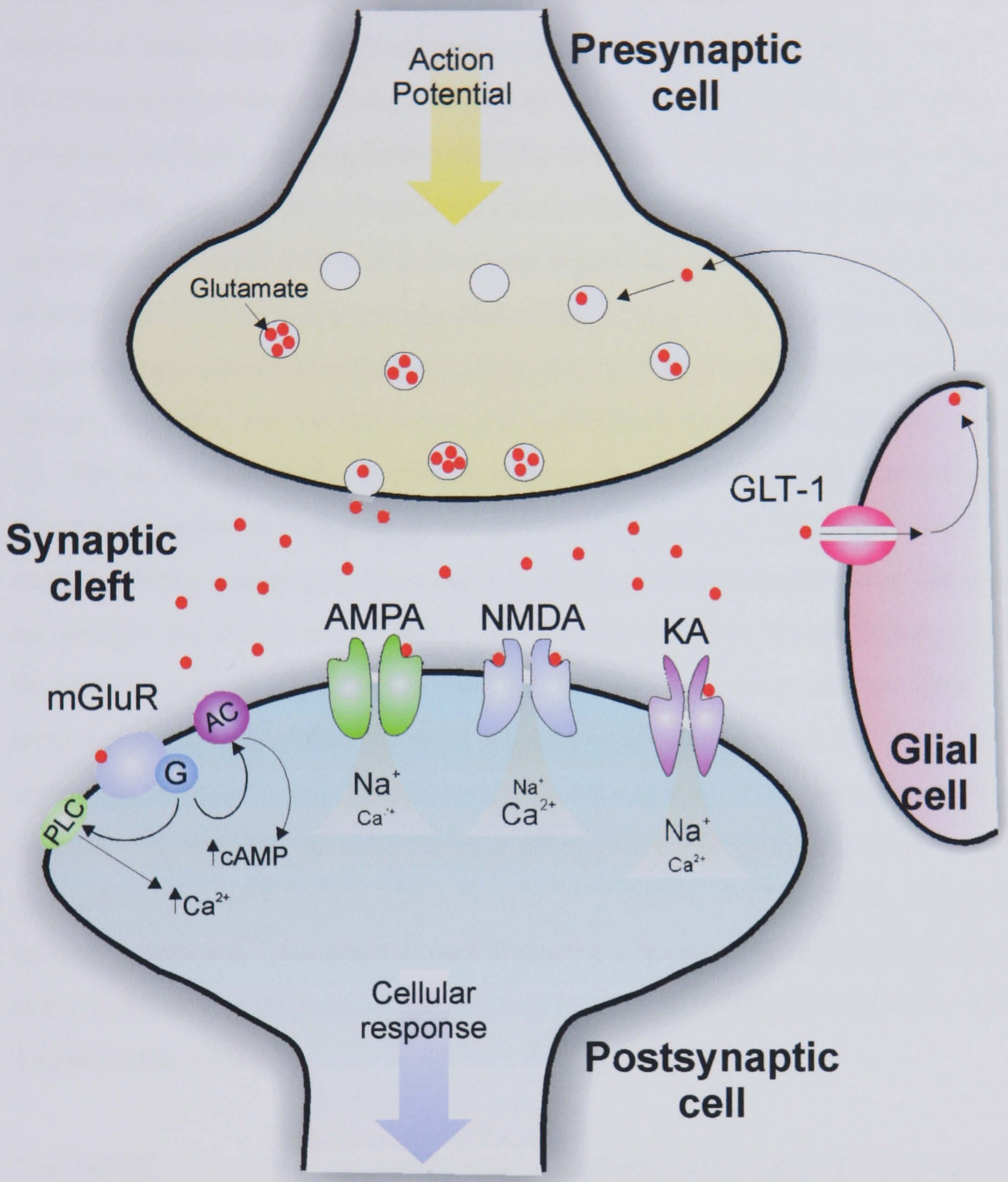

Figure 1.11. Glutamate signalling at CNS synapses. In the presence of an action potential, glutamate-containing vesicles dock with the presynaptic cell membrane and release glutamate into the presynaptic cleft. Glutamate binds to a range of receptors situated on the postsynaptic cell, resulting in numerous cellular responses. Glutamate is removed from the synaptic cleft via glutamate transporters such as GLT-1, terminating the signal and recycled to the presynptic cell.

that glutamate was able to activate inositol phosphate metabolism in striatal and cerebellar granule cells (Sladeczek et al., 1985; Nicoletti, 1986) was supported by the discovery that *Xenopus* oocytes injected with cerebral mRNA develop Cl^- oscillations when stimulated with glutamate, due to inositol-1,4,5-triphosphate (IP_3)-mediated release of intracellular Ca^{2+} (Sugiyama et al., 1987; Murphy and Miller, 1988). Resulting studies identified the existence of a family of metabotropic glutamate receptors (mGluR), ranging from mGluR-1 through to mGluR-8 (for review see Ozawa et al., 1998). The family subtypes are structurally related, but have different selective agonists and activate different downstream signalling events. The mGluR family have also been divided into three groups depending on amino acid sequence, agonist response and subsequent signalling activation. mGluR-1 and mGluR-5 are members of group I, mGluR-2 and mGluR-3 group II and mGluR-4 and 6-8 are members of group III. The structure of different mGluR subtypes are however similar, consisting of an N-terminal approximately 550 amino acid residues in size, a 250 amino acid 7 transmembrane spanning domain and a C-terminal domain the length of which depends on subtype (for review see Hollmann and Heinemann, 1994; Dingledine et al., 1998). The mGluR subtypes that are members of the same group show similar signal transduction characteristics. Group I receptors stimulate Ca^{2+} release from intracellular stores via phospholipase C (PLC)-mediated formation of IP_3 (Masu et al., 1991). The mGluR-1 receptor also activates the signalling molecules cyclic adenosine monophosphate (cAMP) and arachidonic acid (Aramori and Nakanishi, 1992), whilst mGluR-5 does not. The group II and III mGluRs function by inhibiting adenylate cyclase and as a consequence reduces the formation of cAMP (Tanabe et al., 1992; Tanabe et al., 1993).

The mGluRs function in numerous neuronal actions, including neuronal excitability by the inhibition of K^+ currents resulting in depolarisation (Charpak et al., 1990), suppression of presynaptic neuroligand release (Nakanishi, 1994) and synaptic plasticity in the cerebellar cortex (Ozawa et al., 1998).

1.6.2 Ionotropic Glutamate Receptors

In contrast to the mGluRs, ionotropic glutamate receptors (iGluR) are ligand-gated ion channels, which on activation cause an influx of ions into the cell directly through the

receptor (for reviews see Hollmann and Heinemann, 1994; Ozawa et al., 1998; Dingledine et al., 1999). Three families of iGluRs exist, all of which are named after their pharmacological agonist. These families are: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), N-methyl-D-aspartate (NMDA) and kainate. AMPA receptors are found ubiquitously throughout the CNS, although different regions do show varying levels of expression (Monaghan et al., 1984). Kainate receptors demonstrate similar local expression patterns to AMPA receptors, although strong levels are observed in hippocampal regions (Represa et al., 1987). NMDA receptors are found throughout the brain but are primarily located in the forebrain (Monaghan et al., 1989). The iGluRs all have similar structure, consisting of an extracellular N-terminal and intracellular C-terminal and three transmembrane spanning domains named M1, 3 and 4 as M2 is re-entrant loop that faces the cytoplasm and does not transverse the membrane. Amino acid residues within the re-entrant loop are thought to manage the ion permeability of each receptor type. The S1 region, located before M1 and the S2 region, located after M3 are believed to be the agonist-binding site (see Fig. 1.12). The primary function of iGluRs lies in the formation of experienced-based learning and memory, via a complex molecular mechanism referred to as long-term potentiation (LTP).

1.6.2.1 AMPA and Kainate receptors

AMPA receptors are present at most synapses in the CNS and trigger fast excitatory neurotransmission. Four AMPA subunits have so far been identified. The first, GluR1 was isolated from a rat brain cDNA library (Hollmann et al., 1989) and lead to the discovery of three further structurally related subunits, GluR2, 3 and 4, all of which are approximately 900 amino acids in size (Hollmann and Heinemann, 1994) which form either hetero or homo pentamer receptors. All of the AMPA subunits are able to exist as two different variants, termed "flip" and "flop", which are created via alternative splicing of a 115bp extracellular region above the M4 domain (Sommer et al., 1990). This feature, in conjunction with other RNA-mediated modifications of subunit amino acid sequence, results in the AMPA receptor being exceptionally diverse and able to produce a wide range of functional responses.

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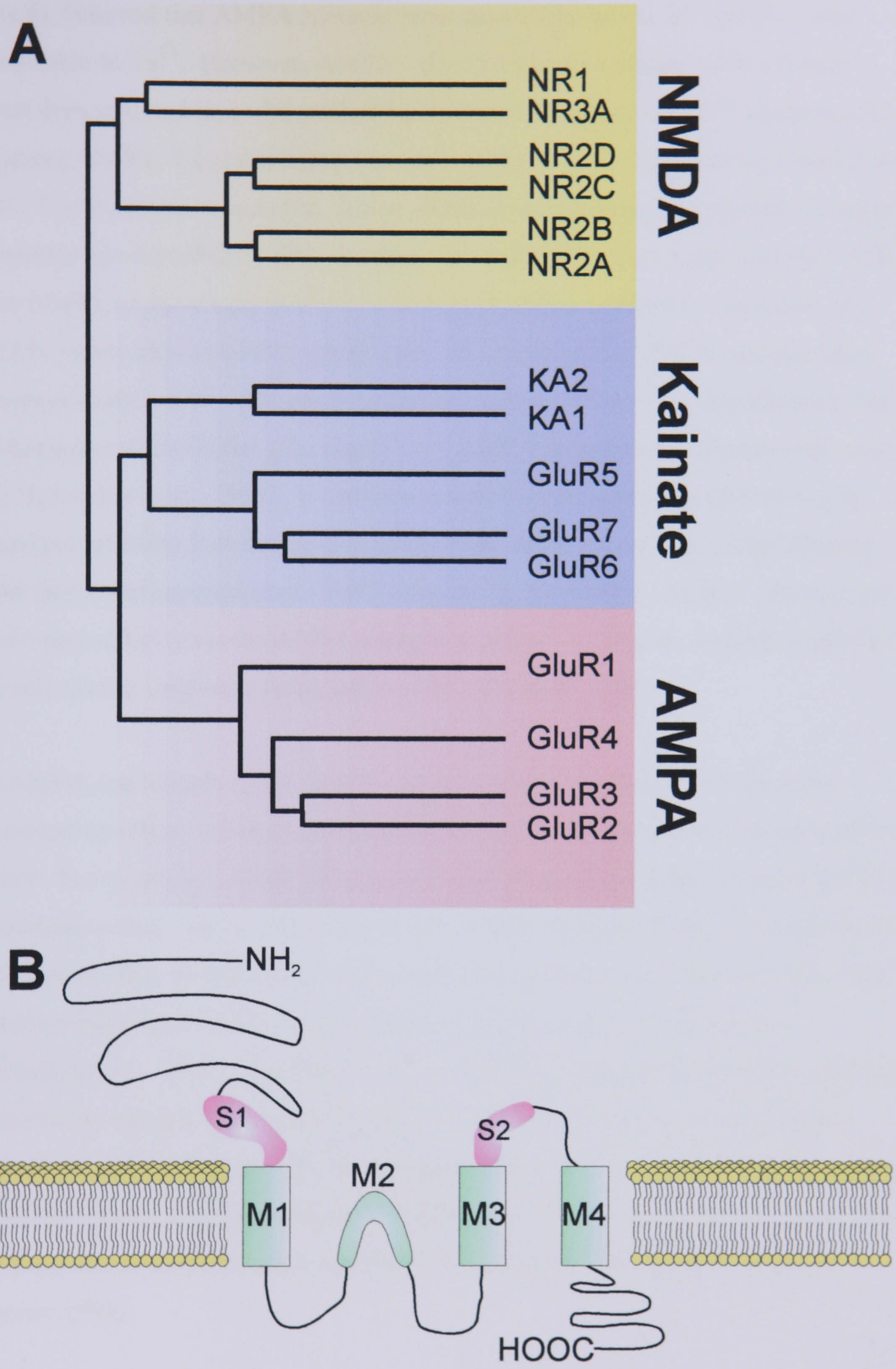

Figure 1.12 The iGluR family. (A) Alignment of the members of the iGluR gene family based on amino acid sequence (adapted from Das et al, 1998). (B)The generalised structure of iGluR subunits, demonstrating the 3 transmembrane (M1, 3 and 4) and the re-entreant loop (M2) and the agonist binding sites (S1 and 2).

The diversity of the AMPA receptor is demonstrated by cation permeability. It was originally believed that AMPA subunits were permeable only to K^+ and Na^+, and impermeable to Ca^{2+}. However, AMPA subunit expression studies using *Xenopus* oocytes demonstrated that whilst GluR2 homomeric receptors are Ca^{2+} impermeable, homomeric GluR1, 3 and 4 receptors are permeable to Ca^{2+}. Expression of GluR2 with GluR1, 3 or 4 greatly reduces Ca^{2+} permeability, demonstrating that the GluR2 subunit is dominant in controlling AMPA receptor Ca^{2+} influx (for review see Seeburg, 1993). Native AMPA receptors are likely to be heteropentamers of GluR1-4 subunits. As GluR2 is widely expressed throughout the CNS, many of the AMPA receptors have low permeability to Ca^{2+}. However, in certain regions of the brain, especially the rat hippocampus and cerebellar glia, highly Ca^{2+} AMPA receptors are present (Iino et al., 1990; Burnashev et al., 1992). It can therefore be hypothesised that differences in calcium permeability between regions of the brain and even cell-to-cell differences may be due to the expression of GluR2 subunits by the AMPA receptor. An increase in Ca2+ permeability via the AMPA receptor enables it to mediate synaptic plasticity, especially during long-term potentiation (LTP) (Jia et al., 1996).

Both AMPA and kainate act as AMPA receptor agonists, although AMPA has a desensitisation effect, which is considered to be due to the subunit composition of the receptor. For example, a GluR4 flop homopentamer receptor channel has the quickest desensitisation time, whilst GluR3 flip is the slowest. Heteropentamers comprised of subunits in the flop variation tend to desensitise quicker than flip, demonstrating that alternative splicing of AMPA receptor subunits control the receptor kinetics (Mosbacher et al., 1994). The differences in AMPA receptor desensitisation, which are determined by subunit composition, can have profound effects on the excitatory postsynaptic current (ESPC). The time it takes for the ESPCs to decay varies between neuronal cell types, depending on their function. It is believed that the differences in ESPCs are due to desensitisation, possibly via AMPA receptor subunit structure (Barbour, 1994).

The kainate family of receptors are composed of five subunits GluR5, 6, 7, KA1 and KA2, of which alternative splicing of the GluR5 subunit and mRNA editing of GluR5 and GluR6 occurs, increasing the receptor diversity (for review see Hollmann and Heinemann, 1994). The fact that kainate elicits an affect on AMPA receptors has

resulted in lack of understanding of the kainate receptors functional properties. However, recent advances in the field of pharmacological AMPA antagonists have ensured that the physiological properties of the kainate receptor can now be investigated. Similar to the AMPA receptor, the native kainate receptor composed of heteromers of the five subunits, also desensitises rapidly, although it is highly permeable to Ca^{2+} (Egebjerg et al., 1991; Kohler et al., 1993). The role of the kainate receptor in the CNS would appear to be in slow glutamatergic neurotransmission (Vignes and Collingridge, 1997), though it also regulates presynaptic neurotransmitter release (Malva et al., 1995; Chittajallu et al., 1996).

1.6.2.2 The NMDA receptor

The NMDA receptor was initially considered to be composed of five receptor subunits, comprising a selection of NMDA receptor (NR) 1 and NR2A-D, until the discovery of two NR3 subunits A and B (previously referred to NMDAR-L or χ-1) (for reviews see Hollmann and Heinemann, 1994; Ozawa et al., 1998; Dingledine et al., 1999). All of these receptor subunits are encoded from three genes, each of which is present on a different chromosome. The stoichiometry of the subunits that form the native NMDA receptor remains a point of contention. The NR1 subunit is fundamental to receptor function (Moriyoshi et al., 1991) and is able to form homomeric channels with NMDA receptor characteristics, but display reduced amplitude current responses compared to receptors in vivo. This indicated that the NR1 subunit co-localises with the other subunits when forming functional receptor channels. It remains unclear as to whether the NMDA receptor consists of four (tetrameric) or five (pentameric) subunits and how many NR1 subunits are present in this receptor (Fig. 1.13). By co-expressing NR1 and NR2 subunits in *Xenopus* oocytes and measuring the number of different conductances, Behe and co-workers (1995) concluded that the NMDA receptors were tetramers, composed of two NR1 subunits and two NR2 subunits. Premkumar and Auerbach (1997) then carried out the same experiments, only to hypothesise the formation of pentamers made up from three NR1 subunits and two NR2 subunits. The finding that post-translational subunit changes affecting receptor conductance may lead to an over-exaggeration of subunit number has further complicated these ideas (Laube et al., 1998).

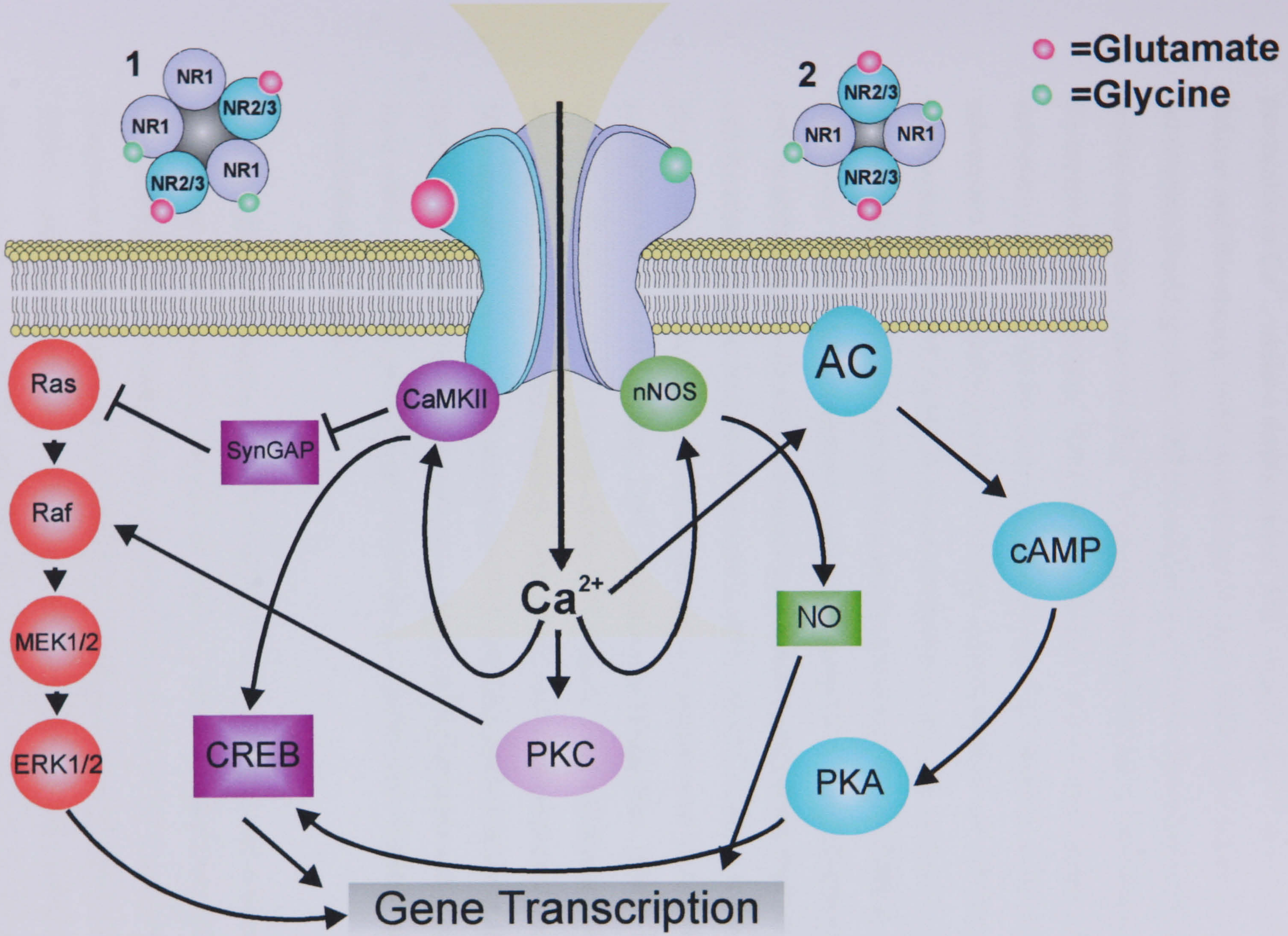

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Figure. 1.13. Simplified schematic diagram of NMDA receptor structure and downstream signalling events. The NMDA receptor is a heteromer, composed of either five (1) or four (2) subunits. On binding glutamate and glycine, the receptor is activated, resulting in calcium influx. Increases in intracellular Ca^{2+} activates CaMKII, PKC, nNOS and adenylate cyclase (AC), activating a wide range of signalling molecules such as MAPK and the transcription factor CREB. These molecules subsequently target transcription factors important in the regulation of synaptic plasticity.

A number of characteristics of the NMDA receptor differ to that of the AMPA and kainate receptors. One difference is ion permeability, as the NMDA receptor is highly permeable to Ca^{2+}, whilst a similar cation, Mg^{2+} actually blocks the receptor channel (Mayer and Westbrook, 1987; Ascher and Nowak, 1988). The binding of Mg^{2+} to and subsequent blocking of the NMDA receptor is a voltage-dependant mechanism. At resting membrane potential, Mg^{2+} blocks the NMDA receptor, preventing the action of the receptor at the synapse. When the membrane becomes depolarised, possibly via activation of AMPA, on the post-synaptic cell, the Mg^{2+} block is removed and subsequent calcium influx occurs. This may explain NMDA and AMPA receptor co-localisation, as AMPA shows an almost immediate activation on agonist binding followed by desensitisation and subsequent long-lasting effect of NMDA receptor activation. The NMDA receptor-mediated calcium influx has profound effects on the cell by activating a wide range of signalling cascades, generating changes in neuronal cell function. Another unique characteristic of the NMDA receptor is the fact that glycine acts as a co-agonist with glutamate and is a requirement for NMDA receptor activation (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). The glycine-binding site appears to be located on the NR1 subunit, whilst glutamate binds to NR2, which possibly explains the reason behind the reduced conductance of the homomeric NR1 receptors compared to heteromeric NR1/NR2 receptors (Sucher et al., 1996). The fact that the NMDA receptor conductance increases as glutamate and glycine bind to their relative subunits provides an acceptable explanation as to the slow activation and desensitisation kinetics.

NR3 is a distant relation to the other NMDA receptor subunits and is the most poorly understood and its function is unlike any other. NR3A co-immunoprecipitates with other NMDA receptor subunits and when co-expressed with NR1 and NR2A in *Xenopus* oocytes, whole cell (Ciabarra et al., 1995) and single channel (Das et al., 1998) conductances and Ca^{2+} influx were markedly reduced. Das and co-workers confirmed these findings by generating NR3 knockout mice that displayed a 3-fold increase in channel current. These results identified the NR3 subunit as a modulator of NMDA receptor function, acting as a "volume control" by reducing the activity of the receptor, the only ligand-gated ion channel known to do so. Parkinson's disease is believed to be due to NMDA receptor hyper-activation due to excessive amounts of

extracellular glutamate. Therefore the NR3 subunit, acting to reduce this level of activation, may have pertinent therapeutic implications.

NMDA receptor subunit composition determines the properties of the receptor channel, greatly increasing its functional diversity (for review see Cull-Candy et al., 2001). NMDA receptor heteromers composed of NR1 and one of the four NR2 subunits demonstrate a great diversity in receptor desensitisation time, ranging from milliseconds (NR1/NR2A) to seconds (NR1/NR2D) (Wyllie et al., 1998; Vicini et al., 1998). NR2A and B are also more sensitive to the blocking action of Mg^{2+} compared to NR2C and D (Momiyama et al., 1996) and although differences in Ca^{2+} permeability are negligible (Burnashev et al., 1995), disparity in Mg^{2+} sensitivity is likely to affect Ca^{2+} conductance. During postnatal development, the NR2B subunit is replaced by NR2A, resulting in faster desensitisation and subsequently quicker NMDA-mediated ESPC decay, which plays a role in synaptic plasticity (Constantine-Paton and Cline, 1998). NR1/NR2C receptors also exist, participating in synaptic plasticity via long-term depression (LTD), the process opposing LTP, mainly in cerebellar granule cells (Hrabetova et al., 2000), however NR1/NR2D heteromers do not exist in the synapse (Cull-Candy et al., 1998).

The effect of Ca^{2+} influx via the activated NMDA receptor regulates the action of intracellular kinases and phosphatases controlling numerous cellular events. NMDA-mediated activation of downstream signalling molecules, of which the most important are Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), PKC and PKA (for review see Dingledine et al., 1998; Kennedy, 2000), underlie cellular responses at the CNS synapse. These molecules in-turn are able to phosphorylate serine and threonine residues situated on the NMDA receptor, enhancing its function by decreasing Mg^{2+} affinity and opening probability (Chen and Huang, 1992; Leonard and Hell, 1997). It therefore becomes possible to understand how the NMDA receptor is able to initiate LTP by protein phosphorylation, rather than longer-term gene transcription. Signalling molecules are organised as a protein complex at the membrane of the postsynaptic cell, called the postsynaptic density (PSD), which is visible by transmission electron microscopy (TEM) (Gray, 1959). It is likely that the components of the PSD vary between synapses, however some signalling molecules are highly abundant in the protein complex, the identification and function of which shall be

discussed in a later chapter. The MAPK pathway culminates in gene transcription and protein phosphorylation of fundamental importance to LTP (English and Sweatt, 1997; Sweatt et al., 2001). NMDA-mediated MAPK activation is considered to be via several signalling proteins, the most important of which is CaMKII (Kurino et al., 1995; Xia et al., 1996; Perkinton et al., 2002). CaMKII inhibits SynGAP, a synaptic Ras GTPase-activating protein. SynGAP is believed to inactivate GTP-ras, the major positive regulator of the MAPK pathway (Scheffzek et al., 1996). Therefore, CaMKII releases the SynGAP-mediated constraints on the MAPK pathway by SynGAP inactivation (Chen et al., 1998). The NMDA receptor-mediated increase in intracellular Ca^{2+} also leads to the activation of PKC and PKA, both potent activators of downstream signalling events. Other signalling molecules involved in LTP appear to be CaMKII-dependent, including the transcription factor cAMP response element binding protein (CREB) and nitric oxide (NO), the latter being activated by way of neuronal nitric oxide synthase (nNOS) (Fig. 1.13).

The signalling mechanisms utilised by glutamate receptors during processes such as synaptic plasticity, are by no-means specific to the CNS. Indeed, the presence of glutamate receptors on any cell type is likely to have profound effects on all aspects of cell function.

1.7 Glutamate Signalling in Peripheral Tissues

The identification of glutamate receptors expressed by cells outside of the CNS, raises new possibilities of glutamate-mediated cellular functions in numerous diverse tissues (for reviews see Skerry and Genever, 2001; Gill and Pulido, 2001). Compelling evidence exists for the function of GluRs in bone, skin and pancreas, whilst glutamate receptors have also been identified in the heart, thymus, testis, taste buds, liver, kidney and gastro-intestinal tract. iGluRs appear also to function in plant cells, as mediators of light signal transduction (Lam et al., 1998), raising the possibility that the mammalian glutamate receptor may have evolved from a more primitive receptor type.

1.7.1 Glutamate signalling in bone

Bone is perhaps the most widely accepted and understood location of peripheral glutamatergic signalling. This signalling process was first hypothesised on the basis of a differential RNA display, in which changes in the expression of the glutamate transporter GLAST-1 following *in vivo* bone loading (Mason et al.: 1997). This transporter was identified in osteocytes (bone matrix cells) and osteoblasts but not osteoclasts, leading to further studies focusing upon the discovery of other components associated with glutamate-mediated signalling. These investigations proved to be extremely productive, identifying a range of glutamate receptors expressed by bone cells *in vivo*, by primary cells and cell lines (Patton et al.; 1998: Chenu et al., 1998). *In vitro*, bone cells treated with the specific non-competitive NMDA receptor antagonist MK-801 resulted in the reduction of osteoblastic bone formation (Dobson et al., 2000; Taylor et al.; 2000), whilst inhibiting osteoclastic-mediated bone resorption (Peet at al, 1999). AMPA receptor antagonism also resulted in increased adipocytic lineage commitment from marrow stromal cells compared to osteoblast differentiation. The ability of glutamate signalling to determine cell lineage from primitive multipotent and progenitor cells has not currently been investigated and may prove extremely exciting.

Attempts to demonstrate glutamate receptor function in bone by agonist-mediated response has proved less conclusive. However, electrophysiological studies on osteoblastic cell lines and primary cells has demonstrated glutamate receptor-like gating characteristics, but similarity of these results to the CNS glutamate receptor remains a point of controversy (Laketic-Ljubojevic et al.; 1999; Gu et al.; 2000). Patch clamping experiments also identified the existence of NMDA receptor currents in osteoclasts (Peet et al.; 1999; Espinosa et al.; 1999).

It has been established recently that osteoblasts also express components required for glutamate release in the CNS synapse. These include SNAREs and associated proteins involved in presynaptic vesicle docking and glutamate release. Osteoblasts also demonstrate regulated and spontaneous glutamate exocytosis, prevention of which leads to inhibition of cell survival and differentiation (Bhangu et al., 2001; Genever and Skerry, 2001). Further examination of osteoblastic glutamate signalling has revealed all the necessary components required for LTP in the CNS postsynaptic cell

(G.J. Spencer, personal communication). These include many of the structural and signalling proteins present in the PSD of which CaMKII is particularly prevalent. Treatment of osteoblastic cells with glutamate resulted in CaMKII activation, inhibition of which reduced osteoblastic differentiation and mineralisation *in vitro*.

1.7.2 Glutamate signalling in other tissues

A high level of NR1 expression by proliferative cells in the resting basal epidermis of skin, raises the possibility that the NMDA receptor is involved in the differentiation of keratinocytes. MK-801 inhibition of human keratinocyte NMDA receptors induced the cells to differentiate (Morhenn et al., 1994; Genever et al., 1999b). In addition to NMDA receptors, the GluR2/3 family of AMPA receptors are localised to the basal surfaces of rat keratinocytes, which also express the glutamate transporters GLT-1 and EAAC-1 and various mGluRs (Genever et al., 1999b). NMDA receptor expression in migrating epidermal cells is reduced during re-epithelialisation following wounding, implying that glutamate signalling is involved in the process of skin development. The supply of glutamate in skin remains unknown, although there are several possible sources, including the possibility of keratinocyte glutamate recycling and release (Davidson et al., 1997) and glutamate containing nerves that are present in skin (Kinkelin et al., 2000).

Pancreatic cells express glutamate receptors of the NMDA, AMPA and kainate families. Electrophysiological patch clamp experiments revealed that the characteristics of these receptors were identical to those found in neuronal cells (Gonoi et al., 1994; Molnar et al., 1995), with the function of the AMPA and kainate receptors possibly determining insulin secretion from pancreatic cells. Glutamate receptor expression has been demonstrated throughout the gastro-intestinal tract especially NR1, which is present in the oesophagus, stomach mucosa and mast cells (Gill and Pulido, 2001). Glutamate-mediated activation of NMDA receptors present in ileum results in smooth muscle contraction, a process responsible for gastro-intestinal motility (Shannon and Sawyer, 1989), which is blocked by Mg^{2+} and MK-801. The presence of glutamate receptor types and function in other tissues is summarised in Figure 1.14.

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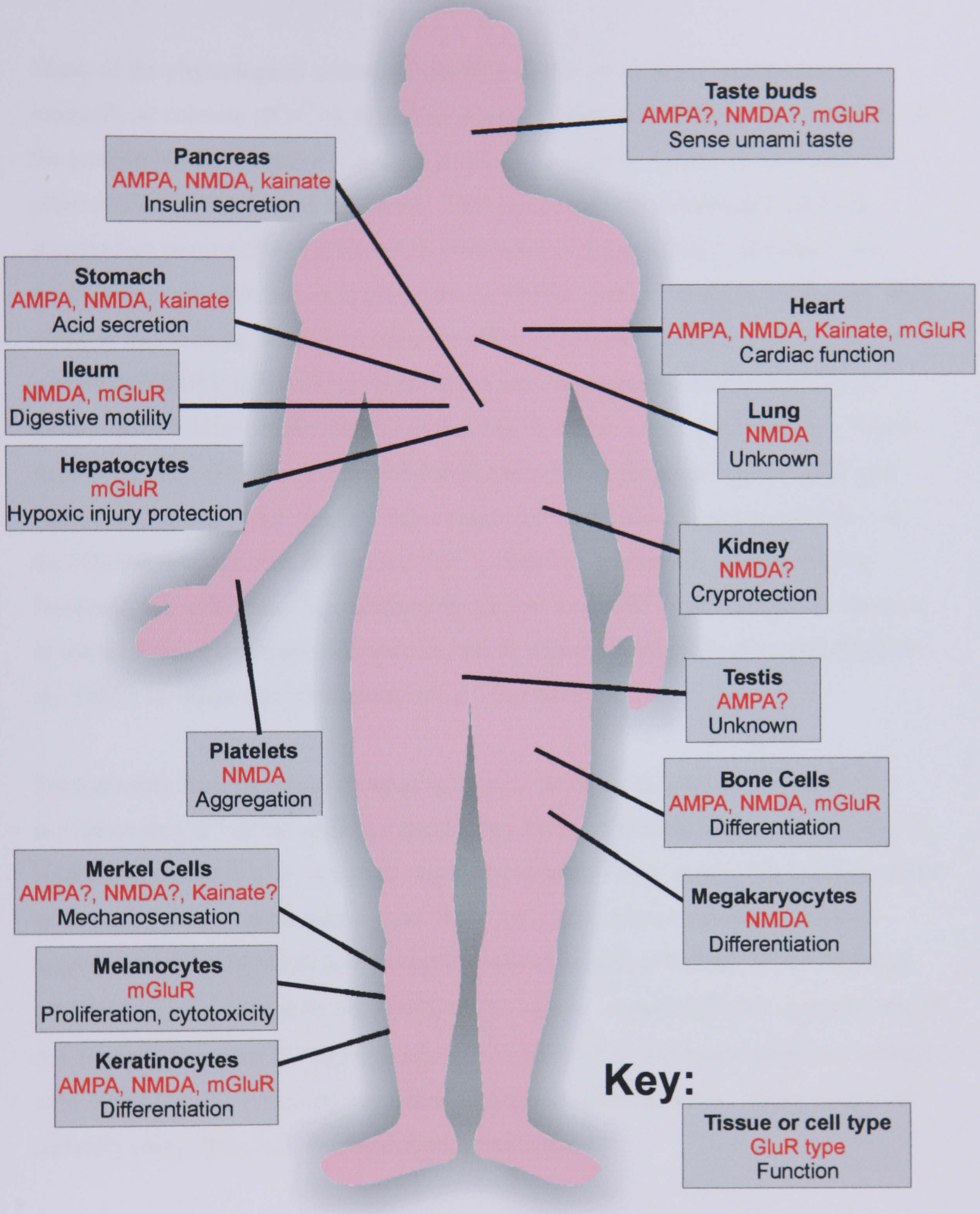


Figure 1.14. Glutamate signalling in selected peripheral tissues. Numerous human tissues of widely varying function express glutamate receptors. In these tissues, glutamate signalling appears to play fundamental roles in their function (for review see Skerry and Genever, 2001)

1.8 The Megakaryocytic Glutamate Receptor

Many of the physiological actions of platelets depend upon the concentration of intracellular calcium ($[Ca^{2+}]_I$), including activation and aggregation. The discovery of the platelet NMDA receptor raises the possibility of this receptors' involvement in platelet $[Ca^{2+}]_i$ (Franconi et al., 1996). The addition of arachidonic acid (AA) to platelet rich plasma (PRP) is known to induce platelet aggregation. However, AA application in conjunction with glutamate or NMDA prevents platelet activation. This effect is shown to be NMDA receptor-specific, as MK-801 addition prior to AA and glutamate/NMDA application prevented the anti-aggregative effects. Addition of glutamate or NMDA in the absence of AA had no effect on platelet function. Similar dose-dependent effects were observed with other pro-aggregation agents, ADP and platelet-activating factor (PAF). There responses were not expected as activation of the NMDA receptor should increase $[Ca^{2+}]_I$, therefore promoting platelet activity. However, this effect may be explained by the fact that NMDA also caused an increase in intracellular cAMP concentration, known to inhibit platelet activation (Bushfield et al., 1985), an effect that was absent in Ca^{2+}-free medium.

Further study from the same group investigated the effect of NMDA receptor activation on Ca^{2+} influx and thromboxane B_2 (TxB_2) synthesis (Franconi et al., 1998). NMDA increased $[Ca^{2+}]_i$ in a dose-dependent manner whilst completely inhibiting the synthesis of the pro-aggregation agent TxB_2, providing more evidence of the anti-aggregation effect of NMDA. The significant therapeutic potential of these findings was demonstrated by combining NMDA with aspirin. Aspirin (20μM) alone decreased AA-mediated human PRP aggregation to 54% from 84%. However, aspirin combined with 100nM NMDA reduced maximum aggregation to around 1%. These results are certainly compelling and warrant further investigation.

The megakaryocytic NMDA receptor was first identified in 1999 (Genever et al., 1999). However, it seems surprising that Franconi and co-workers' discovery of the functional platelet NMDA receptor in 1996 did not lead to a logical investigation of megakaryocytic glutamate signalling. Immunohistochemical staining of bone marrow with an antibody raised against the cytoplasmic domain of the NR1 subunit identified

strong expression by megakaryocytes in rat bone marrow. Further studies revealed NR1 expression by human megakaryocytes and the megakaryocytic cell line MEG-01. NR1 protein expression was confirmed firstly by RT-PCR of rat bone marrow mRNA and MEG-01 cells, using gene specific primers to rat and human brain NR1 respectively. RT-PCR also amplified products in rat marrow and MEG-01 cells that corresponded to the brain NR2D subunit, but not NR2A, B or C. Interestingly, western blot analysis of NR1 expression by MEG-01 cells with and without the differentiation-promoting agent phorbol myristate acetate (PMA), confirmed that the subunit was in a deglycosylated state compared to brain control. This finding is of particular significance, as glycosylation is known to provide NMDA receptor membrane stability, allowing for orientation only in specific areas of the postsynaptic cell. However, deglycosylated receptors would probably distribute evenly over the cell surface, as seen in the megakaryocyte. Human megakaryocytes derived from $CD34^+$ umbilical cord blood cells were also shown to express NR1 by northern blot analysis of total RNA. All acetylcholinesterase (AChE)-positive cells, a megakaryocyte lineage marker, in rat bone marrow also expressed NR1, indicating that the receptor is present at all stages of megakaryocyte maturation.

By treating animals with radiolabelled MK-801 ($[^3H]$-MK-801), which selectively binds to and blocks open NMDA receptor channels followed by autoradiographic examination of bone marrow, it was identified that NMDA receptor subunits formed functional channels *in vivo*. This binding was significantly reduced when animals were pre-treated with an excess of non-radioactive MK-801. The function of the megakaryocytic NMDA receptor was ascertained by treating megakaryocytic cell lines, with MK-801. PMA-mediated increases in cell size and expression of the megakaryocyte-specific marker CD41 were significantly reduced following MK-801 application compared to controls. MK-801 also inhibited cellular adhesion by approximately 50% compared to controls, without affecting cell viability. The changes in cellular adhesion were also observed following application of a non-competitive NMDA receptor antagonist D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5) and using another megakaryoblastic cell line CMK.

These data provided compelling evidence of a functional NMDA receptor in megakaryoblastic cells and identified the presence of this receptor on megakaryocytes

in vivo. Taken in conjunction with the finding that a number of uncharacterised cells in the bone marrow express the glutamate transporter GLT-1 (Mason et al., 1997). enabling the termination of paracrine glutamate signalling, it is possible to theorise an entire glutamatergic signalling system within the bone marrow microenvironment. The effect of NMDA receptor inhibition on megakaryoblastic differentiation supports the identification of a previously unrecognised megakaryocyte signalling system. At these relatively premature stages, this work does however ask more questions than it answers. This thesis aims to answer some of these questions.

1.9 Primary aims

- To determine the NMDA receptor subunit and associated proteins expression by megakaryocytic cell lines and human primary megakaryocytes.

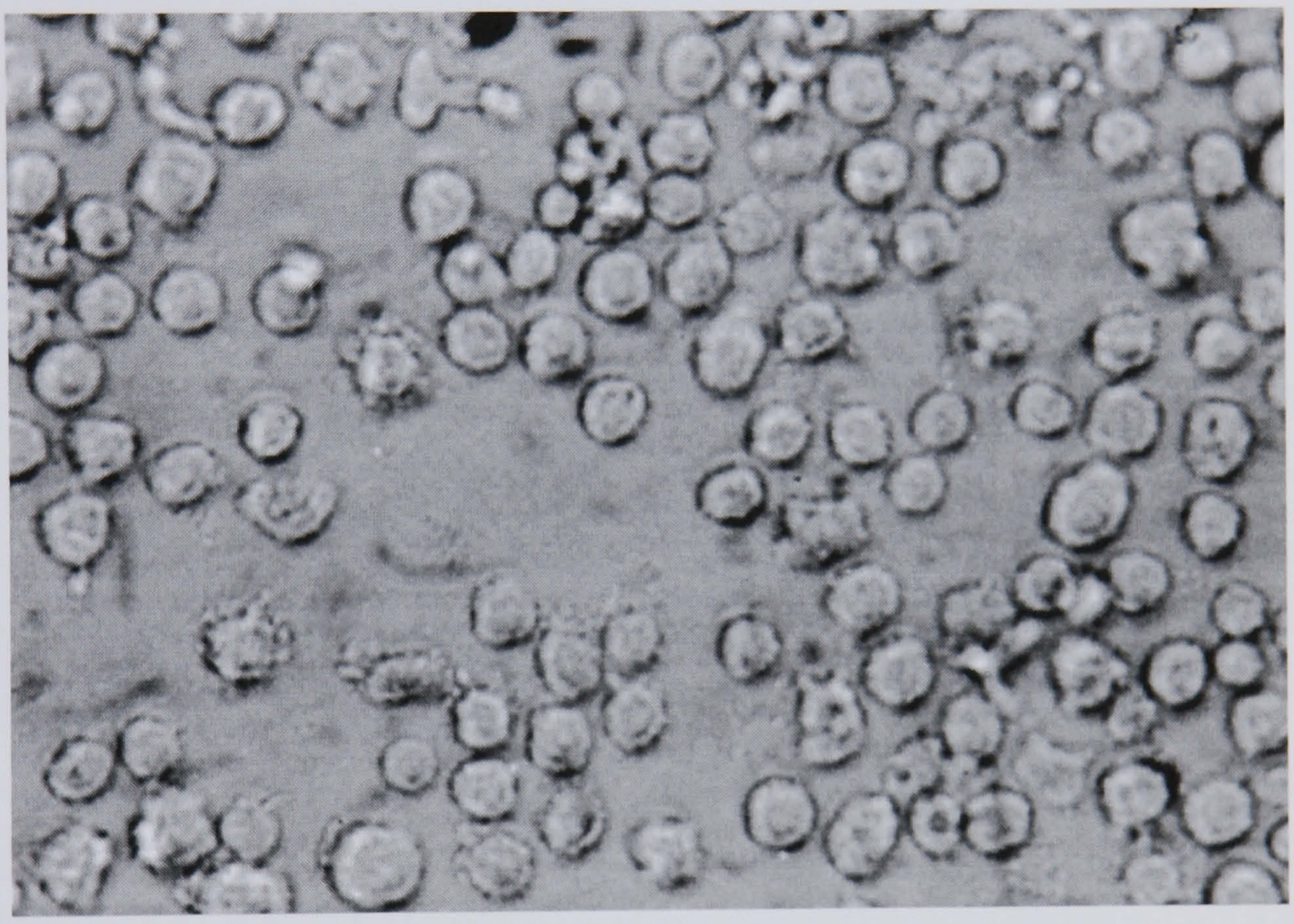
- Establish the function of the megakaryocytic NMDA receptor on human primary cells and megakaryocytic cell line differentiation by using the specific NMDA receptor antagonist MK-801.

- Investigate possible sources of glutamate within the bone marrow microenvironment with which to stimulate the megakaryocytic NMDA receptor.

- Determine the intracellular signalling cascades mediated by the NMDA receptor in the modulation of megakaryocyte differentiation.

- To determine any changes in megakaryocyte number and possible bleeding abnormalities in NR1 hypomorphic mice compared to wild types.

Chapter 2



General Materials and Methods

Freshly immunoisolated umbilical cord blood $CD34^+$ cells (original magnification x400)

Chapter 2

General Materials and Methods

2.1 Cell Culture

2.1.1 Cell culture plasticware and reagents

Tissue culture plasticware and reagents were purchased from Life Technologies (Paisley, UK) unless stated otherwise. Cells were maintained in a humidified atmosphere at 37°C in 5%CO_2/95% air. All media was supplemented with 100U/ml penicillin, 100μg/ml streptomycin and 2mM L-glutamine unless otherwise stated.

2.1.2 Cell line culture

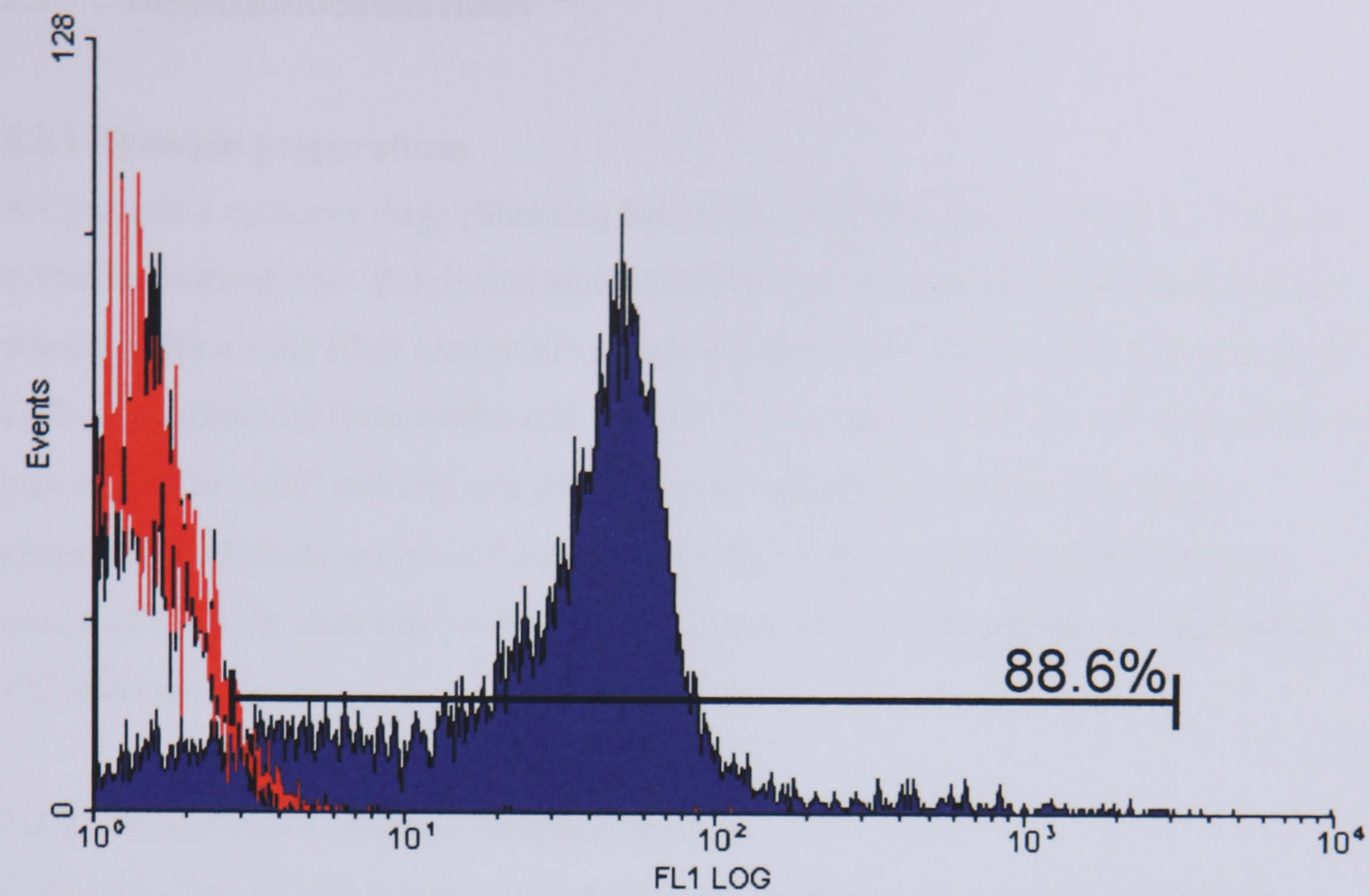
MEG-01 and human erythroleukaemia (HEL) human clonal megakaryoblastic cell lines were cultured according the standard ATCC protocol, in RPMI 1640 medium with 10% foetal bovine serum (FBS). For experiments investigating responses to glutamate, cells were incubated overnight in glutamate-free Neurobasal™ media with serum replacement N2 supplement (Gibco). Both cell lines are non-adherent and media was changed every 3 days, and passaged by centrifugation (400g, 5 min) and replated at $1x10^5$ cells/ml. To induce megakaryocyte differentiation, both cell lines were treated with 100nM phorbol myristate acetate (PMA; dissolved in ethanol) for 72 hours. In non-PMA treated samples, equivalent volume of ethanol was added.

2.1.3 Human CD34-positive-derived megakaryocytes

Umbilical cord blood was obtained from the umbilical cord and placenta from pre-term mothers giving birth by caesarean section. Blood was collected into heparinised tubes, a maximum of 5 minutes after birth. Approximately 50-100ml was extracted from each placental preparation. Blood was only taken after the mothers granted informed consent, at least 12 hours before surgery. CD34-positive ($CD34^+$) cell separation took place within 1 hour of blood collection. Umbilical cord blood was diluted 2:1 vol/vol. with Hanks buffered saline solution (HBSS) and overlaid on Ficoll paque (Fischer, Loughborough, UK). The preparation was centrifuged at 400g for 35 minutes and the mononuclear cells removed from the Ficoll interface and washed

twice by resuspending cell pellet in 10ml cold "buffer A" (phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) (Sigma) and 5mM EDTA). 100µl of cells were taken for purity analysis at this stage and remaining cells were centrifuged at 400g for 10 minutes at 4°C. $CD34^+$ cells were isolated using a magnetic immunoselection protocol (Miltenyi Biotec GMBH, Germany). Washes were performed by adding 10ml of buffer A to the cells followed by centrifugation at 400g for 10 minutes at 4°C. Mononuclear cells were suspended in 100µl of IgG blocking reagent and 100µl mouse anti- human $CD34^+$ primary antibody against the Q-BEND isotype and incubated at 4°C for 15 minutes. After washing, 100µl of mouse anti-human $CD34^+$- fluorescein isothiocyanate I (FITC) conjugated antibody against HPCA-1 isotype was added and incubated for 15 minutes at 4°C and washed. The cell pellet was resuspended in 400µl buffer A and 100µl of magnetic anti-hapten secondary antibody, incubated at 4°C for 15 minutes and washed. The cells were resuspended in 500µl of buffer A and passed through a MS^+ immunoselection column attached to the magnetic base unit (Miltenyi Biotec) and 3x500µl of buffer A passed through the column to remove the CD34-negative ($CD34^-$) populations. The column was removed from the magnetic source and the $CD34^+$ population collected by flushing 1ml of buffer A through the column. 25µl of cells were taken from both $CD34^+$ and $CD34^-$ for purity analysis by flow cytometry. Average purity of $CD34^+$ cells was 91%, ranging from 85.3% to 94.7% (see Fig. 2.1). $CD34^+$ cells were cultured in Iscove's modified Dulbecco's medium (IMDM; supplied containing 500µM L-glutamate) supplemented with 2mM glutamine, 2mg/ml sodium pyruvate, 1% Minimum Essential Medium vitamin solution, 1% non-essential amino acids, 0.1mM β-mercaptoethanol and 2mg/ml L-asparganine. The medium was also supplemented with 10% cord blood plasma, 0.2% BSA and 25ng/ml thrombopoietin (TPO) (Calbiochem, Nottingham, UK). Cord blood plasma was isolated from undiluted umbilical cord blood, centrifuged at 800g for 35 minutes at 4°C and the supernatant plasma fraction removed. Cells were plated at $5x10^4$ cells/ml in 24-well plates and replated at this concentration following 7 days in culture. An additional 25ng/ml TPO was added at this point, and the cells were cultured for a further 7 days in culture.

Original in colour



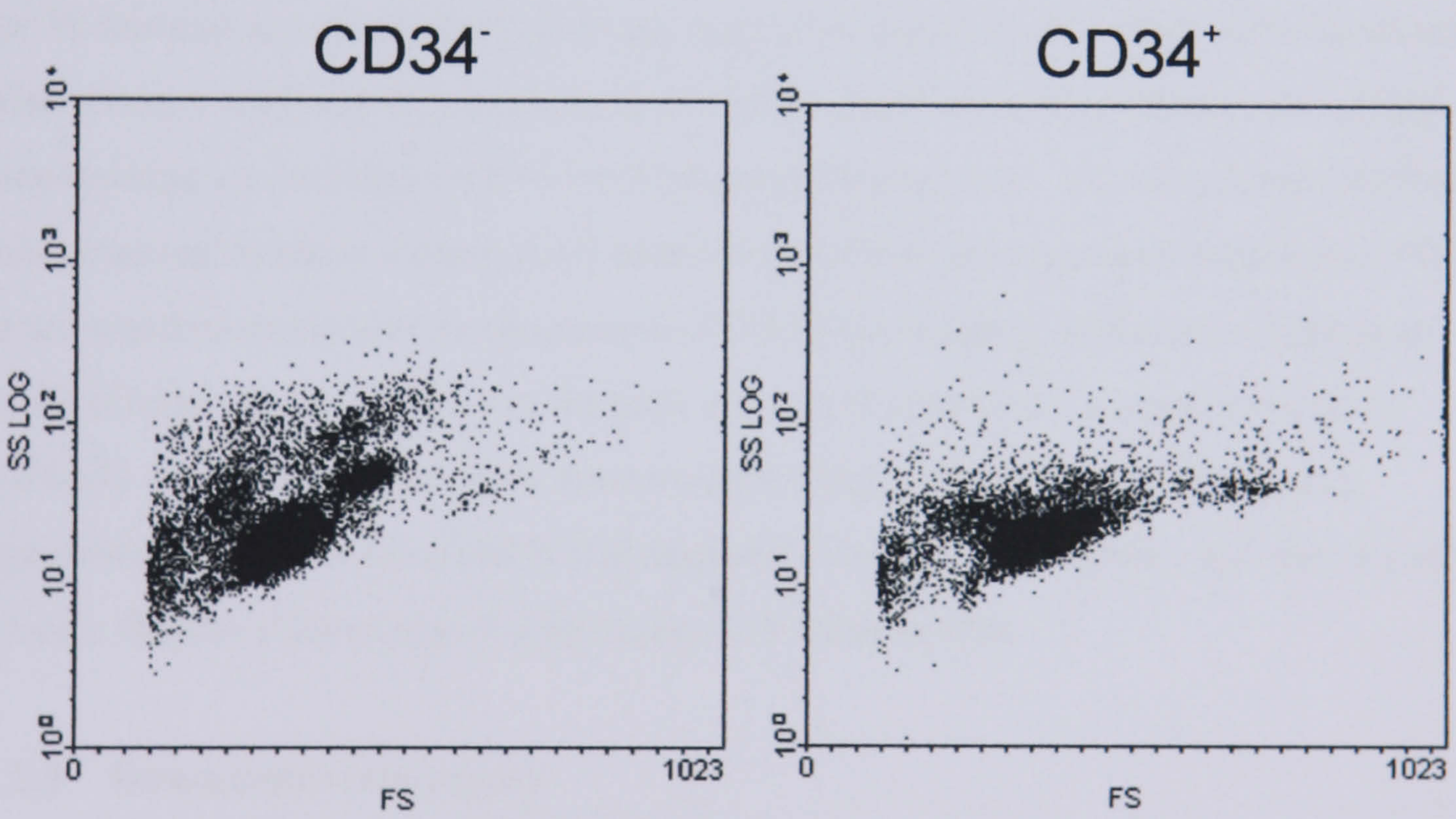


Figure 2.1. Purity analysis of isolated CD34⁺ haematopoietic cells following MACS separation from umbilical cord blood. CD34⁺ cells were labeled with a mouse anti-human CD34 antibody prior to MACS separation. Following separation, CD34⁻ effluent was collected prior to CD34⁺ flow-through and both samples tested for purity by flow cytometry. CD34-positivity typically ranged from 85-92% (blue filled peak) compared to CD34⁻ (red peak) and antibody control (black peak).

2.2 Immunolocalisations

2.2.1 Cytospin preparations

A Cytospin 3 cytocentrifuge (Shandon Scientific Ltd, Runcorn, UK) was used for all cytospin preparations. Polylysine slides (BDH) were cleaned in alcohol, labelled and placed under a slide filter card in the sample chamber and placed in the cytospin head. Cells were removed from media and washed 3 times with PBS. The cell concentration was altered to $1x10^5$ cells/ml and 200µl of cell suspension added to the sample chamber. Cells were spun for 5 minutes at 100g, "LO" acceleration and the slides removed from the slide clip, immediately fixed in 4% paraformaldehyde and kept at 4°C until use.

2.2.2 Fluorescent immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 5 minutes at room temperature and washed 3 times with PBS. The cells were then incubated in 10% goat serum (Sigma) for 30 minutes at room temperature and excess serum removed. Cells were incubated with primary antibody or control IgGs for either 1hr at room temperature for cytospin preparations, or overnight at 4°C for fibronectin bound cells. Excess primary antibody was removed, washed 3 times for 5 minutes with PBS and incubated with either FITC or tetramethyl rhodamine isothiocyanate (TRITC) secondary antibodies (Sigma) at 1:100 dilution for 45 minutes in the dark at room temperature. Excess secondary antibody was removed and cells were washed 3 times with PBS for 10 minutes. Specimens were then mounted in Vectorshield (Vector Laboratories) and viewed using a Leica DMLA microscope (Leica) under U.V illumination.

2.2.3 Immunohistochemistry

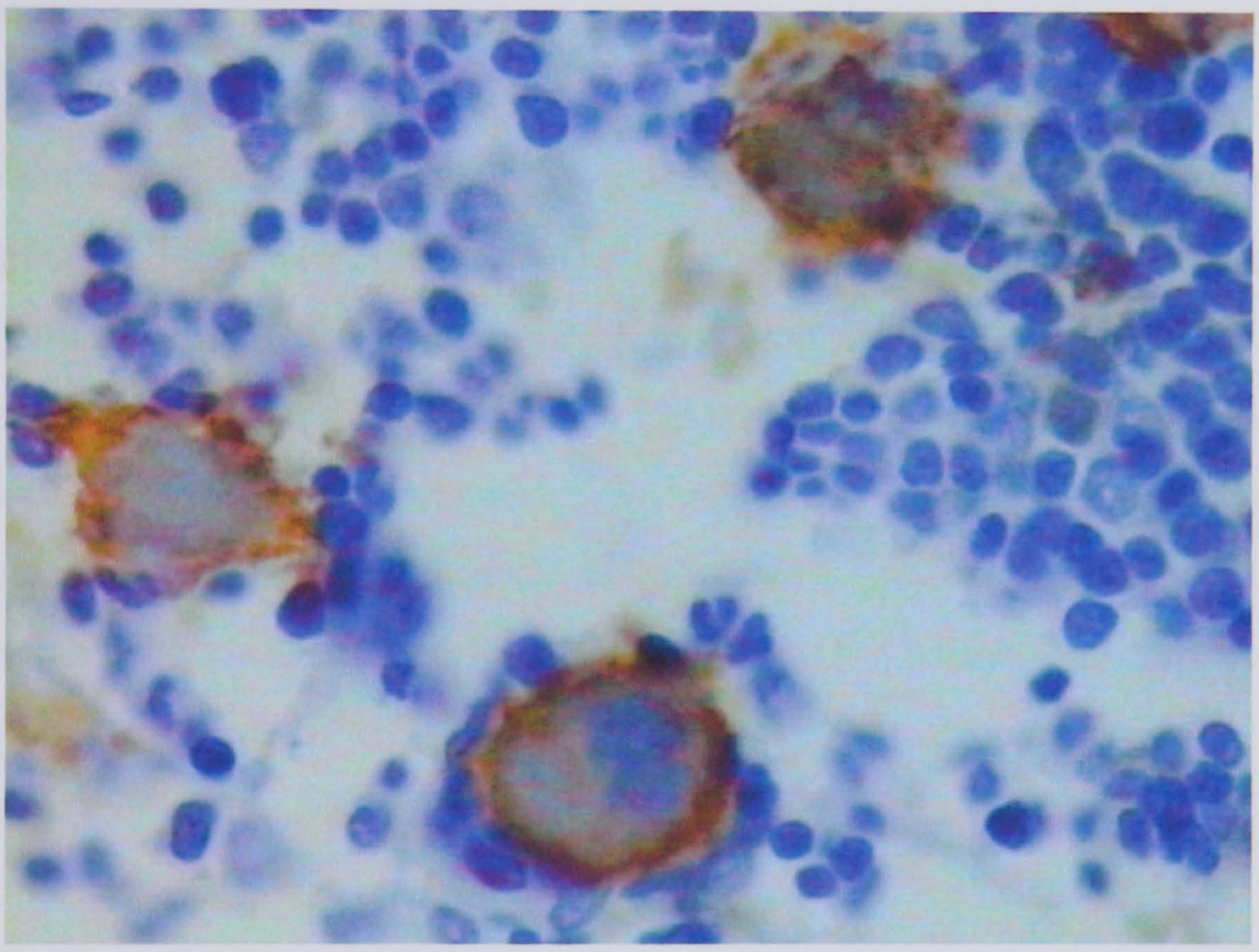
2.2.3.1 Tissue collection and cryosectioning

Spleen, tibiae and femora were removed from adult mice and 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 in chilled isopentane. Frozen sections were cut using a Bright OTF500 cryostat (Bright) fitted with a tungsten carbide tipped knife, collected on polylysine slides and stored at -20°C until required.

2.2.3.2 Peroxidase immunolocalisations

Sections were fixed in 4% paraformaldehyde for 5 minutes before the depletion of endogenous peroxidase activity with 3% hydrogen peroxide (Sigma) for 30 minutes. Non-specific avidin binding to endogenous biotin was prevented using the Vector Avidin / Biotin Blocking Kit (Vector Laboratories) according to the manufacturer's protocol. For sections incubated in non-mouse raised primary antibodies, 10% blocking serum was applied to the tissue sections, chosen to match the species in which the secondary antibody was raised. Sections were incubated with primary antibody for 30 minutes, 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 minutes. The sections were counterstained with haematoxylin before mounting in 90% v/v glycerol/PBS and viewed using a Leica DMLA microscope. Antibody negative controls received identical treatment apart from the application of normal mouse IgGs (Vector Laboratories; 1μg/ml) instead of the primary antibody. All incubations were performed at room temperature with 3 x 5 minute PBS washes between incubations.

Chapter 3



Megakaryocytic NMDA Receptor Expression

Expression of the essential NR1 subunit by a rat bone marrow megakaryocyte.
Brown staining indicates positivity (magnification x400)

Chapter 3

Megakaryocytic NMDA Receptor Expression

3.1 Introduction

NMDA receptor subunit organisation has a profound effect on receptor activity and conductance (see chapter 1.6.2.2). The functional importance of NMDA receptor subunit diversity is indicated by organisational changes during development. mRNA taken from the developing rat brain, demonstrates that NR2D expression is restricted to the brainstem and diencephalons (region of forebrain underlying cerebral hemispheres), whilst NR2B is expressed throughout the brain (Monyer et al., 1994). Shortly after birth, NR2A is found in most regions of the brain, whilst NR2C expression is limited to the cerebellum. It appears therefore that in the CNS, NR2B expression is of greatest importance in the early embryo, which decreases during development to be superseded by the NR2A subunit (Akazawa et al., 1994). This occurrence is believed to underlie the speed of NMDA receptor-excitatory postsynaptic currents (EPSC), the phenomenon linked with memory forming synaptic plasticity (Constantine-Paton, 1998).

Alterations in NMDA receptor subunit composition are not however limited only to regions of the brain. Indeed, single cells also display differences in subunit organisation. One such example is again, the NR2A: NR2B ratio in relation to NMDA receptor-EPSC. Receptors composed of mainly NR2A subunits will display rapidly decaying EPSC compared to cells composed mainly of NR2B subunits. The functional importance of this is explained by studies of rat visual cortex NMDA receptor-EPSCs. Animals reared in the dark and subsequently exposed to light, results in the rapid insertion of new NMDA receptors with an increased NR2A: NR2B ratio, reducing sensitivity by decreasing the time taken for NMDA receptor-EPSC decay (Quinlan et al., 1999).

As previously mentioned (chapter 1.6.2.2), evidence exists for the participation of NR2A-, NR2B- and NR2C containing heteromeric NMDA receptors at the CNS

synapse. The NR1/NR2D receptors have not however been identified at the CNS synapse, even though recombinant data indicted that they would be easy to identify by their extremely slow deactivation kinetics (Cull-Candy et al., 1998). This theory was confirmed by patch-clamp experiments on Purkinje cells (neuronal cells that carry output from the cerebellum) expressing pure NR1/NR2D receptors, as glutamate-mediated receptor activation leads to extremely slow deactivation (Misra et al., 2000a). Although other cells express NR2D, NMDA receptor-EPSC remains fast, demonstrating the lack of pure NR1/NR2D receptors at the synapse (Misra et al., 2000b). This does not however rule out the existence of triheteromeric receptors, such as NR1/NR2B/NR2D that are found in the midbrain (Dunah et al., 1998), which would not display pure NR1/NR2D kinetics.

The above findings clearly reveal that NMDA receptor subunit composition results in functionally different receptors. These are subsequently expressed at different times during the development of the embryonic CNS and by different regions of the adult brain. It is therefore apparent that the structure of the NMDA receptor is directly correlated with its required function on the cell by which it is expressed.

In addition to NMDA receptor subunit composition contributing to its function and subsequent downstream effects, proteins binding to the receptor can regulate its localisation and its signalling. As previously mentioned, NMDA receptor activation underlies the process of synaptic plasticity, by LTD (reduction of synaptic efficacy) and LTP (increase of synaptic efficacy) (chapter 1.6.2.2). These mechanisms are achieved by changes in synaptic strength, which is in turn achieved by modifications in postsynaptic NMDA receptor sensitivity and clustering on the postsynaptic membrane, activation of silent receptors and transcriptional changes (Bliss and Collingridge, 1993; Bear and Malenka, 1994). The molecules involved in these synaptic changes are organised into the post-synaptic density (PSD). The first and most abundant protein identified at these peripheral condensations was PSD-95 (also known as synaptic associated protein-90 (SAP-90)). PSD-95 belongs to a conserved cytoskeletal protein family known as membrane associated guanylate cyclases (MAGUKs), of which other principle members are chapsyn-110/PSD-93, SAP102 and SAP97/hDlg. All MAGUKs contain characteristic domains known as PDZ (**P**SD-95/**D**lg/**Z**O-1) and SH3, which mediate protein-protein interactions forming cell

membrane protein scaffolds (Craven et al., 1998). PSD-95 binds to the long C-terminus of NR2 subunits (Kornau et al., 1995; O'Brien et al., 1998) via one of its three PDZ domains (for review see Fujita and Kurachi, 2000; Sheng, 2001). PSD-95 has a variety of multi-faceted roles in NMDA receptor function, including localisation of the receptor in the PSD (Steigerwald et al., 2000), anchoring the receptor to the postsynaptic membrane (Sheng et al., 2000) and receptor-mediated activation of associated signalling pathways, of which the MAPK pathway is amongst the most critical in synaptic plasticity (Migaud et al., 1998). PSD-95 is able to modulate the MAPK pathway by interacting with CaMKII, which inhibits the synaptic GTPase activating protein SynGAP (Komiyama et al., 2002). In addition to PSD-95, another member of the MAGUK family, chapsyn-110, has been implicated in the coupling of nNOS to the NMDA receptor (Harris and Lim, 2001). Chapsyn-110 facilitates the nNOS activation by linking nNOS to the vicinity of the NMDA receptor, which is subsequently activated by the calcium influx resulting from receptor activation.

Although these four MAGUK proteins account for the majority of the PSD, novel PDZ domain-containing MAGUKs have recently been identified. MALS, the mammalian homologue of the *Caenorhabditis elegans* LIN-7 protein (Simske et al., 1997), contains a single PDZ domain and associate to the NMDA receptor / PSD-95 complex, possibly also to the c-terminal of the NR2 subunit (Jo et al., 1999). Jo and co-workers suggest that MALS is likely to participate in the clustering of NMDA receptors and possibly other glutamate receptors in the PSD. Another novel MAGUK protein is CASK, the mammalian homologue of the *Caenorhabditis elegans* protein LIN-2. The PDZ domain of CASK binds to numerous other PSD proteins, but also specifically to the transmembrane adhesion protein syndecan (Cohen et al., 1998). Interestingly, CASK also contains two guanylate kinase (GUK) motifs, neither of which possesses enzymatic activity. However, a recent study discovered that CASK could translocate from the plasma membrane to the nucleus, and associates via its GUK domain to the transcription factor T-brain-1 (Tbr-1) (Hseuh et al., 2000), which greatly enhances the transcription of Tbr-1-dependant reporter genes. The capability of CASK to directly modulate transcription makes it unique in the PSD-MAGUK family.

SHANK (SH3 domain **ank**ryin repeat) is a common protein in the cytoplasmic face of the PSD (adverse to PSD-95 which is located toward the membrane side of PSD), a location that puts this molecule in prime position to interact with cytoplasmic proteins and endoplasmic reticulum. SHANK has also shown to bind to interact with the actin-binding protein cortactin, raising the possibility that SHANK may be involved in PSD-mediated regulation of the cellular cytoskeleton (Naisbitt et al., 1999). These results have since been confirmed by overexpression of SHANK in cultured neurones, which results in the enlargement of the dendritic spines (Sheng, 2001).

The PSD also contains other non-MAGUK proteins that mediate glutamate signalling. Yotiao, which gains its name from having a similar coiled structure to a popular Chinese breakfast, is highly abundant both at CNS synapses and neuromuscular junctions that associates with the c-terminal tail of specific NR1 splice variants (Lin et al., 1998). Yotiao expression has been confirmed in several tissues apart from brain. Abundant signal was demonstrated in both skeletal muscle and pancreas, whilst lower levels of expression were also identified in heart and placenta, suggesting that the protein may have alternative non-glutamatergic roles. Very little evidence exists on the function of Yotiao. The fact that NR1 subunits are able to cluster in the postsynaptic membrane in the absence of NR2 subunits (Ehlers et al., 1995) and subsequently lacking well characterised NR2-associated clustering proteins, suggests that Yotiao may be involved in NR1 clustering. Lin and colleagues, who demonstrated that Yotiao fractionates with cytoskeletal-associated proteins, confirmed this hypothesis, therefore providing the link between NR1 and the cytoskeleton (Lin et al., 1998). Yotiao has also been revealed as being responsible for recruiting PKA and protein phosphatase 1 (PP1) to the NMDA receptor and appear to counteract each other in the modulation of NMDA receptor channel conductance. In normal conditions, PP1 is continually active, inhibiting the activity of the receptor channel. However, increases in cellular cAMP results in PKA phosphorylation of the receptor, conferring an increase in receptor channel activity (Westphal et al., 1999). Therefore the function of the Yotiao protein is one of NR1 receptor subunit clustering and receptor channel modulation.

It is evident that the presence of the many multi-functional proteins in the PSD gives a different picture of the NMDA receptor at the CNS synapse. Rather than single

receptor channels acting independently to increase intracellular calcium concentration. NMDA and other glutamate receptors are present and linked to a mass of various scaffold and signalling proteins, which together modulate synaptic plasticity. The aim of this chapter is to investigate the expression of the regulatory NR2 and NR3 subunits and PSD proteins are expressed by megakaryocytes using well-characterised megakaryocytic cell lines and primary human megakaryocytes. This would provide a greater understanding of how the NMDA receptor may function in the megakaryocyte compared to its neuronal counterpart.

Basic exploratory research is often performed on megakaryocytic cell lines, two of the most common being used during this research are MEG-01 and human erythroleukaemia (HEL) cells (for review see Saito, 1997). Megakaryocytic cell lines are often derived from the bone marrow of patients with leukaemia and display many of the characteristics of megakaryocytopoiesis and differentiated human primary megakaryocytic cells. MEG-01 cells were derived from a patient suffering with megakaryoblastic crisis of chronic myeloid leukaemia (CML), whilst marrow taken from a patient with acute myelogenous leukaemia resulted in the development of HEL cells. In an undifferentiated state HEL cells possess features common to macrophages, erythrocytes and megakaryocytes (Long et al., 1990). However when stimulated by low concentration doses of PMA, the HEL cell gains a more megakaryocytic phenotype including expression of the megakaryocyte-specific markers CD41 and CD42a, as well as the typical 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), whilst stimulation of HEL cells with erythropoietin (EPO) results in erythrocyte development (Chu et al., 1998). These characteristics make the HEL cell line a useful model in lineage determination studies.

The MEG-01 cell line exhibits megakaryocytic properties (CD41, CD61, CD42a, von Willebrand factor (vWF)) whilst lacking markers for other lineages (Ogura et al., 1985). Expression of CD41, CD42a and vWF by MEG-01 cells is increased by PMA stimulation, which also induces a highly differentiated morphology (Ogura et al., 1989). Some reports indicate that MEG-01 cells produce platelet-like particles that are of similar size and display other features of circulating platelets (Takeuchi et al.,

1998). Unlike HEL cells, MEG-01 cells do not express the TPO receptor c-Mpl. Therefore, PMA is the only known differentiation-promoting agent for MEG-01 cells, whilst HEL cells can be stimulated to differentiate by culture in the presence of TPO (Matsumura et al., 1996).

It is important at this point to consider the potential problems of using phorbol esters to induce differentiation of megakaryocytic cell lines. Prolonged exposure to phorbol esters causes the almost complete depletion of certain PKC isoforms by proteolysis (Hug and Sarre, 1993). Therefore, by following the standard protocol of treating the cell lines for 72 hours in the presence of 100nM PMA to ensure total differentiation (see section 2.1.2), it is not surprising that many of the PKC isoforms are down-regulated following their activation. PMA treatment of MEG-01 cells, although rapidly activating the majority of PKC isoforms, subsequently leads to the down-regulation of the PKC-α, -ε and -θ isoforms following 1-2 hours of treatment. However, the βI and II PKC isoforms are down regulated very slowly, suggesting that the activation of PKC-α, -ε and -θ initiate differentiation whilst PKC-βI and βII are important in the maintenance of differentiation (Nagata et al., 1996).

Recently, other signalling molecules have been identified as having phorbol ester binding sites, namely chimaerins, protein kinase D (PKD)-1, Ras guanyl-releasing protein (GRP), DAG kinase-γ and Munc13 (for review see Brose and Rosenmund, 2002). These signalling molecules can regulate a range of downstream events, such as the MAPK pathway, which are able to stimulate megakaryocyte differentiation. The existence of these phorbol ester-binding molecules makes it important to take into account that non-PKC molecules may also be mediating PMA-induced megakaryocyte cell line differentiation. However, considering that the PKC antagonists GF-109203X and Ro-31-8220 prevent PMA-mediated megakaryocytic cell line differentiation (which may not however be specific PKC inhibitors (Alessi, 1997)), it appears that PKC remains the primary activator of megakaryocyte cell line differentiation (Lumelsky and Schwartz, 1997).

The development of stem cell separation techniques combined with the 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*. There are three main sources of human $CD34^+$ cells, umbilical cord blood (UCB), bone marrow and peripheral blood (for review see Majka et al., 2001). Although peripheral blood is the most accessible source of these cells, the megakaryoblast recovery after 11 days of culture is significantly less than the other sources. Megakaryoblast recovery is greatest in bone marrow, however collection of this tissue is invasive and restrictive. Consequently, 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, 1995; Majka et al., 2000).

The megakaryocytic cell lines and primary cells were used to determine the expression of NMDA receptor subunits and PSD proteins.

3.2 Materials and Methods

3.2.1. RNA isolation

Cells were washed twice with PBS before total RNA was extracted by adding 1ml TRIzol® (GibcoBRL) / $5x10^6$ cells, transferred to a 1.5ml Eppendorf tube and incubated for 5 minutes at room temperature. 200 µl of chloroform (Sigma) was added to the TRIzol® preparation and samples vortexed for 15 seconds before centrifugation at 12,000g for 20 minutes at 4°C. The upper aqueous phase was removed and placed into a new 1.5ml Eppendorf containing 500µl of isopropanol and incubated at -20°C for 20 minutes. RNA was precipitated in the isopropanol and pelleted by centrifugation at 12,000g for 10 minutes at 4°C and supernatant removed. The RNA pellet was washed in 70% v/v ethanol, centrifuged at 4°C for 5 minutes at 7,500g, air-dried and resuspended in 11µl RNase free H_20 (Life Technologies). RNA quality and yield was quantified by using an Ultraspec 2000 spectrophotometer by adding 1µl of purified RNA to 99µl of RNase free H_2O at 260nm and RNA quality analysed by running samples on a 1% TBE agarose electrophoresis gel. All RNA samples were stored at -80°C until use.

3.2.2 cDNA synthesis from total RNA

Genomic DNA was removed from RNA samples by using the DNA-free™ DNase digestion kit (Ambion) according to the manufacturers protocol. Briefly, total RNA was incubated with 0.1 volume of 10x DNase buffer and 1µl of DNase I for 30 minutes at 37°C. 0.1 volumes of DNase activation reagent was then added to the RNA samples and incubated at room temperature for 2 minutes, before the DNase activation reagent was pelleted by centrifugation at 12,000g for 1 minute. cDNA was synthesised from 10µg of DNase treated total RNA using the SuperScript II RT-PCR system (Life Technologies). 1µl oligo $(dT)_{12-18}$ was added to the RNA, the volume adjusted to 12µl using RNase-free H_2O and denatured at 70°C for 3 minutes. A master-mix preparation was prepared containing 10x first strand synthesis buffer, 25mM $MgCl_2$, 10mM dNTP mix, 0.1M DTT, 200U/µl SuperScript II and RNase-free H_2O, added to the RNA and incubated at 42°C for 1hr. The reaction was terminated by heating to 80°C for 5 minutes and RNA digested by treating with RNase H for 20

minutes at 37°C. cDNA samples were diluted 1:5 in RNase-free H_2O and stored at -20°C until use. cDNA integrity was confirmed by performing a reverse transcriptase polymerase chain reaction (RT-PCR) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.2.3 RT-PCR

RT-PCR master mix was prepared containing 0.5U Platinum Taq polymerase, 1x PCR buffer, 0.2mM dNTP mix, 1.5mM $MgCl_2$, 0.2µM forward and reverse primers (Genosys) and 5µl of 1:5 diluted cDNA. The final reaction mix was adjusted to a volume of 50µl/reaction using RNase-free H_2O. The sequence, position, size of product and annealing temperatures for each specific primer pair are listed in Table 1. RT-PCR reactions were performed in a DNA engine PTC200 thermal cycler (M. J. Research) and 10µl of PCR reaction product analysed by gel electrophoresis using a 1% agarose gel containing 0.2µg/ml ethidium bromide and visualisation by a U.V. lamp.

3.2.4 Northern blot analysis

3.2.4.1 Denaturing gel electrophoresis and northern transfer

10µg of total RNA samples were denatured at 65°C for 10 minutes before loading in 10µl of formamide, 3.5µl formaldehyde (37% v/v) and 1µl ethidium bromide. Samples were then fractionated on 0.8% denaturing gels containing 1x MOPS (3-[N-morpholino] propanesulphonic acid) and 3.7% v/v formaldehyde. Following electrophoresis, gels were washed twice in 10x SSC and RNA transferred by capillary blotting on to Hybond N+ nylon membrane (Amersham Biosciences, Amersham, UK) overnight in 10x SSC. The nylon membrane was subsequently washed twice in 2x SSC and the RNA permanently fixed to the membrane by U.V. irradiation using a UVC-508 Ultraviolet Crosslinker (Ultra Lum) and stored at room temperature until required.

Primer	Sequence	Position	Size	Tm	Cycle number
NMDAR1	F-5'-GGAAGGCGCCCCCCAGAAGC-3' R-5'-CGAAGCCCGAGCGGAAAAACAGC-3'	2953-3368	415bp	65℃	35
NMDAR2A	F-5'-CCGGCCTGGGTTGCTCTTC-3' R-5'-AGTTCGCTTTGGATTCTGTGCCTCA-3'	2875-3332	457bp	68℃	35
NMDAR2B	F-5'-CTGCCGGACATCACCACCACAACA-3' R-5'-CATCACGCGACCCACAGCCTTACC-3'	4283-4727	441bp	65℃	33
NMDAR2C	F-5'-GAACGGCATCATTGGGGAGGTGTA-3' R-5'-CGTGTAGCTGGCGAGGAAGATGAC-3'	1629-2088	459bp	68℃	35
NMDAR2D	F-5'-CCGCCGTGTGGGTGATGATGTTCG-3' R-5'-ACGCGGGGCTGGTTGTAG-3'	1836-2310	474bp	65℃	35
NMDAR3	F-5'-CTGCCGGACATCACCACCACAACA-3' R-5'-CATCACGCGACCCACAGCCTTACC-3'	1836-2310	474bp	65℃	35
Yotiao	F-5'-AAGGGAAGAAGAATTTGGTGTTGA-3' R-5'-TGAGGATCTGTTGTTTGGCTTGTA-3'	629-1034	405bp	60℃	37
PSD-95	F-5'-ACGGCGGGTTGAGCGACGAGAGT-3' R-5'-CAGGGAGCGGGGGCGGATGAA-3'	2461-2953	492bp	68℃	37
Chapsyn-110	F-5'-GCTCGGCTGTATGTGCGTTAGAAG-3' R-5'-GAGGCCTGGTGTAGTCGTCGTC-3'	805-1306	501bp	60℃	36
MALS	F-5'-CCACCCCCGCCCCAATCT-3' R-5'-CTCTTTTGAGGCCTCCGTGTCTTTGCAG-3'	143-647	504bp	76℃	36
CASK	F-5'-GCACTACTAGCCGCTGTGTCAAGTCAC-3' R-5'-TCGCCGTTTAAATAGGGAGAGGTG-3'	946-1439	493bp	65℃	35
SHANK	F-5'-AGGACCGGGGACTTCTTGATTGAG-3' R-5'-GATTCCTTGGCGTTCGTACACAGAGTT-3'	239-664	425bp	68℃	37
GAPDH	F-5'-GGTGAAGGTCGGWGTCAACGG-3' R-5'-GGTCATGAGYCCTTCCACGAT-3'	33-552	519bp	63℃	34

Table 1. Primer sequence, gene position, product size, annealing temperature and cycle number used by RT-PCR analysis.

3.2.4.2 Probe synthesis and hybridisation

RT-PCR of RNA derived from MEG-01 and HEL cells was used to generate templates for the labelling reaction. Radiolabelled probes were synthesised from 10ng of template cDNAs in 10mM TRIS HCl (pH 8.0), 1mM EDTA by incubating in random prime labelling mix (Rediprime^{TM}II, Amersham Biosciences) containing 50μCi α^{32}P-dCTP at 37°C for 20 minutes. Gel filtration (G-50 sephadex spin column. Boehringer Mannheim, (Roche, Basal, Switzerland)) was used to remove radiolabelled probes from unincorporated α^{32}P-dCTP and mixed with 100μl sheared salmon sperm DNA (10mg/ml, Boehringer Mannheim, (Roche)). Northern blots were incubated in 12ml Quickhyb buffer (Stratagene, CA) for 20 minutes at 68°C and probes, previously denatured at 95°C for 2 minutes, added to the blot. Hybridisation was performed at 68°C for 1 hour and membranes then washed twice in 2x SSC, 0.1% v/v SDS at room temperature and twice in 0.1x SSC, 0.1% v/v SDS at 60°C. BioMAX films (Kodak) were exposed to the northern blots with two intensifying screens (Kodak) for a minimum of 18 hours at -80°C. Films were developed using a X-ograph automated developer.

3.2.5 Immunolocalisation

Cytospin preparation and immunocytochemistry of MEG-01 cells in the absence and presence of PMA were performed as previously described (chapter 2.1.2). Cytospins were incubated with anti-NMDAR1 monoclonal antibody (Pharmingen; 1μg/ml) for 30 minutes followed by FITC-conjugated goat anti-mouse secondary antibody (Sigma; 1:500 dilution). Antibody-negative controls were incubated with non-specific mouse IgGs (Santa Cruz; 1μg/ml) in the place of primary antibody. Stained preparations were mounted in Vectorshield (Vector Laboratories) and fluorescence viewed immediately using a Leica DMLA microscope (Leica)

3.2.6 Laser microdissection and single cell RT-PCR

Rat tibiae were removed and snap frozen in chilled isopropanol. Specimens were mounted in Cryo-m-bed embedding compound (Bright, UK) on brass chucks and frozen in chilled isopentane. Slide frames and foils (Leica) were U.V. irradiated for 30 minutes using a UVC-508 Ultraviolet Crosslinker (Ultra Lum) to remove all residual RNAses and to reduce the electrostatic charge of the foil. 7μM thick frozen

sections were collected on slide foils, using a Bright OTF500 cryostat (Bright) fitted with a tungsten carbide tipped knife and used immediately. Specimens were air-dried and stained with 0.1% toluidine blue. U.V. laser microdissection (LMD) was performed using a Leica DMLA light microscope fitted with a Leica AS LMD microdissection unit (Leica). A single megakaryocyte, identified by size, morphology and multilobed nucleus was excised from rat bone and collected in a 200µl Eppendorf containing 12 µl of lysis buffer (50mM TRIS HCl (pH 8.0), 100mM NaCl, 5mM $MgCl_2$, 0.5% Triton X-100, 1mM DTT and 100 units RNAse out (Invitrogen)). Samples were centrifuged at 12,000g, 4°C for 10 minutes, chilled rapidly on dry ice and stored at -80°C until use. Synthesis of cDNA and RT-PCR master mix preparation was performed as previously described in this chapter. Identical primers (see Table. 1) were also used to amplify products by single cell RT-PCR, with cycle number increased to 50.

3.3 Results

3.3.1 mRNA expression of NMDA receptor subunits and associated proteins by megakaryocytic cell lines

3.3.1.1 mRNA expression of NMDA receptor subunits by megakaryocytic cells

RT-PCR was used to confirm expression of NR2D by MEG-01 cells in the absence and presence of PMA (Fig. 3.3.1). However, the expression of other NMDA receptor subunits was also identified. In MEG-01 cells, RT-PCR identified expression of NR2A (+PMA only), NR2C (weakly +/- PMA), NR2D (+/- PMA) and NR3 (-PMA only). Expression of NR2B by MEG-01 cells was not detected. These findings would insinuate expression of specific NMDA receptor subunits only, levels of which appear to change during differentiation.

Parallel investigation of NMDA receptor subunit expression by HEL cells by RT-PCR also confirmed the expression of select subunits during PMA-mediated differentiation (Fig. 3.3.1). Similar to MEG-01 cells, NR2B expression was not detected, whilst NR2C mRNA expression was identified in PMA-treated and untreated cells, with an apparent increase in NR2C expression following PMA exposure and a similar pattern of NR2D expression was also identified. In HEL cells, NR2A expression was not detected, contrasting to MEG-01 cells. It was also found that HEL cells lacked NR3 subunit expression at both immature and differentiated stages. All subunits were expressed by human foetal forebrain positive controls. Water controls were carried out in parallel to the above experiments and no products were amplified.

Northern blot analysis was used to quantify expression of NR2D by HEL cells during differentiation. 10μg of total HEL RNA resulted in the identification expression of a 4.7 kb species in both PMA-treated and untreated samples (Fig. 3.3.2). Expression of NR2D increased following PMA treatment by approximately 45% when analysed by densitometry, normalising with 28S RNA.

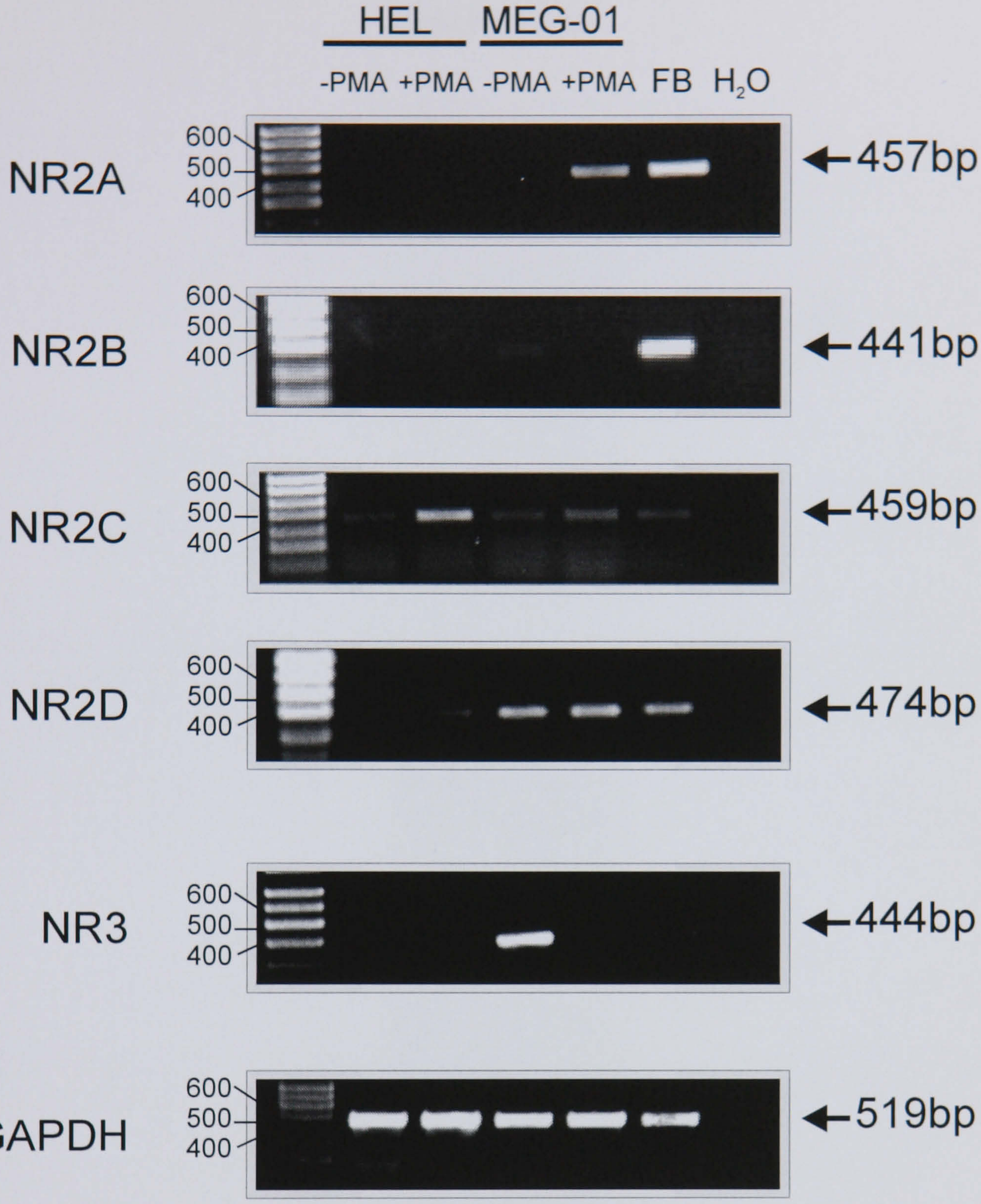


Figure 3.3.1. Expression of NMDA receptor subunits by megakaryoblastic cell lines in the absence and presence of PMA. RT-PCR was used to demonstrate mRNA expression of NR2A-D and NR3 receptor subunits by HEL and MEG-01 megakaryocytic cell lines in the absence and presence of PMA. Human foetal forebrain (FB) was used as a positive control and expressed all NMDA receptor subunits. Control reactions were performed in parallel, in the absence of cDNA (H_2O). Results are representative of 2 independent experiments.

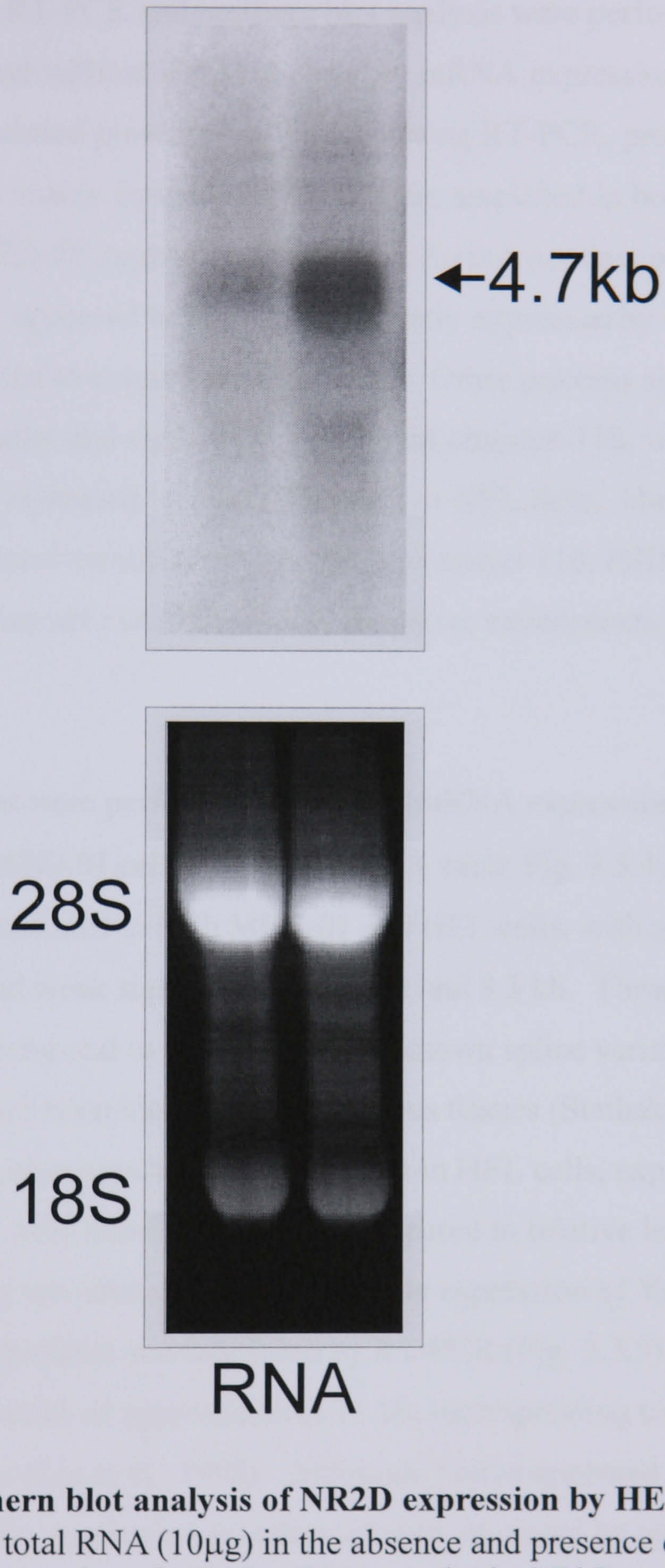


Figure 3.3.2. Northern blot analysis of NR2D expression by HEL cells. Northern blot analysis of HEL total RNA (10μg) in the absence and presence of PMA was used to identify relative expression of NR2D mRNA. A single 4.7 kDa mRNA species was identified, which corresponds in size to the NR2D subunit previous identified in human brain. NR2D expression was increased following PMA treatment. Equal loading was demonstrated by comparing total RNA. Experiment was performed once.

3.3.1.2 mRNA expression of NMDA associated proteins by megakaryocytic cell lines

Similar studies using RT-PCR and northern blot analysis were performed on MEG-01 and HEL cells with and without PMA. to compare mRNA expression of specific NMDA receptor associated protein (Fig. 3.3.3). Using RT-PCR, products identical in size to those found in human forebrain PSD-95 were amplified in both HEL and MEG-01 samples. PSD-95 expression appeared to be independent of differentiation in HEL cells, whereas it appeared to be more abundantly expressed by PMA-treated MEG-01 cells compared to control untreated cells. Other proteins associated with NMDA receptor function and signalling, Yotiao and chapsyn-110, were not detected by RT-PCR as being expressed by either MEG-01 or HEL cells. Human foetal forebrain positive control identified expression of chapsyn-110, PSD-95 and Yotiao. Water controls were carried out in parallel to the above experiments and no products were amplified.

Northern blot analyses were performed to confirm mRNA expression of PSD-95 and Yotiao by HEL and MEG-01 cells (10µg total RNA each; Fig. 3.3.4). PSD-95 expression was demonstrated in both MEG-01 and HEL cells, with an abundant signal identified at 4.2 kb and weak signals present at 6.2 and 8.3 kb. These signals of varying sizes may correspond to three of the four known splice variants of the PSD-95 homologue, which have been identified in numerous tissues (Stathakis et al., 1997). Whilst PSD-95 expression appeared to be constant in HEL cells, expression was increased in MEG-01 cells treated with PMA compared to relative levels of 28S RNA. Northern blot analysis was also used to demonstrate expression of Yotiao by HEL cells, even though no product was amplified by RT-PCR (Fig. 3.3.5). Northern blot analysis revealed a species of approximately 11 kb, corresponding to that found in brain and other tissues (Lin et al., 1998). Although Yotiao appeared to be weakly expressed by HEL cells, expression was also notably increased by exposure of HEL cells to PMA when compared to relative intensity of 28S RNA.

The above results confirmed identification of NR2D expression and provided evidence of NR2 and NR3 subunit expression by megakaryocytic cells lines.

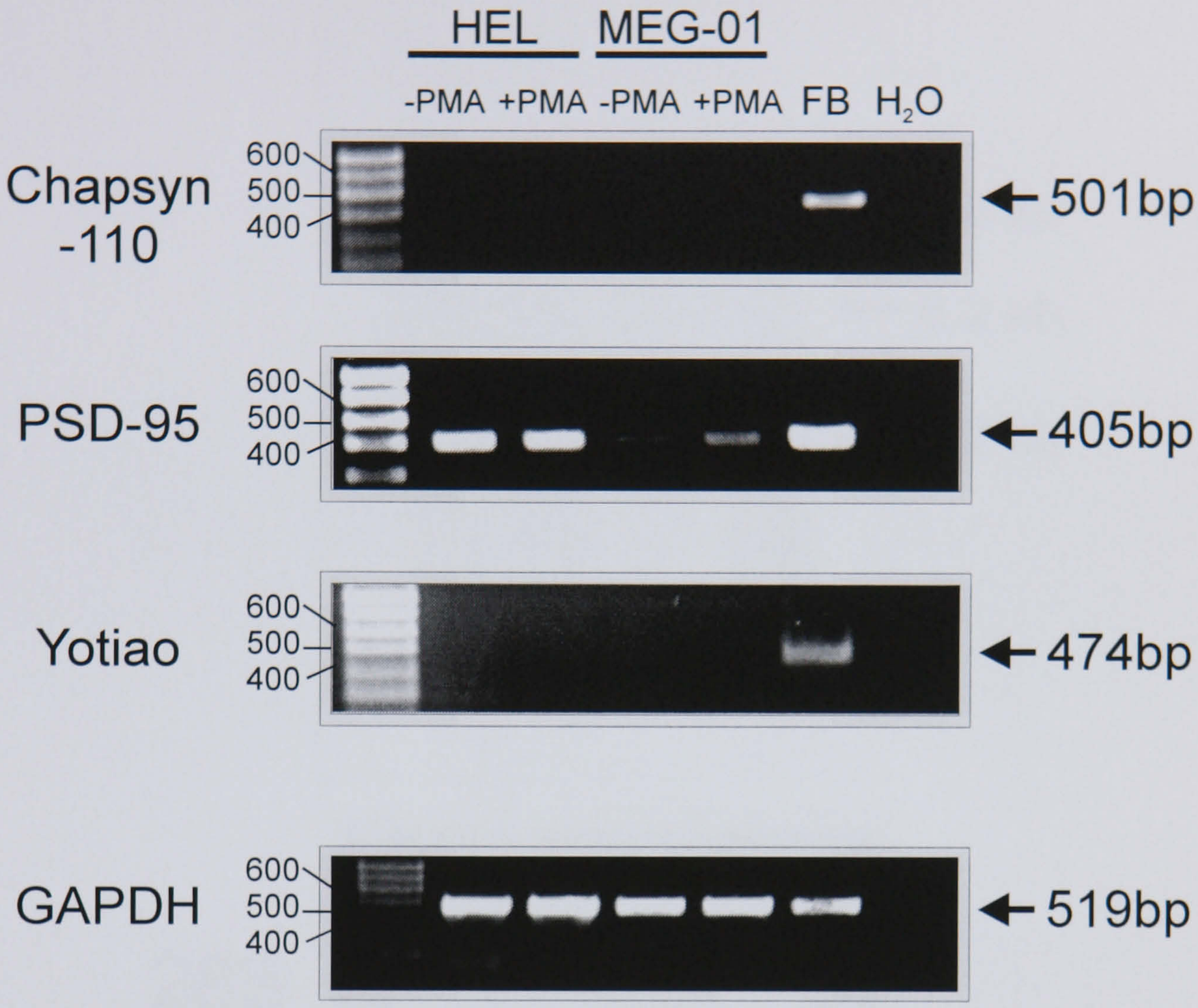


Figure 3.3.3. Expression of NMDA receptor associated proteins by megakaryoblastic cell lines in the absence and presence of PMA. RT-PCR was used to determine expression of NMDA receptor-associated protein mRNA expression by MEG-01 and HEL cells in the absence and presence of PMA. HEL cells were shown to express PSD-95 with and without PMA treatment, whilst MEG-01 cells only in the presence of PMA exhibited weak PSD-95 expression. Expression of Chapsyn-110 and Yotiao was not detected in HEL and MEG-01 cells. Human foetal forebrain (FB) was used as a positive control and expressed all NMDA receptor subunits. Control reactions containing were performed in parallel, in the absence of cDNA (H_2O). Results are representative of two independent experiments.

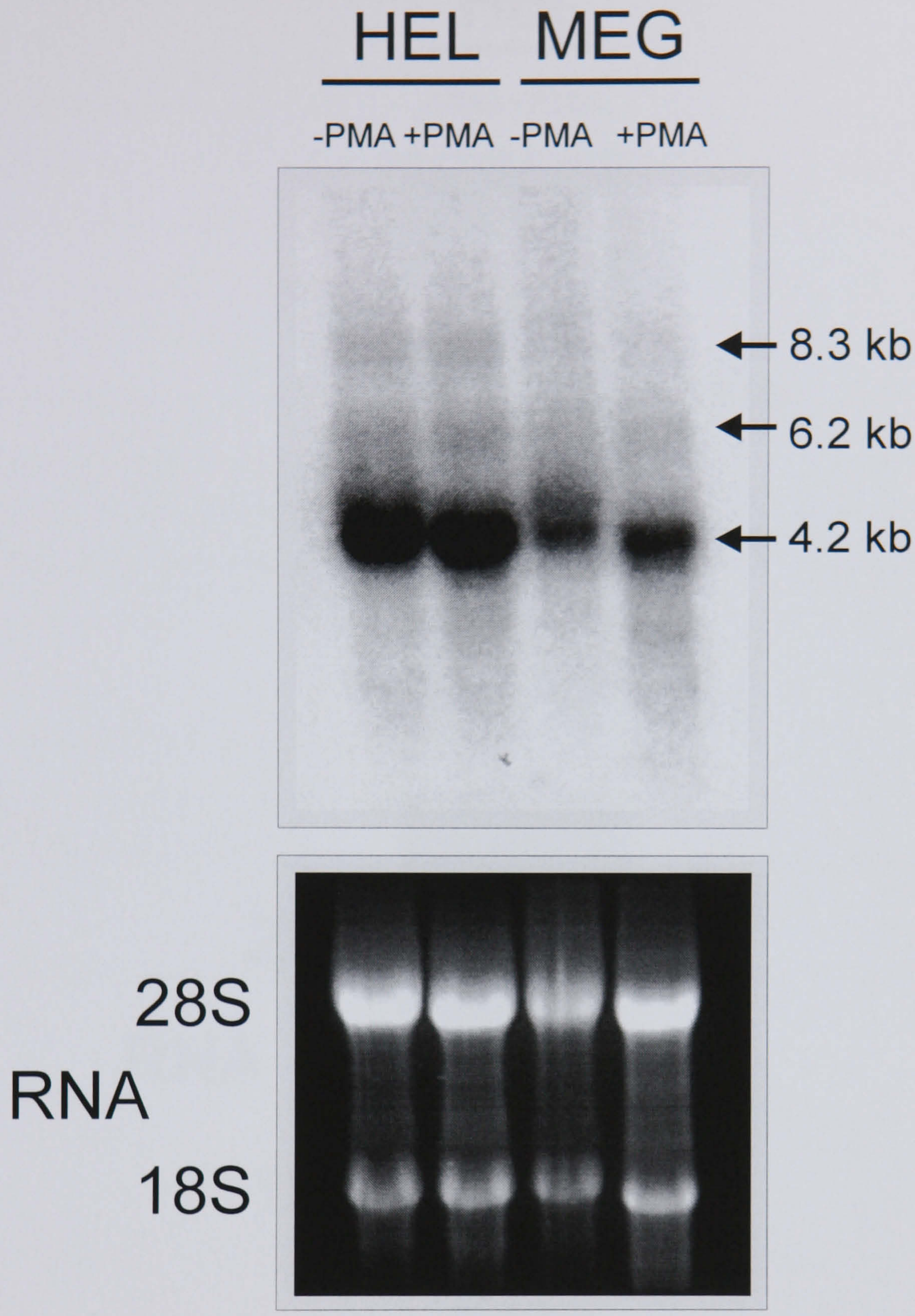


Figure 3.3.4. Northern blot analysis of PSD-95 expression by HEL and MEG-01 cells. Northern blot analysis of total RNA (10μg) was used to demonstrate expression of PSD-95 by both HEL and MEG-01 cells. PSD-95 expression by MEG-01 cells, when compared to total RNA, appears to increase slightly by treating cells with PMA, whilst very little difference is observed in between treatment of HEL cells. Experiment was performed once.

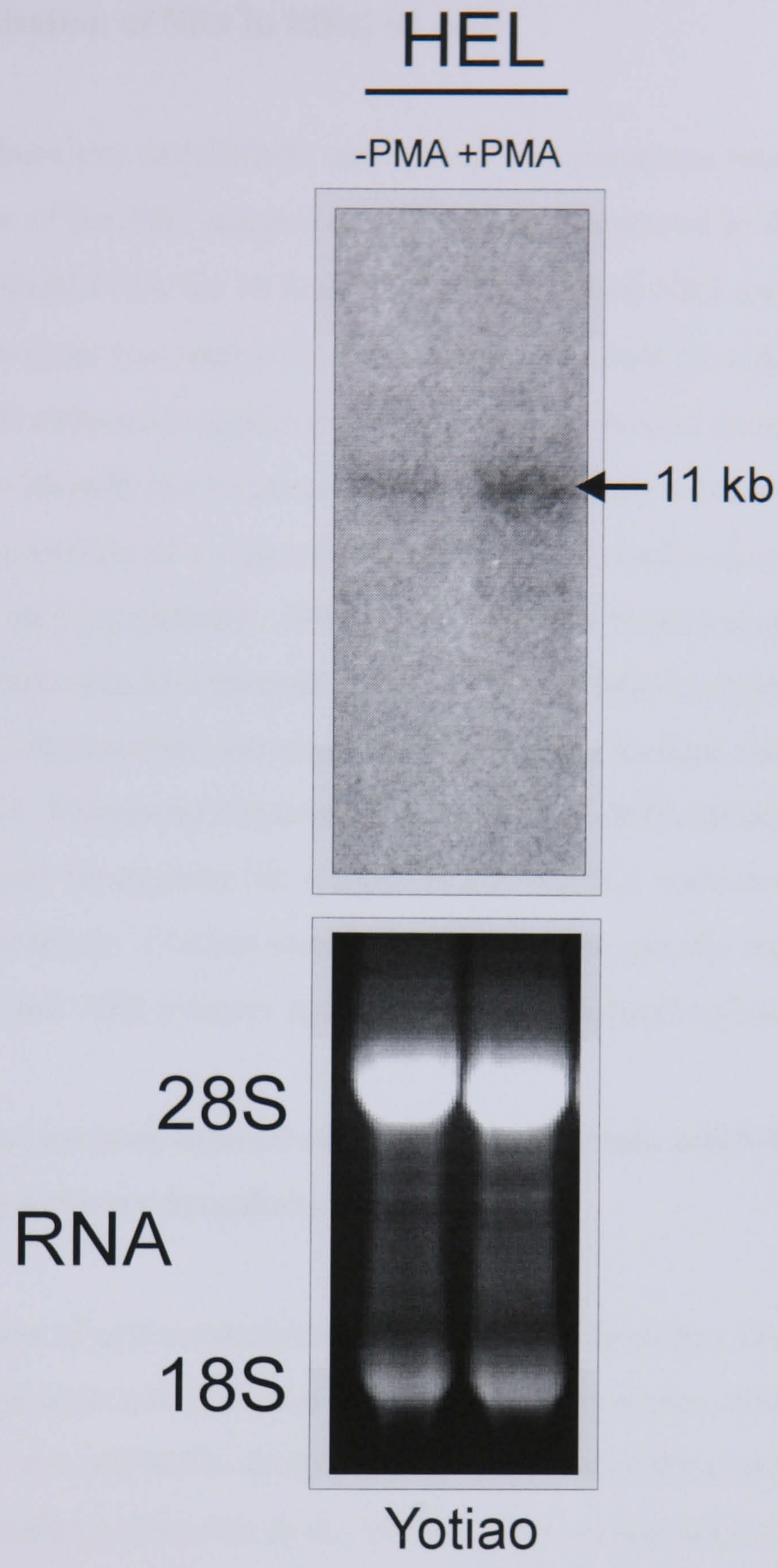


Figure 3.3.5 Northern blot analysis of Yotiao expression by HEL cells. Northern blot analysis of total RNA (10μg) was used to demonstrate expression of Yotiao by both HEL cells. Yotiao expression by HEL cells was shown to have increased following PMA treatment, determined by comparing Yotiao expression to total RNA. Experiment was performed once.

3.3.2 Localisation of NR1 in MEG-01 cells

Immunocytochemistry of MEG-01 cell cytospin preparations was used to determine the localisation of the NR1 subunit in MEG-01 cells cultured in the absence and presence of 100nM PMA for 72 hours (Fig. 3.3.6). Anti-NR1 antibodies (1μg/ml) were used in conjunction with a FITC-secondary antibody in order to accurately localise subunit expression (green positive staining). Nuclei were counterstained with DAPI (blue) to identify the location of the nucleus. Cells were examined by fluorescent microscopy at a magnification of x40 and results shown illustrate representative staining patterns. NR1 expression was restricted usually to a single small cytoplasmic area in untreated MEG-01 cells. PMA-treatment of MEG-01 cells resulted in the characteristic increases in cytoplasm to nucleus ratio, cell size and nuclear volume. Following PMA-mediated MEG-01 differentiation, NR1 was distributed evenly throughout the cytoplasm and was not restricted to any particular cytoplasmic locations. Control samples, in which non-specific mouse IgGs were used in place of the anti-NR1 primary antibody, exhibit the level of background staining.

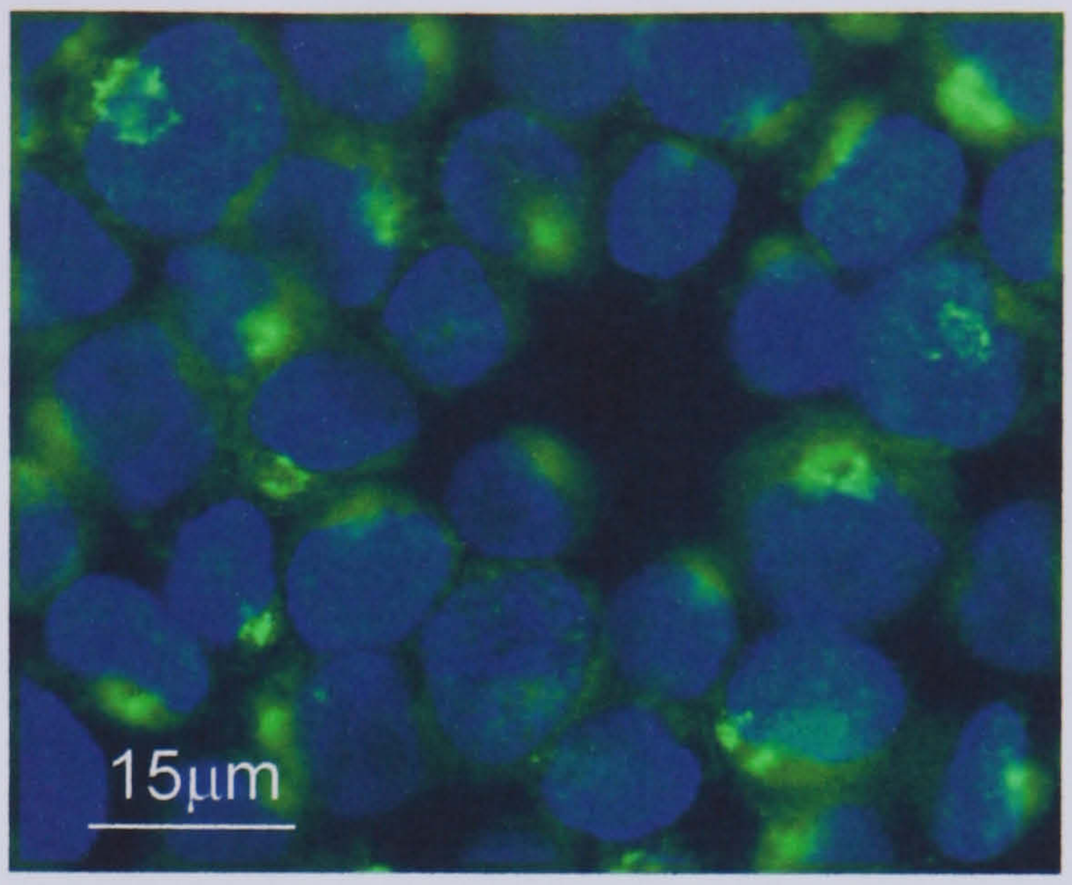
3.3.3 NMDA receptor subunit and associated protein mRNA expression by human primary megakaryocytes

The development of cell separation and culture techniques has made it possible to investigate large numbers of human primary megakaryocytes, derived from UCB. This technique was utilised to establish the expression of the essential NR1 subunit by northern blot analysis (Genever et al., 1999). This section aims to ascertain if primary human megakaryocytes express mRNA for the regulatory NR2 subunits and NMDA receptor associated proteins.

RT-PCR analysis was used to determine expression of NR2 subunits and NMDA receptor associated proteins by human primary megakaryocytes, derived from $CD34^+$ UCB cells, following exposure to 25ng/ml TPO for 14 days. Gene-specific primers to human brain NR2A-D, Yotiao, PSD-95, chapsyn-110, CASK, MALS, and SHANK and RT-PCR programmes used in these experiments were identical to those previously described in chapter 3.3.1.1 and listed in Table 1. Products from primary human

Original in colour

MEG-01
-PMA
NR1



MEG-01
+PMA
NR1

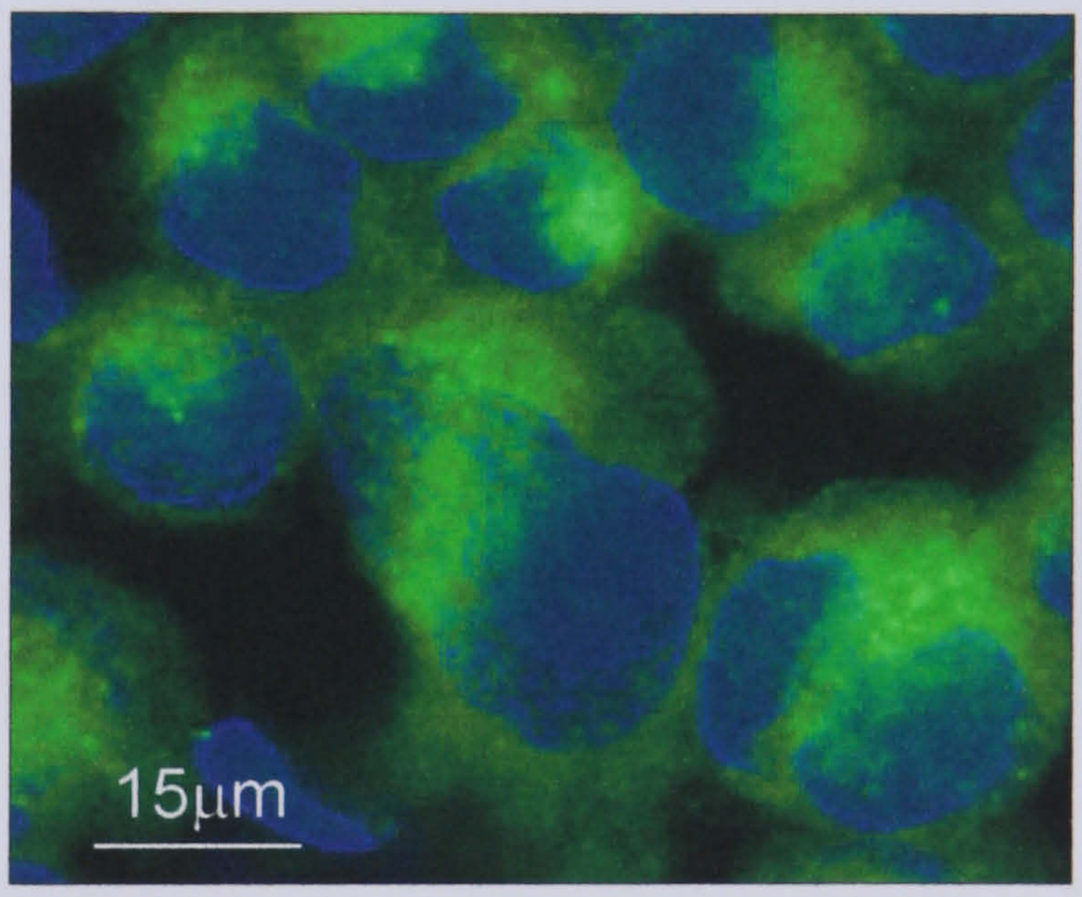


Figure 3.3.6. Immunolocalisation of NR1 in MEG-01 cells in the absence and presence of PMA. Cytospin preparations of MEG-01 cells cultured in the absence or presence of PMA for 72 hours. Untreated MEG-01 cells expressed NR1 (green staining) which was localised at distinct cytoplasmic regions, whilst NR1 was prominently expressed throughout the cytoplasm of PMA-treated MEG-01 cells. Nuclei were stained blue (DAPI). The experiment was performed once.

megakaryocytes identical in size to those found in human foetal cerebellums were amplified for NR2A and NR2D. No product was observed for NR2B and C (Fig. 3.3.7 A). Human foetal cerebellum was used as a positive control all four subunits.

RT-PCR amplified products for the receptor scaffold and signalling proteins Yotiao and PSD-95 only (Fig, 3.3.7 B). mRNA expression of other NMDA receptor associated proteins, chapsyn-110, CASK, MALS and SHANK were identified in human foetal cerebellum only. Water controls were carried out in parallel to the above experiments and no products were amplified (data not shown).

3.3.4 mRNA expression of NMDA receptor subunits by a single ex vivo rat bone marrow megakaryocyte

The recent development of microdissection technology has enabled the excision of target tissue from histological samples. Accurate laser microdissection facilitates the removal of different sized specimens, from a single cell to large cellular groups, identified by high power microscopy and sterile excision allows subsequent expression analyses such as single cell RT-PCR. This method was utilised for the removal of a single megakaryocyte, identified by the cells large size, morphology and multilobed nucleus compared to other cells in the rat bone marrow for subsequent analysis of NMDA receptor subunit expression by RT-PCR (Fig. 3.3.8 A).

Single cell RT-PCR analysis (chapter 3.2.3) using primers specific to NR1, NR2A and NR2D genes in human brain (Table 1), identified weak mRNA expression of NR2A only (Fig. 3.3.8 B). Products for NR1 and NR2D were amplified in human foetal forebrain only. In control reactions lacking reverse transcriptase, no products were amplified.

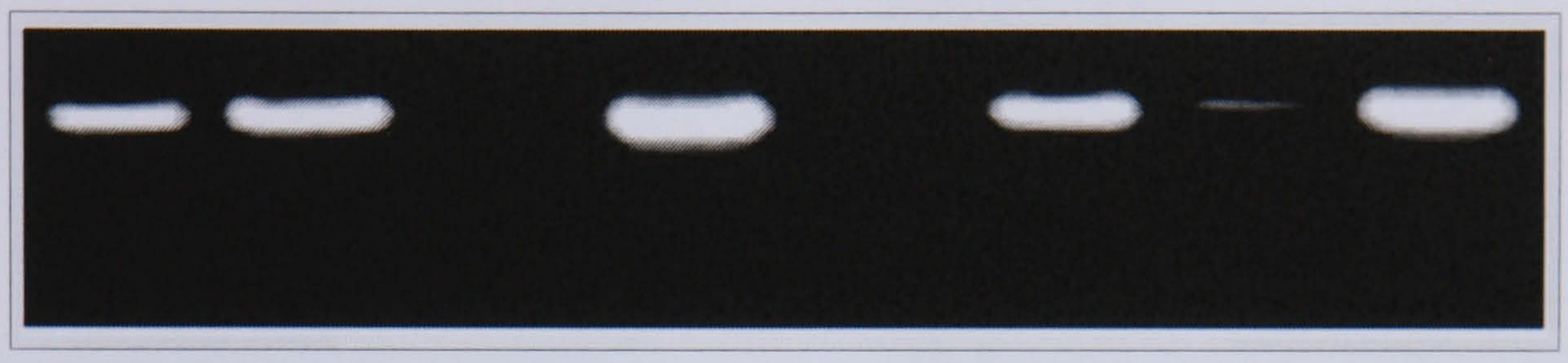


Figure 3.3.7. Expression of NMDA receptor subunits and associated proteins by human primary megakaryocytes. RT-PCR was used to amplify products for NR2A and NR2D from human $CD34^{+}$-derived megakaryocytes (MK), whilst NR2B and C were identified in human foetal cerebellum (CB) only (A). RT-PCR was also identified expression of NMDA receptor associated proteins Yotiao and PSD-95 (B). Other associated proteins, chapsyn-110, CASK, MALS and SHANK were amplified in human foetal cerebellum only. Results are representative of three independent experiments.

Original in colour

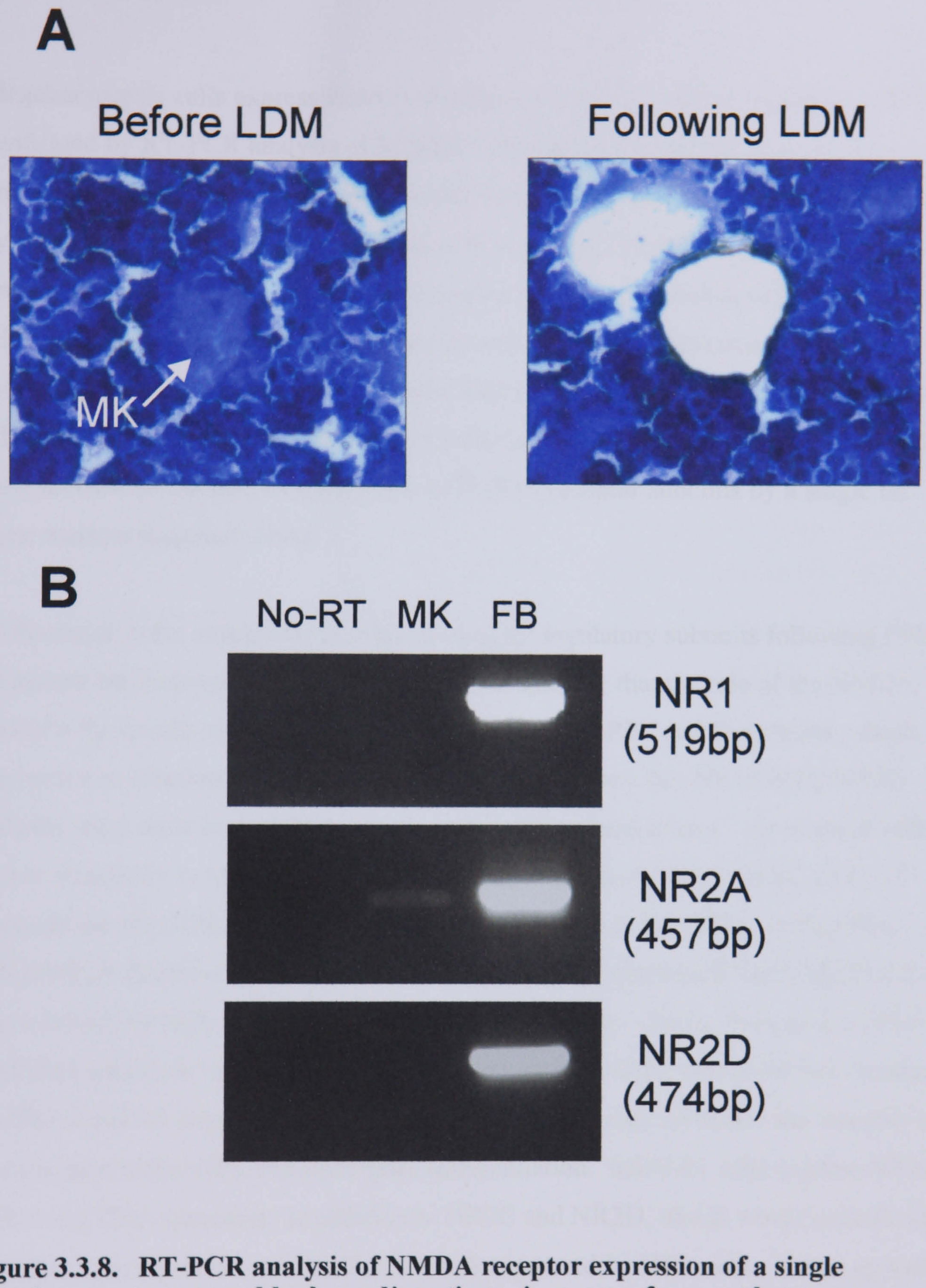


Figure 3.3.8. RT-PCR analysis of NMDA receptor expression of a single megakaryocyte removed by laser dissection microscopy from rat bone marrow. Laser dissection microscopy (LMD) was used to extract a single megakaryocyte from toluidine blue stained rat bone marrow (A). Total RNA was extracted from the megakaryocyte and RT-PCR identified weak expression of NR2A. NR1 and NR2D were identified in human foetal forebrain only (FB). In control reactions lacking reverse transcriptase (No-RT), no products were amplified. Experiment was performed once.

3.4 Discussion

Megakaryocytic cells express functional, channel-forming NMDA receptors, a finding confirmed by RT-PCR analysis of MEG-01 cells and bone marrow, immunohistochemistry, *in vivo* studies and northern blot analysis for NR1 expression by $CD34^+$-derived primary cells (Genever et al., 1999). However, for a greater understanding of NMDA signalling in megakaryocytes, expression of the regulatory NR2 and 3 subunits during megakaryocytic cell line differentiation and by primary megakaryocytes is required. This chapter addresses the expression of these subunits whilst also confirming the expression of proteins identified in the PSD and for the first time focuses on the mRNA expression of NMDA receptor subunits by a single rat bone marrow megakaryocyte.

Differences in the expression of NMDA receptor regulatory subunits following PMA treatment and between HEL and MEG-01 cells indicate that the role of the NMDA receptor these cells may vary. HEL cells express the NR2C and D subunits, which, if expressed as diheteromers with NR1 (there is no evidence that NR1/NR2C/NR2D triheteromers exist in the CNS) would confer a "low-conductance" ion channel with a lower sensitivity to Mg^{2+} and slow deactivation (~300ms for NR1/NR2C and ~1.7 seconds for NR1/NR2D; Behe et al., 1999; Momiyama et al., 1996). In the CNS, NR1/NR2A diheteromers, in contrast to the previously mentioned NR1/NR2C or D, have extremely high conductance, fast deactivation time (~50ms; Behe et al., 1999) and high sensitivity to Mg^{2+}. The apparent increase in NR2C and confirmed increase in NR2D mRNA expression following PMA treatment may influence the function of the receptor as the HEL cell undergoes differentiation. MEG-01 cells express NR2A following PMA treatment, in addition to NR2C and NR2D, which would provide this receptor with a broader activity than the one expressed by HEL cells. It also appears that NR2A and NR2C expression is increased by PMA treatment, implying an increased role for these receptor subunits either during differentiation or in fully differentiated cells, however these findings require confirmation by northern blot analysis. The lack of NR2B expression by either HEL or MEG-01 cells may suggest that an NMDA receptor composition of "intermediate" activity is not utilised by the megakaryocyte.

The functions of various NR2 subunits can be assessed by the use of subunit selective pharmacological agonists and antagonists. One such example is the non-competitive NMDA receptor channel block, MK-801, which shall be discussed at greater depth in chapter 4. Recombinant NR1/NR2A has a higher sensitivity to MK-801 than NR1/NR2B and C (no data exists for recombinant NR1/NR2D: Chazot et al., 1994), which may have implications on the functional studies described in chapter 4, due to MK-801 being employed as a pharmacological NMDA receptor antagonist. Many of the subunit-specific inhibitors target NR2B-containing receptors, however. cis-1-[phenanthren-2yl-carbonyl] piperaxzin-2, 3-dicarboxylic acid (PPDA) specifically blocks NR2C and D containing receptors (Hrabetova et al., 2000) and *N,N,N',N'*-tetrakis-[2-pyridmethyl]-ethylenediamine (TPEN) inhibits only NR2A-containing NMDA receptors (Paoletti et al., 1997). Electrophysiological experiments, which have previously been carried out on megakaryocytes (Thomas et al., 2001), could exploit these subunit-specific inhibitors to give a greater idea of megakaryocyte receptor composition.

Expression of the NR3 subunit by non-PMA treated MEG-01 cells further increases the diversity of the NMDA receptor in this cell type. This subunit has the ability, when co-assembled with the high calcium conductance NR1/NR2A channel (i.e. formation of a NR1/NR2A/NR3 receptor), to lower calcium influx by five-fold (Das et al., 1998; Perez-Otano et al., 2001). The expression of this subunit by MEG-01 cells only in an undifferentiated state may imply that the receptor is being kept in a relatively unresponsive state until the cell becomes differentiated. The reduction in NR3 expression in addition to apparent increase in the expression in NR2A by the differentiated MEG-01 cell, suggests that the increase in receptor conductance is required only in the mature cell type. The lack of NR3 expression by HEL cells may be correlated to the absence of NR2A expression, given that, as the receptor would already have a low conductance, hence the "volume control" effect of the NR3 subunit would have a minimal effect.

The confirmation of PSD-95 and Yotiao expression by HEL cells and PSD-95 expression by MEG-01 cells adds to the NMDA receptor-signalling complex in the megakaryocyte. PSD-95 provides an association between the functional

megakaryocytic NMDA receptor and activation of downstream signalling pathways such as MAPK and nNOS. The presence of PSD-95 may also lead to NMDA receptor clustering in the megakaryocyte, although whether interaction is essential in megakaryocytes as it is for synaptic plasticity in the postsynaptic neurons remains unclear. PMA treatment may also result in a small increase in PSD-95 expression by MEG-01 cells, which could be due either to an increase in PSD-95-mediated signalling, or directly correlated to increases in NR2 subunit expression. It is also noteworthy that both MEG-01 and HEL cells express three different PSD-95 transcripts, which may be of functional importance in non-neuronal tissues (Stathakis et al., 1997). Only the ubiquitously expressed 4.2kb transcript has been identified in brain, whilst expression of both 6.2 and 8.3kb transcripts found in megakaryocytes has also been demonstrated in pancreas, prostate, testis and ovary. Stathakis and colleagues state no function for PSD-95 in these tissues, however it is interesting to note that glutamate signalling was subsequently identified in many of the tissue samples they investigated. The PMA-mediated increase in Yotiao expression by HEL cells raises the possibility that NMDA receptor activity can undergo greater regulation in mature megakaryocytes. Again, whether Yotiao is also implicated in receptor clustering, requires further investigation.

Indeed, a greater level of NMDA receptor clustering following PMA treatment is not supported by the findings of NR1 immunolocalisation in MEG-01 cells in the absence and presence of PMA. It was demonstrated that NR1 expression appeared to be evenly distributed throughout the cytoplasm following PMA treatment, and not at distinct membrane regions as observed in postsynaptic neurons. NMDA receptor clustering to the postsynaptic plasma membrane is vital for correct function. It seems confusing therefore that PMA treated MEG-01 cells display NR1 diffusely distributed throughout the cytoplasm. Matsuda and Hirai demonstrated that disrupting actin and microtubule organisation in human embryonic kidney (HEK) 293 cells resulted in profound rearrangements of NR1 distribution (Matsuda and Hirai, 1999). Interestingly, in actin filament-disrupted cells, NR1 localisation shifted from the plasma membrane and formed cytoplasmic "macroclusters", akin to those formed in non-PMA treated MEG-01 cells. However, in microtubule-disrupted cells, NR1 was distributed evenly throughout the cytoplasm, comparable to the findings of PMA-treated MEG-01 cells. The work by Matsuda and Hirai reveals the importance

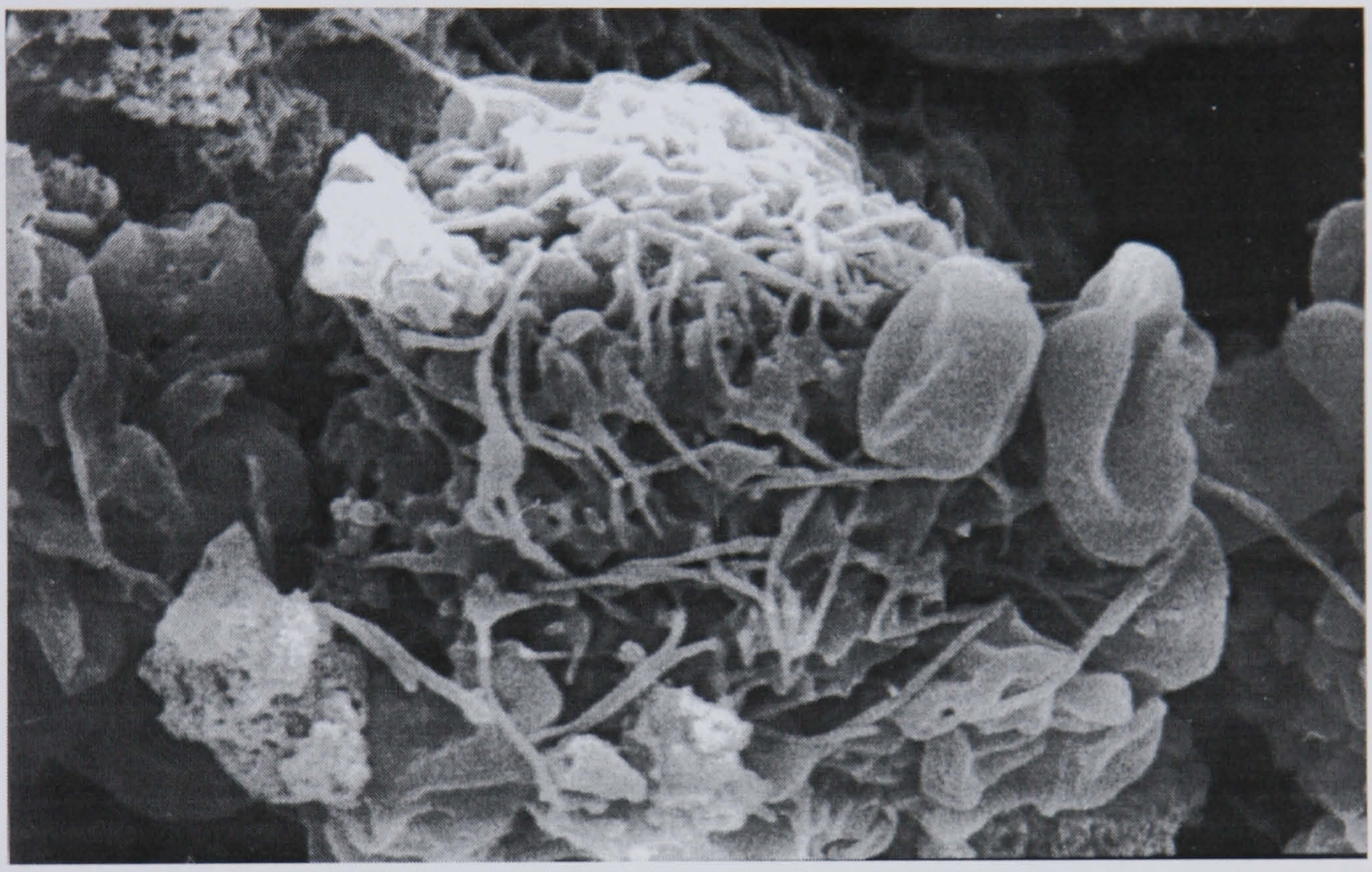
of the cytoskeleton in NMDA receptor clustering, however the changes in cytoskeletal organisation observed during MEG-01 cell differentiation may alter NR1 localisation. However, NR1 immunohistochemistry performed on primary bone marrow megakaryocytes indicate a more membrane located receptor distribution (Genever et al., 1999). This may signify differences in cytoskeletal organisation between cell lines and primary cells, leading to different receptor localisations.

RT-PCR analysis for NMDA receptor subunit and PSD mRNA identified expression of NR2A, NR2D, PSD-95 and Yotiao by human primary megakaryocytes for the first time. Therefore, in a manner similar to the megakaryocytic cell lines described above, expression of NR1/NR2A or NR1/NR2D diheteromers would confer receptors of different conductance and magnesium sensitivity and again an "intermediate" receptor type expressing NR2B or C would not form. These findings also imply that primary cell functional studies using the NMDA receptor antagonist MK-801, described in subsequent chapters, should have a high affinity for these receptors. Further work regarding primary cell subunit expression should focus on northern blot analysis at different time pints to identify changes in receptor subunit composition during differentiation. The same conclusions can be drawn regarding the expression of PSD-95 and Yotiao. Other PSD proteins, chapsyn-110, CASK, MALS and SHANK appear not to be expressed or expressed at negligible levels, implying that the primary megakaryocytes express only the components for "specialised" NMDA receptor signalling.

Laser microdissection and subsequent single cell RT-PCR analysis is the first example of this technology carried out on a bone marrow megakaryocyte. This technique confirmed mRNA expression of the NR2A subunit, but unfortunately due to time and technical constraints, it was not possible to optimise the single-cell RT-PCR protocol, possibly leading to NR1 and NR2D products not being detected. The absence or presence of NMDA receptor subunits expressed by bone marrow megakaryocytes cannot be confirmed until an exact protocol for laser dissected-megakaryocyte single cell RT-PCR is achieved. Future use of this technique may provide a greater understanding of bone marrow megakaryocyte mRNA expression.

This chapter has established that many of the components that encompass the NMDA receptor signalling system in the CNS are expressed by megakaryocytes. As megakaryocyte NMDA receptor research is still in its infancy, it currently has to be assumed that the receptors function in a similar manner to those found in the CNS. Such assumptions may however be imprudent. Due to the nature of neuronal cells, the function of the NMDA receptor in the CNS is highly unlikely to be identical to those expressed by megakaryocytes. The presence of regulatory receptor subunits would suggest however, that regulation of the receptor activity is of importance, especially before, during and after differentiation. I would also propose that megakaryocytic PSD-95 acts as a NMDA receptor associated signalling protein rather than distinct receptor clustering. This work still requires clarification. This would include analysis of the expression of all NMDA receptor subunits and PSD proteins by cell lines and by primary cells at various stages of differentiation by northern blot analysis, in addition to receptor subunit/PSD protein co-localisations and co-immunoprecipitations. However, the exact nature of the receptor will only be clarified by electrophysiological investigation of agonist response and receptor conductance. Such confirmation would provide a stronger grounding on which to tackle the main aim of this research, how this receptor affects megakaryocyte function.

Chapter 4



Function of the Megakaryocytic NMDA Receptor

The formation of spindle-like proplatelet structures on the surface of human primary megakaryocytes demonstrated by scanning electron microscopy (magnification x 10,000)

Chapter 4

Function of the Megakaryocytic NMDA Receptor

4.1 Introduction

The characterisation of the megakaryocytic NMDA receptor as described in chapter 3, confirms the existence of NMDA receptor components required for receptor function. These data therefore provoke intriguing questions regarding the function of NMDA receptors in megakaryocytes. Exploratory studies by Genever and co-workers identified some functions of the NMDA receptor in MEG-01 cells related to PMA-mediated differentiation and adhesion (Genever et al., 1999[a]). These findings, in addition to numerous others directed at determining NMDA receptor function in neuronal cells and peripheral tissues, used the specific NMDA receptor antagonist dizocilpine, otherwise known as MK-801 (Peet et al., 1999; Genever et al., 1999[b], Huang et al., 1999; Garcia-Zaragoza, 2000; Sin et al., 2002). By using this antagonist, it is possible to identify the effect on a particular cell as a direct consequence of aberrant NMDA receptor function.

NMDA receptor antagonist research has predominantly focused on the ability of these compounds to prevent neuronal degeneration in acute brain injury conditions such as epilepsy, head trauma and stroke, which are a result of excessive NMDA receptor activation (for review see Hickenbottom and Grotta, 1998). The neuroprotective actions of the receptor antagonists do however have psychotic side effects, such as schizophrenia in humans (Javitt and Zukin, 1991) and neurotoxicity in rats (Olney et al., 1991; Jevtovic-Todorovic et al., 1998; Farber et al., 2002). Indeed, antagonist-induced NMDA receptor hypofunction strongly resembles naturally occurring events in the ageing human brain, such as Alzheimer's disease (Olney and Farber, 1995; Wozniak et al., 1998). As such, extensive research into the function of NMDA receptor antagonists has been able to identify the detailed actions of these compounds.

MK-801 is an extremely potent, non-competitive antagonist that binds only to NMDA-type glutamate receptors (Wong et al., 1986). As a result, MK-801 is a highly effective neuroprotectant against extremely high doses of NMDA, but also results in extreme psychotic side effects (Woodruff et al., 1987). MK-801 acts by binding to a site within the open NMDA receptor ion channel, thereby blocking receptor-mediated calcium entry (DeLorenzo et al., 1998; Ikonomidou et al., 1999), a mechanism that is also shared by the common anaesthetic agent ketamine (Stone and Addae, 2002).

The results of MK-801 treatment on MEG-01 differentiation as described by Genever and co-workers, demonstrated that NMDA receptor inhibition might inhibit the PMA-mediated differentiation of these cells. However, other functions of the receptor in these cells remain unanswered and will be a subject of this chapter. Although immunological, morphological and molecular determination of megakaryocytic cell line differentiation is an effective tool by which to start functional investigations, certain characteristics of these cell lines are not comparable with primary bone marrow megakaryocytes. A more representative *in vitro* model of megakaryocyte differentiation may be achieved by generating primary human megakaryocytes from $CD34^+$ HSCs, a technique that has been exploited in this chapter.

One characteristic of the terminally differentiated $CD34^+$-derived megakaryocyte, which is rarely observed in megakaryoblastic cell lines, is the formation of proplatelet structures in culture (Haller and Radley, 1983; Cramer et al., 1997; see chapter 1.2.3). Identifying the mechanisms regulating platelet production is a key aim of megakaryocyte research, therefore the initial events governing the formation of proplatelets has recently attracted a significant level of interest. Much study has focused on the profound cytoskeletal reorganisation required in the production of these structures, although the molecular mechanisms involved in proplatelet production remain unclear (Leven and Yee, 1987; Cramer et al., 1997). Microtubule stabilisation causes the formation of abnormal proplatelets (Tablin et al., 1990), whilst disruption greatly inhibits proplatelet formation (Handagama et al., 1987) suggesting a vital role for microtubules in the formation of proplatelets. Two tubulin isoforms, α and β, integrate to form into filaments, which in turn combine to form microtubules (Downing and Nogales, 1998). The β1 tubulin isotype is specific to haematopoietic

cells, and along with other cytoskeletal components, is considered to be under the control of the transcription factor NF-E2 (Lecine et al., 2000).

The role of actin in proplatelet formation remains confusing as inhibiting actin polymerisation causes both the enhancement (Tablin et al., 1990) and inhibition (Handagama et al., 1987) of platelet production in different systems. However, a recent study identified that inhibition of PKCα altered the actin dynamics, reducing the number of proplatelets formed from TPO-stimulated proplatelet production (Rojnuckarin and Kaushansky, 2001). Considering that TPO and other haematopoietic cytokines appear to have no control over proplatelet formation, identification of NMDA-mediated effects of megakaryocyte platelet production *in vitro* is a key aim of this chapter.

$CD34^+$-derived megakaryocytes also display comparable characteristics *in vitro* to bone marrow megakaryocytes. They increase greatly in size during TPO-mediated differentiation, with a terminally differentiated cell being around 25-35 μm in diameter (see Fig. 1.9). They also express a range of megakaryocyte lineage-specific markers including CD61 and CD41, but also express higher levels of platelet-specific markers such as CD42a and CD62P at terminal differentiation (Lepage et al., 2000; Perez et al., 2001), unlike the currently available cell lines.

These cells also display similar ultrastructural characteristics to bone marrow megakaryocytes (Schmitt et al., 2001). Terminally differentiated primary cells have multi-lobed indented nuclei, indicting that normal nuclear maturation has taken place. Characteristic cytoplasmic maturation also occurs, with the invasion of dilated demarcation membrane and the formation of cytoplasmic α-granules, both of which are essential in the process of platelet release and subsequent platelet function. TEM and SEM have also previously been used to determine extent of proplatelet formation. Such studies have revealed that mature platelets are actually released from the tips of proplatelet structures with extensive branching and bending greatly increasing the number of proplatelet ends (Italiano et al., 1999).

Although the use of MK-801 to determine the function of the NMDA receptor is a practical and widely used method. it is also important to determine the effect of agonist-mediated receptor activation on target cells. NMDA receptor agonist responses are regularly studied in neuronal cells (Anderson et al., 2002: Allgaier. 2002) and have also been employed in the study of glutamate signalling in bone cells (Laketic-Ljubojevic et al., 1999; Gu et al., 2002). NMDA receptor activity in such studies is characterised by agonist-induced calcium influx, typically measured using the patch-clamp method. However, other methods do exist such as fluorescent-based calcium measurements (Pal et al., 1999), as used in this chapter or spectrophotometry/microscopy-based calcium imaging which has previously been used in the study of calcium oscillations in rat megakaryocytes (Mason et al.. 2000).

For agonist–mediated NMDA receptor activation to occur, a source of extracellular glutamate is required. In the CNS, glutamate is released from presynaptic cells following depolarisation, which results in the activation of membrane-bound receptors on the postsynaptic cell (see chapter 1.6), whilst keratinocytes and nerve cells have been identified as candidates for glutamate release and recycling in skin (Davidson et al., 1997; Kinkelin et al., 2000). Glutamate release has also recently been studied in osteoblastic cells (see chapter 1.7.1). As previously stated, osteoblasts express the functional glutamate transporter molecule GLAST (EAAT1), which has been implicated in glutamate uptake in neuronal cells (Mason et al.. 1997). As osteoblasts have also been shown to express SNARE and other vesicular proteins associated with glutamate release in neuronal cells (Bhangu et al., 2001), it is possible to hypothesise that the osteoblast may be responsible for glutamate release and recycling in bone.

A more recent study adds further weight to these claims by demonstrating that several osteoblastic cell types spontaneously release glutamate at concentrations comparable to neuronal cells (Genever and Skerry, 2001[a]). The concentration of glutamate released was also significantly increased during differentiation. However, unlike neurons, osteoblastic K^+-mediated depolarisation inhibited glutamate release, suggesting that voltage-mediated osteoblastic calcium entry reduced the level of glutamate exocytosis. Treating osteoblastic cells with the glutamate release inhibitor riluzole (1-10μM), reduced the osteogenic differentiation without affecting viability.

However, addition of higher concentrations (≥25μM) of riluzole, which usually has 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, 2001[b]).

The vesicular glutamate transporter characterised as brain-specific Na^+-dependent inorganic phosphate (Pi) transporter I (BNPI), now referred to as VGLUT1 (vesicular glutamate transporter 1; Ni et al., 1994) has also recently been identified as being expressed by osteoblasts (Hinoi et al., 2002). Although VGLUT1 does cause an increase in Na^+-dependent Pi uptake, giving the protein its original name, it is now believed that its major role is to load recycling vesicles with glutamate in presynaptic glutamatergic axon terminals (Bellocchio et al. 1998; Bellocchio et al., 2000; Takamori et al., 2000). VGLUT1-positive immunoisolated vesicles display enhanced levels of glutamate uptake, whilst over-expression of VGLUT1 in non-glutamatergic neurons results in these cells releasing glutamate, indicating that VGLUT1 expression is sufficient to define glutamatergic phenotype. However, VGLUT1 expression was not identified in some presynaptic cells demonstrated to be releasing glutamate, suggesting the existence of other vesicular glutamate transporters (Bellocchio et al., 2000). This lead to the cloning of a VGLUT1 homologous protein called differentiation-associated BNPI (DNPI; later named VGLUT2), which was upregulated in expression when pancreatic cells are stimulated to differentiate in neuronal cells (Aihara et al., 2000). Subsequent studies revealed that VGLUT2 was also functionally homologous to VGLUT1 (Takamori et al., 2001). VGLUT2 was previously thought not to be expressed by osteoblasts (Hinoi et al., 2002) however, recent unpublished evidence suggests otherwise (P.G. Genever, personal communication).

These findings demonstrate the intricacies of the glutamate signalling system in bone, displaying some of the similarities and differences compared to signalling in the CNS. Whether such a system exists in megakaryocytes or the bone marrow microenvironment is important to consider, as currently no evidence exists as to the source of glutamate for the activation of megakaryocytic NMDA receptors. The existence of uncharacterised GLT-1 positive cells in the bone marrow (Mason et al.,

1997) indicates that glutamate recycling in the vicinity of the megakaryocyte is feasible although the glutamate source and release mechanisms remain elusive.

This chapter addresses a number of diverse questions. These include further determinations of the functional role of NMDA receptors in megakaryocytic cell line differentiation, in addition to expanding the study to the role of the receptor in human primary megakaryocytes. This work also includes for the first time, investigations to identify the effects on MEG-01 cells of agonist-induced NMDA receptor activation and finally provides evidence of megakaryocytic glutamate release mechanisms.

4.2 Materials and Methods

4.2.1 Cell culture

MEG-01 and HEL cell lines were cultured as previously described (chapter 2.1.2). MK-801 (Tocris; Bristol, UK, 50μM unless otherwise stated) was added to cells at the same time as PMA and cultured for a period of 72 hours. Human CD34+-derived megakaryocytes were cultured as previously described (chapter 2.1.3) and 50μM MK-801 added 15 minutes prior to the addition of TPO at day 0 and day 7 of culture.

4.2.2 Glutamate binding assay

Prior to each glutamate binding experiment, MEG-01 cells were removed from normal culture medium, washed three times in PBS and transferred to glutamate/aspartate-free medium (Neurobasal™) containing N2™ supplement for four hours. Cells were then washed twice in cold binding buffer (100mM NaCl, 5mM KCl, 2mM $CaCl_2$ in Hanks' balanced salt solution (HBSS)) and cell number adjusted to $1x10^6$ cells /ml. Cells were then incubated with 250μCi [^{3}H]-glutamate (Amersham Pharmacia) with or without non-radioactive glutamate, at concentrations ranging from $1x10^{-3}$M to $1x10^{-8}$M and with or without MK-801 (50μM) in 500μl of cold binding buffer for 4 hours at room temperature. Cells were washed twice in cold binding buffer and solubilised in 0.1% sodium dodecylsulphate (SDS) before the extract was diluted with scintillant (Ultragold, Packard) and relative remaining radioactivity counted using a Coulter counter (LS-60001C; Beckman Coulter).

4.2.3 MEG-01 fibronectin adhesion assay

4.2.3.1 Fibronectin-mediated cellular adhesion and NR1 immunolocalisation

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

in dH_2O and blocked in 0.1% BSA solution for 1 hour at 37°C before being removed. Cells were then plated directly onto FN coated coverslips in 24-well plates at an initial cell concentration of $1x10^5$ cells/ml. 500µl of cell suspension was added to each well and incubated for 72hrs in the presence of 100nM PMA, with or without 50µM MK-801. Media and non-adherent cells were then removed and remaining cells washed 3 times with PBS, fixed in 4% paraformaldehyde for 5 minutes and blocked in 10% goat serum (diluted in PBS) for 30 minutes. Cells were then incubated overnight at 4°C with mouse anti-active β-1 integrin (1:500; Pharmingen) and antibody binding was identified by incubation with FITC-conjugated goat anti-mouse secondary antibody for 45 minutes. Following washing (3x PBS), coverslips were mounted with Vectorshield mountant (Vector Laboratories) on microscope slides and fluorescence viewed under UV illumination. Antibody controls were performed using identical antibody concentrations of non-specific mouse IgGs instead of primary antibody.

4.2.3.2 Adhesion assay

Fibronectin coating of well bases of 96-well culture plates was performed as described above (chapter 4.3.3.1). MEG-01 cells were seeded at a density of $1x10^4$ cells/well and cultured the absence or presence of 100nM PMA and 50µM MK-801 and cultured for a period of 72 hours. Culture media and non-adherent cells were removed by gentle washing with PBS and remaining adherent cells were fixed in 4% paraformaldehyde for 5 minutes at room temperature and washed three times in PBS. Adherent cells were stained with 0.5% crystal violet in 70% ethanol for 30 minutes at room temperature and all non-bound dye was removed by washing thoroughly with PBS. Cells were lysed by adding 200µl 0.01% Triton-X in 0.1M sodium hydroxide and incubated for 30 minutes at room temperature until all bound dye was eluted. Absorbance was measured using a Dynex MRX5000 plate reader (Dynatech, Billinghurst, UK) at a wavelength of 570nm. Relative absorbance was compared between control and treated cells and background absorbance from an average of "blank" wells, subjected to identical treatment in the absence of cells, removed from each sample.

4.2.4 Immunoisolation of NR1-positive MEG-01 cells

MEG-01 cells were cultured as previously stated (chapter 2.1.2) in the absence and presence of 100nM PMA. Following 72 hours of culture, adherent cells were removed by the addition of 5mM EDTA and all cells were washed three times with PBS. Cell concentration was altered to $1x10^7$ cells /ml and resuspended in 2 ml magnetic activated cell sorting (MACS) buffer (PBS (pH 7.2), supplemented with 0.5% BSA and 2mM EDTA). Cells were then incubated with anti-NMDAR1 monoclonal antibody (Pharmingen; 1µg/ml) for 30 minutes at 4°C. Cells were washed three times (by adding 10x volume MACS buffer to cell suspension, centrifuged for 5 minutes at 400g and supernatant removed) and subsequently incubated for 15 minutes at 4°C in horse anti-mouse biotinylated secondary antibody (Vector Laboratories; 1:200) diluted in MACS buffer. Following three washes, cells were resuspended in 80µl of MACS buffer and 20µl of MACS anti-biotin microbeads (Miltenyi Biotec), mixed well and incubated at 4°C for 15 minutes. Cells were washed three times and resuspended in 500µl of MACS buffer prior to magnetic separation by applying the cell suspensions over a MACS MS^+ column (Miltenyi Biotec). Negative cells were allowed to pass through and collected along with the effluent from three subsequent column washes (NR1-negative). The column was then removed from the magnetic source and positive fraction flushed out by application of 1ml MACS buffer (NR1-positive).

4.2.5 MTT assay

To determine cell viability, the methylthiotetrazole (MTT) assay was used. Four parallel populations; -PMA NR1-negative, -PMA NR1-positive, +PMA NR1-negative and +PMA NR1-positive isolated by MACS were plated into 96-well culture plates at a concentration of $1x10^4$ cells/ml and cultured in RPMI 1640 without phenol red with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine for 72 hours. MTT (Sigma) was then added to each well at a final concentration of 1mg/ml and the cells incubated at 37°C for a further 3 hours. Cells were lysed using 0.05M HCl containing 10% SDS and the absorbance measured at 570nm on a plate reader (Dynatech MRX5000).

4.2.6 Primary megakaryocyte morphological characterisations

4.2.6.1 Total cell and proplatelet counts

Human $CD34^+$-derived megakaryocyte cell numbers were determined after 14 days of culture, by counting the number of visible cells (magnification x200) in eight randomly selected fields of view / well using a Leica DMIL inverted light microscope (Milton Keynes, UK). All available wells were counted, for MK-801 treated and untreated cells. One proplatelet forming cell was identified by one cell body, forming at least 2 cytoplasmic proplatelet extensions in each randomly selected field of view taken for cell number counting. Relative proplatelet formation was quantified by percentage of proplatelet forming cells compared to total cell number. Experiments were carried out blind to treatment and control cell groups.

4.2.6.2 Cell size quantification

$CD34^+$-derived megakaryocytes were cytospun onto microscope slides following the method described in chapter 2.4.1. Cells were fixed in 4% paraformaldehyde for 5 minutes at room temperature and stained with Mayer's haematoxylin solution (Sigma) for 5 minutes before washing gently in tap water and counter-stained with 1% eosin (Sigma) for 10 minutes. Eosin was then removed by washing slides in tap water and mounted in PBS containing 10% glycerol. The cell area was digitally quantified using Leica Quantimet image analysis system (Leica Q500win standard version 2.2 Leica Imaging Systems) and the area of the cells in six random fields determined using a Leica DMLA light microscope.

4.2.6.3 Transmission electron microscopy

Day 14 megakaryocytes were washed twice in PBS before fixation in 4% paraformaldehyde/ 2.5% glutaraldehyde in 100mM phosphate buffer (pH7.0) for 90 minutes at room temperature. Cells were then washed three times for 10 minutes in PBS and subjected to secondary fixation in 1% osmium tetroxide (OsO_4) for 1 hour on ice and washed twice for 10 minutes in PBS. The cells were dehydrated through graded ethanols, dried over a molecular sieve and washed twice in epoxypropane for 5 minutes each. Embedding was performed by adding 60% epoxypropane / 40% epon araldite for 30 minutes and left to desiccate with silica gel overnight. 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 (JLJEM 1200 EX; Tokyo, Japan).

4.2.7 Antigen expression determination by flow cytometry

Following media removal, cells were washed 3 times in buffer A (see chapter 2.1.3) to prevent clumping. 100µl ($1x10^5$) of cell suspension was used per sample, and non-specific binding blocked by incubating with normal relevant IgGs (Pharmingen) at a concentration of 1µg/ml for 10 minutes and washed. All washes were performed by adding 1ml of buffer A to the cell suspension, followed by centrifugation at 400g for 5 minutes at 4°C and removal of supernatant from the cell pellet. Primary-FITC conjugated antibodies, raised against CD61, CD41 (both Pharmingen) and CD42a (Sigma) were added to the cells (10µl per 10^6 cells) and incubated at 4°C for 30 minutes in the dark. Negative controls were treated with non-specific-FITC conjugated antibodies. Cells were washed and remained at 4°C in the dark until analysis on a Coulter XL flow cytometer emitting an excitation light at 488nm from an argon laser. FITC signal was detected by FL1 with an emission maximum of 520nm. Dead cells and doublets were excluded by light scatter properties and positive staining for propidium iodide (PI) after a 30 second incubation. The percentage of live cells expressing the different antigenic markers was determined by setting a positive gate at 1% on negative control samples.

4.2.8 Apoptosis detection by flow cytometry

Apoptotic cells were identified using an Annexin V-FITC apoptosis detection kit (Oncogene, MA). The kit works on the principle that phosphatidyl serine (PS) is exposed on the surface of cells undergoing apoptosis. In the presence of calcium, PS binds rapidly to the anti-Annexin V-FITC conjugated antibody, indicating the early stages of apoptosis. PI is used as an indicator of late apoptosis by its ability to enter cells only through porous membrane and stain DNA red, enabling the identification of various stages of apoptosis. The procedure was carried out according to the

manufacturer's protocol. Cell suspension concentration was adjusted to $1x10^6$ cells/ml, without removing media, therefore preventing the onset of apoptosis by serum starvation. 500µl of cell suspension was transferred to microcentrifuge tubes and 10µl of media binding reagent added, followed by the addition of 1.25µl of Annexin V-FITC antibody. Cells were incubated in the dark at room temperature for 15 minutes. The cell suspension was then centrifuged at 400g for 5 minutes at room temperature and the media removed. The cells were then gently resuspended in cold 1x binding buffer and 10µl of PI added. Samples were kept on ice in the dark and analysed by flow cytometry immediately, using a Coulter XL flow cytometer with an argon laser exciting at 488nm. The FITC signal was detected at 520nm on FL1 and the PI at 620nm on FL2, adjusting to ensure minimal overlap between the two measurements. Log of annexin V-FITC was plotted on the x-axis and log of the PI readings on the y-axis.

4.2.9 Ploidy analysis by flow cytometry

Megakaryocyte aneuploidy was analysed using a PI DNA staining solution (Quest Biomedical, Solihull, UK), according to the manufacturer's protocol. Cells were removed from media and washed 3 times in buffer A and the cell concentration adjusted to $1x10^6$ cells in a volume of 200µl of buffer A. 500µl of PI solution containing 25U/ml of RNase H and detergent was added and the cells were incubated in the dark at room temperature for 10 minutes in a horizontal position. The cells were then passed through a 25-gauge needle to ensure only single cells were counted and were kept on ice and in the dark until flow cytometric analysis. Single cells only were analysed by their light scatter properties and PI positive readings were taken on the FL2log setting.

4.2.10 Intracellular calcium measurements

Intracellular calcium measurements were recorded according to established methods developed specifically to cell suspensions (Nelemans: Calcium Signalling Protocols. Methods in Molecular Biology: Volume 114). MEG-01 cells were treated with 100nM PMA for 72hrs and washed 3 times in PBS to remove media and PMA before

being transferred to Neurobasal™ media containing 1% N2 supplement and incubated overnight. Cells were washed 3 times in HBSS (without Mg^{2+}, containing 1mM Ca^{2+}) and cell concentration adjusted to $5x10^6$/ml. Cells were then loaded with 1μM indo-1-acetyloxymethyl ester (AM) (Molecular Probes, Oregon) for 30 minutes at 37°C. Excess dye was removed by washing 3 times in HBSS and resuspended at a cell concentration of $5x10^5$ cells/ml. Indo-1-AM fluorescence was analysed using an LS55 luminescence spectrometer (Perkin Elmer, Cambridge, UK). 2.5ml of cell suspension was added to each cuvette with the ambient temperature kept at 37°C and continually agitated by magnetic stirring. Following the addition of 1-10μM NMDA with 100nM-1μM glycine calcium influx was monitored with an excitation wavelength of 330nm and emission wavelengths at 405nm for calcium bound dye and 485nm for calcium free dye with 5nm slit width. Relative changes in fluorescence were expressed as a ratio of bound and free indo-1-AM. 500nM ionomycin (Calbiochem, Nottingham, UK) was added to achieve a maximum figure for bound dye and 5mM EDTA to chelate free calcium, thereby determining fluorescence for calcium free dye. $[Ca]_i$ can be calculated by measuring fluorescent intensity using the following equation;

$$\mathbf{[Ca^{2+}] = K_d \times [(R - R_{min}) / (R_{max} - R)] \times (S_{f2}/S_{b2})}$$

Where R is the experimentally measured ratio of fluorescent intensities; R_{min} is the measured fluorescence ratio intensity in the absence of calcium; Rmax is the measured fluorescent ratio of Ca^{2+} saturated dye; the term (S_{f2}/S_{b2}) is the signal ratio of fluorescence measured at emission wavelength 485nm in the absence of calcium (f2) and at calcium saturation (b2); and Kd is the dissociation constant for indo-1-AM (=0.23). NMDA and AMPA receptor-mediated changes in $[Ca^{2+}]_i$ by adding NMDA and glycine or the AMPA receptor antagonist CFM-2 (Tocris) at varying concentrations at recorded time points.

4.2.11 Glutamate release assay

Glutamate release from MEG-01 cells was determined using an enzyme-linked fluorimetric assay. This assay has previously been employed in the study of neuronal

glutamate release (Nicholls et al., 1987; Bezzi et al., 1998) and has recently been modified for the study of glutamate release from osteoblastic cells (Genever and Skerry, 2001). The assay is based on the principle that addition of glutamate dehydrogenase (GDH) and β-nicotinamide adenine dinucleotide phosphate ($NADP^+$; both Sigma) will result in released glutamate being oxidised to α-ketoglutarate, resulting in the formation NADPH that can subsequently be determined fluorimetrically. MEG-01 cells were grown as previously stated (chapter 2.1.2) in the absence and presence of 100nM PMA for 72 hours. Following culture, cells were washed three times in PBS to remove any traces of exogenous glutamate and replated in blacked 96-well plates (μclear, Greiner Labortechnik Ltd., Stonehouse, UK) at various cell densities ranging from 1-5 $x10^4$ cells/ml. Cells were incubated in release buffer (NaCl (120mM), KCl (3mM), NaH_2PO_4 (1.25mM), HEPES-Na (25mM), glucose (4mM), MgCl2 (1mM), $CaCl_2$ (2mM) and $NADP^+$ (1mM), pH7.4)) prewarmed to 37°C prior to use and the reaction initiated by the addition of GDH (40U/ml). Fluorescence was then measured using a Dynex MFX fluorescent plate reader (Dynatech) at 37°C, at an excitation wavelength of 355nm and emission wavelength of 460nm. Standard curves were created by the application of known concentrations of glutamate for each assay and protein concentrations determined by the BCA protein assay (Pierce).

4.2.12 VGLUT2 immunolocalisation

MEG-01 cells and cytospin preparations, rat tibiae cryosectioning and immunolocalisations were performed as previously stated (chapter 2.3.3). Rabbit anti-VGLUT2 primary antibody (Synaptic Systems, Gottingen, Germany) was used at a 1:500 dilution with anti-rabbit biotinylated secondary antibody (1:200 dilution; Vector Laboratories). For co-immunolocalisation of VGLUT2 and NR1 in rat megakaryocytes, VGLUT2 immunodetection was performed prior to incubating with mouse anti-NMDAR1 (1:400; Pharmingen) overnight at 4°C and antibody binding detected following a 45 minute incubation using FITC-conjugated goat anti-mouse secondary antibody (1:100; Sigma).

4.3 Results

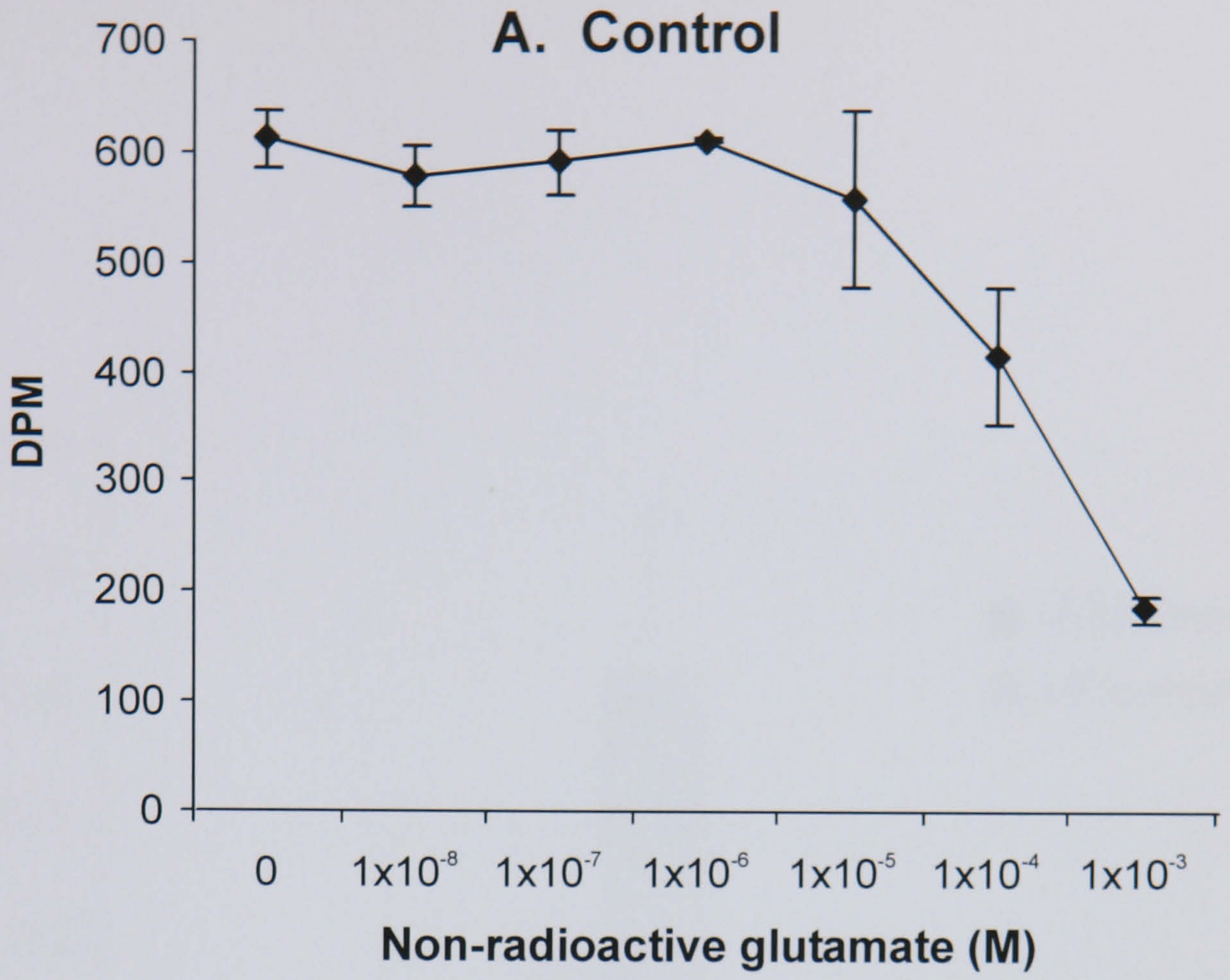
4.3.1 Glutamate binding by MEG-01 cells

The ability of NMDA receptors on MEG-01 cells to bind glutamate was determined by radioactive binding assay. The addition of increasing concentrations of non-radioactive glutamate resulted in the competitive inhibition of specific [^{3}H]-glutamate (Fig. 4.3.1 A). Glutamate competed for binding with [^{3}H]-glutamate with an IC_{50} of approximately $5x10^{-3}$M, a higher concentration than that observed in both osteoblastic cells (Laketic-Ljubojevic et al., 1999) and neuronal cells (Davies et al., 1981). Pre-incubation of MEG-01 cells with 50μM MK-801 prior to application of non-radioactive and [^{3}H]-glutamate, significantly reduced the levels of specific glutamate binding (Fig. 4.3.1 B).

4.3.2 Functional importance of NMDA receptor activity in megakaryocyte differentiation

4.3.2.1 Function of NMDA receptors in MEG-01 cells

Some of the morphological characteristics of MEG-01 cells treated with the NMDA receptor antagonist MK-801 were previously characterised (Genever et al., 1999). Fibronectin, a substratum glycoprotein, has been shown to greatly increase MEG-01 adhesion and elongation by the stimulation of cytoskeletal actin reorganisation (Yamazaki et al., 1999). PMA-mediated adhesion of MEG-01 cells to fibronectin was reduced to basal levels following MK-801 (50μM) treatment. MEG-01 cells grown in the absence of PMA and fibronectin, displayed only low levels of adhesion (Fig. 4.3.2). Megakaryocyte adhesion to fibronectin is mediated through the integrin α5β1 (Schick et al., 1998). Using an antibody directed against the activated conformation of β1 integrin, immunoreactivity was most prominent at the swollen terminals of pseudopodia in MEG-01 cells adhered to fibronectin in the presence of PMA only (Fig. 4.3.3). However, MK-801 treatment prevented the formation of pseudopodia by MEG-01 cells and reduced the activity of β-1 integrin expression by these cells (Fig. 4.3.3).



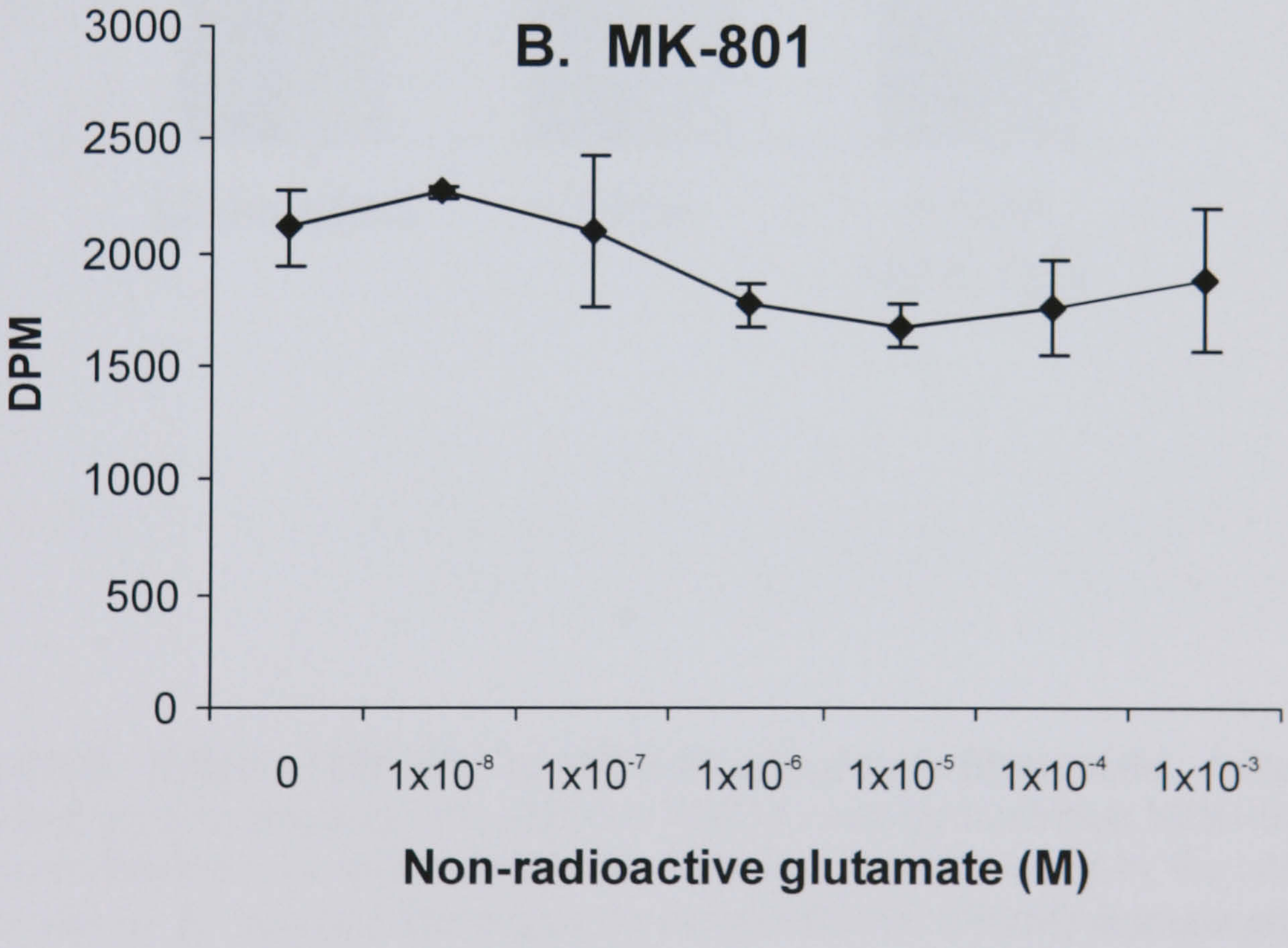


Figure 4.3.1. Glutamate binding by MEG-01 cells. The binding of ^{3}[H]-glutamate by MEG-01 cells was competed against increasing concentrations of non-radioactive glutamate. The levels of bound glutamate are similar to those found in neuronal cell cultures (A). Glutamate binding kinetics was altered following the application of the NMDA receptor antagonist MK-801 (50μM; B). Results are representative of two independent experiments.

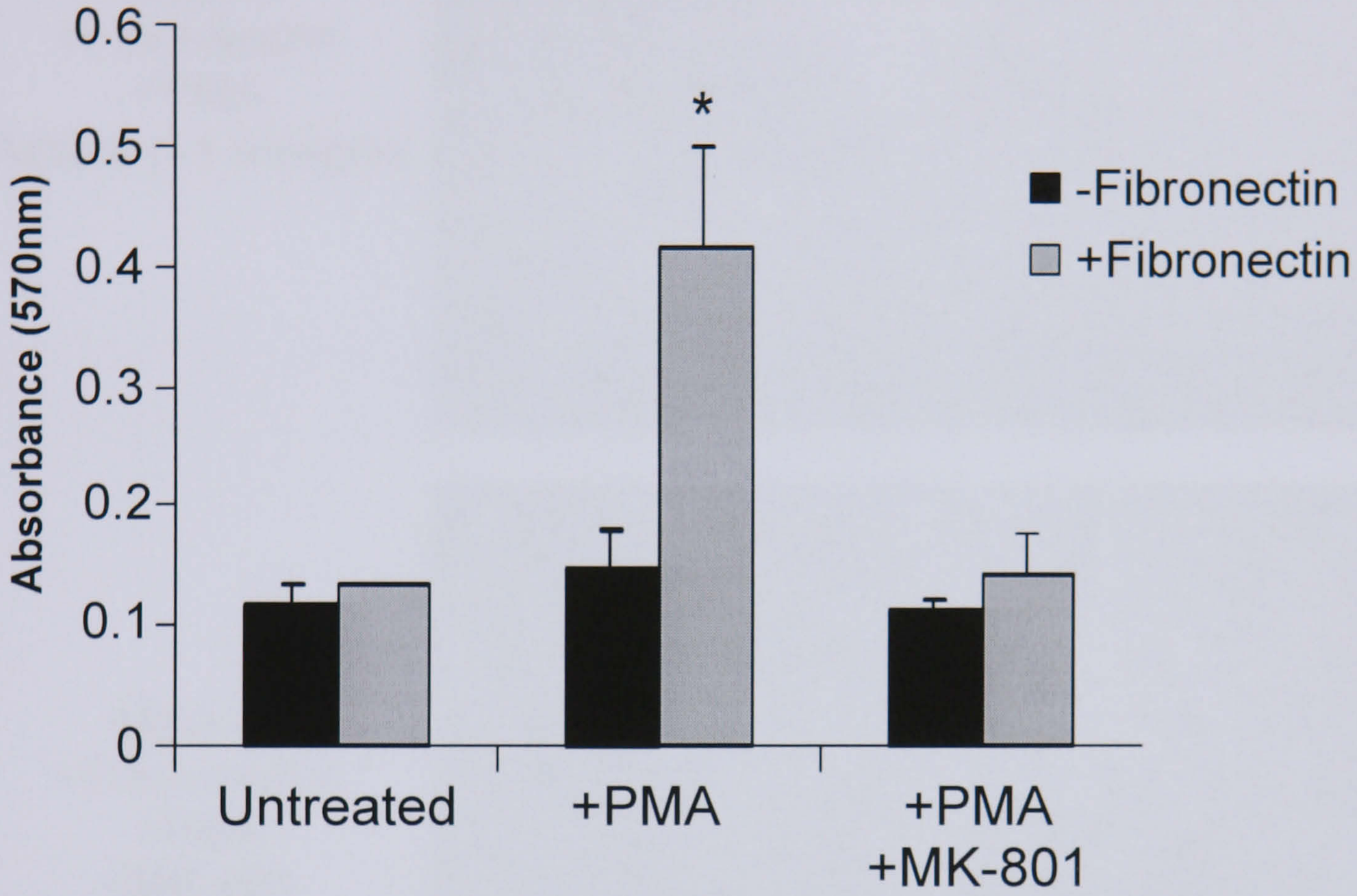


Figure 4.3.2. Effect of MK-801 on MEG-01 adhesion to fibronectin. Adhesion assays were used to determine the effect of NMDA receptor inhibition MEG-01 adhesion to fibronectin-coated substratum. Adhesion was increased by the addition of PMA and the presence of fibronectin, however MK-801 (50μM) dramatically reduced levels of MEG-01 adhesion to fibronectin (*=$p<0.05$; one way ANOVA). Results represent two independent experiments.

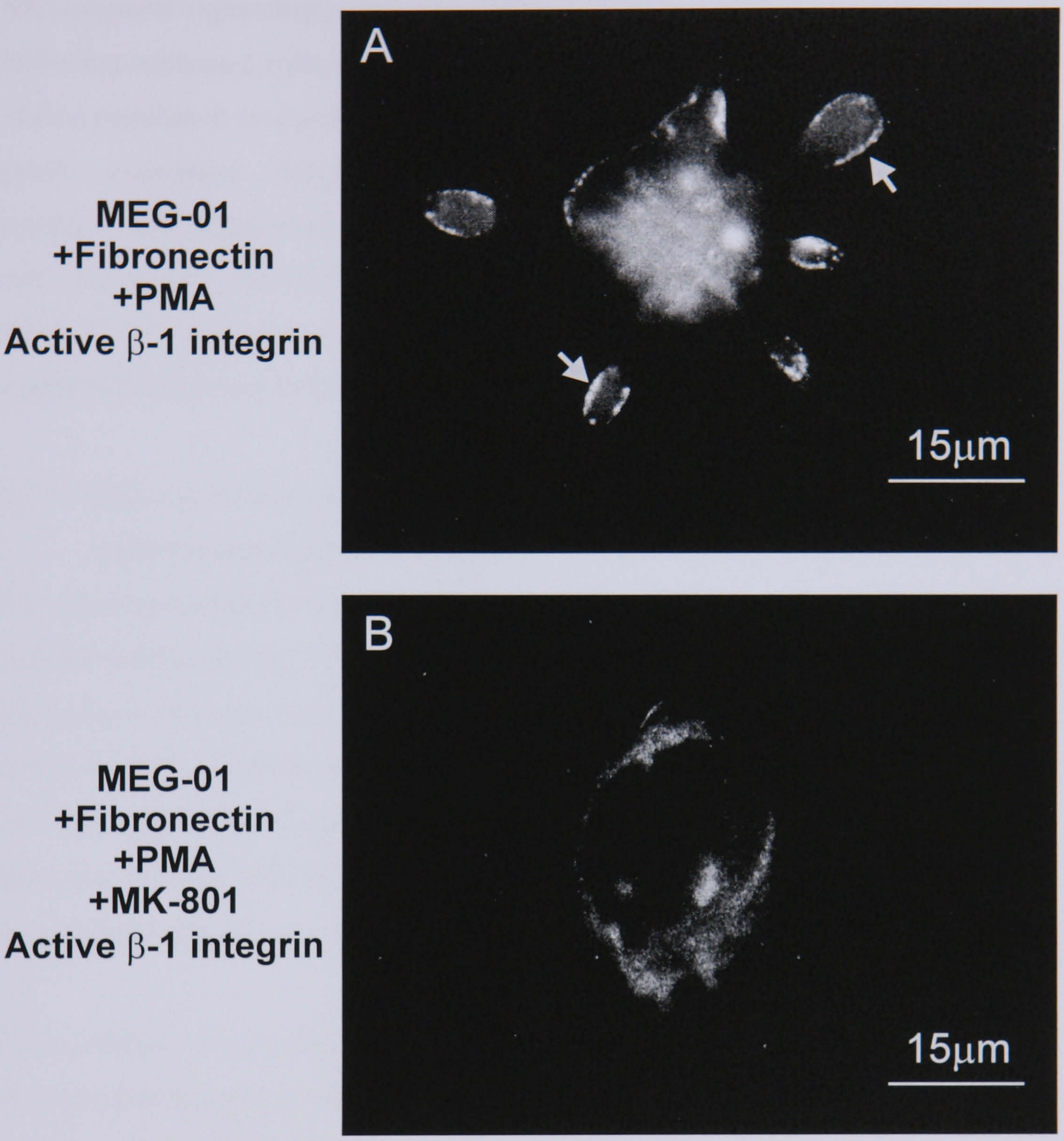


Figure 4.3.3. Effect of NMDA receptor inhibition on localisation of active β-1 integrin in fibronectin adhered MEG-01 cells. Immunolocalisation of activated β-1 integrin by MEG-01 cells grown on fibronectin in the absence and presence of MK-801. PMA-treated MEG-01 cells displayed extensive cytoplasmic elongations with active β-1 integrin expression highly expressed at the rounded ends of these structures (arrows; A). MK-801 treated cells lacked cytoplasmic extensions and β-1 integrin appeared to be significantly weaker (B). Results are representative of two independent experiments.

To determine the effect of NR1 expression on MEG-01 viability, MEG-01 cells grown in the absence and presence of 100nM PMA were separated into NR1-positive and NR1-negative expressing populations by MACS. The MTT-assay was then performed following continued culture of the separated cell populations for 72-hours. The PMA-treated population revealed basal levels of viability, as did the PMA-untreated NR1-positive population. Only the NR1-negative population in the absence of PMA exhibited significant levels of viability, similar to those found in unseparated MEG-01 cells (Fig. 4.3.4). As PMA is not thought to cause cell death in MEG-01 cells, (P. G. Genever, personal communication of unpublished data) it is likely that the decrease in viable cells observed in this experiment is due to reduced levels of proliferation.

4.3.2.2 Effect of NMDA receptor inhibition on morphological characteristics of primary human megakaryocytes

The primary megakaryocyte undergoes a great number of morphological transformations during differentiation, some of which were investigated in this section. Increases in cell size, indicative of megakaryocyte differentiation, were studied by image analysis of human primary megakaryocytes cytospin preparations (Fig. 4.3.5). All cells were cultured in the presence of TPO (25ng/ml) and in the absence or presence of 50μM MK-801 for 14 days. MK-801 almost halved the average megakaryocyte cell surface area, from approximately 500μm^2 to 300μm^2.

The formation of proplatelets by primary megakaryocytes is an indicator of normal terminal megakaryocyte differentiation. Indeed, by the latter stages of *in vitro* megakaryocyte differentiation (>10 days of culture), proplatelet structures can be clearly observed. The percentage of megakaryocytes producing these structures in the absence and presence of 50μM MK-801 was analysed blind and by two independent experimenters at day 11 and day 14. After 11 days, MK-801 had no significant effect on cell number (Fig. 4.3.6 A), but reduced the percentage of cells producing proplatelets from an average of 3.5% to less than 1% in MK-801-treated cells (Fig. 4.3.6 B). Following 14 days of culture, MK-801-treatment significantly reduced cell number (Fig. 4.3.7 A) and reduced the number of proplatelet forming cells from an average of 8.5% to 2% (Fig. 4.3.7 B and C).

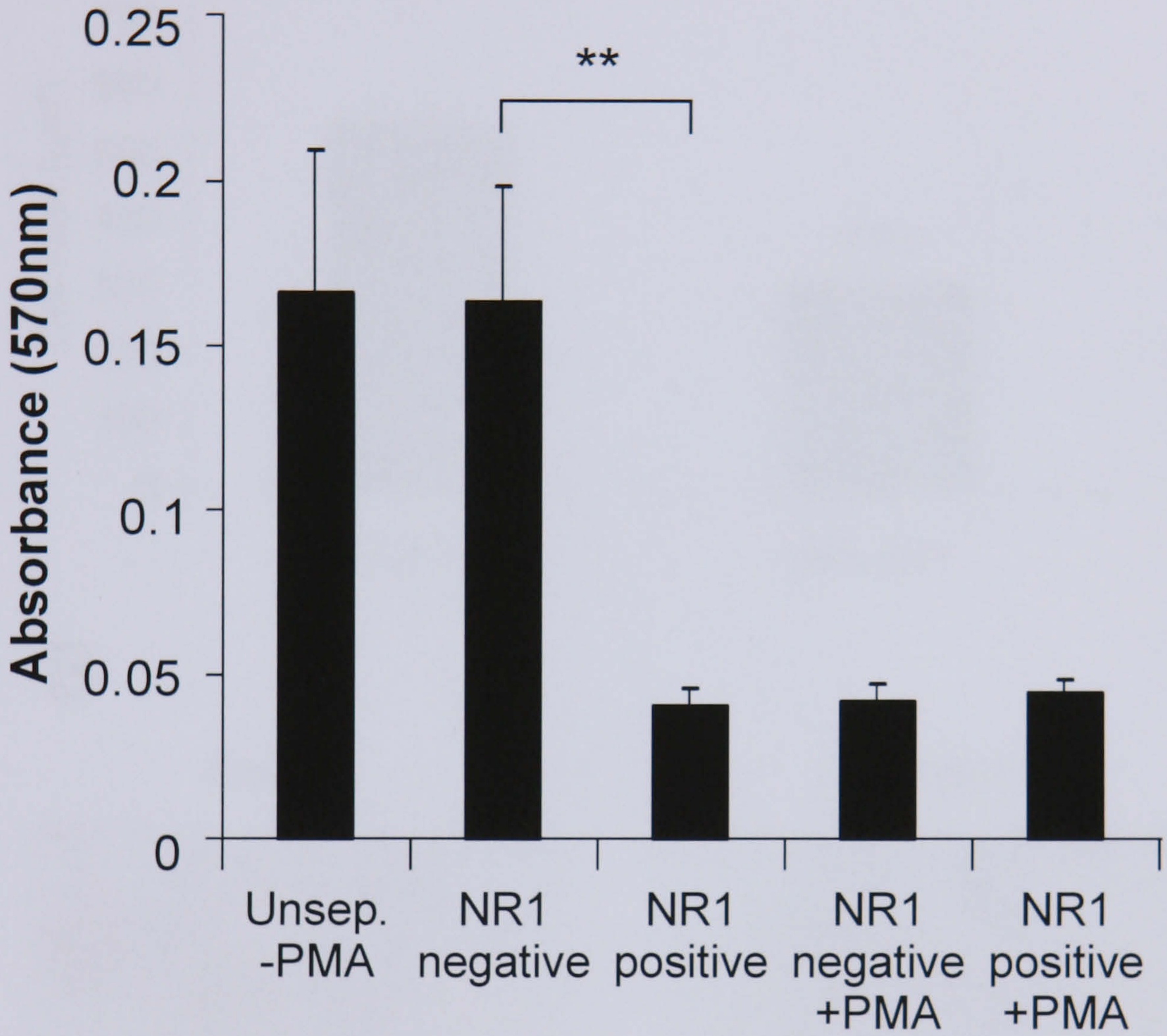


Figure 4.3.4 Proliferation of MEG-01 cells following MACS for NR1-positive cells in the absence and presence of PMA. MTT assay was used to investigate the effect of the NMDA receptor expression on MEG-01 cell proliferation. NR1-positive isolated MEG-01 cells failed to proliferate following 72 hours of culture, whilst both NR1-negative and unseparated (Unsep.) cells exhibited significantly (** = $p<0.01$; one way ANOVA) greater levels of proliferation. PMA-treatment resulted in both NR1-negative and positive cells failing to proliferate. Results are representative of two independent experiments.

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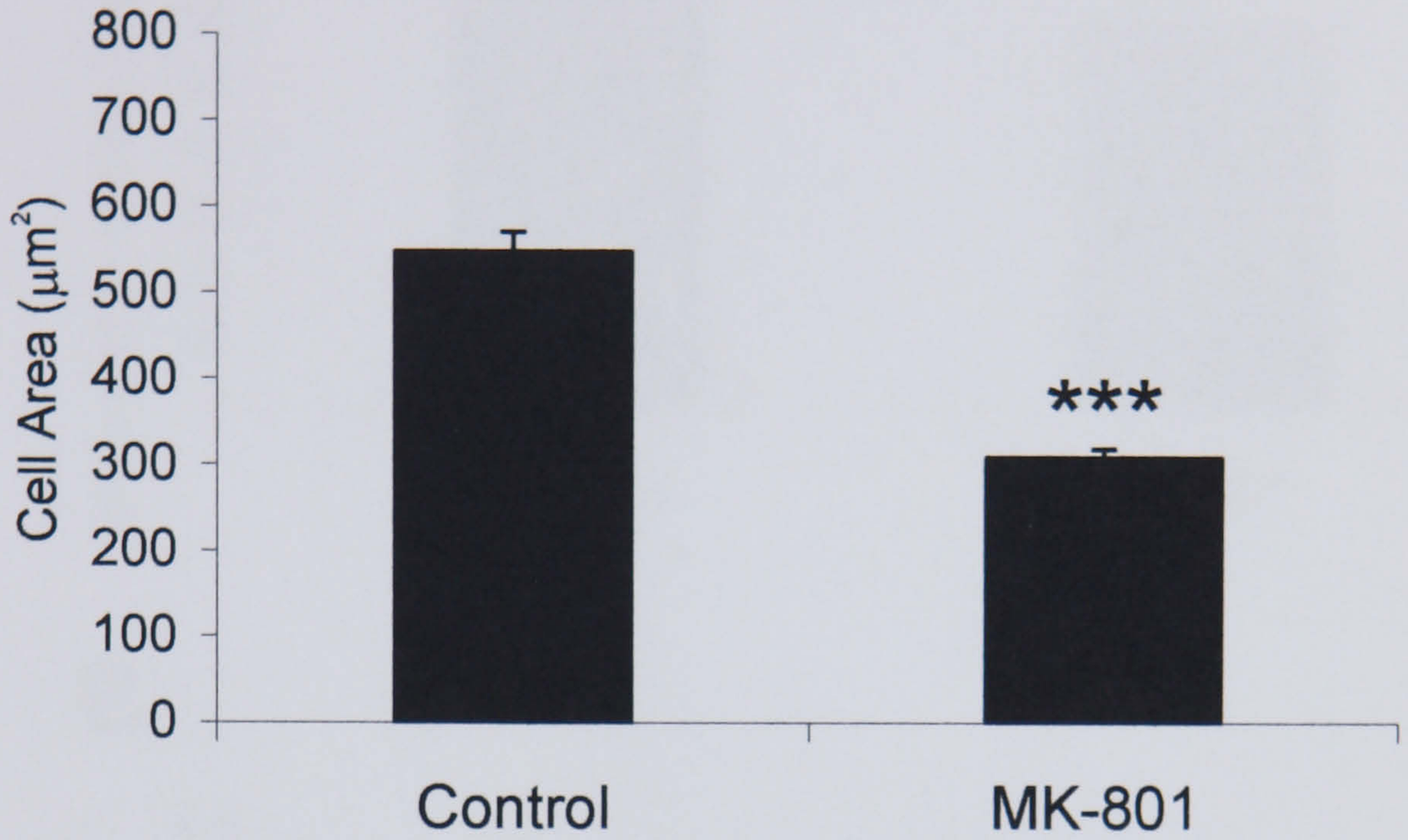

Figure. 4.3.5 Cell area of human primary megakaryocytes with and without MK-801 following 14 days of culture. Cytospin preparations of human megakaryocytes cultured in the absence and presence of MK-801, were digitally analysed for cell surface area (μm^2). Cell surface area was almost halved compared to control following MK-801 treatment (A, $p < 0.001$; students t-test). Example of haematoxylin/eosin stained cytospin preparations are displayed in (B). Results are representative of 2 independent experiments.

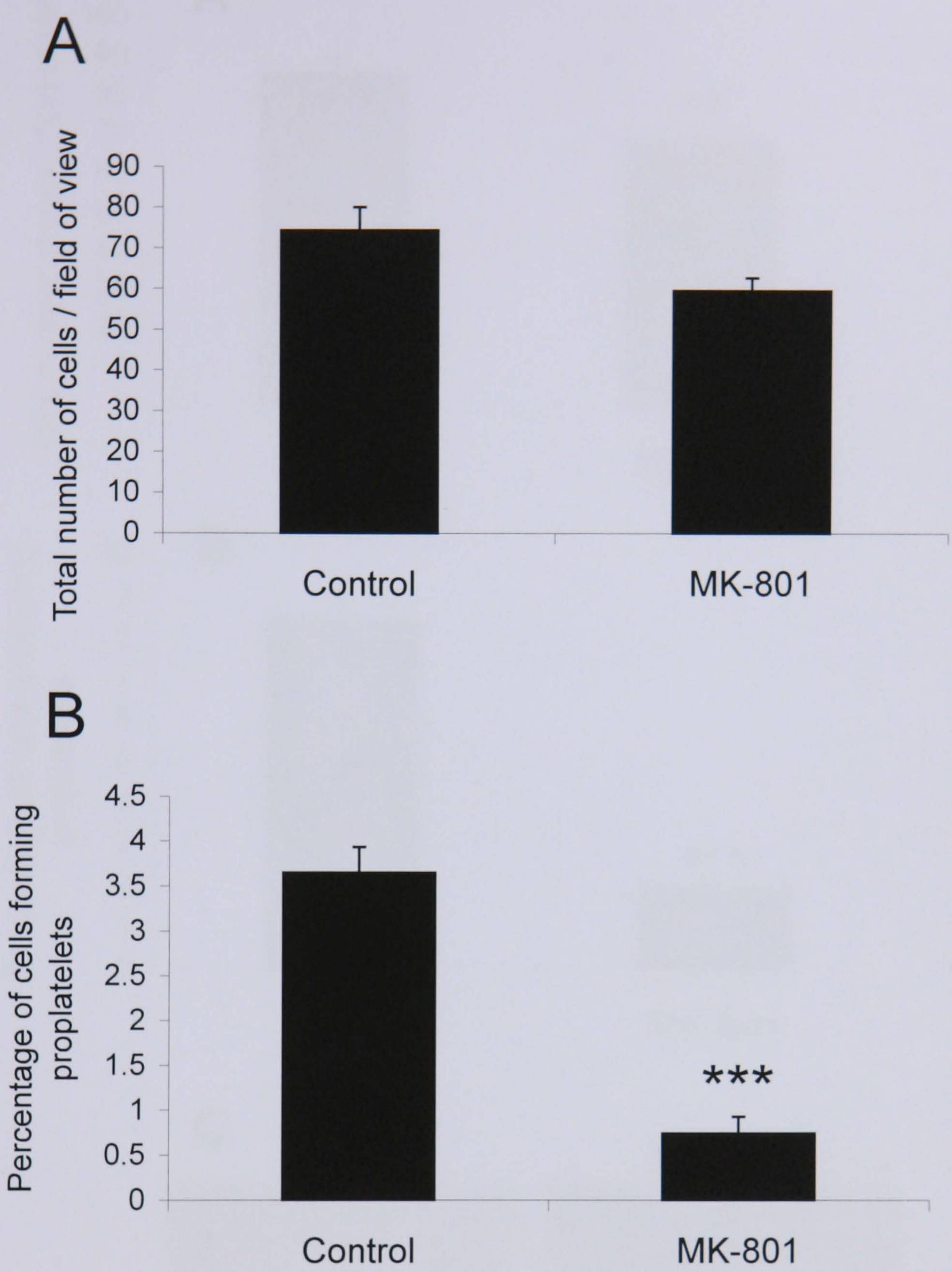

Figure 4.3.6. Effect of NMDA receptor inhibition on megakaryocyte number and proplatelet formation after 11 days of culture. MK-801 treatment had no significant effect on cell number (A), but significantly ($p<0.005$; students t-test) reduced the percentage of cells displaying proplatelet structures (B). Results represent two independent experiments.

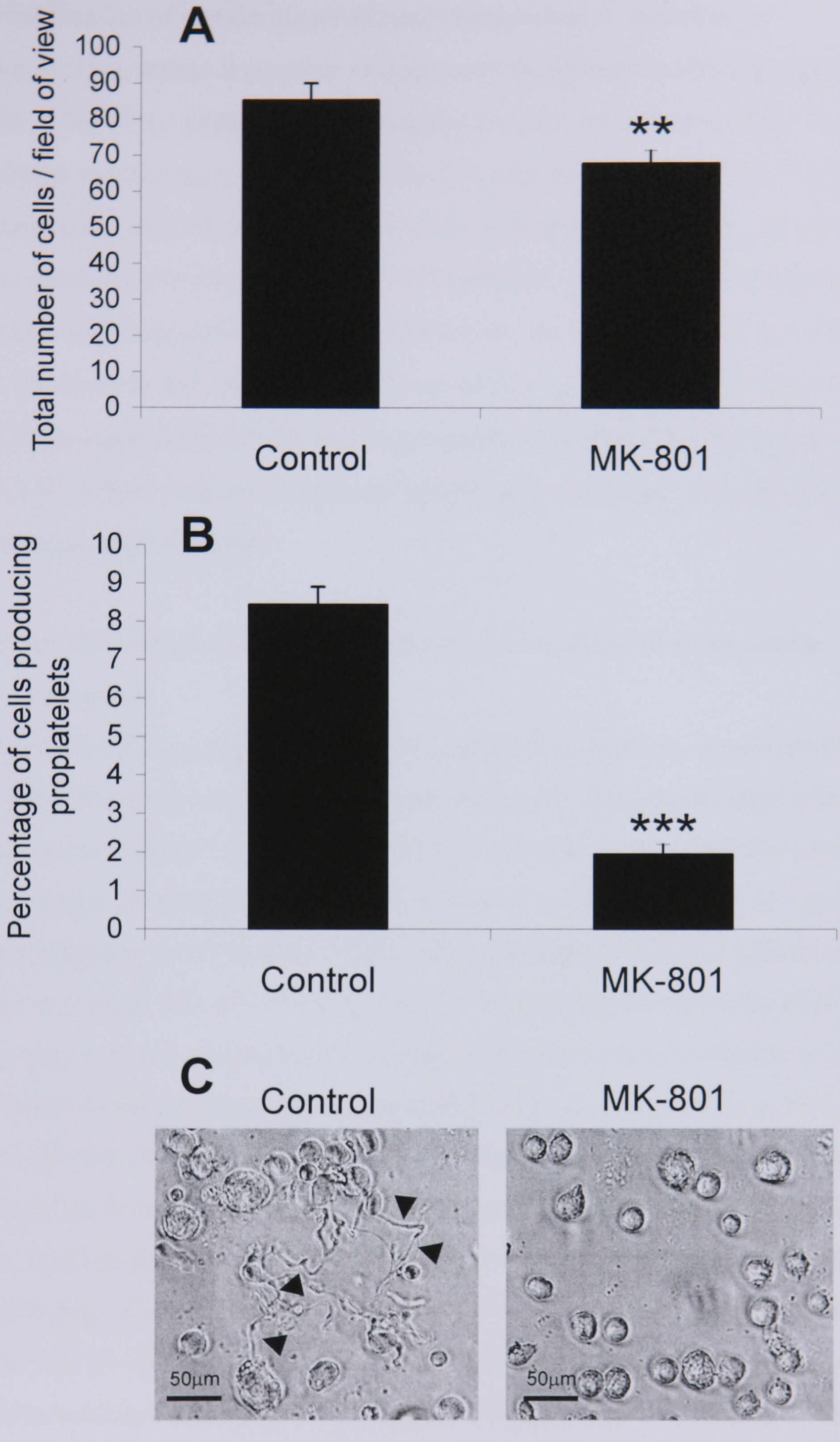

Figure 4.3.7 Effect of NMDA receptor inhibition on megakaryocyte number and proplatelet formation after 14 days of culture. MK-801 treatment significantly reduced megakaryocyte number (A, ** = $p<0.01$; students t-test), and proplatelet formation after 14 days of culture (B and C (arrowheads), *** = $p<0.005$; students t-test). Results represent two independent experiments.

The identification of certain ultrastructural characteristics displayed by megakaryocytes, makes it possible to determine the platelet producing capabilities of the cells. Therefore, primary human megakaryocytes, following 14 days of culture in the presence and absence of MK-801 (50μM), were analysed by TEM. Control megakaryocytes exhibited multi-lobed nuclei, a dilated demarcation membrane system forming platelet territories towards the cell periphery, dense platelet cytoplasmic α-granules and proplatelet structure formation on the outside edge of the cell (Fig. 4.3.8). In contrast, MK-801-treated cells presented rounded, single-lobed nuclei, they lacked demarcation membranes and α-granules and proplatelet structures were absent (Fig. 4.3.9). These cells also displayed significant cytoplasmic abnormalities, in the form of large open cisternae.

4.3.2.3 MK-801 induced inhibition of megakaryocyte differentiation marker expression

The expression of megakaryocyte-specific cell surface markers reveals the extent of TPO-induced expression by primary megakaryocytes. By quantifying the levels of differentiation markers by flow cytometry it is possible to document the process of megakaryocyte differentiation. MK-801 treatment reduced the level of expression of the early megakaryocyte marker, CD61 from an average of 72% of cells in control samples to around 50% of cells (Fig. 4.3.10). Similar figures were also observed for the expression of the intermediate megakaryocyte differentiation marker CD41, which was reduced from an average of approximately 76% of control cells, to 54% of cells treated with MK-801 (Fig. 4.3.11). The most profound reduction in expression was to the marker for terminally differentiated megakaryocyte/platelet specific marker CD42a, from an average of 40% of control cells to 23% of cells following MK-801 treatment (Fig. 4.3.12). Annexin V/ PI flow cytometric analysis confirmed that MK-801 had no significant effect on cell viability, with approximately 91% and 89% of cells remaining viable following 14 days of culture with TPO and TPO with MK-801 respectively (Fig. 4.3.13). In addition, MK-801 had no significant effect on necrosis and early/late apoptosis.

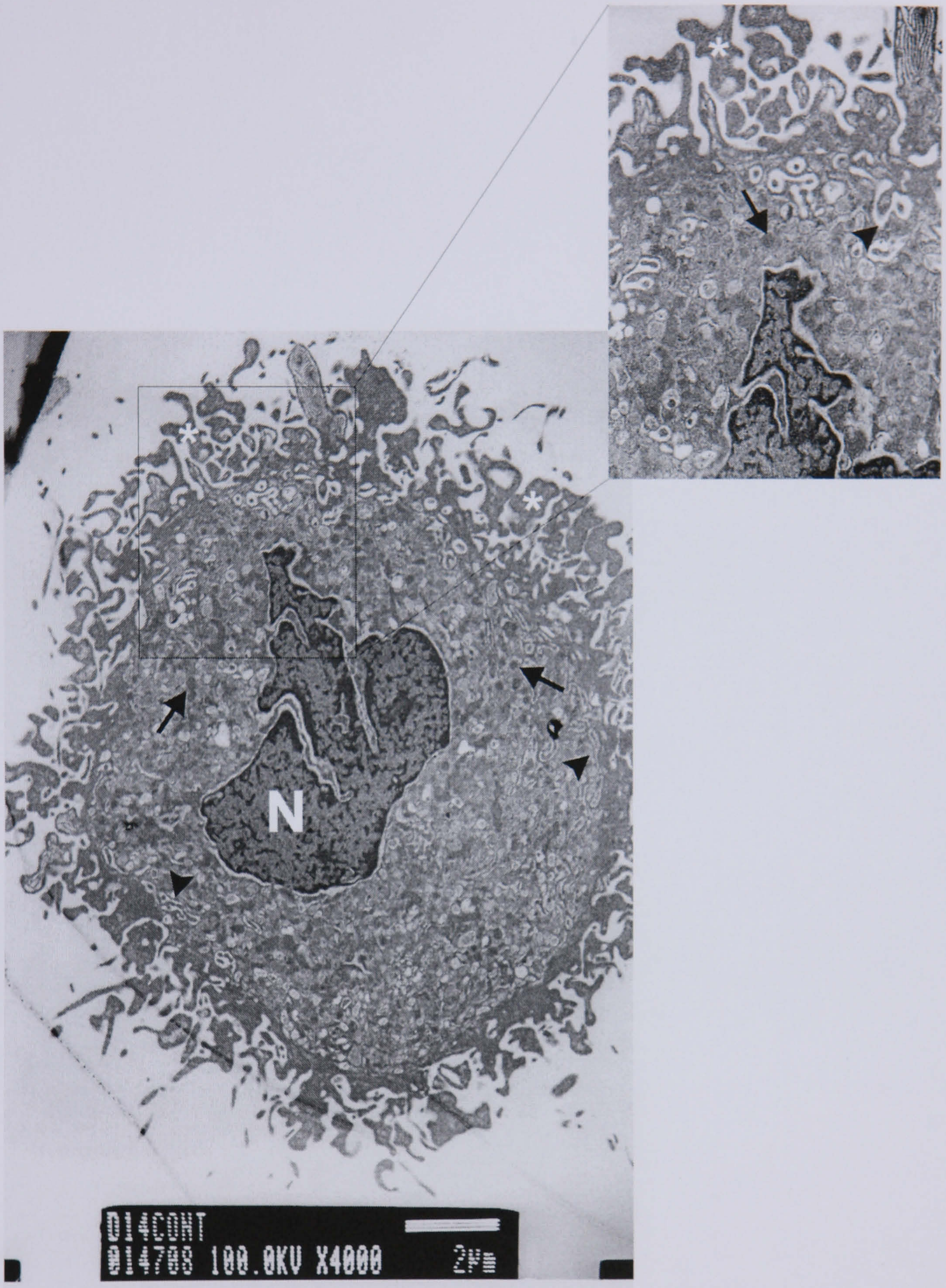

Figure 4.3.8. Ultrastructural morphology of human primary megakaryocytes. Ultrastructural analysis by TEM of human primary megakaryocytes cultured with TPO for 14 days. Cells displayed normal physiological characteristics, including multi-lobed nucleus (N), dilated demarcated membrane (arrowheads), α-granules (arrows) and proplatelet formation at the periphery of the cell (*). Magnification x4000.

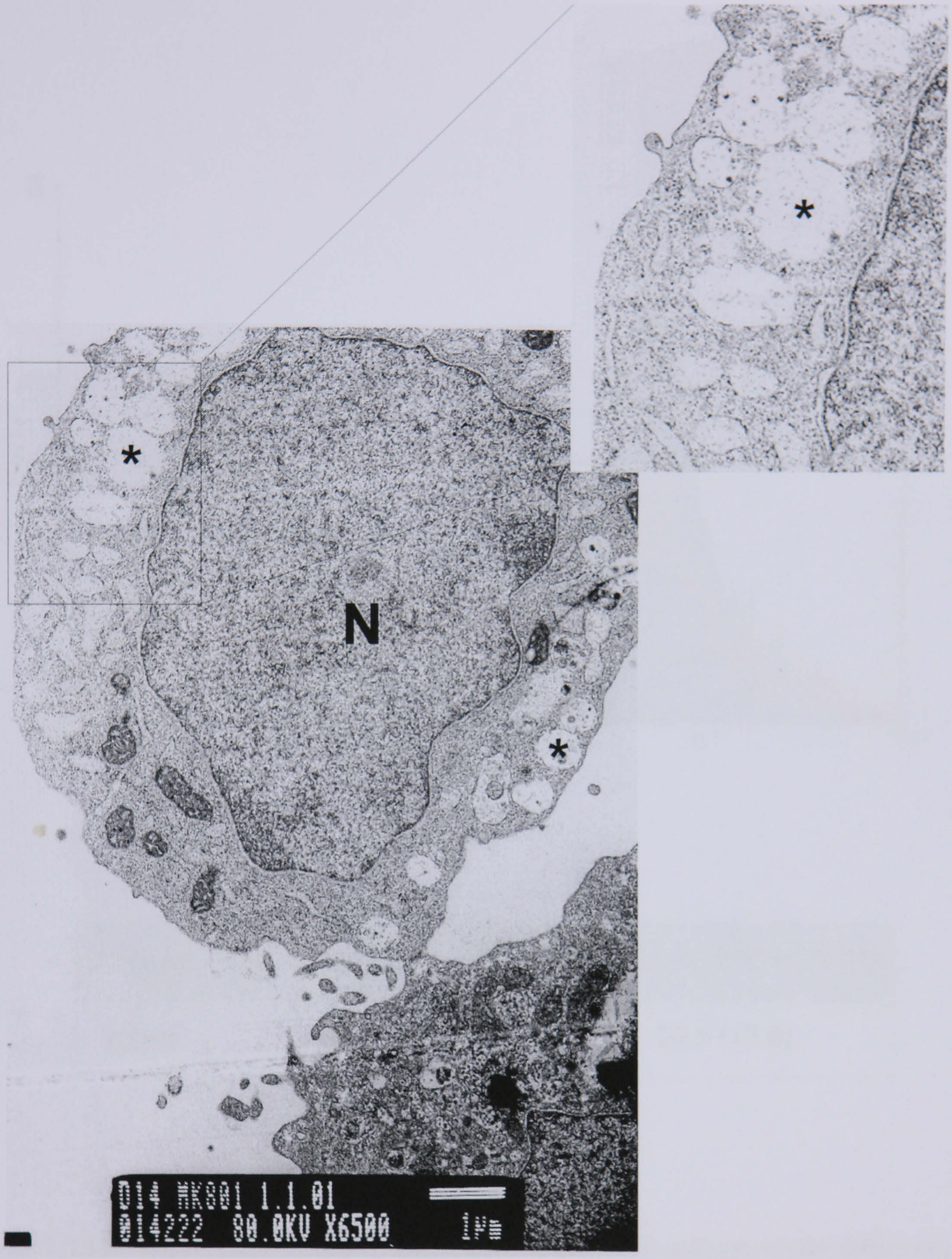

Figure 4.3.9. Ultrastructural morphology of human primary megakaryocytes treated with MK-801. Ultrastructural analysis by TEM of human primary megakaryocytes cultured with TPO and 50μM MK-801 for 14 days. Cells lacked multi-lobed nuclei (N), dilated demarcation membrane, α-granules and proplatelet structures. Large open cisternae were also identified within the cell cytoplasm (*). Magnification x6500.

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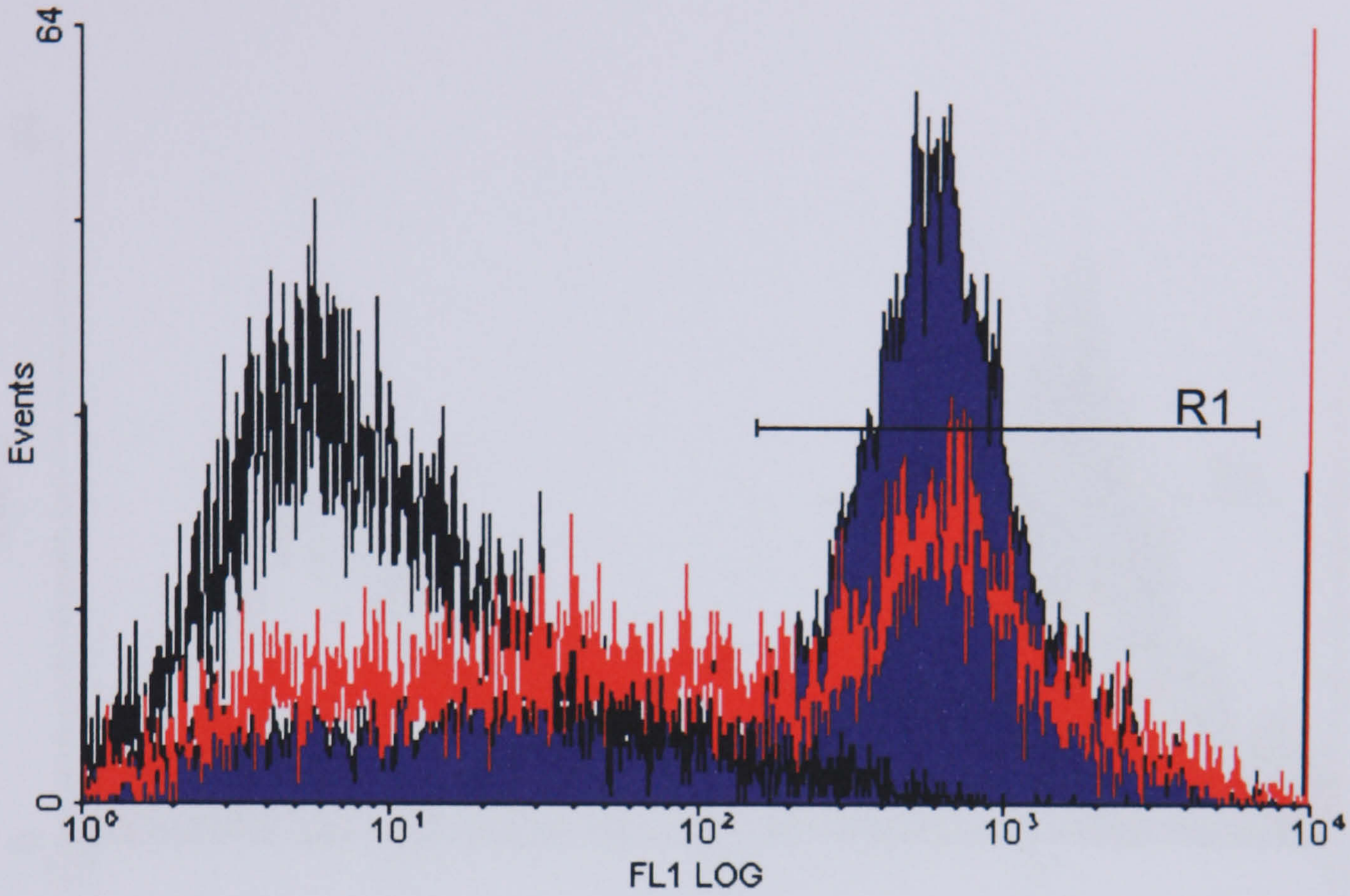

Antigen	% positivity control (±SD)	% positivity MK-801(±SD)
CD61	78 (±7.6)	50.9 (±3.6)

Figure 4.3.10. Expression of CD61 by human primary megakaryocytes following treatment with MK-801. Flow cytometry was used to determine expression of the cell surface marker CD61 by human primary megakaryocytes grown in the presence of TPO for 14days, with and without MK-801. 78% (±7.6) of control cells (solid blue fill) were positive for CD61expression, which was reduced to 50.9% (±3.6) in cells treated with 50μM MK-801(red outline). Positivity is gated at higher than 1% of antibody control (black outline; Region R1) and data shown is typical of 3 independent experiments.

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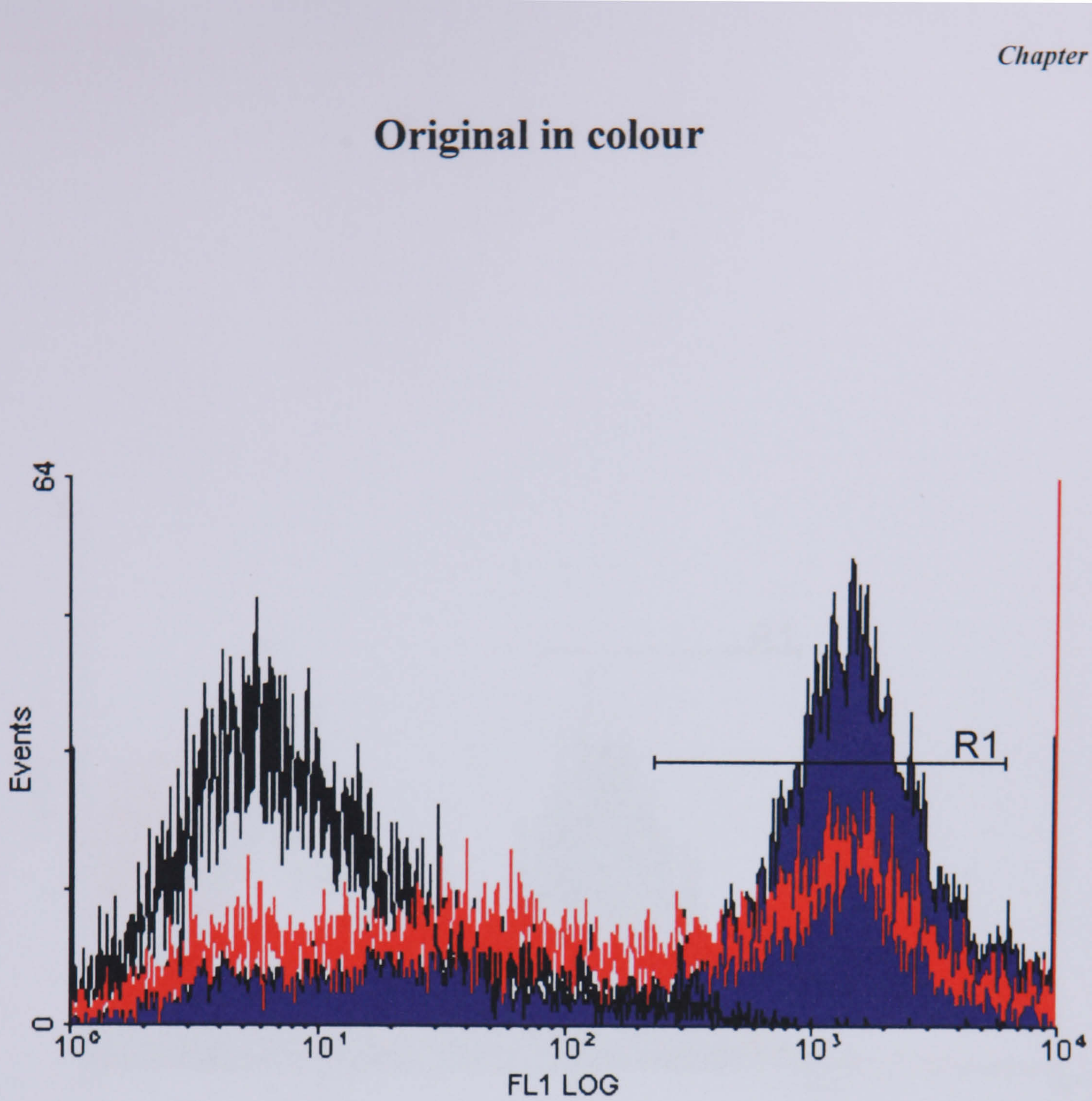

Antigen	% positivity control(±SD)	% positivity MK-801 (±SD)
CD41	76.2 (±7.3)	54.8 (±5.2)

Figure 4.3.11. Expression of CD41 by human primary megakaryocytes following treatment with MK-801. Flow cytometry was used to determine expression of the cell surface marker CD41 by human primary megakaryocytes grown in the presence of TPO for 14days, with and without MK-801. 76.2% (±7.3) of control cells (solid blue fill) were positive for CD41expression, which was reduced to 54.8% (±5.2) in cells treated with 50μM MK-801(red outline). Positivity is gated at higher than 1% of antibody control (black outline; Region R1) and data shown is typical of 3 independent experiments.

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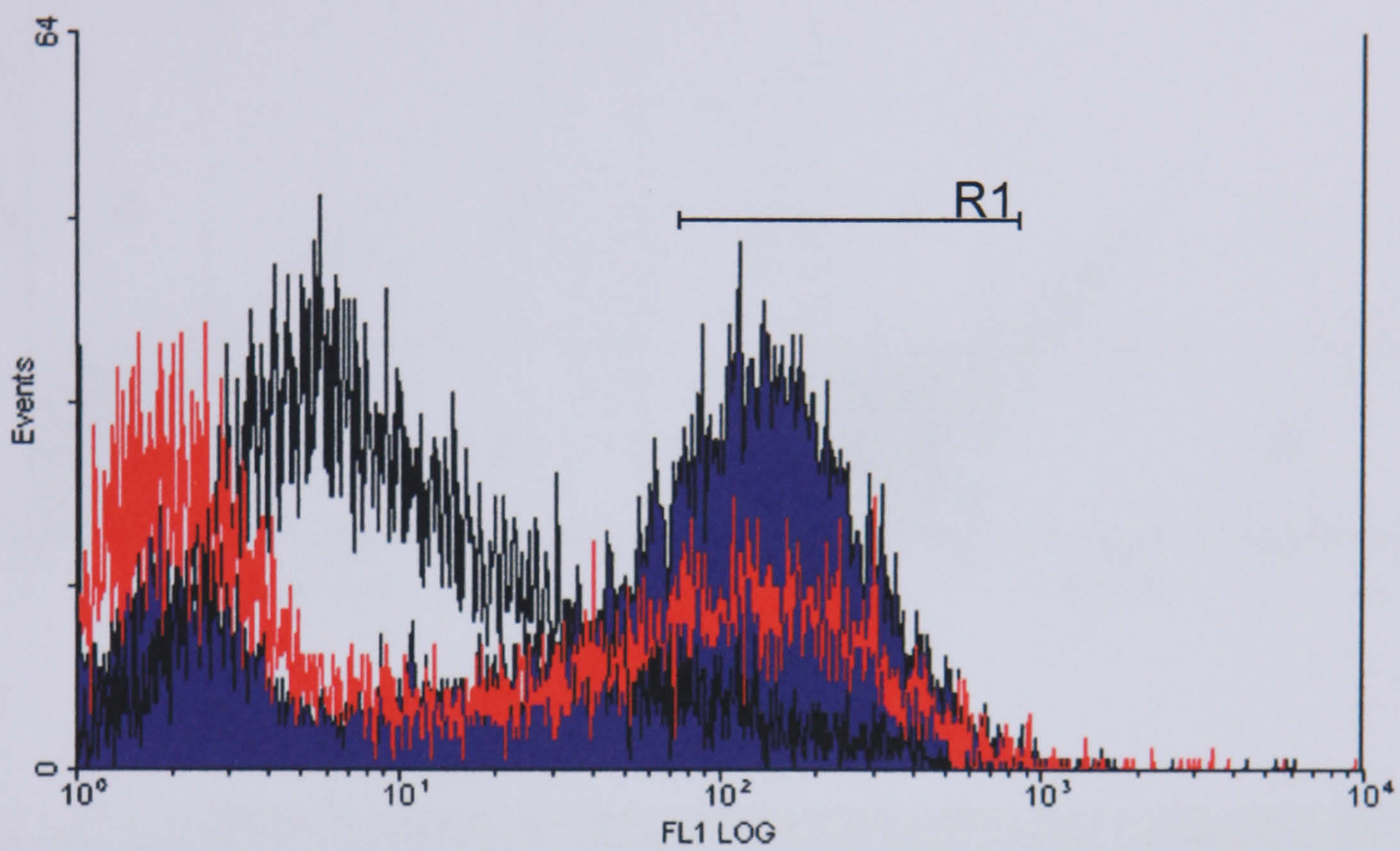
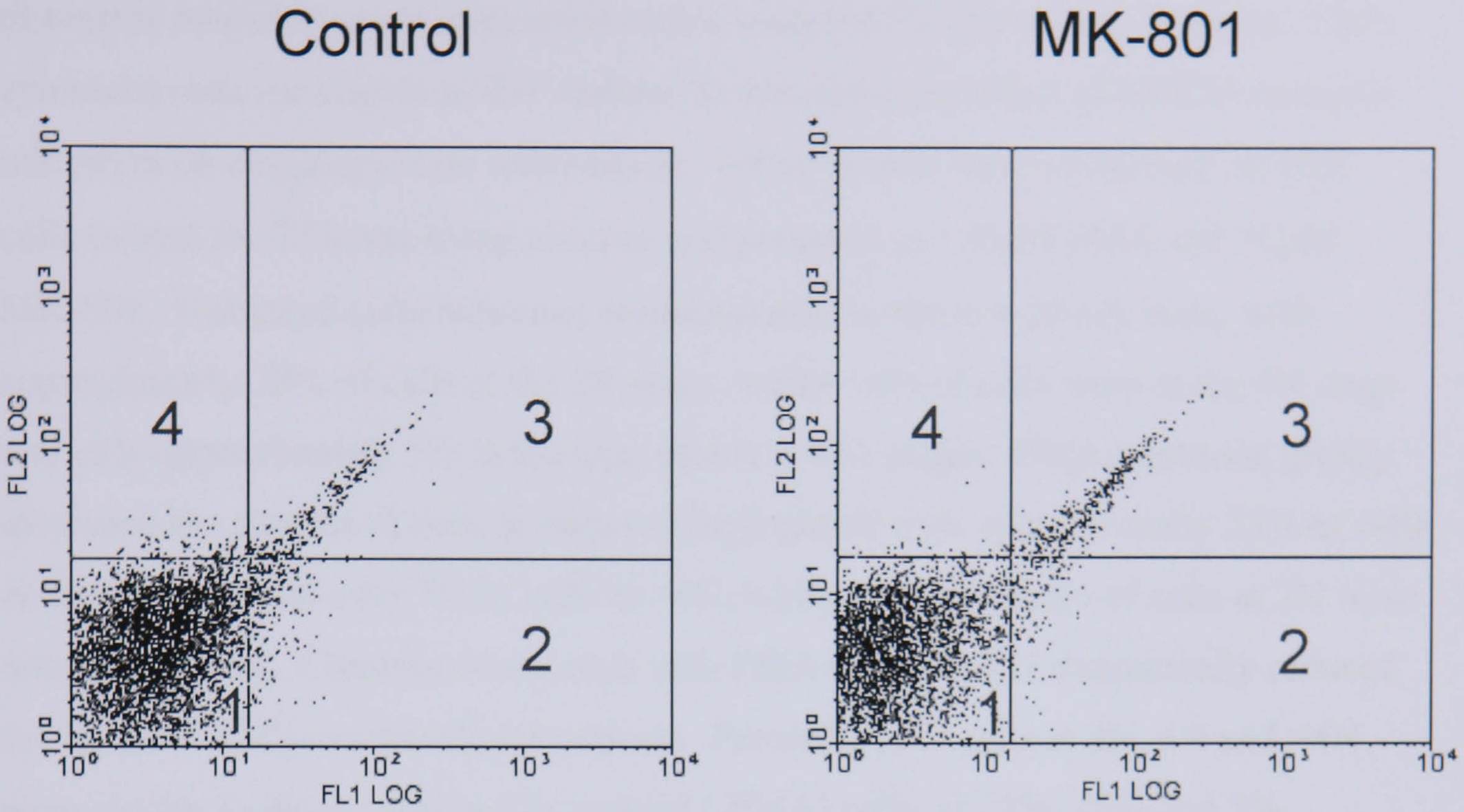


Antigen	% positivity control(±SD)	% positivity MK-801 (±SD)
CD42a	40.7 (±9.5)	23 (±3.8)

Figure 4.3.12 Expression of CD42a by human primary megakaryocytes following treatment with MK-801. Flow cytometry was used to determine expression of the cell surface marker CD42a by human primary megakaryocytes grown in the presence of TPO for 14days, with and without MK-801. 40.7% (±9.5) of control cells (solid blue fill) were positive for CD42a expression, which was reduced to 23% (±3.8) in cells treated with 50μM MK-801(red outline). Positivity is gated at higher than 1% of antibody control (black outline; Region R1) and data shown is typical of 3 independent experiments.



Treatment	Area1	Area2	Area3	Area4
Control	91% (±3.3)	2.46% (±0.8)	4.96% (±0.5)	1.77% (±0.5)
MK-801	88.9% (±5.4)	2.63% (±0.8)	6.28% (±1.5)	2.01% (±0.9)

Figure 4.3.13. Effect of MK-801 on apoptosis of human primary megakaryocytes. Megakaryocytes were labeled with annexin V and PI and analysed by flow cytometry to determine the effect of 50μM MK-801 on apoptosis. No significant differences were observed between control and treated cells, with approximately 90% cells remaining viable (annexin V-negative / PI-negative) following 14 days of culture. Results are representative of three independent experiments.

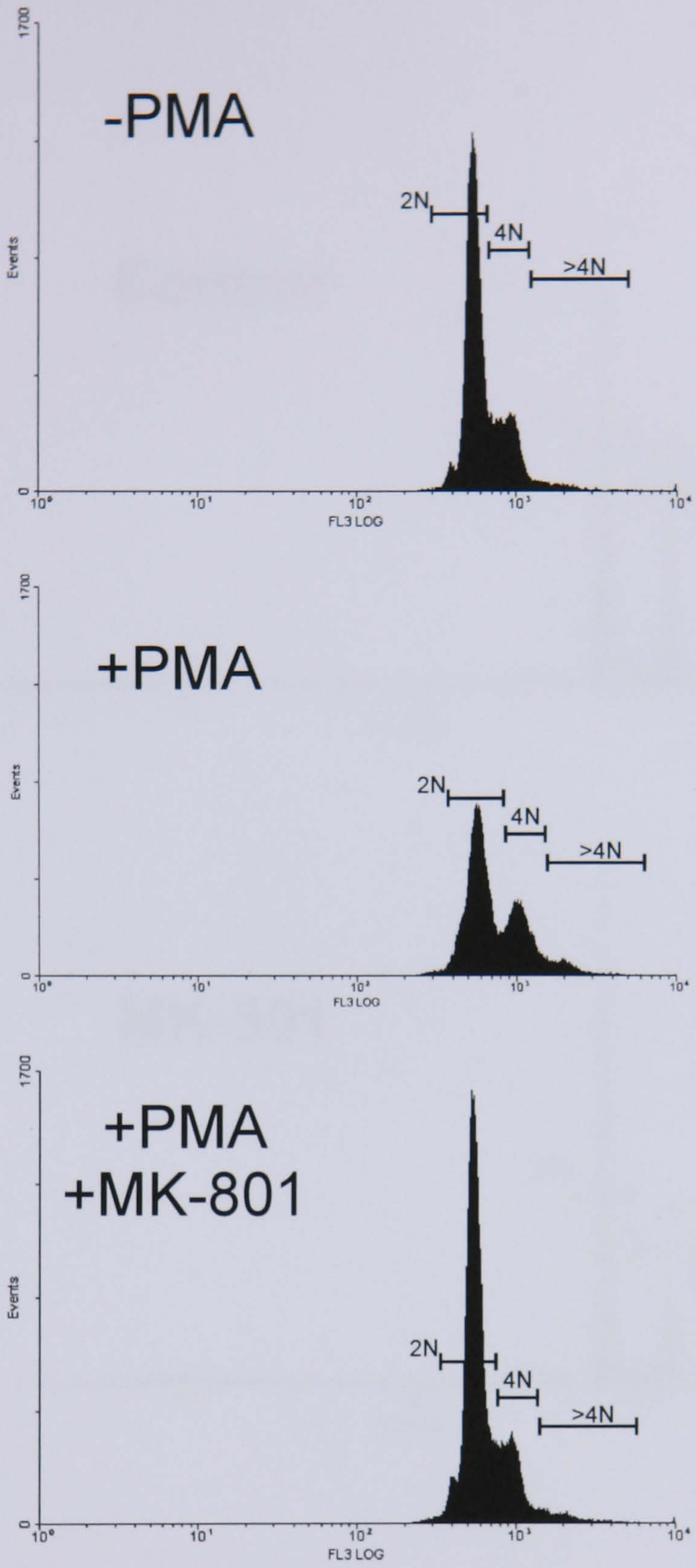
4.3.2.4 Effect of NMDA receptor inhibition on megakaryocyte polyploidy

In addition to cytoplasmic maturation, proplatelet formation and megakaryocyte-specific antigen expression. nuclear maturation is also a characteristic of normal megakaryocyte maturation and is essential for correct cell function. Flow cytometry was used again in this section, to determine the effect of NMDA receptor inhibition on megakaryocyte endomitosis. Initial studies were performed on HEL cells treated for 72 hours in the absence and presence of 100nM PMA and 50μM MK-801. Untreated cells remained predominantly in the low-ploidy state, with approximately 78% of cells at the 2N stage, whilst 16% of cells were at the 4N stage and only approximately 3% in the high ploidy (>4N) stages. PMA treatment greatly increased the number of cells at stages of high ploidy with approximately 25% of cells at the 4N stage and over 7% of cells at >4N, whilst the percentage of cells at 2N were notably reduced. Combined treatment with PMA and MK-801 dramatically reduced the levels of PMA-mediated endomitosis. Percentages of cells at 2N, 4N and >4N were similar to those exhibited by control (-PMA) cells, at 77%, 15% and 3% respectively (Fig. 4.3.14).

The levels of polyploidy were also analysed in human primary megakaryocytes following 14 days of culture with TPO with and without 50μM MK-801 (Fig. 4.3.15). MK-801 treatment reduced the percentage of cells at 4N from 20% to 12% and >4N from approximately 8.5% to 4%. Levels of low ploidy cells (2N) remained higher in MK-801-treated cells compared to control.

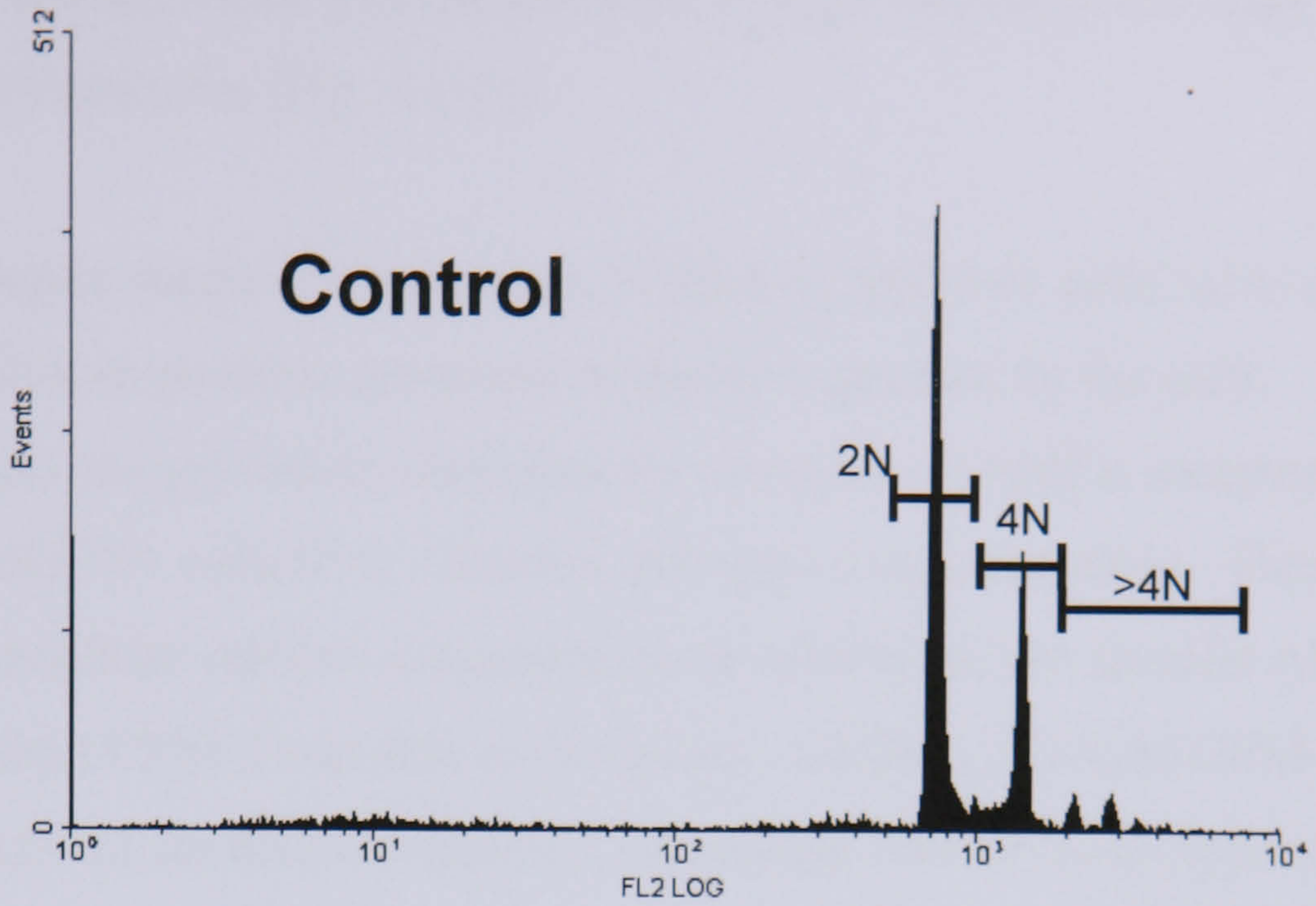
4.3.3 Activation of the NMDA receptor by NMDA and glycine

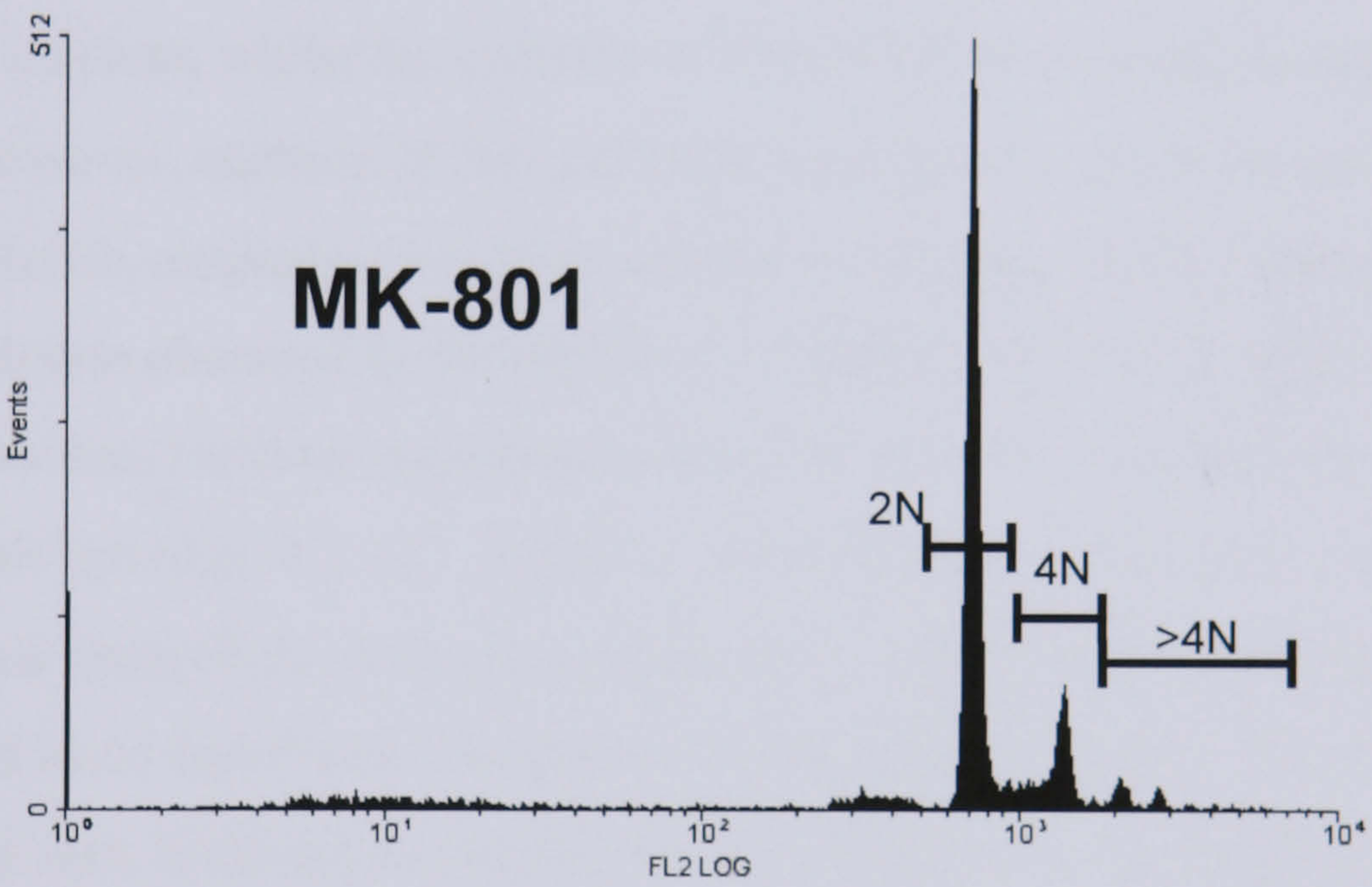
The response of the megakaryocytic NMDA receptor to its pharmacological agonist NMDA and co-agonist glycine has not been previously investigated. To address whether the NMDA receptor is readily activated by varying concentrations of NMDA and glycine, MEG-01 cells were loaded with the calcium indicator indo-1-AM and intracellular calcium concentration monitored using a fluorimeter. Calcium concentration was measured prior to and following agonist addition, assuming that any increase in concentration would be as a result of NMDA receptor activation and calcium influx. The addition of 1μM NMDA/100nM glycine and 10μM NMDA 1μM



	Levels of ploidy		
Treatment	2N	4N	>4N
-PMA	77.9%	16.1%	2.94%
+PMA	61.9%	24.8%	7.18%
+MK-801	77.3%	15.4%	3.26%

Figure 4.3.14 Effect of MK-801 on megakaryocytic cell ploidy. Pliody levels of HEL cells cultured with or without PMA for 72 hours and in the absence or presence of 50μM MK-801 analysed by flow cytometry. PMA treatment considerably increased the percentage of cells displaying levels of high ploidy (4N and greater), whilst MK-801 treatment reduced ploidy to levels comparable to those displayed by untreated cells. Results are representative of two independent experiments.





	Levels of ploidy		
Treatment	2N	4N	>4N
Control	48.3%	20.2%	8.56%
MK-801	58.5%	12.2%	4.16%

Figure 4.3.15 Effect of MK-801 on human primary megakaryocyte ploidy. Pliody levels of human primary megakaryocytes cultured with TPO for 14 days and in the absence or presence of MK-801 analysed by flow cytometry. MK-801 treatment almost halved the percentage of cells displaying levels of ploidy 4N and greater, whilst a greater percentage of cells remained at ploidy levels (2N). Results are representative of two independent experiments.

glycine made no significant difference to basal intracellular calcium concentration, whilst 100μM NMDA/10μM glycine did cause a slight increase from basal intracellular concentration (Fig. 4.3.16).

The lack of receptor-mediated response to NMDA by MEG-01 cells led to the investigation of other possible glutamate receptors expressed by the cells. Unpublished data has previously identified the existence of AMPA receptor subunits expressed by MEG-01 cells (P.G. Genever, personal communication). Therefore, changes in intracellular calcium concentration on addition of the specific AMPA receptor antagonist CFM-2 was also investigated. Addition of 25μM CFM-2 dramatically reduced the concentration of intracellular calcium from approximately 45nM to 20nM (Fig. 4.3.17). Addition of 50 μM CFM-2 resulted in further reductions in intracellular calcium, whilst the addition of 100μM CFM-2 caused no significant differences. However, addition of 200μM CFM-2 produced a small increase in intracellular calcium concentration. In an attempt to confirm whether changes in intracellular calcium observed by the addition of CFM-2 were due to influx of extracellular calcium, identical experiments were performed in the absence of extracellular calcium (Fig. 4.3.18). Addition of 25μM CFM-2 did cause a slight decrease in basal intracellular calcium concentration, whilst further additions of CFM-2 resulted in no significant changes in calcium concentrations. The reduction in intracellular calcium, from approximately 18nM to 15nM following addition of 25μM CFM-2 was however considerably less than the reduction observed in the presence of extracellular calcium.

4.3.4 Megakaryocytic glutamate release

The identification of the glutamate recycling protein GLT-1 expression by unidentified cells in the bone marrow certainly indicates that a release and recycle mechanism may be present (Genever et al., 1999). However, the source of glutamate used by the megakaryocytic NMDA receptor remains unclear. The lack of agonist-induced effects, but clear antagonist effects with regards to intracellular calcium concentration (see chapter 4.3.3), suggests that there may be endogenous glutamate release. The possibility of glutamate being released by the megakaryocyte itself was investigated.

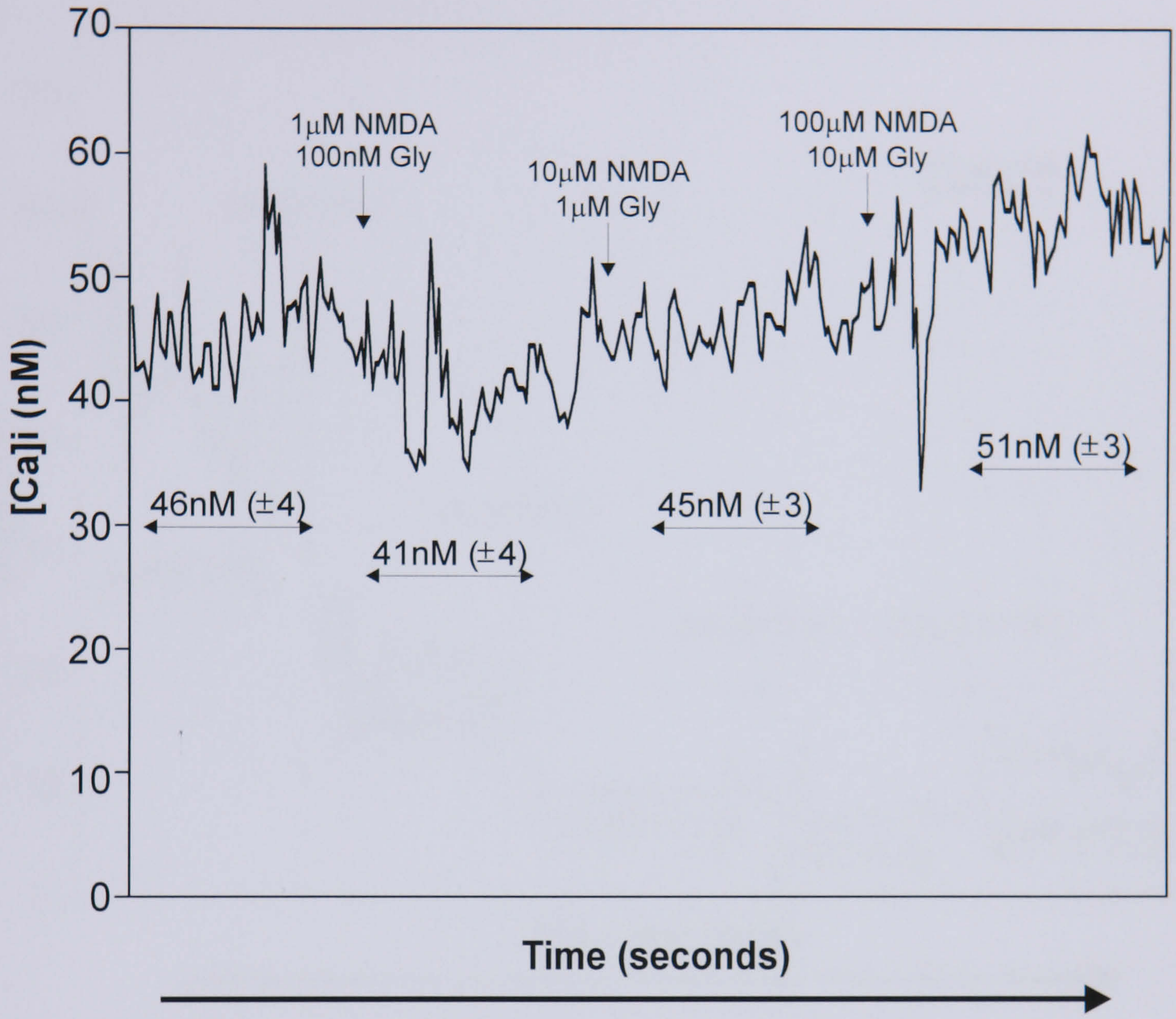


Figure 4.3.16. Changes in intracellular calcium concentration in HEL cells following addition of NMDA and glycine. Intracellular calcium concentration $[Ca]_i$ was monitored in indo-1-AM-loaded HEL cells using a fluorimeter. Average $[Ca]_i$ (±SD), was measured prior to and following the addition of increasing concentrations of NMDA and glycine at known time points. Lower concentrations of NMDA and glycine made no significant difference to $[Ca]_i$, whilst 100μM NMDA, 10μM glycine slightly increased the $[Ca]_i$. Results are representative of three independent experiments.

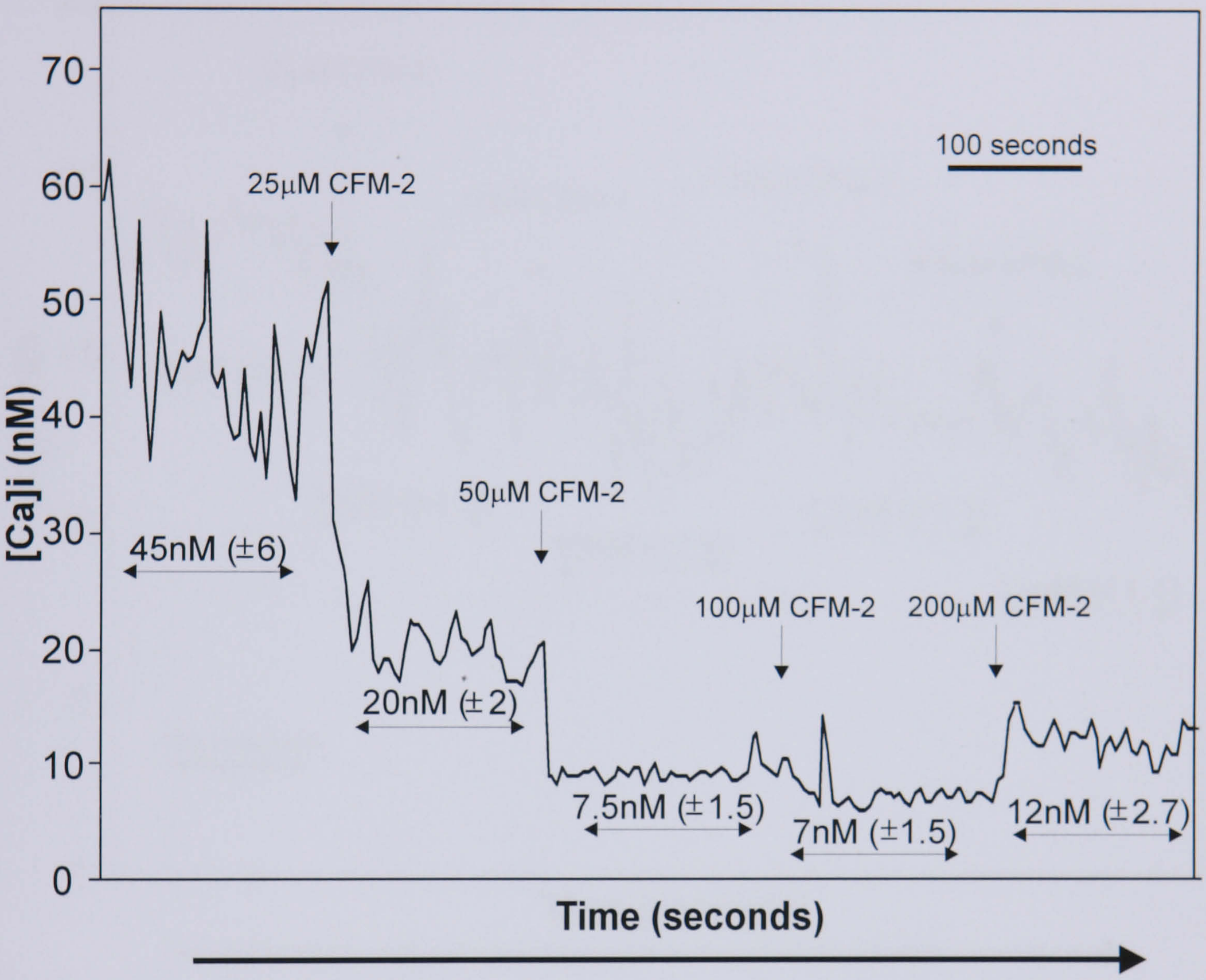


Figure 4.3.17. Changes in intracellular calcium concentration in HEL cells following addition of the AMPA receptor antagonist CFM-2. Intracellular calcium concentration $[Ca]_i$ was monitored in indo-1-AM-loaded HEL cells using a fluorimeter. Average $[Ca]_i$ (±SD), was measured prior to and following the addition of increasing concentrations of CFM-2 at known time points. Upon addition of 25μM CFM-2, $[Ca]_i$ was dramatically reduced. Further reductions were also observed following addition of 50 and 100μM CFM-2, whilst 200μM CFM-2 appeared to increase $[Ca]_i$. Results are representative of two independent experiments.

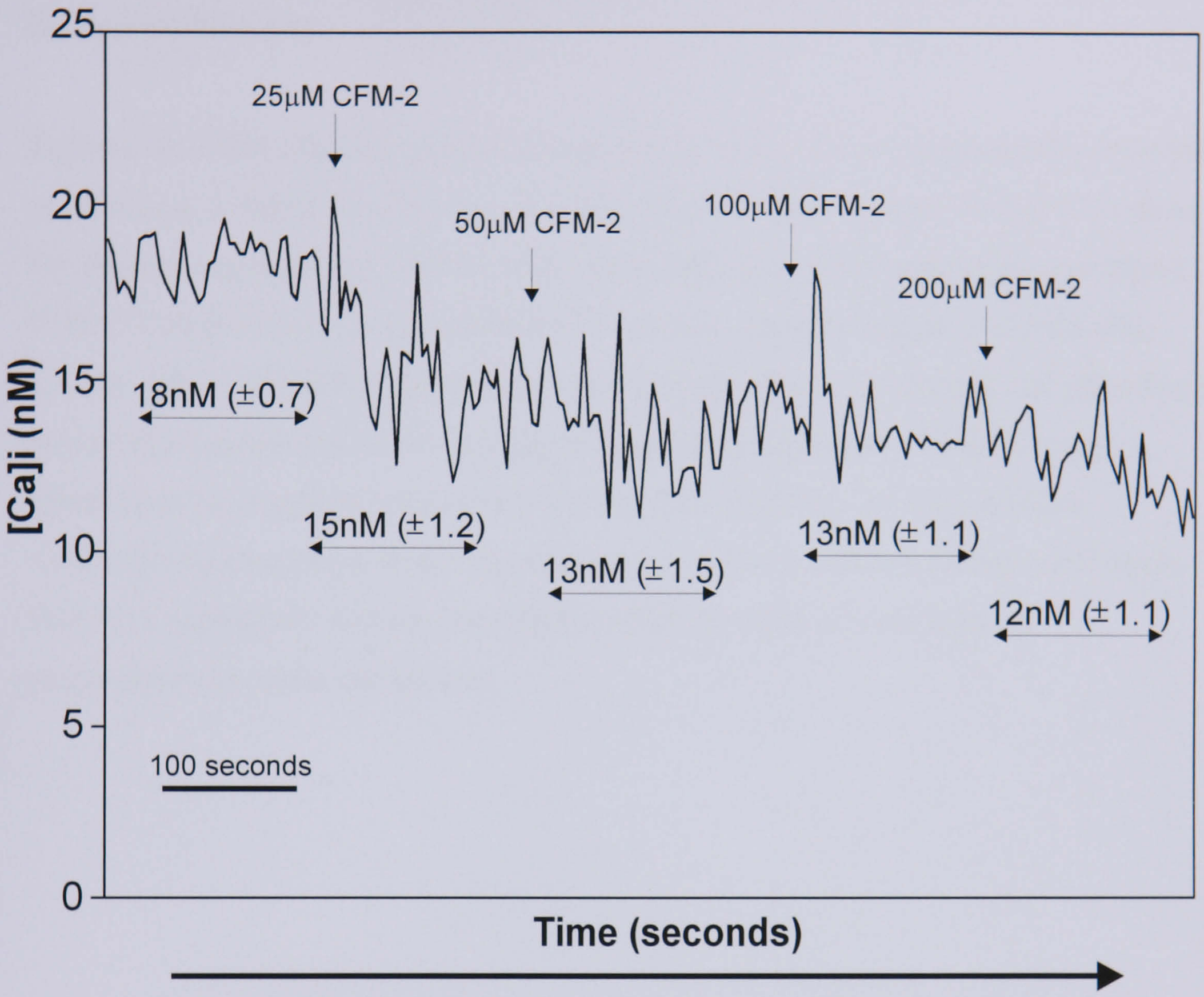


Figure 4.3.18. Changes in intracellular calcium concentration in HEL cells following addition of the AMPA receptor antagonist CFM-2 in a calcium-free environment. Intracellular calcium concentration $[Ca]_i$ was monitored in indo-1-AM-loaded HEL cells in a calcium-starved environment using a fluorimeter. Average $[Ca]_i$ (±SD), was measured prior to and following the addition of increasing concentrations of CFM-2 at known time points. CFM-2 addition in the absence of extracellular calcium caused no significant increase in $[Ca]_i$. Results are representative of two independent experiments.

using a glutamate release assay (Fig.4.3.19). It was demonstrated that MEG-01 cells release low concentrations (around 300pM/μg protein) compared to neuronal (Nicholls and Sihra, 1986) and osteoblastic cells (Genever and Skerry, 2001). Glutamate release from PMA treated cells (B) was not significantly different to those cells cultured in the absence of PMA (A).

Expression of the vesicular glutamate transporter VGLUT2 was confirmed in cytospin preparations of MEG-01 cells (Fig. 4.3.20). MEG-01 cells express VGLUT2 both in the absence and presence of PMA with expression located throughout the cytoplasm. VGLUT2 expression was also identified in rat bone marrow megakaryocytes (Fig. 4.3.21). VGLUT2 immunoreactivity was observed with a cytoplasmic and prominent pericellular localisation in all NR1-expressing megakaryocytes. VGLUT2 also appeared to be expressed exclusively by megakaryocytes in the bone marrow. Antibody negative preparations demonstrated specificity and low background levels. VGLUT1 expression was not identified in MEG-01 cells or bone marrow megakaryocytes (data not shown).

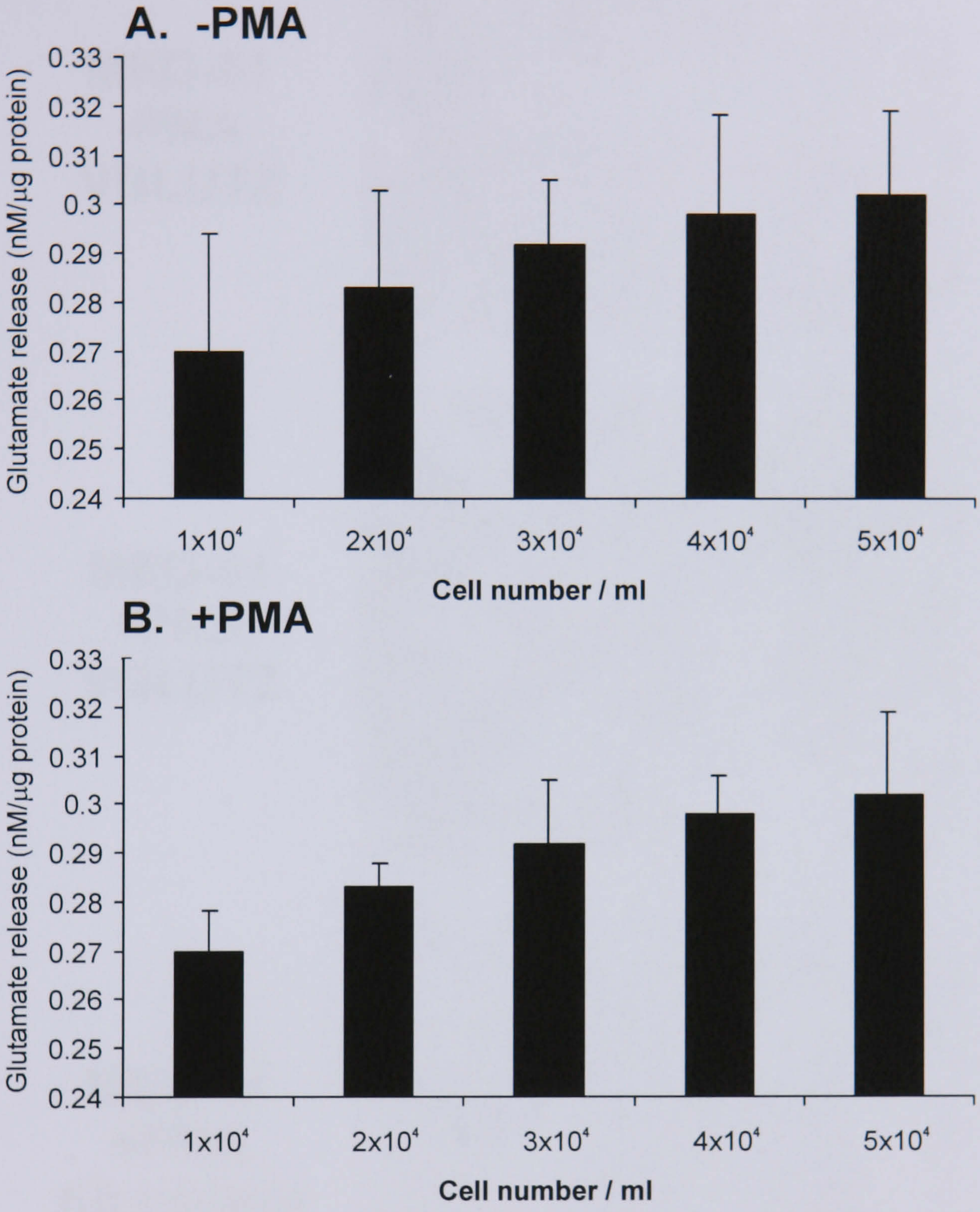


Figure 4.3.19. Glutamate release by MEG-01 cells. Glutamate release assay identified that MEG-01 cells release glutamate proportional to the number of cells in the assay. Control (A) and PMA-treated (B) cells displayed no significant differences in glutamate release. Results are representative of two independent experiments.

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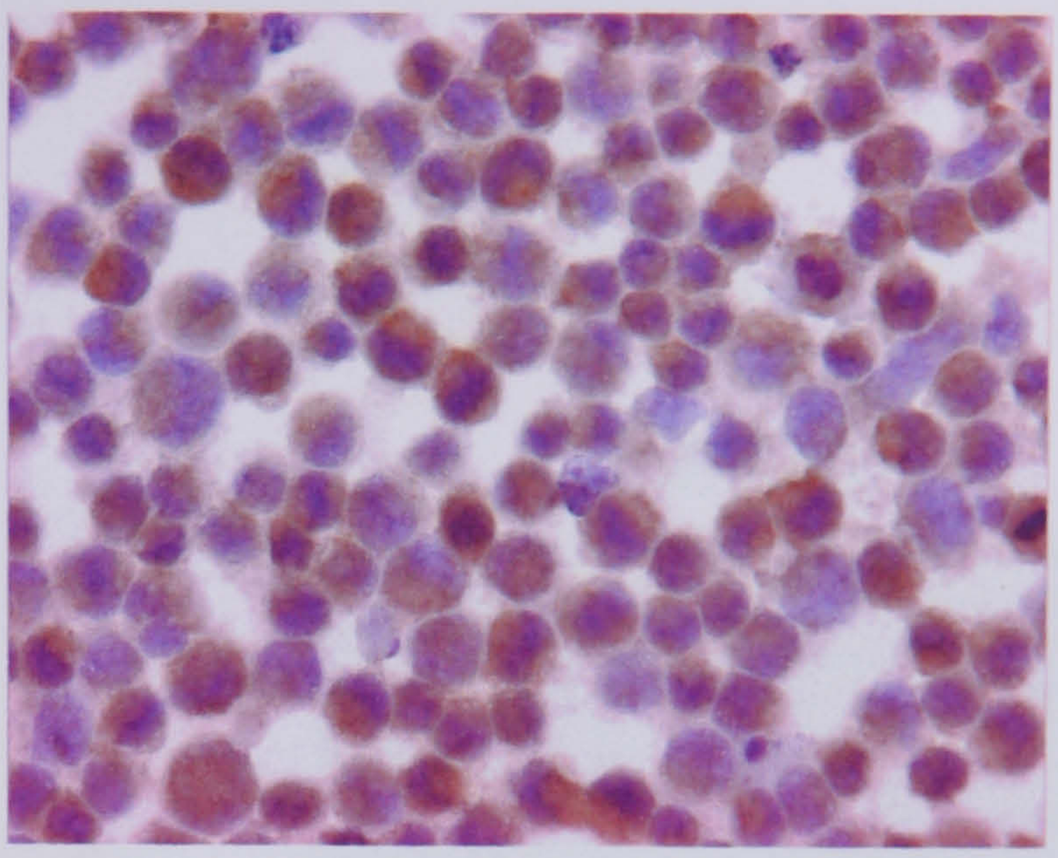
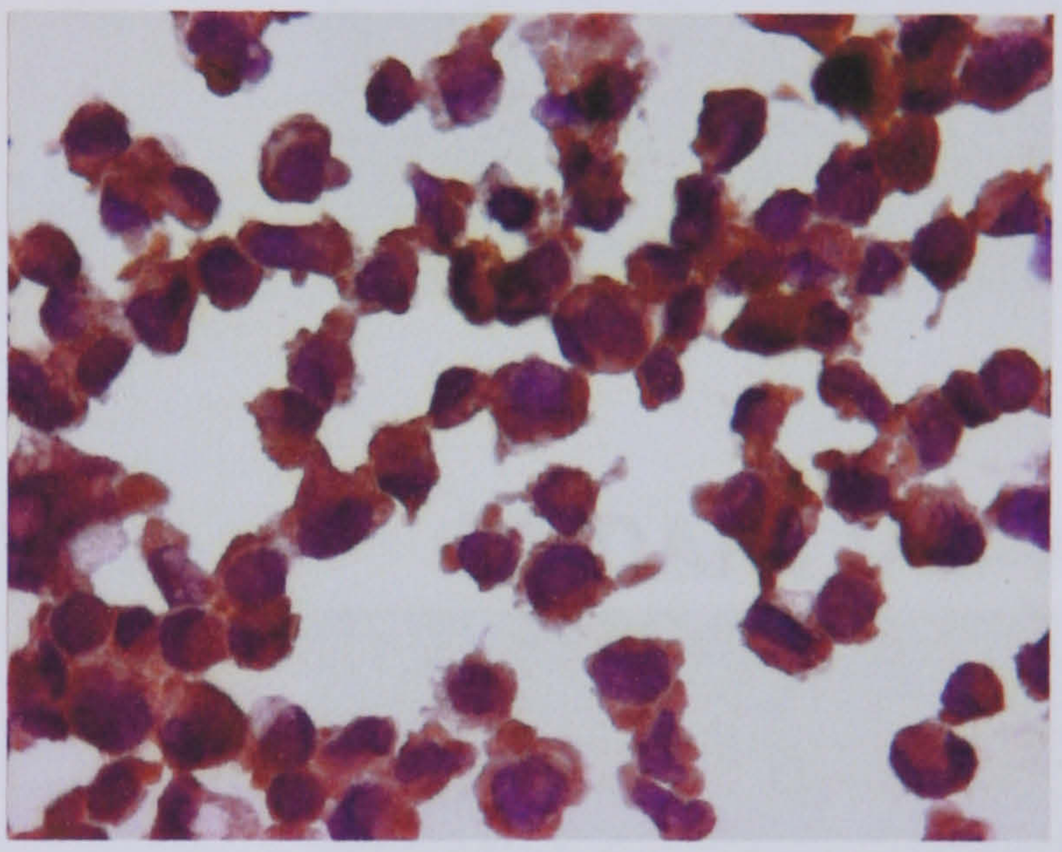
MEG-01
-PMA
VGLUT2



MEG-01
+PMA
VGLUT2



MEG-01
+PMA
Ab control

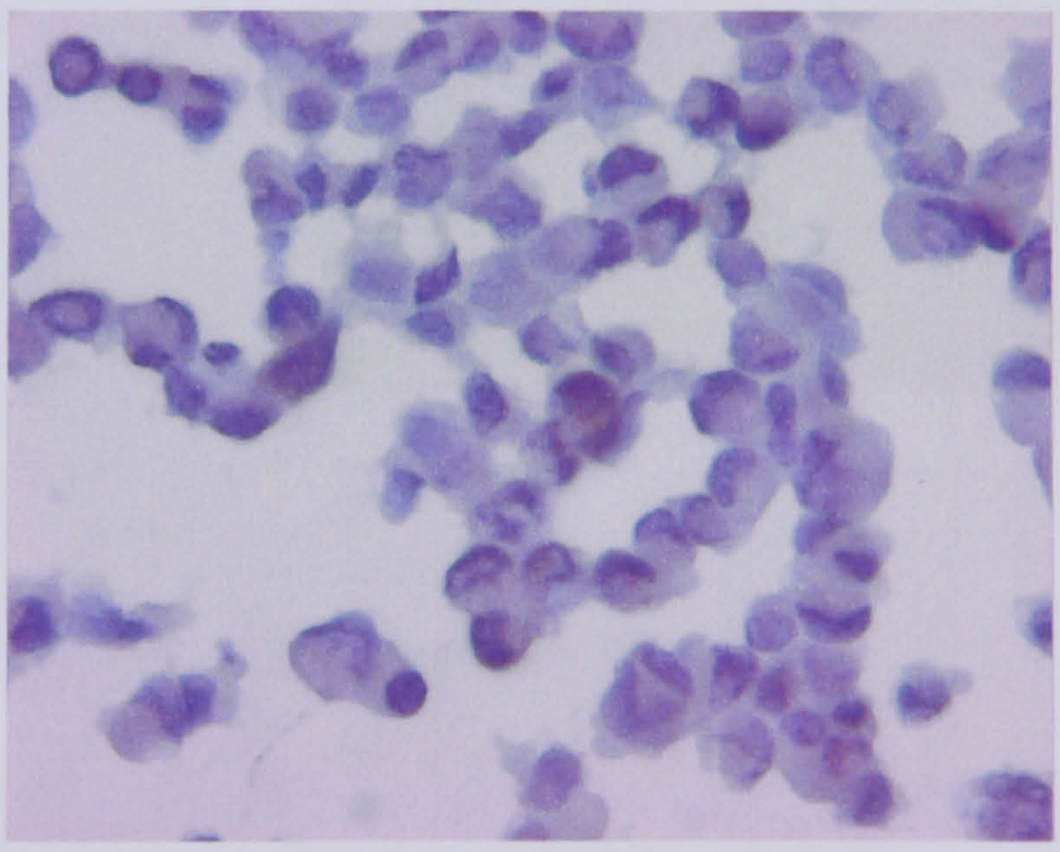


Figure 4.3.20 VGLUT2 expression by MEG-01 cells. Immunocytochemical identification of VGLUT2 expression by MEG-01 cytospin presparation in the absence and presence of PMA. MEG-01 cells with and without PMA exhibit cytoplasmic expression of VGLUT2 (brown staining). Antibody control (Ab control) displays background levels of staining. Results are representative of two independent experiments.

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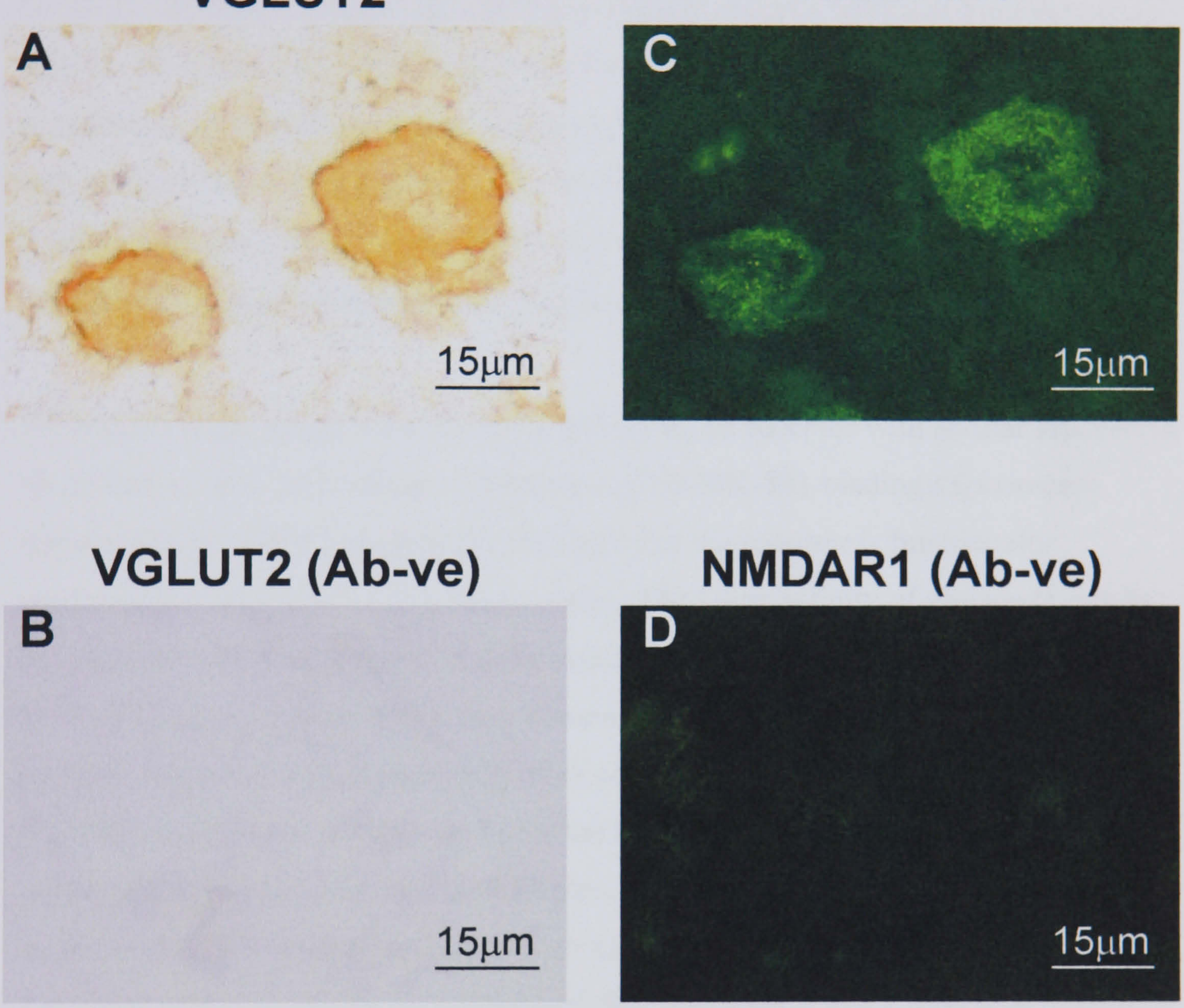


Figure 4.3.21. VGLUT2 expression by rat tibial megakaryocytes.
Immunohistochemical examinations of rat tibial sections identified pericellular expression of VGLUT2 by bone marrow megakaryocytes (brown staining; A). Megakaryocytes were identified as being NR1 positive (green FITC staining; C). Peroxidase and FITC antibody controls (B and D respectively) displayed low levels of background staining. The experiment was performed once.

4.4 Discussion

Previously, evidence concerning the function of the megakaryocytic NMDA receptor was scant. The original investigations regarding glutamate signalling in megakaryocytes identified that by inhibiting the NMDA receptor with MK-801, MEG-01 cell differentiation and adhesion was significantly inhibited (Genever et al., 1999[a]). How this novel signalling system integrates with other, more recognised megakaryocytopoiesis-regulating cytokines, requires a greater understanding of the effects that NMDA receptor inhibition has on megakaryocytes. The aim of this chapter is to provide greater insight into NMDA receptor function in megakaryocytic cell lines, as well as human primary megakaryocytes.

Evidence that glutamate binds to the megakaryocytic receptor with similar kinetics as identified in other cells, along with previous [^{3}H]-MK-801 binding experiments (Genever et al., 1999[a]), support the evidence that the receptor is functionally comparable to that present in neuronal cells. The lower affinity of glutamate binding by MEG-01 cells compared to both neuronal and bone cells (Davies et al., 1981; Laketic-Ljubojevic et al., 1999) may demonstrate one of the differences of this receptor compared to that present in other cell types. It is also of interest to note that MK-801, which does not directly block the binding of glutamate to its binding site, greatly alters the glutamate binding kinetics. The significant role of the NMDA receptor in LTP is widely acknowledged (for review see Dingledine et al., 1998). One such characteristic of LTP is clustering of the membrane NMDA receptors, mediated by PKC phosphorylation of a serine residue in the cytoplasmic domain of either the NR1 or NR2A subunit (Tingley et al., 1997; Zheng et al., 1999). Clustering of the receptor in the megakaryocyte membrane would therefore increase glutamate binding, an effect that is inhibited by NMDA receptor antagonism. Currently, it is only possible to hypothesise that this is the case and requires more focused investigation of receptor subunit phosphorylation and localisation

Fibronectin (FN) is a key element of the extracellular matrix in bone marrow. The role of FN has also been shown to be of major importance in HSC differentiation (Weiss and Reddy, 1981; Verfaillie et al., 1991), providing anchorage by which HSCs

can migrate to specific regions of the bone marrow that promote maturation (Hamilton and Campbell, 1991). Megakaryocytes can produce, and in the presence of thrombin, secrete FN (Schick et al., 1996), the role of which is believed to be in the formation of pseudopodia resembling proplatelet structures (Schick et al., 1998; Yamazaki et al., 1999). The principle integrin involved in the adhesion of megakaryocytes to fibronectin is $\alpha5\beta1$, which binds megakaryocytes to fibronectin both "resting" and "active" (+ thrombin) state (Schick et al., 1998). The data provided here demonstrated that the activation of $\beta1$ integrin was decreased following NMDA receptor blockade offering one explanation as to why MEG-01 adhesion to FN was significantly reduced by MK-801. MEG-01 adhesion to fibronectin is induced through PMA-mediated PKC activation. This suggests that integrin activation could occur either as a direct or indirect result of active PKC, possibly via the receptor for activated C-kinase 1 (RACK1), which interacts with the $\beta1$ integrin subunit (Liliental and Chang, 1998).

These findings however, suggest a role for NMDA receptors in the normal process of integrin activation. Similar investigations regarding $\beta1$ integrin activation in human polymorphonuclear leukocytes, identified that increased intracellular calcium concentration in synergy with direct PKC activation was required for $\beta1$ integrin activation (Rowin et al., 1998). Therefore, it is conceivable that increases in intracellular calcium concentration induced by the activation of the functional NMDA receptor, provides the source of calcium that, in conjunction with activated PKC, activates integrin-mediated FN binding. Inhibition of the NMDA receptor-mediated calcium influx would thus prevent integrin activation. How PKC and calcium interact in the modulation of integrin activation however, remains unknown. These findings have major implications not only on cytoskeletal reorganisation, but also on the possible effects of integrin signalling (for review see Giancotti and Rouslahti, 1999). Adhesion of megakaryocytes to FN has been shown to directly activate the intracellular signalling molecule ERK1/2 (Mizutani et al., 2002), in turn promoting the formation of proplatelet structures (Jiang et al., 2002).

In addition to being important in the adhesion of MEG-01 cells, it is also possible that NMDA receptor activity modulates their proliferative capacity. The role of the NMDA receptor in megakaryocyte differentiation remains unclear. Work described in

this chapter demonstrated that NMDA receptor antagonism caused an inhibition of human primary megakaryocyte differentiation on several levels. The reduction in expression levels of CD61, CD41 and CD42a by primary megakaryocytes in the presence of MK-801 implies that these cells were of a less differentiated phenotype. However morphological studies, confirm that megakaryocytic NMDA receptor activity also has key roles in other aspects of normal megakaryocyte functions. It may be that the NMDA receptor activity is able to modulate the activity of intracellular signalling molecules that are known to promote differentiation. This hypothesis will be addressed in Chapter 5. However, if the receptor expressed by megakaryocytes and neuronal cells is comparable it may be possible to draw analogies, which may have functional implications. One such example is in the dramatic antagonism of proplatelet production *in vitro* brought about by inhibition of the NMDA receptor. The formation of proplatelets is in many ways similar to the process of neuronal outgrowths. Axonal growth is of central importance both during the development of the adult nervous system and the regeneration of neuronal systems following injury. The process of neurite elongation followed by extensive branching (similar to the structures observed by ultrastructural analysis of proplatelet formation (Italiano et al., 1999)) relies upon reorganisation cytoskeletal proteins including microtubules and actin. Indeed, depolymerisation of actin and tubulin reduces the ability of hippocampal neurones to develop the characteristic extensive branching (Allison et al., 2000). The NMDA receptor has recently been demonstrated to modulate neuron outgrowth. MK-801 application to cultured chick neurones inhibits neurite initiation outgrowth and branching in a dose dependent manner, resulting in a reduction of the "neurite tree" complexity (Cuppini et al., 1999). It is possible that the NMDA receptor achieves this by controlling the action of the axonal growth-associated protein GAP-43, with MK-801 significantly reducing the levels of GAP-43 mRNA in hippocampal granule cells (Cantallops and Routtenberg, 1999). Other evidence also suggests that the NR1 and NR2B NMDA receptor subunits directly bind to β-tubulin and modulate microtubule formation (van Rossum et al., 1999).

It is also of interest to note that the recently characterised NR3 subunit has a role in the formation of normal dendritic spines and branches (Das et al., 1998). As NR3A has been shown to decrease the conductance of the NMDA receptor both *in vivo* and *in*

vitro, the expected increase in dendritic spine branching was exhibited in NR3A knockout mice. It is therefore a distinct possibility the megakaryocytic NMDA receptor is also of great importance in the formation of microtubules from β1 tubulin and actin polymerisation, both of which are key to the formation of normal proplatelet structures. The inhibition of proplatelet formation has not only been confirmed by examination of cells *in vitro*, but also by ultrastructural analyses which indicate the profound differences in cytoplasmic maturation between control and MK-801 treated cells. As shown in chapter 3, the expression of the NR3 subunit is restricted to immature cells, therefore providing a possible control mechanism to ensure that proplatelet formation occurs only in mature cells.

These findings are of particular significance when considering that no haematopoietic cytokine has currently been identified that directly promotes the formation of proplatelets *in vitro*. Although these data provide the first evidence of a cell surface receptor with the ability to regulate proplatelet formation, the connections with the other factors involved in proplatelet production, such as the transcription factor NF-E2 and cytoskeletal proteins, need to be investigated. However, if these links are confirmed, the implications to the understanding of how platelets are formed will be significant.

Ultrastructural analyses also confirmed that other characteristics associated with the production of normal platelets are affected by NMDA receptor inhibition. The apparent lack of α-granules in the cytoplasm is of particular interest, as this is a characteristic displayed by peripheral blood $CD34^+$-derived megakaryocytes from patients suffering the rare congenital bleeding disorder, gray platelet syndrome (GPS; Drouin et al., 2001). It would appear that this condition results from molecules vital for platelet function, especially vWF, being released elsewhere in the cell rather than the platelet α-granule, explaining the presence of vWF in GPS megakaryocytes but absence in GPS platelets. As α-granules are only identifiable by TEM due to their dense packaging, empty granules cannot be identified. Whether the pathology of GPS is caused by a lack of or mutation in the megakaryocytic NMDA receptor and how NMDA receptor-mediated signalling may promote α-granule formation warrants further investigation.

Possibly the most striking cytoplasmic abnormality observed in MK-801 treated megakaryocytes is the formation of large open cisternae displayed by many of the cells examined in this study. Since this work was performed, similar characteristics were identified in megakaryocytes treated with monoclonal CD9 antibody (Clay et al., 2001). The function of CD9 is unknown, even though it is expressed on numerous different haematopoietic cells (Boucheix et al., 1985), although it is postulated that it may play a role in several different cellular events including proliferation, adhesion and maturation (Maeker et al., 1997; Langaudriere-Gesbert et al., 1997). In addition to the cytoplasmic abnormalities, suggestive of dysfunctional membrane remodelling, treating megakaryocytes with the monoclonal CD9 antibody increased the levels of proliferation whilst reducing expression of CD41. The apparent morphological and immunological similarities in MK-801 and CD9 antibody-treated megakaryocytes may be coincidental, indirectly linked though other signalling pathways or directly associated in normal megakaryocyte differentiation. However, both lines of work are still in their relative infancy and associations between the two systems warrant further investigation.

In addition to providing essential evidence regarding cytoplasmic maturation, ultrastructural analyses also identified that MK-801 treatment reduced the levels of nuclear maturation. This subsequently led to the quantitative analysis of polyploidisation, which confirmed that the NMDA receptor has the ability to modulate megakaryocytic endomitosis. The mechanism by which this is attained is likely to be via an extensive signalling mechanism downstream of the NMDA receptor, such as the ERK1/2 pathway (Miyazaki et al., 2001), which is able to affect various aspects of the cell cycle that underpin endomitosis. The effect of NMDA receptor inhibition on intracellular signalling will be addressed in chapter 5.

The lack of agonist response in MEG-01 cells was intriguing. The addition of NMDA with the co-agonist glycine to cells expressing the functional NMDA receptor could be expected to induce recordable increases in intracellular calcium concentration. This response underpins many of the functions of the NMDA receptor in the CNS. However, it should be appreciated that the function of the receptor in the megakaryocyte is highly unlikely to be identical to that in the CNS. It is possible that

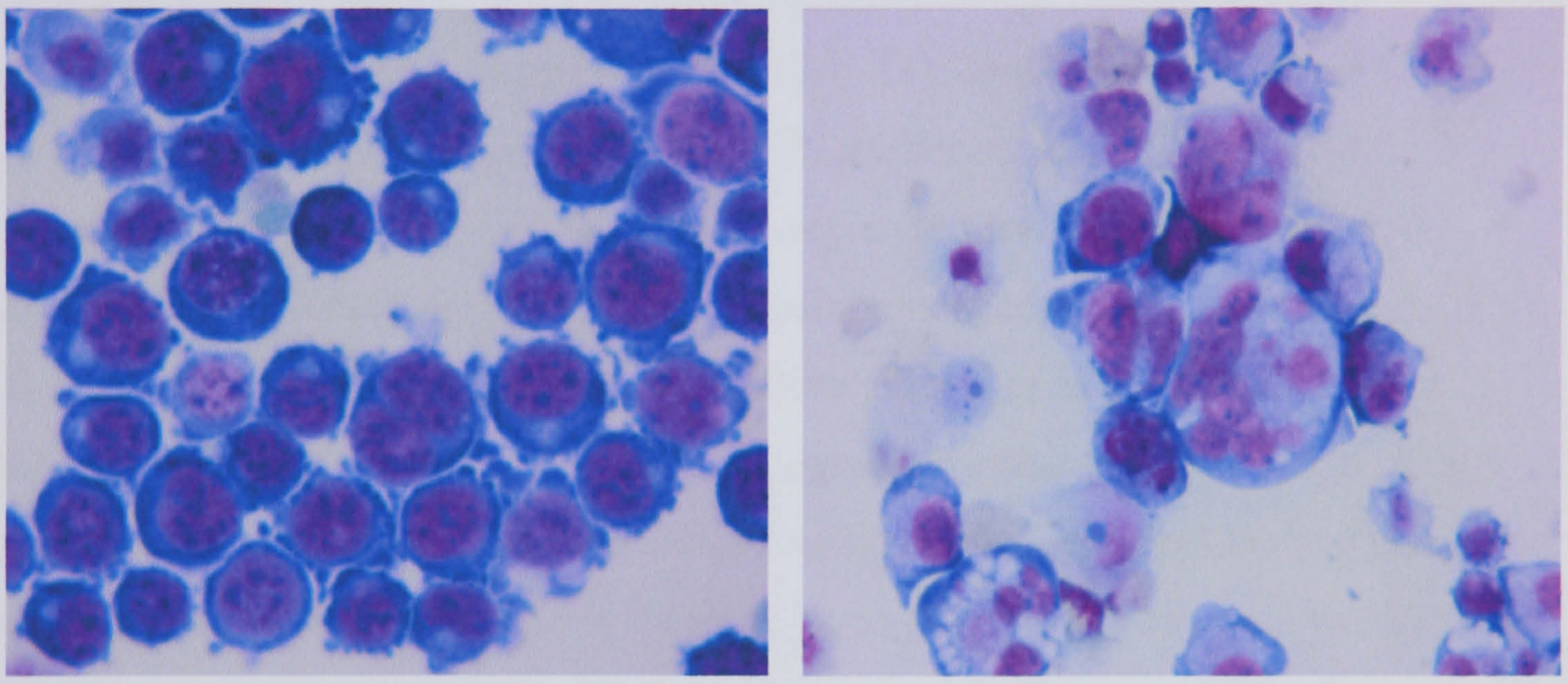
the activation of the megakaryocytic NMDA receptor actually causes very small increases in intracellular calcium, which cannot be measured using the methods described in this chapter. It may be the case that the calcium influx is only required for the activation of the NMDA receptor in the locality, and it is the activated NMDA receptor that directly influences downstream signalling events rather than large increases in intracellular calcium. The presence of the AMPA and kainate receptors has not been studied since their expression was identified by RT-PCR in MEG-01 cells (P.G. Genever, personal communication). It would appear that MEG-01 cells express the AMPA receptor subunit GluR1, as well as the kainate receptor subunit KA2 (for review see chapter 1.6.2.1). The existence of AMPA/NMDA receptor cross-talk is now well established and AMPA receptor activation has been shown to modulate NMDA receptor activity (Bai et al., 2002). As one of the key roles of the AMPA receptor is in depolarisation-induced Mg^{2+} removal from the NMDA receptor, application of the specific AMPA antagonist CFM-2 may indirectly prevent the activation of the NMDA receptor. Therefore, the reduction of intracellular calcium concentration observed in the presence of CFM-2 might either be through inhibition of an uncharacterised functional megakaryocytic AMPA receptor, or by the prevention of Mg^{2+} removal from the NMDA receptor. This is in great need of further investigation. One requirement is to perform more accurate techniques of measuring receptor activation and changes in calcium influx, for example, patch clamping, which has previously been used for the characterisation of the osteoblastic glutamate receptor (Laketic-Ljubojevic et al., 1999). However, the lack of agonist response has since led to the investigation of megakaryocytic glutamate release, which may provide an explanation as to why NMDA and glycine application has very little apparent effect on receptor activation.

The release of glutamate from MEG-01 cells in addition to the identification a protein involved in packaging of glutamatergic vesicles in MEG-01 cells and bone marrow megakaryocytes, is an extremely exciting finding. It may therefore be the case that MEG-01 cells are under constant stimulus of glutamate and starving the cells of glutamate to induce a large response to NMDA and glycine application may therefore not be viable. It may indeed be the case that the cells will have to be treated with the glutamate release inhibitor (such as riluzole) in conjunction with glutamate starving, to allow a detectable agonist response. The glutamate release findings also have

significant implications regarding the bone marrow microenvironment. It is possible to imagine a situation in which megakaryocytes release glutamate primarily for their own use (due to their relative sparseness in the bone marrow) and glutamate is recycled via the GLT-1 positive cells in the vicinity of the megakaryocyte. The fact that megakaryocytes express VGLUT2, which is expressed only by differentiated neuronal cells, and not VGLUT1, suggests that glutamate release may only occur in differentiated megakaryocytes. However, undifferentiated MEG-01 cells do express VGLUT2 and release glutamate at similar levels to differentiated MEG-01 cells. It may therefore be the case that megakaryocytes release glutamate throughout differentiation, possibly mediating other cells in the bone marrow, but the high level of expression of NR3 by the undifferentiated cells prevents the activation of the megakaryocytic NMDA receptor. Currently, this situation is only speculative. However, further investigation, along the same lines as that performed on osteoblastic cells (Genever and Skerry, 2001[a]), would provide a greater understanding of glutamate signalling in the bone marrow microenvironment with regards to the regulation of megakaryocytopoiesis.

The data in this chapter provides a greater understanding of the functional effects of the megakaryocytic glutamate receptor. Inhibition of the NMDA receptor appears to have profound control over many aspects of megakaryocyte differentiation and function and may provide a degree of control similar to the more established haematopoietic cytokines. However, to determine if this is indeed true, a greater understanding of downstream signalling events is required and will be addressed in the next chapter.

Chapter 5



NMDA Receptor-Mediated Signal Transduction in Megakaryocytes

Effects of PKC activation by PMA on MEG-01 cells. Untreated cells (left) are small and rounded compared to the large, multinucleated cells PMA-treated cells (Magnification x400)

Chapter 5

NMDA Receptor-Mediated Signal Transduction in Megakaryocytic Cells

5.1 Introduction

Since TPO, the key cytokine regulating megakaryocytopoiesis became commercially available, intracellular signal transduction studies in megakaryocytes have predominantly focused on the events following TPO application (reviewed in chapter 1.3.1). The subsequent findings have defined the action of TPO and provided compelling evidence as to its role in megakaryocyte survival, proliferation and differentiation. When characterising any membrane receptor signalling system, it is essential to define the downstream intracellular effects of receptor activation and/or inhibition. Doing so will identify how the signalling systems brings about cellular responses as well as potentially allowing pharmacological manipulation of these mechanisms. This chapter determines the effects of NMDA receptor activation and inhibition on intracellular signalling events in megakaryocytic cell lines, by investigating both established megakaryocytopoiesis-promoting pathways and more "neuronal" NMDA receptor mediated signal transduction.

The signal transduction studies in this chapter use the megakaryocytic cell lines MEG-01 and HEL, which are described in depth in chapter 3.1. By using these cell lines, it is possible to investigate the effect of PMA-induced differentiation that occurs in both cell lines, as well as determining the effects of TPO signalling in the c-Mpl expressing HEL cells (Zauli et al., 1997; Kalina et al. 2001). As MEG-01 cells do not express c-Mpl (Rollinger-Holzinger et al., 1998), the PKC-activating agent PMA is used to trigger differentiation, demonstrating the importance of PKC in megakaryocyte differentiation. The phorbol ester PMA competes for the same binding site on PKC as diacylglycerol (DAG) and the reversible kinase activity of PKC relies on the binding of either DAG or phorbol esters (Sharkey and Blumberg, 1985). The region of the PKC regulatory domain responsible for the inhibition of catalytic activity

of PKC is known as the pseudosubstrate site (House and Kemp, 1987) and is inhibited by the binding of DAG or phorbol ester, therefore "activating" the catalytic activity of PKC. Some PKC isoforms, including PKCα that has been shown to have particular importance in megakaryocytopoiesis and platelet production (Rojnuckarin and Kaushansky, 2001), also have a calcium specificity domain that is believed to influence the translocation of PKC to various regions of the cell, such as the plasma membrane and the nucleus (Luo and Weinstein, 1993; Haller et al., 1994). PKCα has also been shown to translocate to the plasma membrane following small increases in intracellular calcium concentration just under the plasma membrane, known as "calcium sparks", which have local intracellular effects (Haller et al., 1989; Berridge and Dupont, 1994; Maasch et al., 2000). By translocating to the plasma membrane, active PKC is able to interact with Raf and stimulate the MAPK pathway.

Compelling evidence now exists that both PMA and TPO-mediated megakaryocytic differentiation is via active PKC, which, initiated by stimulation of Raf, directly leads to the activation of the MAPK pathway that comprises ERK1/2, in addition to stress activated kinases p38 and Jun kinases (Rouyez et al., 1997; Fichelson et al., 1999; Avarham and Price, 1999). In megakaryocytes the effects of inhibiting the ERK1/2 pathway with the specific inhibitor PD98059 are profound. Even in the presence of TPO, megakaryocyte differentiation from HSCs is significantly reduced and the number of immature cells greatly increased following PD98059, indicating that the ERK1/2 pathway is involved in differentiation rather than lineage determination (Fichelson et al., 1999). However, Hong and co-workers have indicated that inhibition of PKC with the bisindolylmaleimide GF109203X promoted erythrocyte differentiation of HEL cells in the presence of PMA, indicating that another non-ERK1/2 PKC downstream signalling mechanism controls megakaryocyte lineage determination (Hong et al., 1996). The application of GF109203X to primary megakaryocytes has also been shown to significantly reduce the levels of TPO-induced nuclear maturation (Rojnuckarin et al., 1999). However the interaction of the ERK1/2 pathway and DNA synthesis in unknown.

The ERK1/2 pathway is also essentially involved in various aspects of neuronal cell function. The stimulation of neuronal NMDA receptors leads to the

calcium-dependent activation of ERK1/2 (Kurino et al., 1995; Xia et al., 1996), which is of great importance in LTP in hippocampal cells and LTD in cerebellar cells (for review see Sweatt, 2001). It is thought that ERK1/2 is able to modulate transient LTP by the regulation of voltage-gated potassium channels (Adams et al., 2000) and late LTP via the activation of transcription factors such as cAMP response element (CRE) binding protein (CREB; Huang et al., 2000). CREB is the best-characterised CRE binding protein, which on activation by the phosphorylation of the serine 133 residue, directly interacts with TFIIB, an essential constituent of the transcriptional machinery (Chrivia et al., 1993). CREB has also been implicated in the promotion of neuronal cell survival (Han and Holtzman, 2000), however, it appears that the PI3-K/AKT pathway may play a more important role in cell survival (Hetman et al., 2000). Interestingly, the CREB transcription factor has also been implicated in the lineage determination and possibly the differentiation of both megakaryocytic cell lines and human primary cells (Zauli et al., 1998). TPO application to HEL cells results in the rapid dose-dependent phosphorylation of CREB in HEL cells, which is not mimicked by the erythrocyte lineage stimulants erythropoietin and hemin. This suggests that CREB activity promotes megakaryocytopoiesis over erythrocytopoiesis and may even be involved in megakaryocyte lineage determination. By the use of pharmacological inhibitors of PKA, PKC and MAPK, it has also been demonstrated that CREB activation in megakaryocytes appears to be MAPK dependent. In human primary cells, high levels of phosphorylated CREB were identified in the nucleus following TPO application, which corresponded with increases in CD61 expression. These data provides significant evidence that CREB may regulate TPO-mediated gene expression, which determines entry to the megakaryocyte lineage from erythrocyte/megakaryocyte bi-potential cells.

Considering the profound effects mediated by ERK1/2 and CREB pathways in both neuronal and megakaryocytic cells, it becomes of great importance to determine whether the NMDA receptor has the ability to modulate these pathways in megakaryocytic cells.

Changes in intracellular calcium concentration are common in the majority of cell types. In neuronal cells, $[Ca^{2+}]_i$ is regulated by of various means, including: ligand-gated ion channels (such as the NMDA receptor), voltage gated ion channels, Ca^{2+}

pumps and exchangers as well as from intracellular stores via ryanodine and IP_3-sensitve channels. $[Ca^{2+}]_i$ increases stimulate the activation of a wide range of signalling events from localised areas, for example "calcium sparks" through to the modulation of gene transcription. An increase in intracellular calcium concentration is detected by calcium sensing proteins, of which the most common is calmodulin (CaM). The binding of Ca^{2+} to CaM to form a Ca^{2+}/CaM complex, results in the functional adaptation of numerous proteins, possibly the most important of which is the serine/threonine kinase CaM kinase (CaMK; for review see Soderling, 2000). CaMK remains in a non-active state by interacting with an autoinhibitory sequence via its catalytic domain. CaMK becomes active by Ca^{2+}/CaM binding adjacent to the autoinhibitory site, disrupting its interaction with the catalytic domain, which results in CaMK switching to its active conformation (Braun and Schulman, 1995). Autophosphorylation of active CaMK results in Ca^{2+} independent prolonged activation. Therefore a transient increase in $[Ca^{2+}]_i$ can lead to autonomous CaMK activity.

CaMKII is by far the most widespread member of the CaMK protein family. CaMKII is comprised of one or more of its 10-12 subunits, of which there are four isoforms, α, β, γ, and δ and it appears that subunit expression regulate CaMKII function in various tissue types (Brocke et al., 1999). Although the αCaMKII subunit isoform appears to be restricted to the CNS, the β, γ and δ isoforms are expressed in a wide range of tissues (Tombes and Crystal, 1997). The isoform present in any given cell is subject to which of the four genes encoding the protein is transcribed and which of a substantial number of alternatively spliced forms of each primary transcript is translated (Means, 2000). Although CaMKII signalling is not restricted to the CNS, it is of interest to note that tissues expressing both α and β CaMKII subunits also express glutamate signalling components. Interaction of CaMKII with the NMDA receptor in the CNS has been documented (Leonard et al., 1999). It has been hypothesised that the β CaMKII subunit binds to F-actin and on NMDA receptor activation, can translocate to the PSD (Shen and Meyer, 1999). Once in the PSD, CaMKII can act on signalling substrates located in the vicinity of the NMDA receptor. One such substrate is the novel Ras-GTPase-activating protein SynGAP, which is phosphorylated and potently inhibited by CaMKII, which leads to the potentiation of

the MAPK pathway (Chen et al., 1998). It is therefore probable that the CaMKII enzyme links the activated NMDA receptor to the MAPK pathway, via SynGAP and plays an essential role during LTP in neuronal cells. It is also believed that CaMKII can lead to the activation of CREB, although whether this activation is direct or via the ERK1/2 pathway remains elusive (Sheng et al., 1991; Mabuchi et al., 2001). However, Wu and McMurray have also suggested that CaMKII can inhibit the function of CREB in neuronal cells by phosphorylating the serine142 residue, which attenuates the mechanism of CREB once bound to DNA (Wu and McMurray, 2001).

Another tissue location where CaMKII activity appears to be of particular importance is in bone (G. J. Spencer, personal communication). Osteoblasts express the neurospecific CaMKII isoforms α and β and on application of glutamate and glycine significantly increase CaMKII activity, demonstrating glutamate receptor-mediated activation of CaMKII in osteoblasts. CaMKII inhibition with KN-62 resulted in a significant reduction in osteoblast differentiation, whilst mechanical loading of clonal osteoblastic cells, which stimulates osteogenesis, produced a rapid increase in CaMKII activity. These findings imply a significant role of CaMKII in osteoblastic differentiation and owing to the expression α and β CaMKII isoforms that are thought to interact with the NMDA receptor, can be triggered by NMDA receptor activation. The NMDA receptor-associated activity of CaMKII in bone leads to similar questions being posed about the signalling mechanisms of the megakaryocytic NMDA receptor.

No evidence exists as to whether CaMKII is expressed by megakaryocytes, probably due to the signalling system still being regarded as "neurospecific". Therefore the aims of this chapter were to determine whether megakaryocytes expressed CaMKII, to identify the association between CaMKII and NMDA receptor activity and determine the functional importance CaMKII in megakaryocyte differentiation. The effects of NMDA receptor activation on adhesion and downstream signalling pathways (ERK1/2 and CREB) were also determined in megakaryocytic cells to identify how ligand binding may be transformed into a cellular response.

5.2 Materials and Methods

5.2.1 Cell culture

MEG-01 and HEL cell lines were cultured as previously described (see chapter 2.1.2). PMA (100nM), TPO (25ng/ml), KN-93 (5μM, Tocris) and KN-92 (5μM, Tocris) treatments were performed for 72 hours unless otherwise stated. For experiments determining ionomycin-induced signal transduction, cells were preincubated with 20μl DMSO, KN-92 or KN-93 for 15 minutes prior to the application of 500nM ionomycin (Sigma) or DMSO. Following 5 minute incubations, media was removed and cells were washed twice with PBS prior to cell lysis. For experiments investigating the effects of NMDA and glycine treatment, MEG-01 cells were incubated with PMA for 72 hours, washed three times to remove exogenous glutamate and cultured overnight in glutamate-free Neurobasal™ media containing 1% N2 supplement (Gibco). Cells were then treated with various concentrations of NMDA and glycine dissolved in PBS, for 15 minutes before media was removed and the cells was twice in PBS prior to lysis.

5.2.2 Western blot analysis

5.2.2.1 Protein extraction

Megakaryoblastic cell lines were plated at an initial concentration of $1x10^5$ cells/ml and were exposed to treatments for a maximum of 72hrs. Cells were then removed from media and washed 3 times in PBS. Cells were lysed with cold kinase lysis buffer (New England Biolabs; 10x = 25 mM Tris-HCl (pH 7.5), 5 mM beta-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) for 5 minutes at 4°C. Lysates were centrifuged at 12,000g for 10 minutes to remove cell debris and protein concentration determined using a 96-well microplate version of a BCA protein assay kit (Pierce). 25μl of BSA standard at concentrations ranging from 25μg/ml to 2,000 μg/ml and unknown samples were mixed with 200μl of working reagent (v/v. 50:1, Reagent A: Reagent B), mixed thoroughly and incubated at 37°C for 30 minutes in a shaking incubator. The plate was allowed to cool to room temperature before absorbance was measured at 570nm using Dynex MRX microplate

reader. Unknown protein concentrations were calculated from the standard curve given by the known BSA protein concentrations. All samples were plated in triplicate and blanks wells, containing only lysis buffer and working reagent, were subtracted from the sample averages.

5.2.2.2 Polyacrylamide gel electrophoresis and protein transfer

10-20μg protein lysates were added to SDS loading buffer and denatured at 100°C for 5 minutes. Protein samples and molecular mass standards were loaded on a 5% stacking gel and separated using a 10% SDS-polyacrylamide resolving gel in running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH8) at 200V, 100mA until the dye-front ran to the bottom of the resolving gel. Gels were removed from the running plates, equilibrated in transfer buffer (25mM Tris, 193mM glycine, 20% methanol) for 10 minutes and separated proteins electroblotted onto PVDF membrane (Amersham Pharmacia Biotech.) at 100V, 250-350mA for 1hr. Membranes were washed twice for 5 minutes in TBS-T (20mM Tris, 137mM NaCl, 0.1% Tween20, pH 7.6) with gentle agitation before immunodetection.

5.2.2.3 Immunodetection

Membranes were incubated in blocking buffer (4% (w/v) skimmed milk powder or 5% (w/v) BSA in TBS-T, according to manufacturer's recommendation) for either 15 minutes at room temperature with gentle agitation and 4°C overnight, or for 1hr at room temperature with gentle agitation. Membranes were then rinsed twice and washed for 15 minutes before incubating with primary antibodies diluted in blocking buffer at the following concentrations; rabbit anti-ERK1/2, rabbit anti-phosphoERK1/2, mouse anti-CREB, mouse anti-phosphoCREB (all 1:1000, New England Biolabs), CaMKII and phospho-CaMKII (both 1:2000, Santa Cruz Biotechnologies) and GAPDH (1:800, Immunodetection Laboratories), and incubated for 1hr at room temperature or overnight at 4°C with gentle agitation. The membranes were then rinsed twice quickly, once for 15 minutes and twice for 5 minutes in TBS-T to remove excess antibody. Peroxidase-conjugated secondary antibodies were diluted in blocking buffer (1:2000, Santa Cruz Biotechnologies) and incubated with the membrane for 1hr at room temperature. Unbound antibody was removed by 2 quick rinses, one 15-minute wash and four 5 minutes washes and the membrane developed

by incubation in ECL chemiluminescent solution (Amersham Pharmacia Biotech) for 1 minute. Membranes were exposed to Hyperfilm (Amersham Pharmacia Biotech) at room temperature, initially for 5 minutes before adjusting exposure time according to clarity of positive results. Hyperfilm was developed using a X-ograph automated developer. Membranes were kept at 4°C in TBS-T until stripped (see below) and re-probed. All washes were performed at room temperature with gentle agitation.

5.2.2.4 Membrane stripping

Bound antibodies were removed from the membrane by incubation with pre-warmed stripping buffer (62.5mM Tris, pH6.8, 0.7% β-mercaptoethanol, 2% SDS) at 60°C for 30 minutes with vigorous agitation. Membranes were then washed twice for 10 minutes in TBS-T before immunodetection was performed on the stripped blots.

5.2.3 DNA profiling of cell cycle by flow cytometry

Cells were grown in the absence and presence of PMA, KN-92 and KN-93 for 72 hours. Cells were then washed three times in PBS, fixed in 70% ethanol for 5 minutes, washed twice in PBS and DNA stained as previously described (chapter 4.2.9). To determine the cell cycle phase, cells were analysed by flow cytometry and positive readings taken on the FL2 setting with the first peak being G0/G1 phase, second peak G2/M phase and the trough in between the peaks being S phase.

5.2.4 Bone marrow megakaryocyte NR1 expression by c-Mpl$^{-/-}$ knockout mice

Frozen tibiae sections from c-Mpl$^{-/-}$ and c-Mpl0$^{+/+}$ mice were a kind gift from Dr. M. Perry, University of Bristol, UK. To avoid non-specific binding and high background caused by using the monoclonal mouse anti-NR1 antibody on mouse tissue, the mouse on mouse (MOM™) staining kit was used (Vector Laboratories) according to the manufacturer's protocol. Prior to application of primary antibody, sections were incubated for 1 hour in M.O.M™ mouse Ig blocking reagent (2 drops/2.5ml PBS), washed three times in PBS and incubated for 5 minutes is M.O.M™ diluent. Excess diluent was removed and subsequent antibody incubations: mouse anti-NR1 (30 minutes: 1:400, Pharmingen) and biotinylated anti-mouse secondary (20 minutes:

1:200, Vector Laboratories), were performed in M.O.M™ diluent. All other steps were performed as previously described (see chapter 2.2.2).

5.3 Results

5.3.1 NMDA receptor-mediated modulation of ERK1/2 activation in megakaryocytic cells

Changes in NMDA receptor-mediated ERK1/2 activation in the megakaryoblastic MEG-01 and HEL cells were determined by western blot analysis of cell protein lysates using a phospho-ERK1/2 monoclonal antibody. ERK1/2 activation, stimulated by PMA (100nM), was reduced dose dependently by increasing concentrations of the NMDA receptor antagonist MK-801 (Fig. 5.3.1). Decreased levels of ERK1/2 phosphorylation were determined by comparing to levels of total ERK1/2.

MEG-01 cells were treated with increasing concentrations of NMDA and glycine in order to clarify the effect of agonist-mediated NMDA receptor activation on ERK1/2 phosphorylation. Addition of 10μM NMDA / 1μM glycine resulted in a small increase in ERK1/2 activation compared to cells treated with PMA only. However, cells treated with 100μM NMDA / 10μM glycine and 1mM NMDA / 100μM glycine resulted in significant decrease in levels of ERK1/2 phosphorylation. Levels of total ERK1/2 demonstrated equal loading (Fig. 5.3.2).

5.3.2 NMDA receptor-mediated activation of CaMKII in megakaryocytic cells

Western blot analysis of MEG-01 whole cell lysates identified expression of the 50kD α isoform as well as the slightly larger β isoform of CaMKII (Fig. 5.3.3). PMA treatment of MEG-01 cells did not appear to increase levels of CaMKII phosphorylation. However increasing concentrations of MK-801 seemed to decrease the phosphorylation of both isoforms. Due to high background levels, it is not possible to accurately determine levels of activated CaMKII.

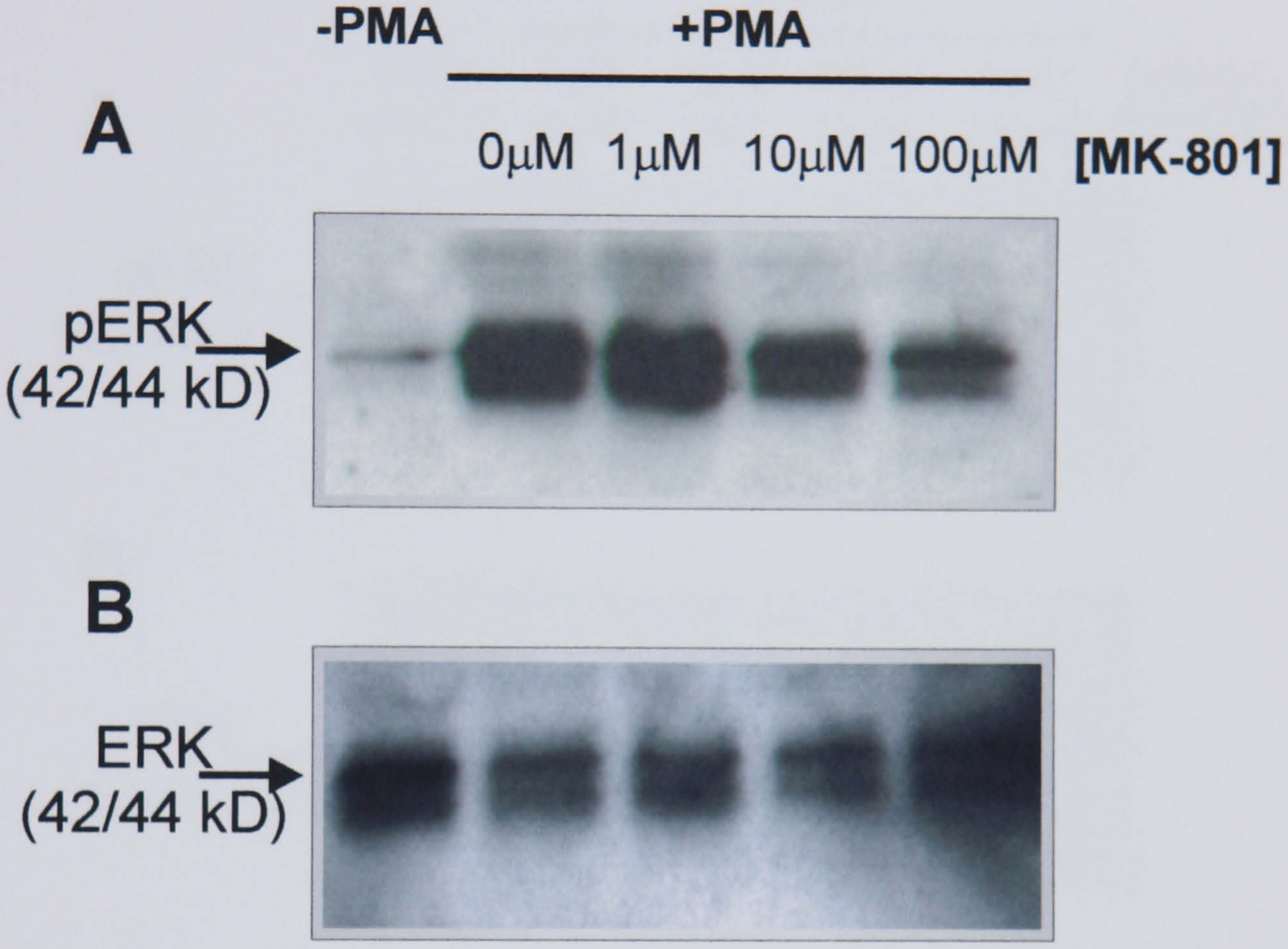


Figure 5.3.1. Western blot analysis of NMDA receptor inhibition-mediated expression of phosphorylated ERK by MEG-01 cells. PMA-mediated phosphorylation of ERK (pERK, A) was significantly reduced by treating cells with increasing concentrations of MK-801. Treatment of MEG-01 cells with 100μM MK-801 reduced levels of pERK by over a half compared to PMA-only treated controls. Equal loading was demonstrated by total levels of unphosphorylated ERK (ERK, B). Results are representative of three independent experiments.

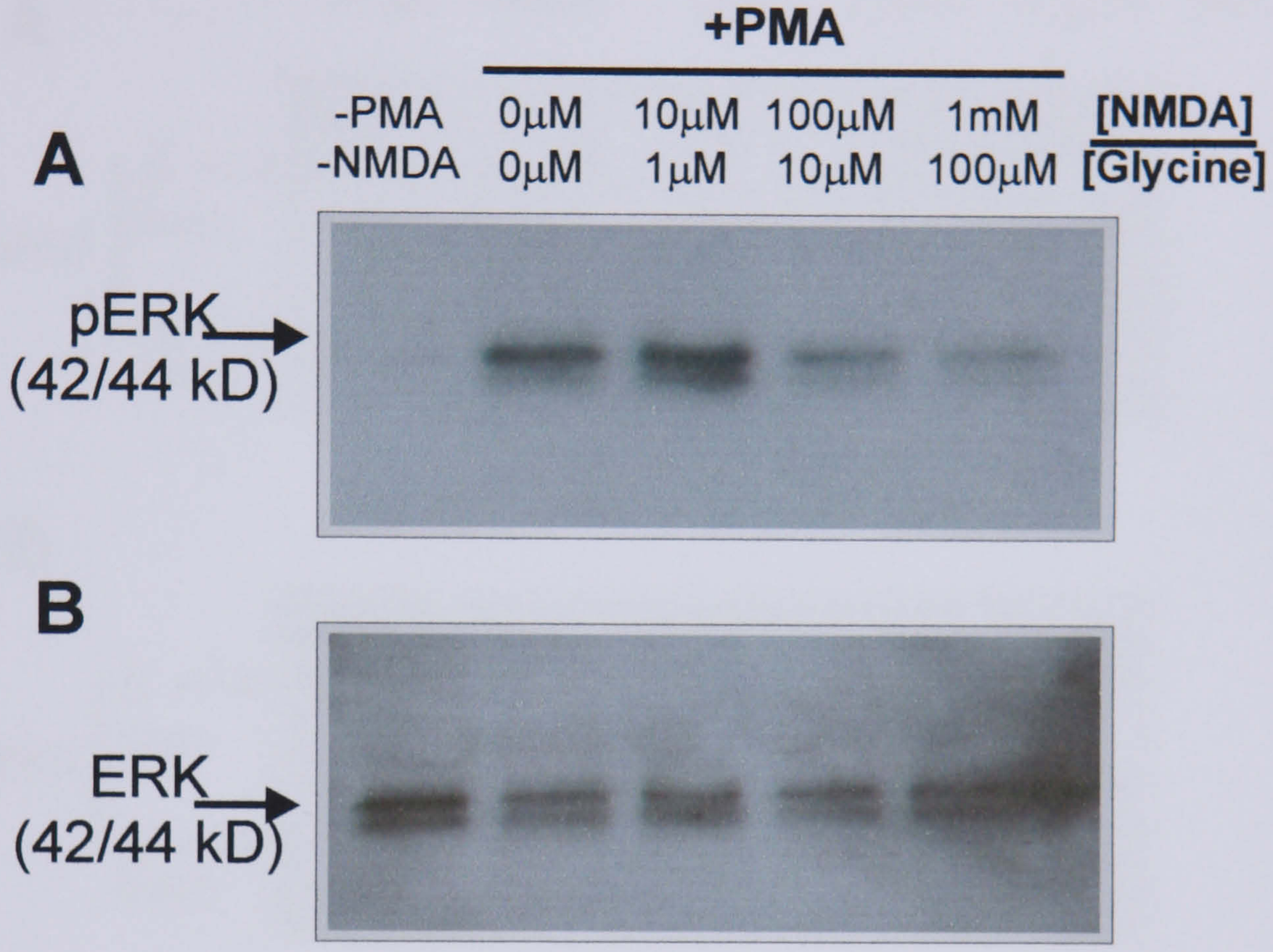


Figure 5.3.2 Western blot analysis of NMDA receptor activation-mediated expression of phosphorylated ERK by MEG-01 cells. PMA-mediated phosphorylation of ERK (pERK, A) was altered in glutamate starved MEG-01cells by treating cells by increasing concentrations of NMDA and glycine. 10μM NMDA with 1μM glycine slightly increased expression of pERK, whilst treatment of MEG-01 cells with 100μM NMDA / 10μM glycine and 1mM NMDA / 100μM glycine dramatically reduced pERK expression levels. Equal loading was demonstrated by total levels of unphosphorylated ERK (ERK, B). Results are representative of two independent experiments.

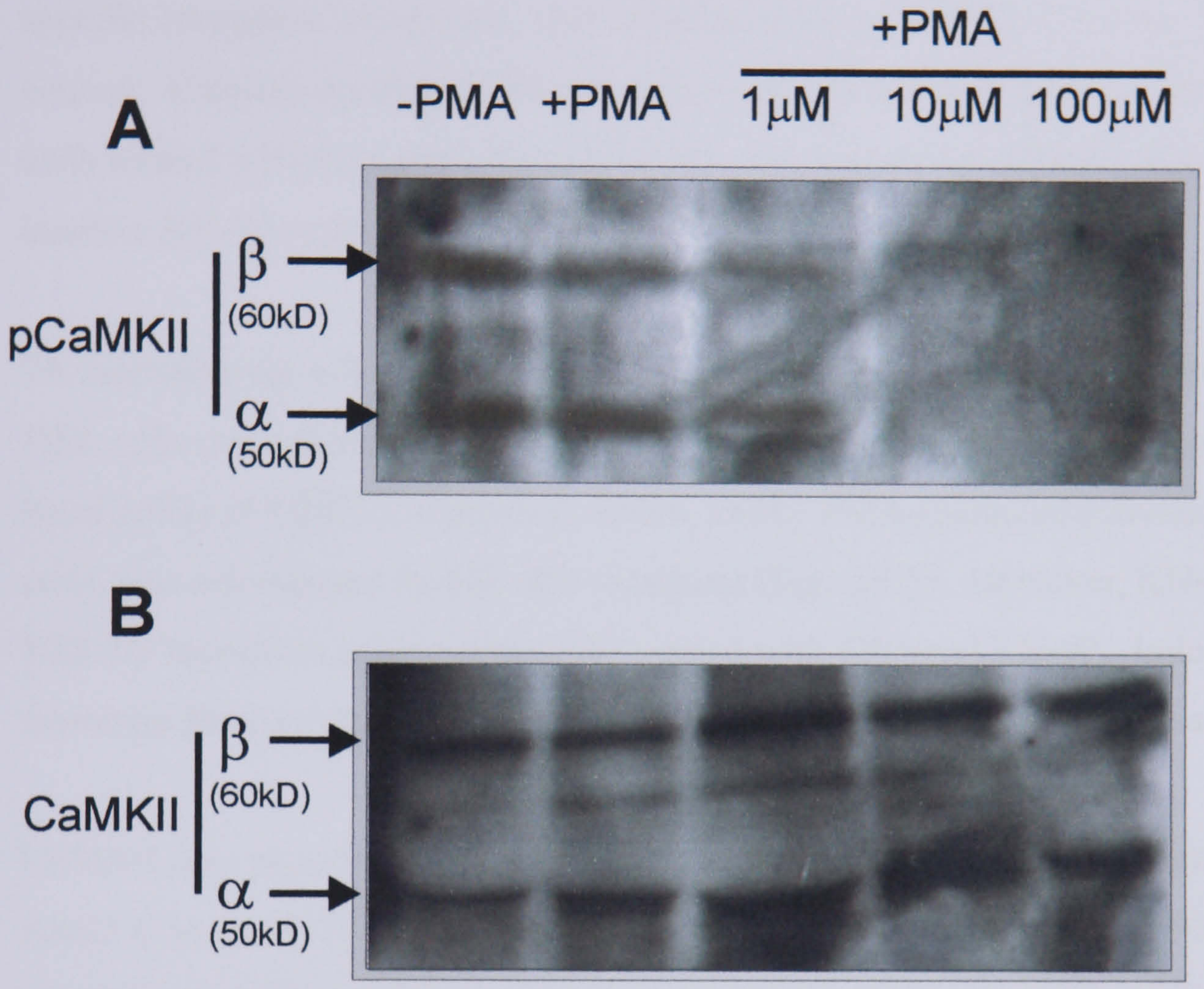


Figure 5.3.3. Western blot analysis of NMDA receptor-mediated activation of CaMKII in MEG-01 cells. Levels of CaMKII activation in MEG-01 cells with and without PMA and MK-801 were analysed by western blotting using an antibody specific to phosphorylated CaMKII isoforms (A). Both α and β isoforms were identified whilst 10 and 100μM MK-801 appeared to reduce levels of CaMKII phosphorylation (pCaMKII) compared to total CaMKII (B). Results are representative of two independent experiments.

5.3.3 CaMKII signalling in megakaryocytes

The identification of megakaryocytic CaMKII expression initiated subsequent investigation of CaMKII-mediated signalling and subsequent effects of CaMKII inhibition on megakaryocyte differentiation. Treating MEG-01 cells with the calcium specific ionophore ionomycin, lead to the activation of ERK1/2 compared to DMSO control. Calcium-mediated ERK1/2 activation was however significantly reduced in cells treated with the CaMKII inhibitor KN-93 compared to DMSO control and the inactive KN-93 isoform, KN-92 (Fig. 5.3.4).

To determine the effect of CaMKII inhibition on TPO-mediated activation of ERK1/2, HEL cells were used as they express the TPO receptor, c-Mpl. TPO greatly increased basal levels of ERK1/2 activation, which, unlike PMA-mediated activation in MEG-01 cells, was not reduced by MK-801 treatment (Fig. 5.3.5). However, KN-93 did reduce ERK1/2 activation compared to cells treated with TPO and KN-92. Indeed, KN-93 treatment lowered expression of phospho-ERK1/2 to approximately basal levels.

CaMKII also plays a significant role in the cell cycle, possibly by the activation of cdc25-C at the G2/M phase of the cell cycle (Patel et al., 1999). To investigate a possible role of CaMKII in megakaryocytic G2/M phase transition, HEL cells were treated with PMA in conjunction with either KN-92 or KN-93 and the cellular DNA content analysed following 72 hours of culture. PMA treatment leads to a small increase in the percentage of cells in the G2/M phase, whilst decreasing the number of cells in G1 and S phases (Fig. 5.3.6). KN-93 treatment in conjunction with PMA caused an increase in the percentage of cells found with a G2/M DNA content, from approximately 19% to 41%. Whilst performing DNA profiling experiments, it was also observed that KN-93 severely inhibited HEL cell adhesion. PMA-mediated increases in cellular adhesion were totally prevented by treatment with KN-93, whilst KN-92 had no clear effect (Fig.5.3.7).

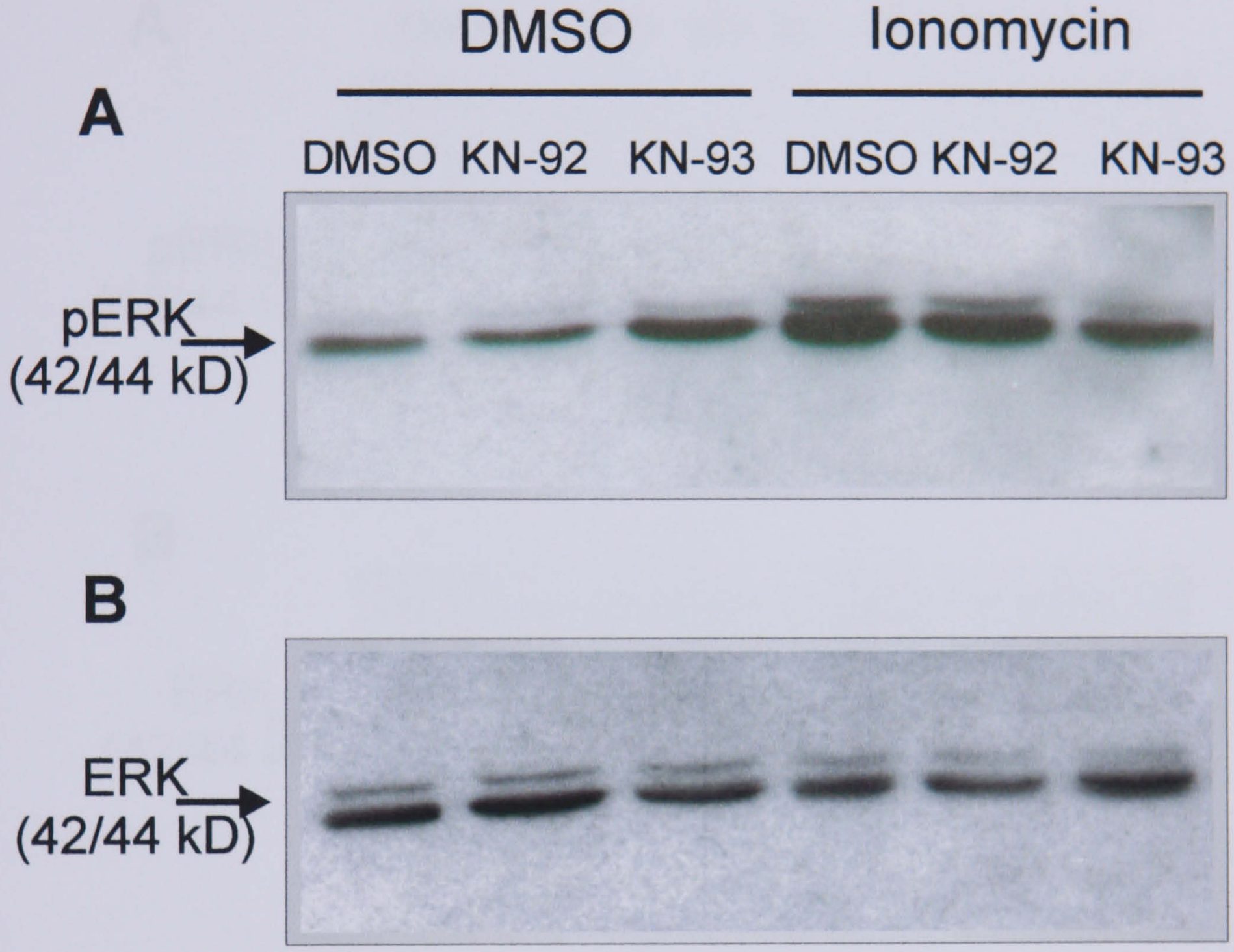


Figure 5.3.4 Western blot analysis of CaMKII-mediated ERK phosphorylation in MEG-01 cells. Ionomycin-mediated activation of ERK in the presence of the specific CaMKII antagonist KN-93 (5μM). KN-93 significantly lowered the levels of ERK phosphorylation compared to the inactive control KN-92 (A). Equal loading was demonstrated by total levels of unphosphorylated ERK (B). Results are representative of three independent experiments.

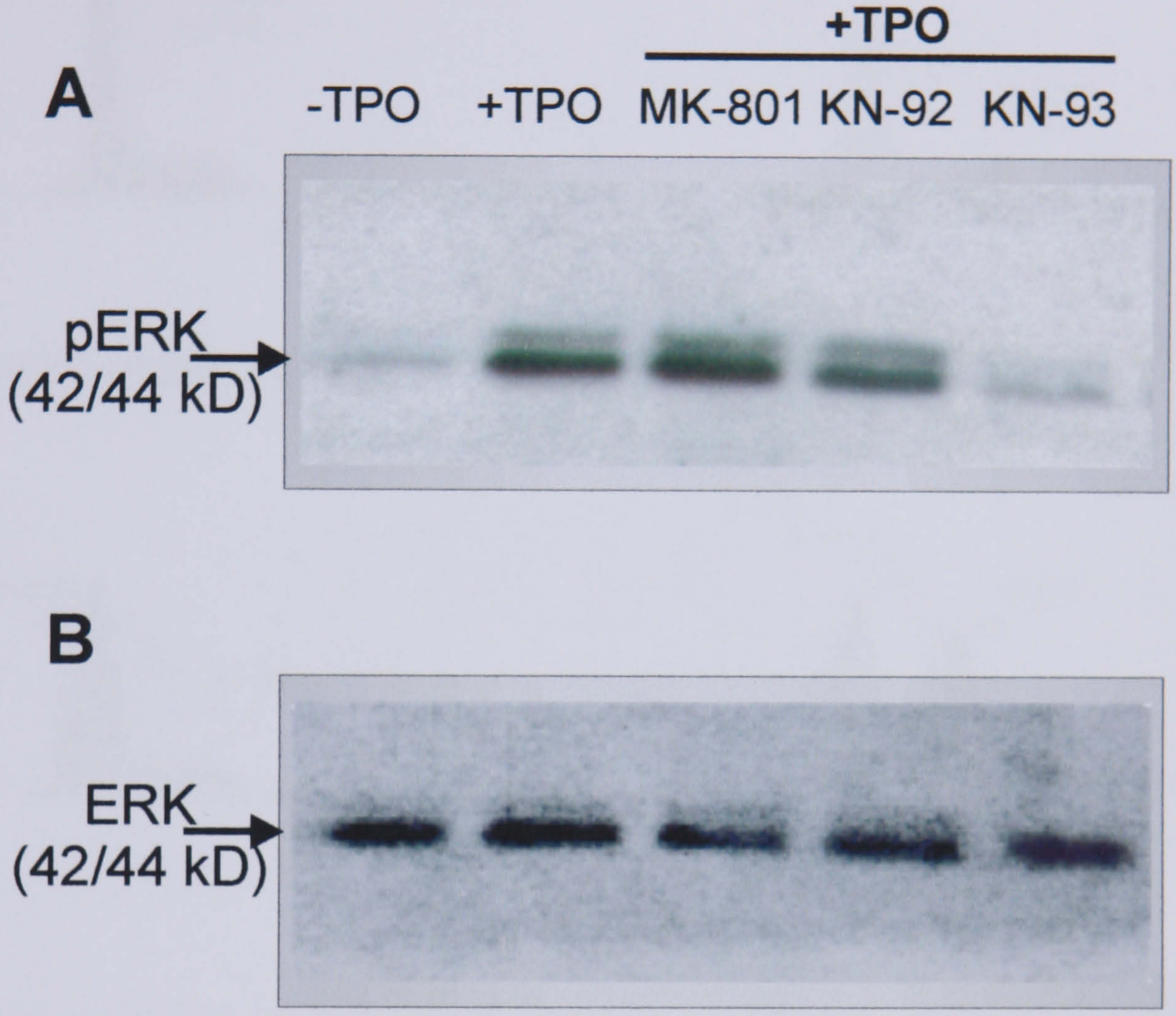
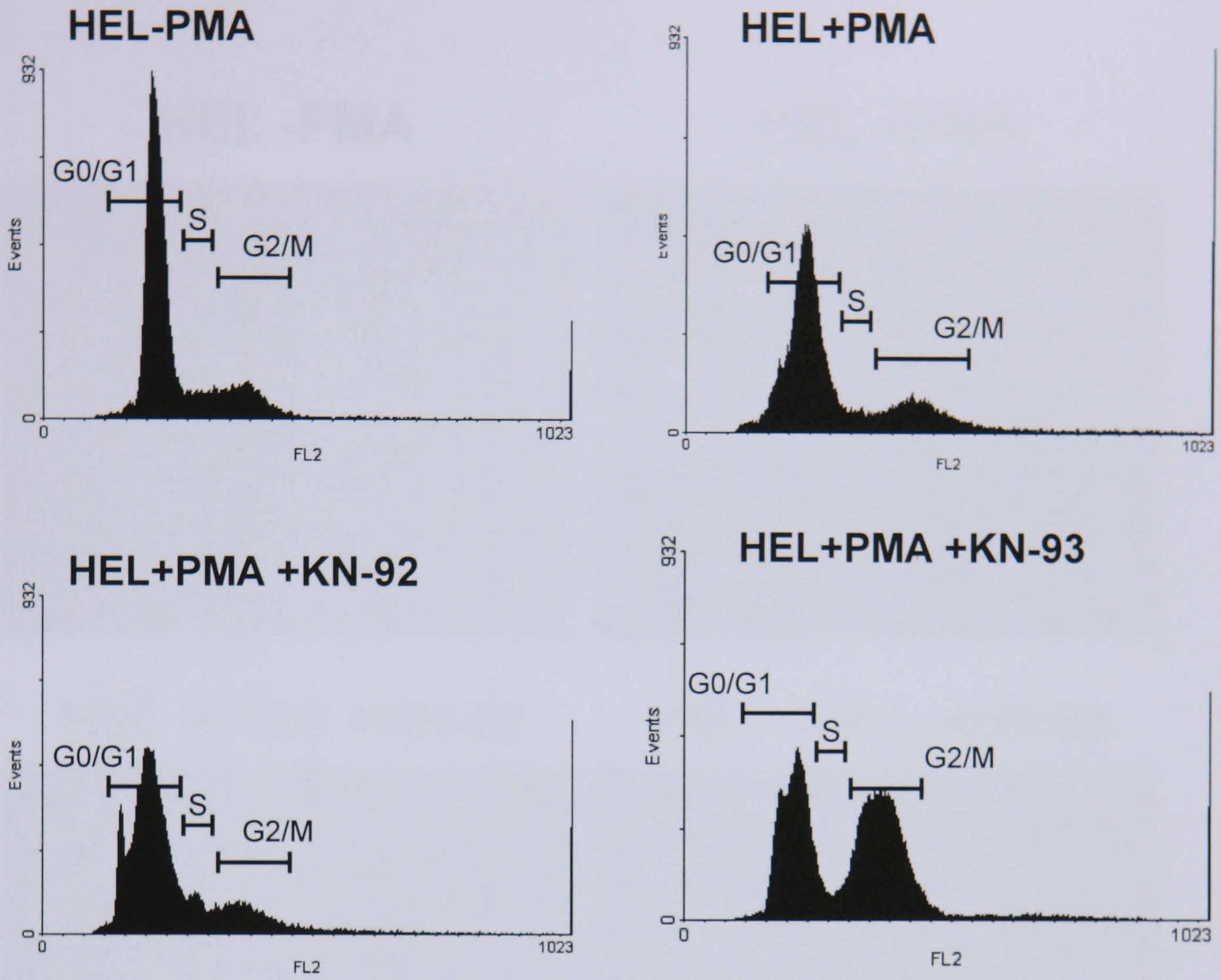


Figure 5.3.5 Western blot analysis of NMDA receptor and CaMKII-mediated ERK phosphorylation in HEL cells. TPO-mediated expression of phosphorylated ERK was dramatically inhibited by the specific CaMKII antagonist KN-93 (5μM) compared to KN-92 (5μM) control (A). MK-801 (50μM) made no significant difference to ERK phosphorylation. Equal loading was demonstrated by total levels of unphosphorylated ERK (B). Results are representative of three independent experiments.



	Cell cycle phase		
Treatment	G0/G1	S	G2/M
-PMA	73.4%	10.9%	12.7%
+PMA	60.9%	7.28%	19.6%
+PMA +KN-92	65.0%	12.9%	8.88%
+PMA +KN-93	42.9%	11.8%	41.8%

Figure 5.3.6 Effect of CaMKII inhibition on HEL cell cycle. Flow cytometry DNA profiling was used for the investigation of PMA-mediated changes in the percentage of HEL cells in different stages of the cell cycle following addition of KN-93. KN-93 dramatically increased the percentage of cells in the G2/M phase of the cell cycle, whilst decreasing the percentage of cells in the G0/G1 phase. These affects were not observed in KN-92-treated cells, although KN-92 did appear to lower the number of cells in the G2/M phase whilst increasing the number in the S phase. Results are representative of three independent experiments.

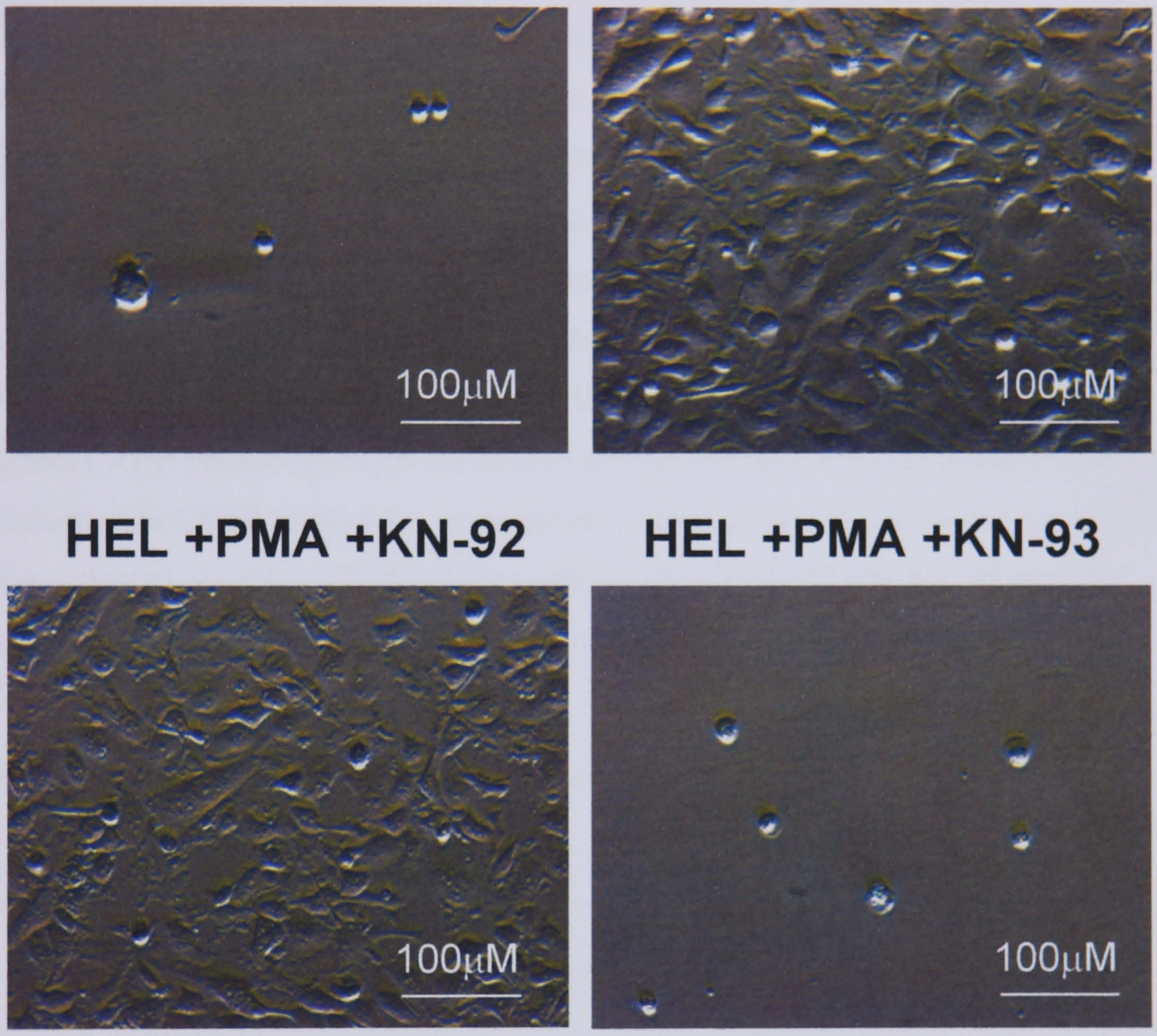


Figure 5.3.7 Effect of CaMKII inhibition on HEL cell adhesion. PMA-induced increases in HEL cell adhesion was dramatically reduced following inhibition of CaMKII with KN-93 compared to cells treated with PMA only and KN-92.

5.3.4 NMDA receptor-mediated modulation of CREB activity in megakaryocytic cells

TPO-mediated activation of CREB transcription factor has recently been identified as being important in the transcription of genes with various roles in megakaryocytic differentiation (Zauli et al., 1998). The NMDA receptor has also been identified as a potent activator of CREB in neuronal cells (Mao and Wang et al., 2002), leading to the investigation of NMDA receptor-mediated activation in MEG-01 and HEL cells. Here it was demonstrated that TPO increased the expression levels of phosphorylated CREB in HEL cells (Fig. 5.3.8). The NMDA receptor antagonist MK-801 failed to affect CREB phosphorylation whilst the CaMKII inhibitor KN-93 caused a significant reduction in CREB activation. Interestingly, although MEG-01 cells express CREB, the activated phospho-CREB isoform was not identified and was not affected by PMA-stimulation (Fig. 5.3.9).

5.3.5 Expression of NR1 by c-Mpl$^{-/-}$ bone marrow megakaryocytes

Mice lacking the gene for the TPO receptor c-Mpl display aberrant megakaryocytopoiesis (Gurney et al., 1994; Alexander et al., 1996). Mice lacking normal TPO-mediated megakaryocyte signalling also display greatly reduced levels of other proteins that are involved in the regulation of megakaryocyte differentiation and proliferation. With this in mind, immunohistochemical analysis of NMDA receptor expression by c-Mpl$^{-/-}$ bone marrow megakaryocytes was determined. Megakaryocyte number was greatly reduced in c-Mpl$^{-/-}$ bone marrow, however all remaining megakaryocytes expressed NR1 at a similar level as wild type (c-Mpl $^{+/+}$) controls. This indicates that the NMDA receptor is expressed in megakaryocytes in the absence of TPO-mediated signalling (Fig. 5.3.10).

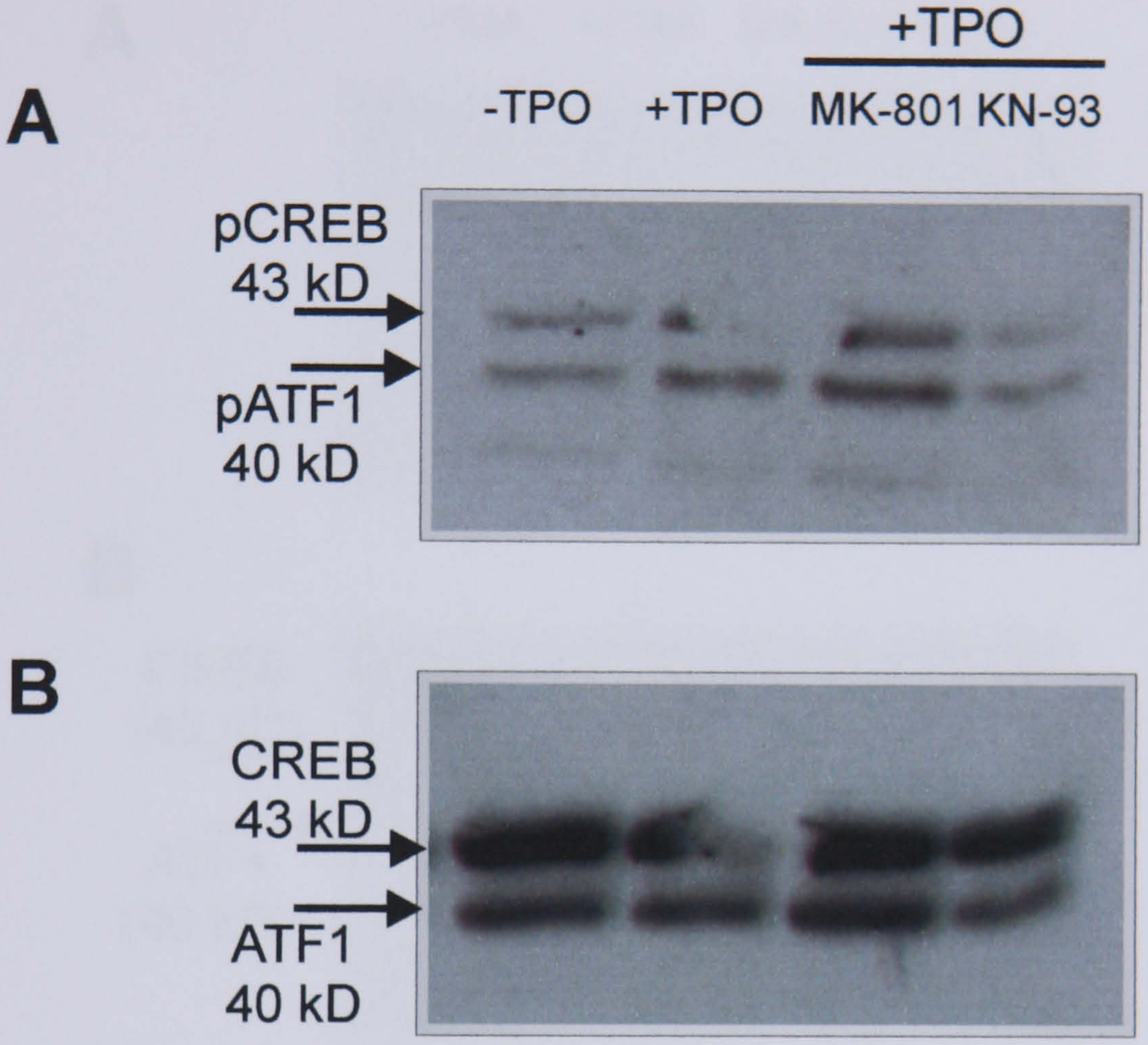


Figure 5.3.8 Western blot analysis of NMDA receptor and CaMKII-mediated CREB phosphorylation in HEL cells. TPO-mediated phosphorylation of CREB (pCREB) and associated transcription factor 1 (pATF1) was reduced to basal levels by KN-93 (5μM) treatment, but remained unaltered by treatment with MK-801 (50μM, A). Equal loading was demonstrated by total levels of unphosphorylated CREB and ATF (B). Results are representative of two independent experiments.

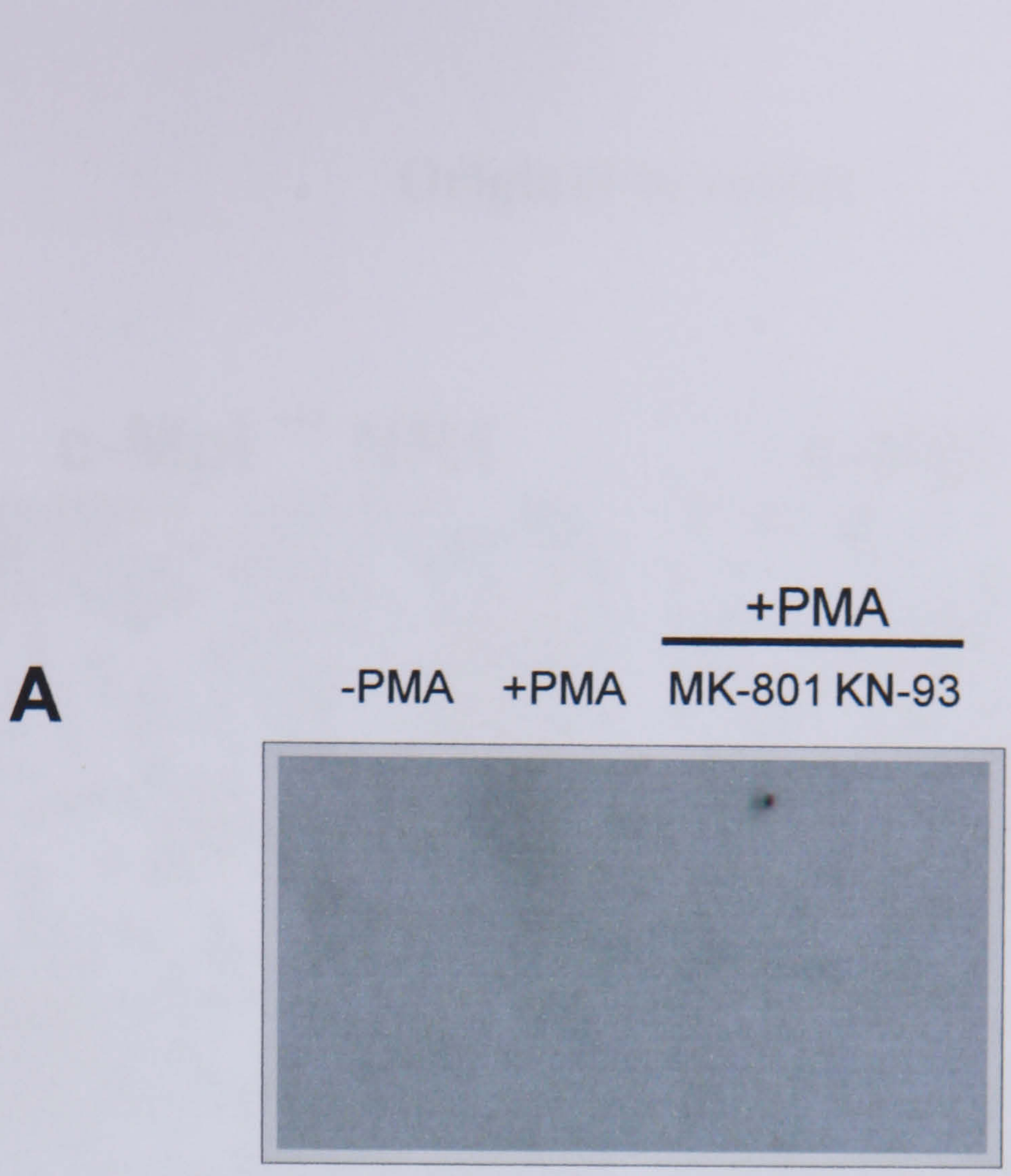


Figure 5.3.9 Western blot analysis of NMDA receptor and CaMKII-mediated CREB phosphorylation in MEG-01 cells. PMA-mediated expression of phosphorylated CREB and ATF1was not identified in MEG-01 cells (A). Total CREB and ATF1were however identified, confirming megakaryocytic expression of the transcription factors (B). Results are representative of two independent experiments.

Original in colour

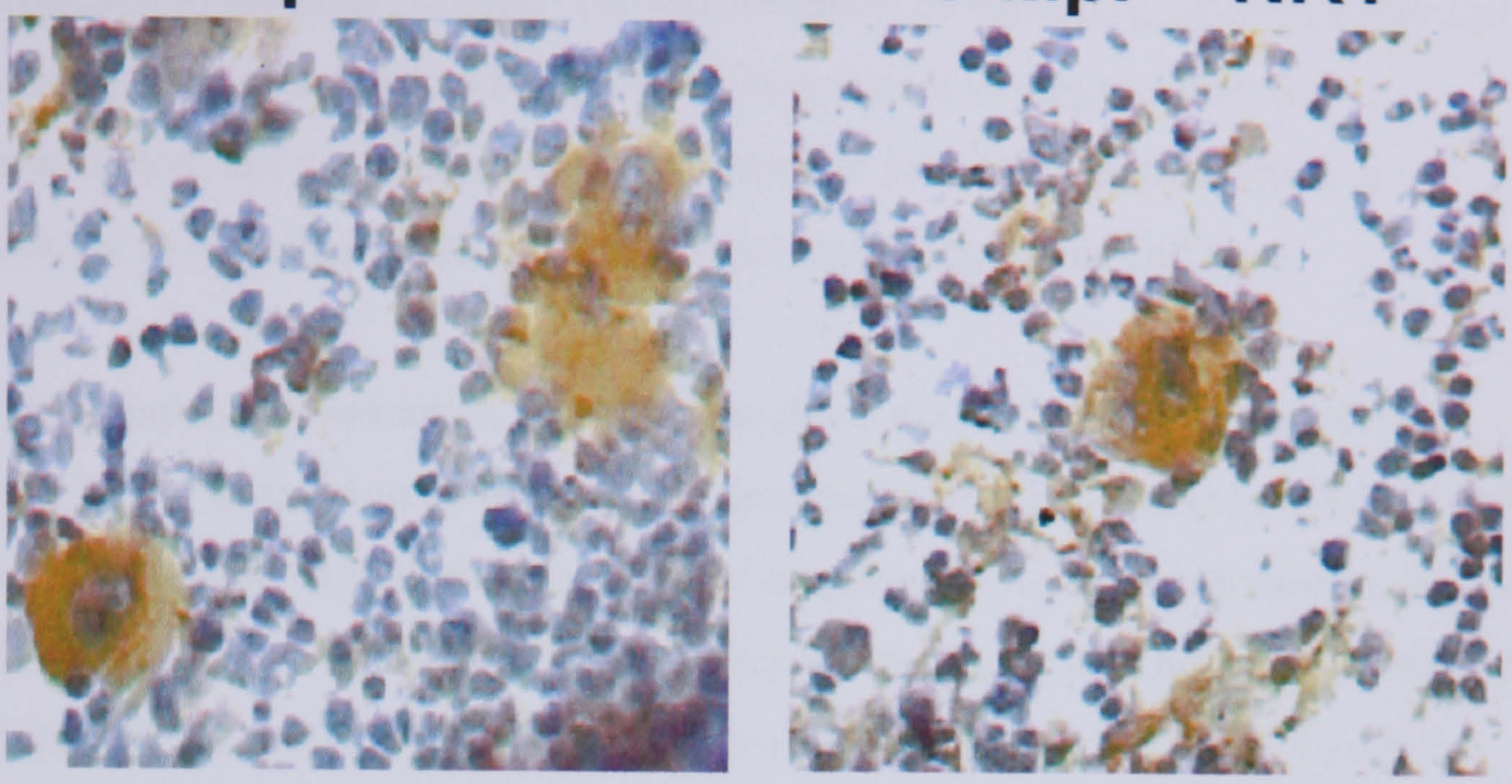


Figure 5.3.10 Immunohistochemical analysis of NR1 expression by c-Mpl$^{-/-}$ mouse bone marrow megakaryocytes. NR1 expression by bone marrow megakaryocytes was compared in c-Mpl wildtype (c-Mpl $^{+/+}$) and knockout (c-Mpl$^{-/-}$) mice. NR1 was maintained in megakaryocytes from knockout mice, compared to wildtypes. Antibody controls (Ab -ve) low background levels. Results are representative of three independent experiments.

5.4 Discussion

This chapter provides the first evidence of NMDA receptor-mediated signal transduction in megakaryocytes. The confirmation of signalling pathways that have previously been identified as being of key importance in megakaryocyte differentiation, are modulated by megakaryocytic NMDA receptors which is significant to our understanding of glutamate signalling in this cell type.

The dose-dependent reduction in PMA-stimulated ERK1/2 phosphorylation (see section 3.1 for discussion on the possible drawbacks of using PMA) in MEG-01 cells by MK-801 would explain the inhibition of megakaryocyte differentiation as described by Genever and co-workers (1999) and observed in chapter 4. The fact that MK-801 inhibits ERK1/2 activation in the presence of PKC activating agent PMA could indicate that the NMDA receptor is either downstream of PKC, possibly involved in the regulation of Raf. It is also possible that the activated NMDA receptor provides the "calcium sparks" required for the translocation of PKC isoforms with Ca^{2+} specificity domains, such as PKCα, to the cell membrane. The latter hypothesis is further supported by the fact that ERK1/2 phosphorylation, although significantly decreased, was not abolished by MK-801 application. This implies that the NMDA receptor does not have absolute control of ERK1/2 activation and PKC retains the ability to activate Raf, possibly by binding with other sources of Ca^{2+} enabling PKC to activate the MAPK pathway. It would be advantageous to confirm the interaction of the megakaryocytic NMDA receptor with PKC, by analysing changes in PKC regulation in the presence of MK-801 and/or NMDA and glycine (Goldfarb et al., 2001), and also determining whether inhibition of NMDA receptor can alter the translocation of PKC from the cytoplasm to the plasma membrane (Hong et al., 1998; Racke et al., 2001).

The effect of NMDA and glycine application on the levels of phospho-ERK1/2 was extremely intriguing. The small increase in phospho-ERK1/2 in response to low concentrations of NMDA and glycine are encouraging, implying that the activation of the NMDA receptor does increase ERK1/2 activation in the presence of PMA. The relatively low levels of NMDA and glycine-stimulated ERK1/2 phosphorylation may

be due to endogenous glutamate released from MEG-01 cells as described in chapter 4 and consequently, these cells were not starved of glutamate, which may mask agonist responses. The effect evoked by high concentrations of NMDA and glycine may demonstrate bi-directional control of ERK1/2 activation by the megakaryocytic NMDA receptor, as observed in neuronal cells (Chandler et al., 2001). In primary cortical neuronal cells the addition of 100μM NMDA reduced the basal levels of active ERK1/2, with 50μM NMDA identified as the optimal concentration for NMDA receptor-mediated ERK1/2 activation. This work perfectly demonstrates the complexity of NMDA receptor interactions with ERK1/2 in the CNS. It appears that the receptor is able to both activate and inhibit the MAPK pathway, depending on the concentration of extracellular glutamate, which may have implications in cell signalling pathways regulating cell survival following pathological extracellular glutamate concentrations as a result of neuronal injury. The NMDA receptor pathway involved in inhibiting ERK1/2 remains unknown. It may therefore be possible that the megakaryocytic NMDA receptor has the ability to act in a similar fashion and is able to modulate rather than just activate the ERK1/2 pathway. It seems unlikely however, that concentrations high enough to inhibit the neuronal ERK1/2 activation mediated by the NMDA receptor exists in the bone marrow and the concentration of glutamate released from MEG-01 cells would only be enough to activate the receptor.

The work by Chandler et al. (2001) also raises interesting points regarding how the neuronal NMDA receptor activates ERK1/2. They demonstrated that the NMDA-induced ERK1/2 activation still occurs in the presence of the PKC inhibitor GF109203X, indicating that activation could take place independently of PKC. They also showed that CaMKII and PI3-K antagonists do inhibit NMDA-mediated ERK1/2 activation, suggesting that these pathways are involved in MAPK activation in the CNS. The modulation of the MAPK pathway by the megakaryocytic NMDA receptor requires greater definition. If it were the case that the receptor does mediate bi-directional regulation of the MAPK pathway, it could provide a unique level of control over megakaryocyte differentiation.

The expression of the CaMKII α and β isoforms by MEG-01 cells further increases the complexity of the megakaryocytic NMDA receptor and provides an alternative

means by which the MAPK pathway can be activated, independent of PKC. This finding also allows comparisons to be drawn between the receptor found in the CNS to that expressed by the megakaryocyte. Whether the NMDA receptor directly activates CaMKII, despite MK-801 possibly lowering the levels of activated CaMKII, is unclear. This can be confirmed by performing a radioactive kinase assay, to determine the activity of CaMKII following application of NMDA and MK-801 to megakaryocytic cells. The function of CaMKII in megakaryocytes is clearer. Ca^{2+} induced ERK1/2 activation, as demonstrated by ionomycin application, is reduced by the CaMKII inhibition. This demonstrated the regulative ability of Ca^{2+} in megakaryocytic cells, in addition to providing evidence indicating that signalling mechanisms influenced by changes in $[Ca^{2+}]_i$ can be mediated by CaMKII. Interestingly, TPO-induced activation of ERK1/2 in HEL cells was profoundly inhibited by CaMKII inhibition, reducing ERK1/2 activity to approximately basal levels. The fact that MK-801 appeared to have no effect on TPO-mediated ERK1/2 activation in these cells, suggests that TPO, unlike PMA treatment in MEG-01 cells, has the ability to activate ERK1/2 signalling independently of PKC, probably via adaptor proteins as described in chapter 1.3.1. The dramatic inhibition of the MAPK pathway caused by KN-93 suggests that the target of CaMKII in megakaryocytic cells is downstream to that of TPO signalling events. One possible target of CaMKII, as in neuronal cells, is SynGAP. The phosphorylation of SynGAP by CaMKII relieves its inhibitory restraints on Ras, therefore inhibition of CaMKII function could prevent TPO-mediated Ras activation. Whether megakaryocytes express SynGAP requires investigation. However, it has recently been shown that osteoblasts do express SynGAP, demonstrating for the first time that this molecule does exist outside the CNS (G.J. Spencer, personal communication). It is also possible that megakaryocytic CaMKII can interact with other megakaryocytic GAPs, thereby having a profound effect of MAPK activation.

The cellular effects of CaMKII inhibition in megakaryocytic cells included interruption of the cell cycle and reducing PMA-induced adhesion. KN-93 induced a cell cycle block at G2 phase, an effect previously reported to also occur in HeLa cells (Patel et al., 1999). Patel and colleagues demonstrate that the changes in the cell cycle were caused by CaMKII-mediated phosphorylation and activation of the tyrosine phosphatase cdc25, which in turn dephosphorylates the protein kinase cdc2, which has

been identified as the key mediator of G_2/M cell cycle phase transition (reviewed in Morgan, 1995). Therefore, by inhibiting the function of CaMKII, the cdc25-mediated dephosphorylation of cdc2 does not occur and phase transition is prevented. This may be of central importance in the endomitotic cell cycle of megakaryocytes, in which highly polyploid cells have been identified as having lowered levels of activated cdc2 (Zhang et al., 1998; Matsumura et al., 2000). The data in this chapter therefore suggests a role for CaMKII in megakaryocytic endomitosis. This role could be one of applying a break to endomitosis and ensuring that normal mitosis progresses during megakaryocytic proliferation. It has now been established that megakaryocytic endomitosis is of fundamental importance in the production of normal platelets (for review see chapter 1.2.2). Therefore, the potential role of CaMKII in the cell cycle of megakaryocytes may also be of great importance in thrombocytopoiesis.

The reduction in megakaryocytic cell adhesion caused by CaMKII inhibition raises other possible functions of this enzyme in these cells. CaMKII has previously been identified as a modulator of $\alpha5\beta1$ integrin adhesion to FN in addition to integrin-mediated inside-out signalling (Bouvard et al., 1998). Interestingly, these investigators showed, by using Chinese hamster ovary (CHO) cells transfected with the α and β isoforms of CaMKII, that CaMKII inhibition maintained the high affinity state of $\alpha5\beta1$ integrin, whilst addition of CaMKII to cell lysates inhibited binding to fibronectin *in vitro*. They also demonstrated that CaMKII did not affect integrin expression, implying that integrin activity was the target of CaMKII in these cells. With regards to the effects of CaMKII inhibition observed in this chapter, the findings of Bouvard and co-workers are intriguing, as they appear to be completely opposite to those found in megakaryocytes. Presently, the effect of CaMKII inhibition on megakaryocyte adhesion remains an interesting observation and requires further analysis before comparisons to the work by Bouvard and colleagues can be accurately drawn (Bouvard et al., 1998). It is also important to recognise the possible limitations of KN-93 as a CaMKII antagonist. KN-93 is a quinolonesulfonamide is thought to act as a CaMKII-specific ATP antagonist (Tokumitsu et al., 1990), although subsequent reports have suggested doubts regarding the specificity of this antagonist (Means, 2000). This work indicates that KN-93 also acts as an antagonist to other members of the CaMK family of enzymes, namely CaMKI and CaMKIV. It is therefore important

to take into consideration that if KN-93 has any cellular effects on megakaryocytes, they may not necessarily be CaMKII-specific.

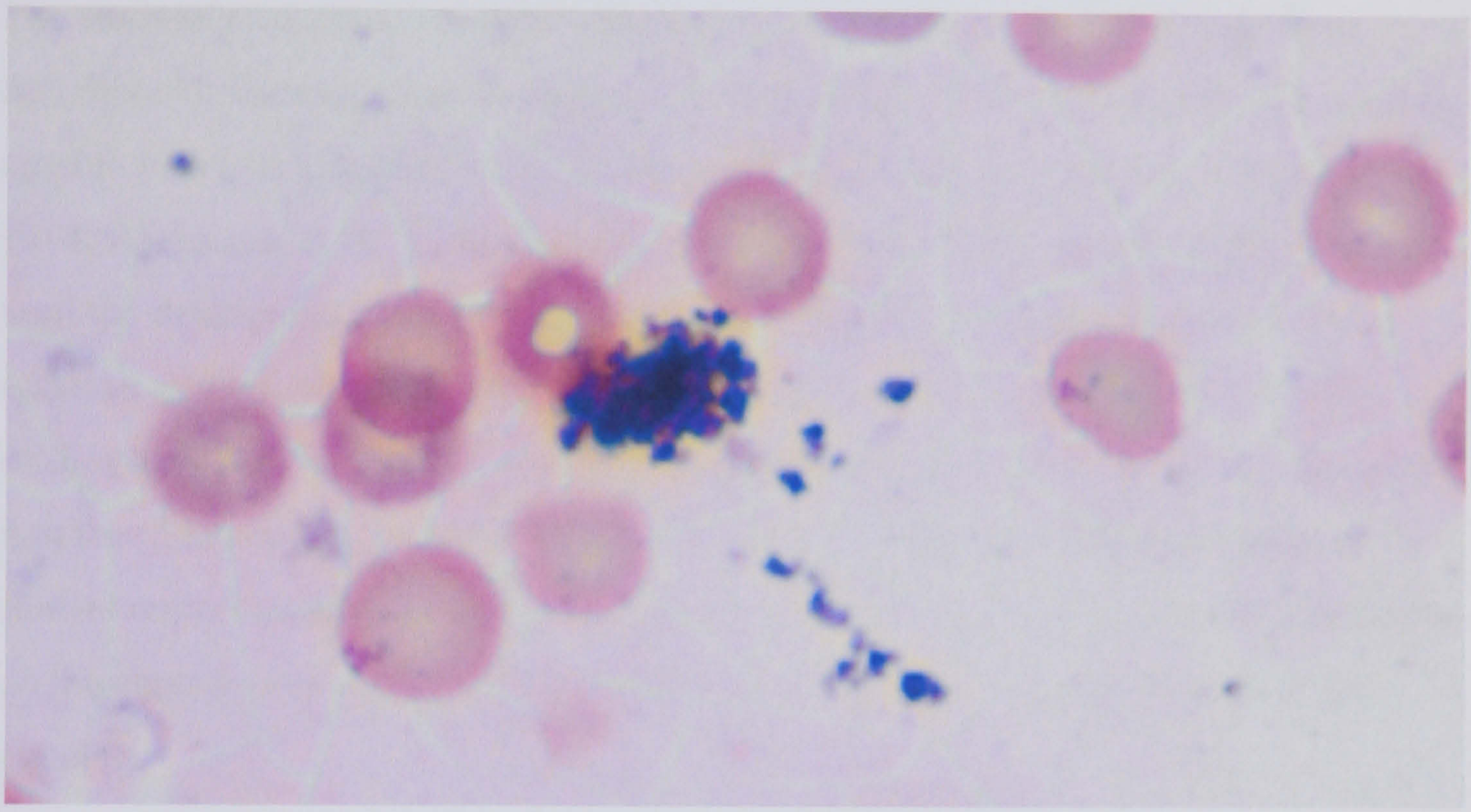
Published data regarding the action of CREB in megakaryocyte differentiation is scarce. However, recent evidence suggests that this transcription factor is activated by TPO binding to the c-Mpl receptor and subsequently has effects on megakaryocyte lineage determination and possibly also differentiation (Zauli et al., 1998). We confirmed the findings that TPO results in CREB phosphorylation in HEL cells, but also found that blocking the action of CaMKII can inhibit this effect. This phenomenon is common in neuronal cells (Huang et al., 2000; Han and Holtzman, 2000), where by blocking CREB activity with CaMKII inhibitors can result in changes in LTP and cell survival. Interestingly, as MK-801 appeared to have no effect on CREB activation, this may be an NMDA receptor-independent effect, implying that CaMKII activation occurs via another calcium source. Although CREB exists in MEG-01 cells, the application of PMA did not result in its activation. Two clear hypotheses emerge from these findings. The first is that CREB activation in megakaryocytes may be PKC independent and occurs following TPO addition via adaptor proteins described previously. The second supports the theory by Zauli and co-workers (1998) that the role of CREB is in megakaryocyte lineage determination. As MEG-01 cells are already of the megakaryocytic lineage, unlike HEL cells that are erythrocyte/megakaryocyte bipotential, there may not be a requirement for CREB activation.

The interaction between-TPO mediated signal transduction through the c-Mpl receptor and NMDA receptor expression is unclear. However, it would appear that expression of the NMDA receptor is unaltered in the absence of c-Mpl-mediated signalling, which has certain implications regarding both signalling mechanisms. The findings that c-Mpl$^{-/-}$ mice produce normal megakaryocytes and platelets suggest that other systems are able to influence differentiation in the absence of TPO (Bunting et al., 1997). As the findings in this and previous chapters demonstrate that the NMDA receptor possesses a level of control over the normal megakaryocyte maturation, it is possible that megakaryocytic NMDA receptor signalling can compensate for the absence of TPO effects, ensuring that the remaining megakaryocytes do differentiate

normally. This theory is strongly supported by the findings in this chapter, which demonstrate that many of the signalling systems activated by TPO that are important for megakaryocytic differentiation, are also modulated by the NMDA receptor.

The aim of this chapter was to bridge the gap between the expression of a functional NMDA receptor and the cellular effects observed when the receptor was antagonised. It has now been demonstrated that the NMDA receptor inflicts control over ERK1/2 activation, possibly in both an inhibitory and excitatory manner, explaining the less differentiated phenotype observed in MK-801 treated cells (chapter 4). The identification of CaMKII expression by megakaryocytes is a significant finding of its own accord, as it appears to influence various aspects of normal megakaryocytic cell maturation. This finding may have implications on megakaryocytic signalling as a whole and with further investigation, could be implicated in megakaryocyte lineage selection, endomitosis, adhesion and inside-out integrin signalling. As the CaMKII isoforms expressed by megakaryocytes were considered to be neuronal and interact with the NMDA receptor in the postsynaptic cell, it is a distinct possibility that the megakaryocytic NMDA receptor may modulate CaMKII function. Currently, this link is tenuous, as the western blot analysis is not clear enough to directly identify the effect of NMDA receptor inhibition on levels of active CaMKII. These studies warrant further investigation, and it may be that if the NMDA receptor is able to activate CaMKII, its influence could have widespread implications for megakaryocyte differentiation. To fully establish the exact signalling downstream of the megakaryocytic NMDA receptors requires focused and dedicated investigation, which due to time restraints was not possible in this study. However, this chapter does provide explanations as to how the NMDA receptor modulates megakaryocyte differentiation, as well as presenting other possible routes for further analysis.

Chapter 6



Megakaryocytes and Platelets in NR1^{neo} hypomorphic mice

May-Wright Giemsa stained peripheral mouse blood film showing pink coloured red blood cells and a clump of blue stained platelets (original magnification x1000)

Chapter 6

Megakaryocytes and Platelets in NR1^{neo} Hypomorphic Mice

6.1 Introduction

The study of transgenic animals with receptor gene ablation can often provide a definitive characterisation of receptor function *in vivo*. One such example, frequently cited in previous chapters, is that of TPO and c-Mpl knockout models (Gurney et al., 1994; Alexander et al., 1996; de Sauvage et al., 1996; Bunting et al., 1997). Indeed, until the development of these knockouts, the process of megakaryocytopoiesis in addition to the function of TPO and its receptor was poorly understood. Similar studies have also been performed on the NMDA receptor to provide greater insights into the mechanisms by which it is able to affect animal behaviour, with most of the studies understandably focusing on learning and memory. This chapter concentrates on the changes evoked by NMDA receptor gene ablation on megakaryocytes and platelets.

Changes in the expression of individual NMDA receptor subunits add to the clarity of function. Several types of NMDA receptor subunit-specific transgenic mice exist, including, global knockouts, in which expression of a functional product of the gene of interest is abolished; conditional knockouts, in which receptor expression is knocked out in only certain regions of the brain; point mutations, which produce complex phenotypic and functional changes in the receptor and knock-ins, in which the receptor protein is over-expressed. These transgenic animals have provided substantial evidence regarding the function of specific NMDA receptor subunits in the CNS. These include demonstrating the importance of the NR2B subunit in synaptic targeting of the receptor (Ito et al., 1997), that NR2D expression is implicated in juvenile epilepsy (Bengzon et al., 1999) and also identifying the role of the NR3A subunit as an NMDA receptor "volume control" (Das et al., 1998). As important as these findings are, the majority of NMDA receptor subunit knockouts are targeted at the

essential NR1 subunit. NR1 knockout animals have demonstrated that the subunit is fundamental for the function of the receptor. In these animals, NMDA receptor-mediated Ca^{2+} influx is totally abolished (Forrest et al., 1994) and several groups have also demonstrated that NR1 is implicated in the formation of somatosensory maps formation, which are involved in the sensing of external stimuli in the foetal brain (Li et al., 1994; Iwasato et al., 1997; Messersmith et al., 1997). However, as much as the NR1 transgenic animals provide insight into subunit and receptor function *in vivo*, the diseases in which NMDA receptor dysfunction has been implicated cannot be investigated, as the animals usually die within 10 hours of birth. The reasons behind neonatal death are believed to be due to defective breathing and suckling mechanisms (Sprengel and Single, 1999).

This fact has frustrated the use of transgenic animals in the research of NMDA receptor related psychotic diseases such as schizophrenia. However, recently a genetically altered mouse line was produced that expressed lowered levels of the NR1 subunit protein (Mohn et al., 1998). The creation of these animals involved using a vector targeted to integrate in to the NR1 locus (designated *Grin 1*) and insert a neomycin (*neo*)-resistant gene into intron 20. The impact of this insertion was to reduce the expression of NR1 mRNA and protein to an average of 8.1% and 7.3% respectively. It was also demonstrated that insertion of the *neo* gene did not produce any point mutations or novel transcripts, indicating that the $NR1^{neo}$ mutation produced a hypomorphic allele. Importantly, although NR1 expression was greatly lowered, it appeared that expression of the receptor was at high enough levels for the animals to progress from the neonatal stage and develop into normal adults. The adult mice were physically normal compared to control (albeit smaller than average, they were still in the normal range) and displayed no obvious abnormalities outside the CNS (although detailed studies have not been performed on peripheral tissues). The $NR1^{neo-/-}$ mice do however have severe psychological abnormalities. The general behaviour of these mice including; increased motor activity, stereotypic behaviour (fixed response to external stimuli, indicative of learning not taking place), abnormal social behaviour and deficient sexual interactions are typical of those seen in animal models of drug-induced schizophrenia (Moghaddam and Adams, 1998). Indeed, drugs previously shown to induce schizophrenia in mice, such as phencyclidine (PCP) and

MK-801, did not have any additional effect on $NR1^{neo-/-}$ mice. This psychotic behaviour of $NR1^{neo-/-}$ was prevented by the administration of the widely used anti-psychotic drugs, clozapine and halperidol. The expression of functional receptors present in the pre-frontal cortex was also determined by [^{3}H] MK-801 binding assay. Competition curves demonstrated that the IC_{50} for both $NR1^{neo+/+}$ and $NR1^{neo-/-}$ were identical, demonstrating that the remaining receptors were functionally normal, however the maximal binding was only 10% of that of the wild type. The findings by Mohn and colleagues support the importance of NMDA receptor function in schizophrenia and reveal the action by which anti-psychotic drugs may evoke their effects. Further studies using these animals will lead to a greater understanding of the role of the NMDA receptor in other diseases of the CNS.

Currently, no investigations have been performed on these animals with regard to the effect of NR1 gene ablation on peripheral tissues. These animals provide an unparalleled opportunity to investigate glutamate signalling in peripheral tissues due to the fact that they survive to adulthood. Not only would the extraction and experimentation on organs and tissues from 10-hour-old neonates prove extremely challenging, age-related defects regarding dysfunctional NMDA receptor signalling in peripheral tissues cannot be determined in NR1 global knockouts. The $NR1^{neo}$ transgenic mouse is the only viable model on which to study the effect of NR1 gene ablation on megakaryocytopoiesis and platelet production for several reasons. Firstly, the site of haematopoiesis changes during foetal development from the yolk sac, to the aorta-gonad-mesonephros (AGM) and finally to the liver (Zon, 1995; Traver et al. 2001). Indeed it is not until 4-7 days after birth that haematopoiesis, including megakaryocyte and platelet production appears in the bone marrow. Secondly, many of the experiments performed to determine the level of platelet production involves the bleeding of the animals and measuring the clotting time (see chapter 6.2.3) and also taking a comparatively large amount of peripheral blood for automated or histological analysis (see chapter 6.2.4 and 6.2.5). Such procedures would be impossible to perform on neonates.

This chapter is the first to determine the effects on peripheral tissues observed in the $NR1^{neo-/-}$ mouse by focusing on megakaryocytopoiesis and platelet production. The

experiments performed on these animals are comparable to those carried out in the c-Mpl and TPO knockout models to determine effect on megakaryocyte and platelet function. These investigations should provide a penetrating insight into the role of the NMDA receptor in the proliferation and maturation of megakaryocytes and also the in the production of functional platelets *in vivo*.

6.2 Materials and methods

6.2.1 Generation of NR1 $^{neo-/-}$ mice

Transgenic animals were generously provided by Dr. Bev Koller (University of North Carolina). These mice were generated as described previously (Mohn et al., 1999). Briefly, cells were transfected with JNS2 vector containing *neo* gene and positive selection markers. Targeted clones were identified by Southern analysis and injected into blastocysts to generate chimeras, which were then bred to B6D2 animals, obtaining NR1$^{neo+/-}$ animals. Intercrossing between the wild type and heterozygous colonies generated NR1$^{neo+/-}$ animals and a breeding colony established. All mice used in the following experiments were generated from intercrossing the first 2 heterozygous animals. The NR1$^{neo-/-}$ mice expressed 5-10% of the normal levels of NR1 compared to wild type, suggesting a hypomorphic NR1 allele enabling the *neo* homozygous mice to survive to adulthood.

6.2.2 Acetylcholinesterase staining of NR1$^{neo-/-}$ megakaryocytes

Tibiae, femora and spleen were removed from NR1$^{neo+/+}$, NR1$^{neo+/-}$ and NR1$^{neo-/-}$ mice, frozen in liquid nitrogen and sections collected and stored as method 2.4.4.1. Acetylcholinesterase (AChE)-positive cells within these tissues were identified as megakaryocytes. Substrate was prepared freshly before use by adding 5mg of acetylcholine iodide (ACI: Sigma) to 6.5ml 0.1M Na_2HPO_4 (pH6.0). The following was added, in order to the substrate whilst stirring;

0.5ml 0.1M $C_6H_5Na_3O_7.2H_2O$ (sodium citrate)
1.0ml 30mM $CuSO_4$
1.0ml H_2O
1.0ml 5mM $K_3Fe(CN)_6$
= 10ml AChE staining solution

AChE staining solution was applied to the slides and incubated for 2 hrs at room temperature in a humidified chamber. Slides were rinsed with 0.1M Na_2HPO_4 and

fixed in 4% paraformaldehyde for 5 minutes, washed 3 times for 5 minutes in PBS and counterstained for 5 minutes in Mayer's haematoxylin. Slides were mounted in 90% v/v. glycerol/PBS and viewed using a Leica DMLA microscope. Spleen sections used were approximately of the same size (15mm x 5mm) and all positively stained splenic megakaryocytes were counted for each section. Counts were performed blind.

6.2.3 Bleeding time assay

Mice were anaesthetised by intraperitoneal injection of avertin, made by mixing equal volumes of tribromyl ethyl alcohol and tertiary amyl alcohol (2.5% dilution), at 250mg/kg body weight. 5mm of the distal end of the mouse-tail was then removed and immediately placed in 0.9% saline solution maintained at 37°C. The bleeding time was measured as 15 seconds after the time taken for all traces of bleeding to cease. If bleeding continued for over 20 minutes, platelet plug formation was promoted by applying pressure to cut region using 3MM paper.

6.2.4 Whole blood cell count

200µl of blood was removed from anaesthetised mice via cardiac puncture of the left ventricle in syringes containing 20µl of 50mM EDTA to prevent clotting. 12µl of whole blood was then counted using a Heska's animal blood counter (Heska, CO). 20µl remaining blood was used in the production of blood films, by spreading a thin layer of blood over polylysine slides and fixing in 70% methanol for 5 minutes and allowed to air dry and May-Wright Giemsa counter-stained.

6.2.5 Transmission electron microscopic analysis of platelets and bone marrow

Whole blood was collected by cardiac puncture as described in section 6.2.4 from $NR1^{neo+/+}$, $NR1^{neo+/-}$ and $NR1^{neo-/-}$ mice. Platelet rich plasma (PRP) was collected from whole blood by centrifugation at 150g for 10 minutes and supernatant collected as PRP. To prevent platelet activation, platelets were firstly fixed lightly by adding 100µl of PRP to 1 ml of 0.2% glutaraldehyde in 0.1M sodium cacodylate at pH 7.4 and incubated at room temperature for 30 minutes. Platelet suspension was then

centrifuged at 800g for 10 minutes, the supernatant removed and the platelet pellet resuspended in 4% glutaraldehyde in 0.1M sodium cacodylate at pH 7.4. Samples were then kept at room temperature until TEM preparation. For bone marrow examinations, tibiae and femora were extracted from NR1$^{neo+/+}$ and NR1$^{neo-/-}$ and immediately fixed in 4% glutaraldehyde in 0.1M sodium cacodylate at pH 7.4 and kept at room temperature until TEM sample preparation.

TEM sample preparation was performed as previously described (chapter 4.2.6.3). Approximately 40 platelet clusters were analysed at various magnifications and 15 photographs taken for each group at 12,000 and 25,000-x magnification. Due to the rarity of the megakaryocyte in the bone marrow, it was possible to examine every megakaryocyte in each sample and approximately 15 photographs of each sample taken at 4,000 and 10,000 X magnification.

6.3 Results

6.3.1 Splenic megakaryocyte number in NR1^{neo} mice

The generation of the NR1 hypomorph, NR1^{neo}, has made it possible to determine the effect of NR1 down-regulation in adult mice. With regard to possible affects on megakaryocyte number, spleens removed from wildtype (NR1$^{neo\ +/+}$), heterozygous (NR1$^{neo+/-}$) and knockout (NR1$^{neo\ -/-}$) mice were snap frozen and 7μm cryosections of comparable size taken and stained for the megakaryocyte-specific marker AChE. Megakaryocyte counts, performed blind, demonstrated a significant decrease in megakaryocyte number, from an average of 82/section in NR1$^{neo+/+}$ to 62/section in NR1$^{neo\ -/-}$ (Fig. 6.3.1). Interestingly, megakaryocyte number was significantly increased to an average of 128/section in NR1$^{neo+/-}$ animals.

6.3.2 Bleeding time and platelet counts of NR1^{neo} mice

As changes in megakaryocyte number may directly alter the number of circulating platelets, consequential variations in clotting time were established. In the following experiments, the tail cut assay was used as a method of quantifying clot formation in age-matched NR1$^{neo\ +/+}$, NR1$^{neo\ +/-}$ and NR1$^{neo\ -/-}$ mice. Bleeding times of NR1$^{neo-/-}$ were increased nearly four-fold compared to NR1$^{neo\ +/+}$, from an average of 2 minutes 47 seconds to 9 minutes 34 seconds (Fig. 6.3.2). The bleeding times of NR1$^{neo\ +/-}$ mice were not significantly different to wildtype controls.

As the common cause of increased bleeding time is a reduction in the number of platelets, platelet counts and subsequent histological examination of peripheral blood smears were performed. Peripheral blood was removed via cardiac puncture and kept in 10mM EDTA solution to prevent activation until platelet counts were performed. Whole blood counts in NR1$^{neo\ +/+}$, NR1$^{neo\ +/-}$ and NR1$^{neo\ -/-}$ revealed no significant difference in circulating red blood cell number, haemoglobin, haematocrit or platelet

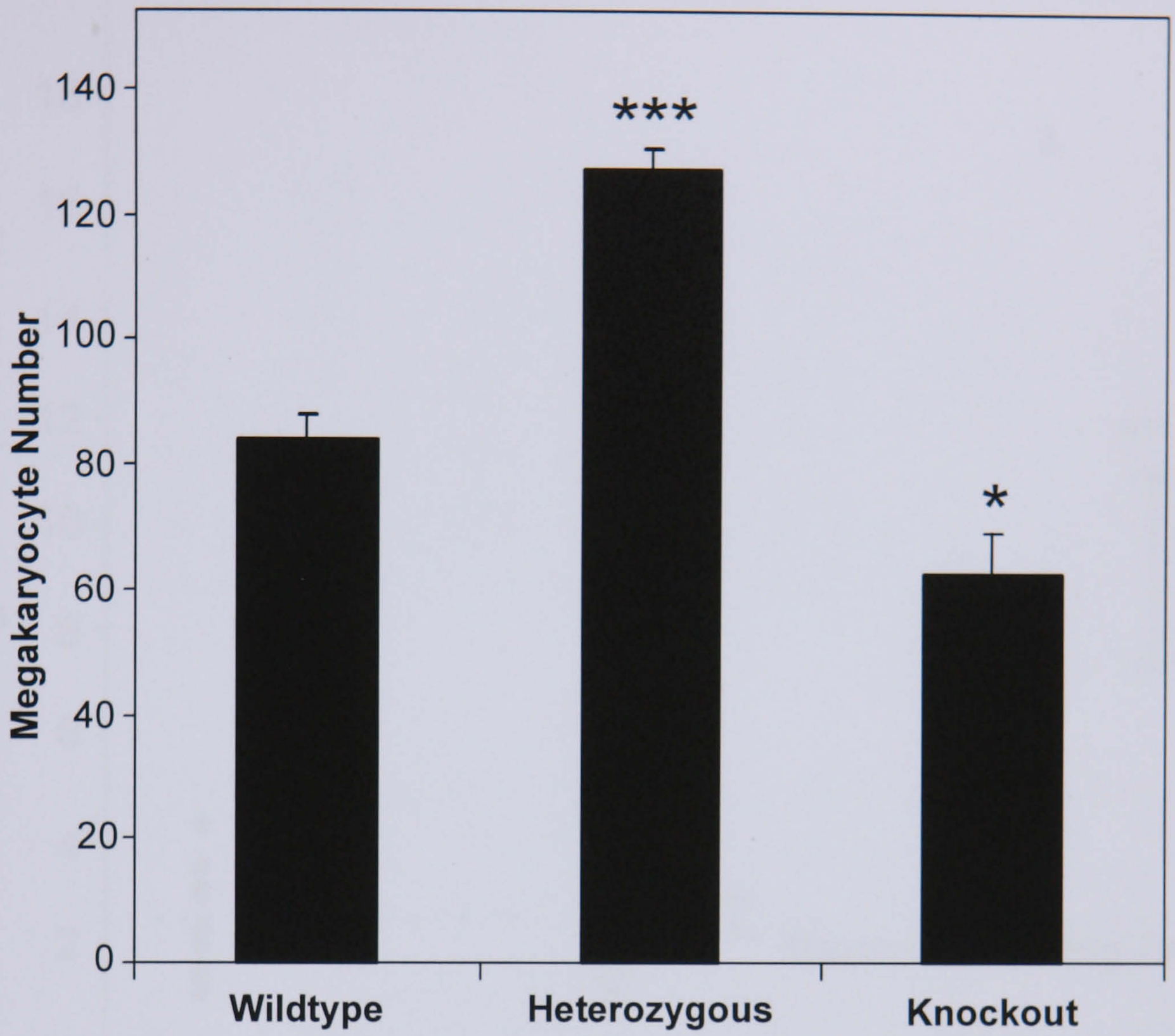


Figure 6.3.1. Splenic megakaryocyte number in NR1^{neo} wildtype, heterozygous and knockout mice. Megakaryocyte number was quantified by counting AChE-positive cells present in the spleen of wildtype, heterozygous and knockout mice. Experiments were performed blind and revealed a decrease in megakaryocyte number in knockout animals compared to wildtype , but interestingly a significantly increased in heterozygotes. (* $p<0.05$, ***$p<0.001$; one way ANOVA).

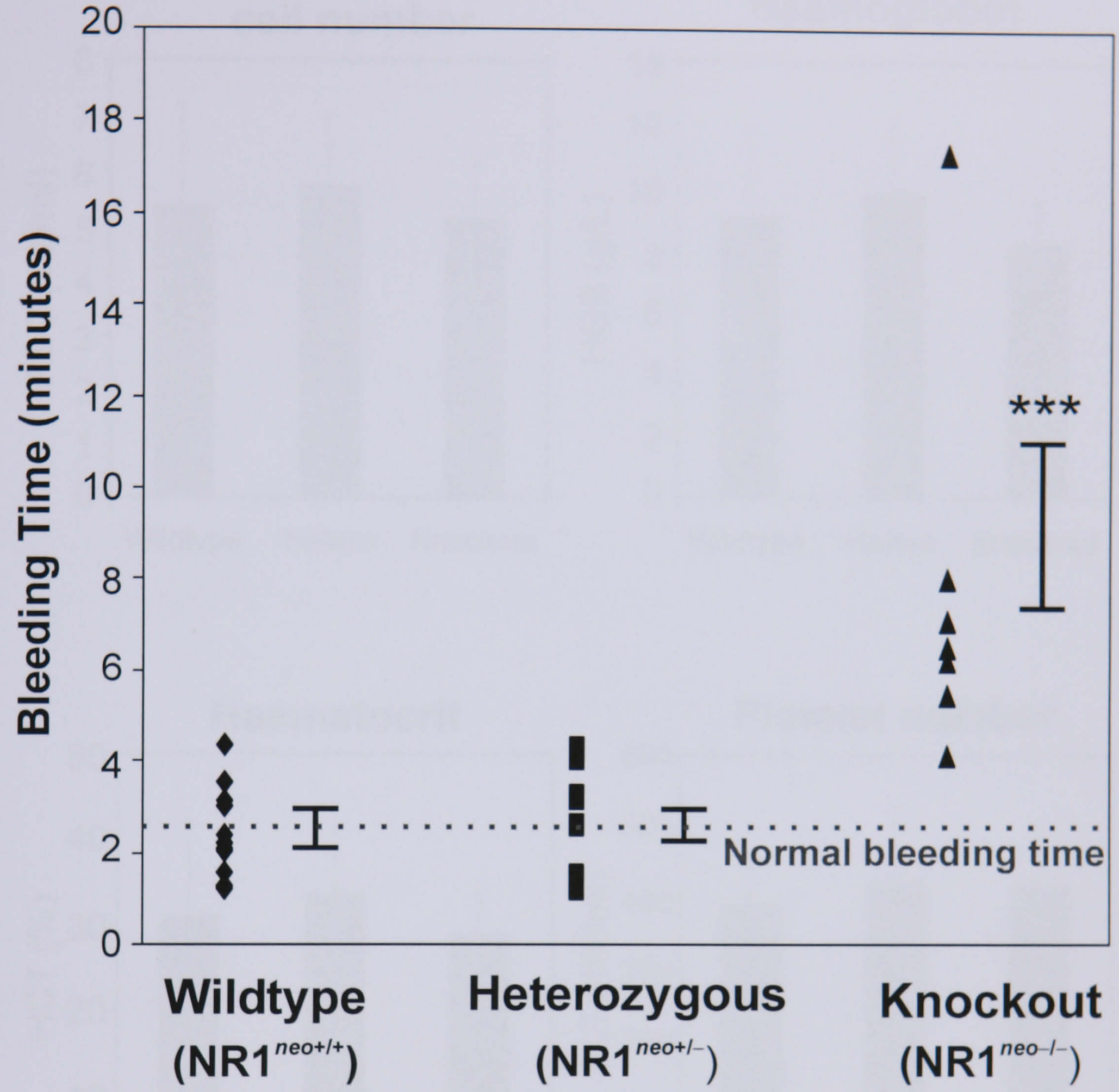


Figure 6.3.2. Bleeding times in wildtype ($NR1^{neo+/+}$), heterozygote ($NR1^{neo+/-}$) and knockout ($NR1^{neo-/-}$) mice. Average bleeding time, measured by tail cut assay, was significantly increased in knockout mice compared to wildtype and heterozygous. The average bleeding time of wildtype was 2.47 minutes (±S.D. 1.02 minutes) and 2.17 minutes (±S.D. 1.10 minutes) in heterozygous, whilst bleeding time was increased to an average of 9.34 minutes (±S.D. 5.42 minutes) in knockouts. (*** = $p<0.001$; Mann-Whitney rank sum test)

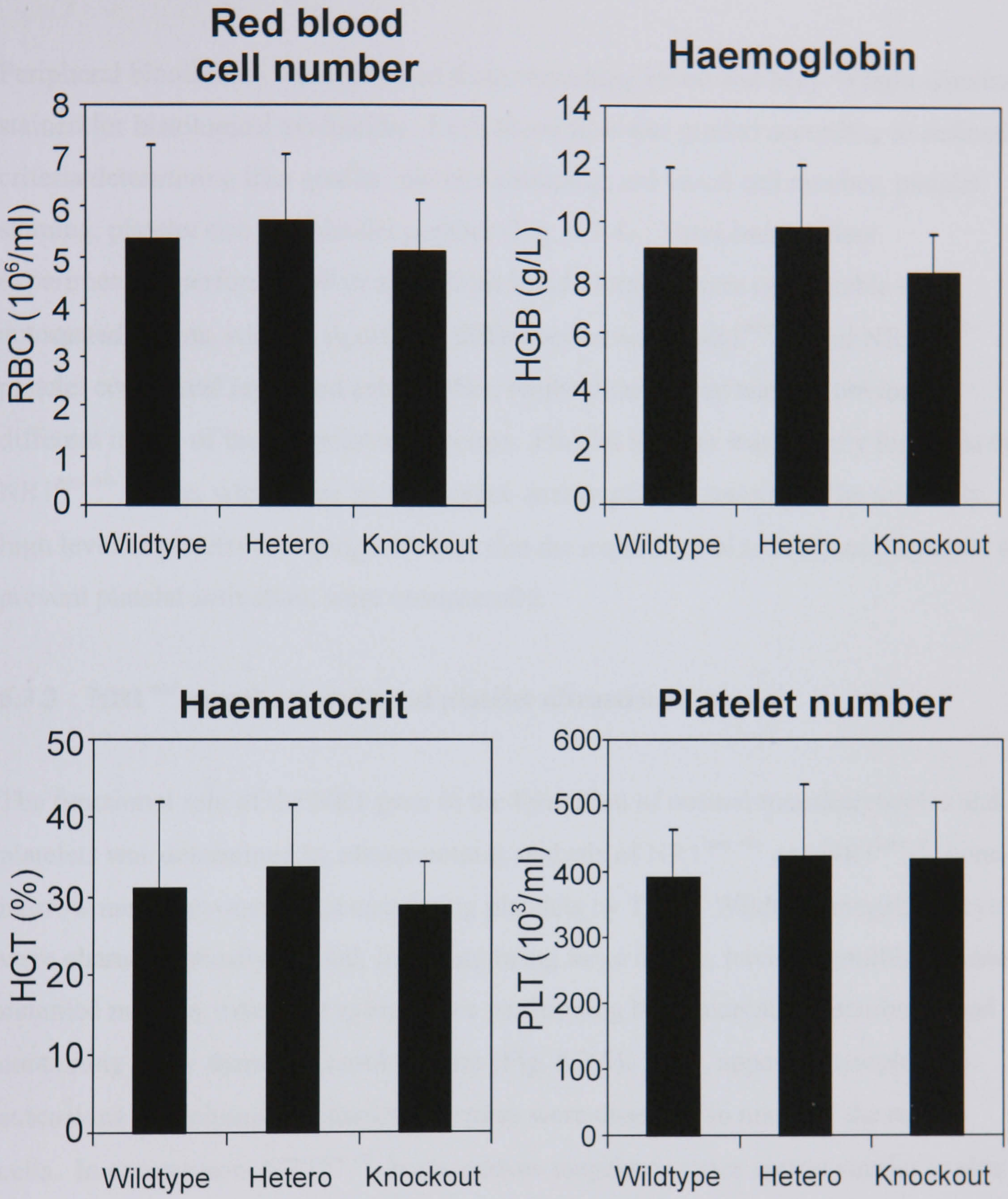


Figure 6.3.3. Complete blood counts from NR1^{neo} mice. Peripheral blood taken by cardiac puncture was subjected to automated counting. Counts were performed on red blood cells (normal range 5-12x10^6/ml), haemoglobin (normal range 11-18g/L), haematocrit (normal range 36-52%) and platelet number (normal range 140-600x10^3/ml). No significant difference was observed between wildtype, heterozygous (hetero) or knockout in any of the selected counts (one-way ANOVA). Error bars = standard deviation, n=8 for wildtpye, heterozygous and knockout.

number (Fig. 6.3.3). Counts for all other circulating blood cells were also similar in all three groups.

Peripheral blood films were prepared from remaining blood and May-Wright Giemsa stained for histological evaluation. Each blood film was graded according to defined criteria determining film quality, platelet clumping, red blood cell number, platelet staining, platelet size and platelet number (Fig. 6.3.4). Three independent experimenters performed all examinations blind. Results were comparable to automated counts, with no significant difference between $NR1^{neo\ +/+}$ and $NR1^{neo\ -/-}$ platelet counts and red blood cell number, whilst platelet size was not obviously different in any of the experimental groups. Platelet number was slightly higher in the $NR1^{neo\ +/-}$ group, which may be due higher quality platelet staining. The relatively high level of platelet clumping indicates that the experimental techniques employed to prevent platelet activation, were unsuccessful.

6.3.3 $NR1^{neo}$ megakaryocyte and platelet ultrastructure

The functional role of the NR1 gene in the formation of normal megakaryocytes and platelets was determined by ultrastructural analysis of $NR1^{neo\ +/+}$ and $NR1^{neo\ -/-}$ bone marrow megakaryocytes and circulating platelets by TEM. Wildtype megakaryocytes were characteristically normal, including being large in size, having a multilobed and indented nucleus, extensive cytoplasmic partitioning by demarcation membrane and containing many dense α-granule bodies (Fig. 6.3.5). Also, apparent cytoplasmic extensions resembling proplatelet structures were observed in many of the mature cells. In comparison, $NR1^{neo\ -/-}$ bone marrow megakaryocytes appear similar in size and nuclear maturation and cytoplasmic α-granules were clearly present (Fig. 6.3.6). However, the levels of demarcation membrane invasion into the cytoplasm appeared less perfuse in many mature cells leading to larger platelet territories. Proplatelet structures were also not observed in these cells.

The ultrastructure of circulating platelets obtained via cardiac puncture and isolation of platelet-rich plasma were also analysed by TEM. The general morphology of all platelets indicated that they have been activated (non-activated morphology is a

uniform discoid shape), again indicating the technical failure to prevent platelet activation (Fig. 6.3.7). However. morphological differences between $NR1^{neo\ +/+}$, $NR1^{neo\ +/-}$ and $NR1^{neo\ +/+}$ were not apparent, with normal numbers of α-granules and marginal bands of microtubules observed in all platelets observed. There was also no obvious difference in platelet size between groups.

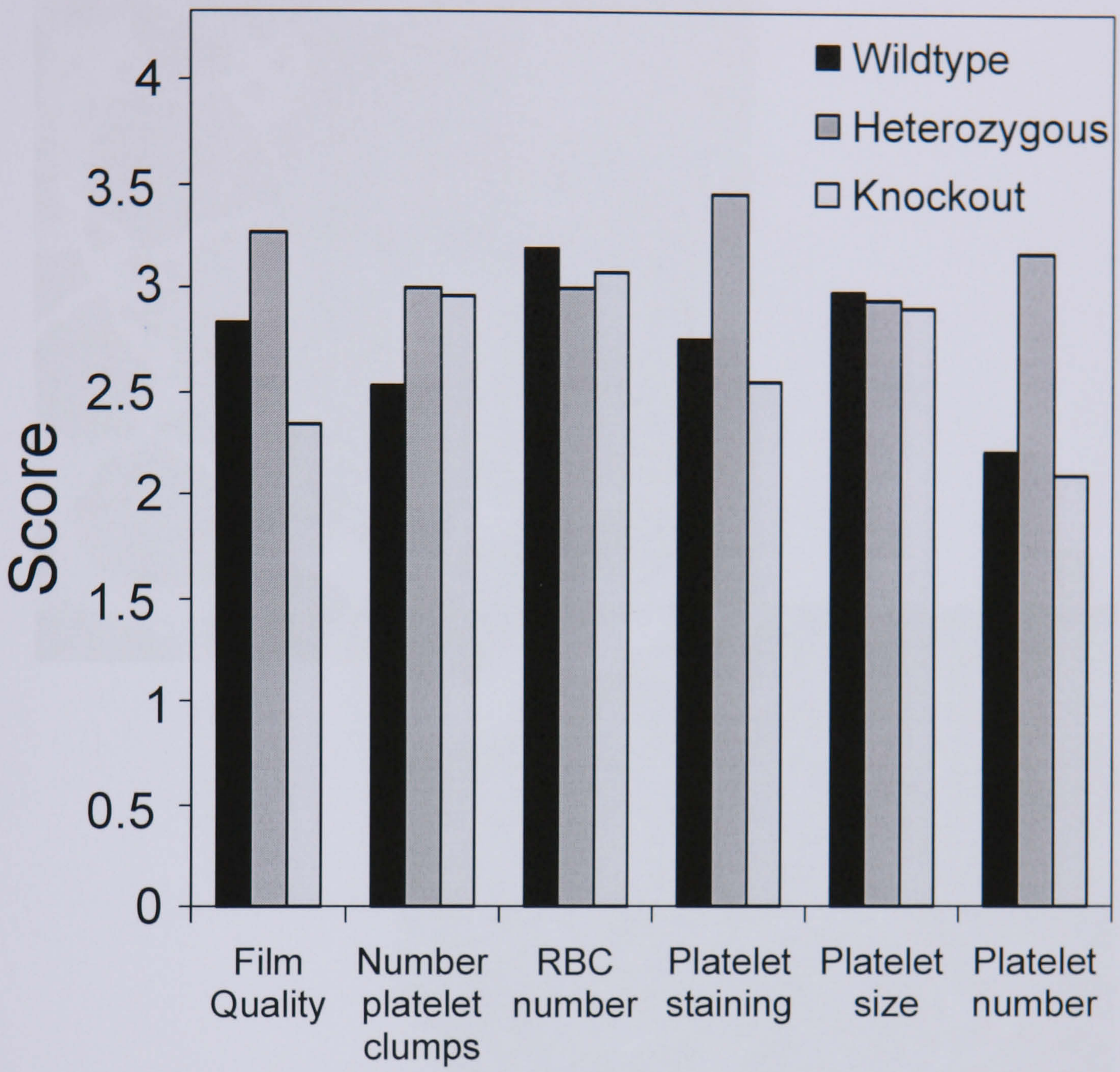


Figure 6.3.4. Histological grading of peripheral blood smears from NR1^{neo} wildtype, heterozygous and knockout mice. Histological examinations performed blind by three independent experimenters revealed no differences in red blood cell (RBC) number, platelet clumping, size or number in knockouts compared to wildtype. Platelet number may be slightly increased in heterozygous mice, although this could be due to the films being of a higher quality and better platelet staining.

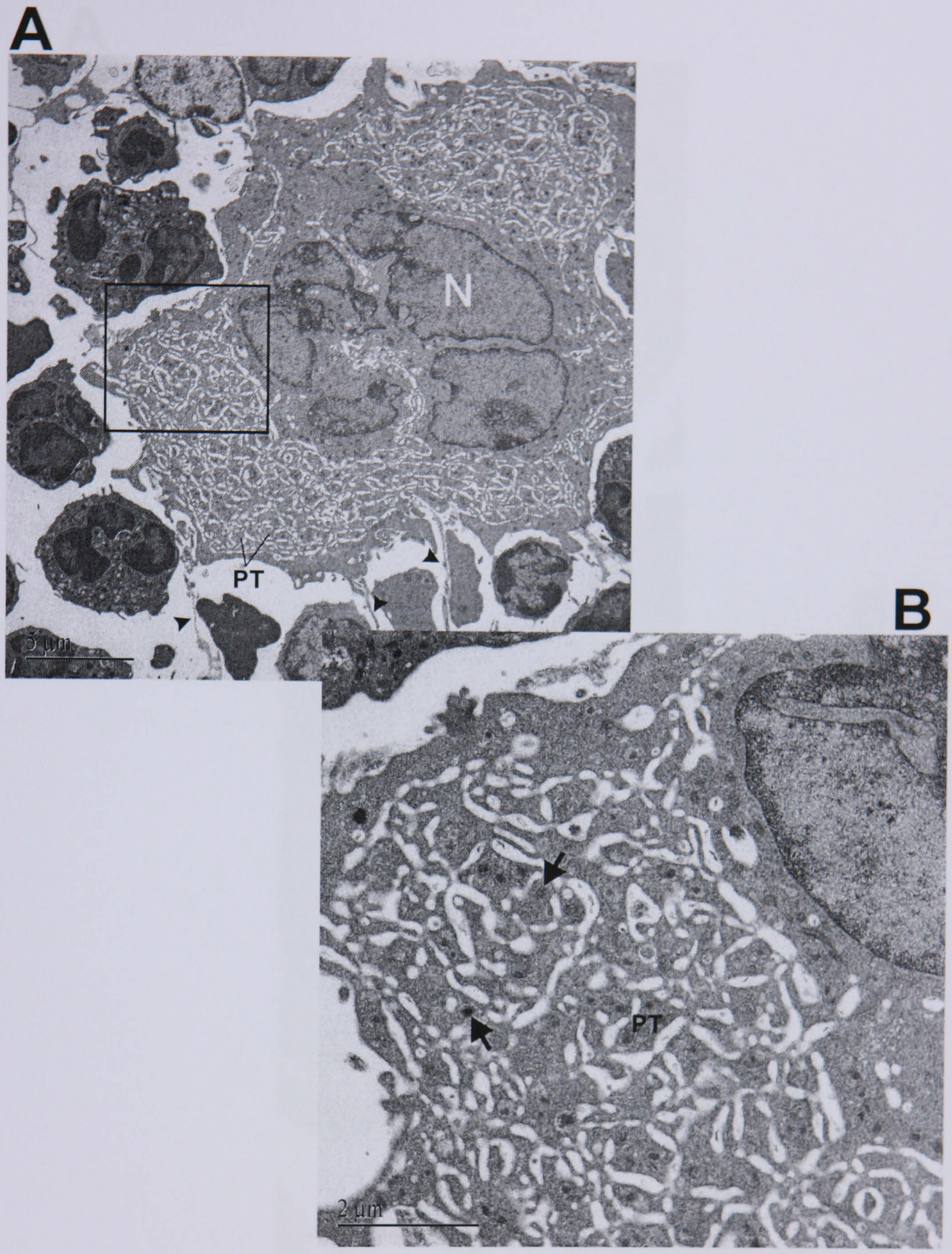


Figure 6.3.5. Ultrastructure of NR1$^{neo\ +/+}$ bone marrow megakaryocytes. Ultrastructural determination of NR1$^{neo\ +/+}$ bone marrow megakaryocyte morphology by TEM. Megakaryocytes display normal physiological morphology, including multi-lobed, indented nuclei (N), extensive demarcation membrane system leading to numerous small platelet territories (PT) and the presence of proplatelet structures (A; arrowheads) . Higher magnification images reveal normal platelet-territories and α-granule formation (B; arrows).

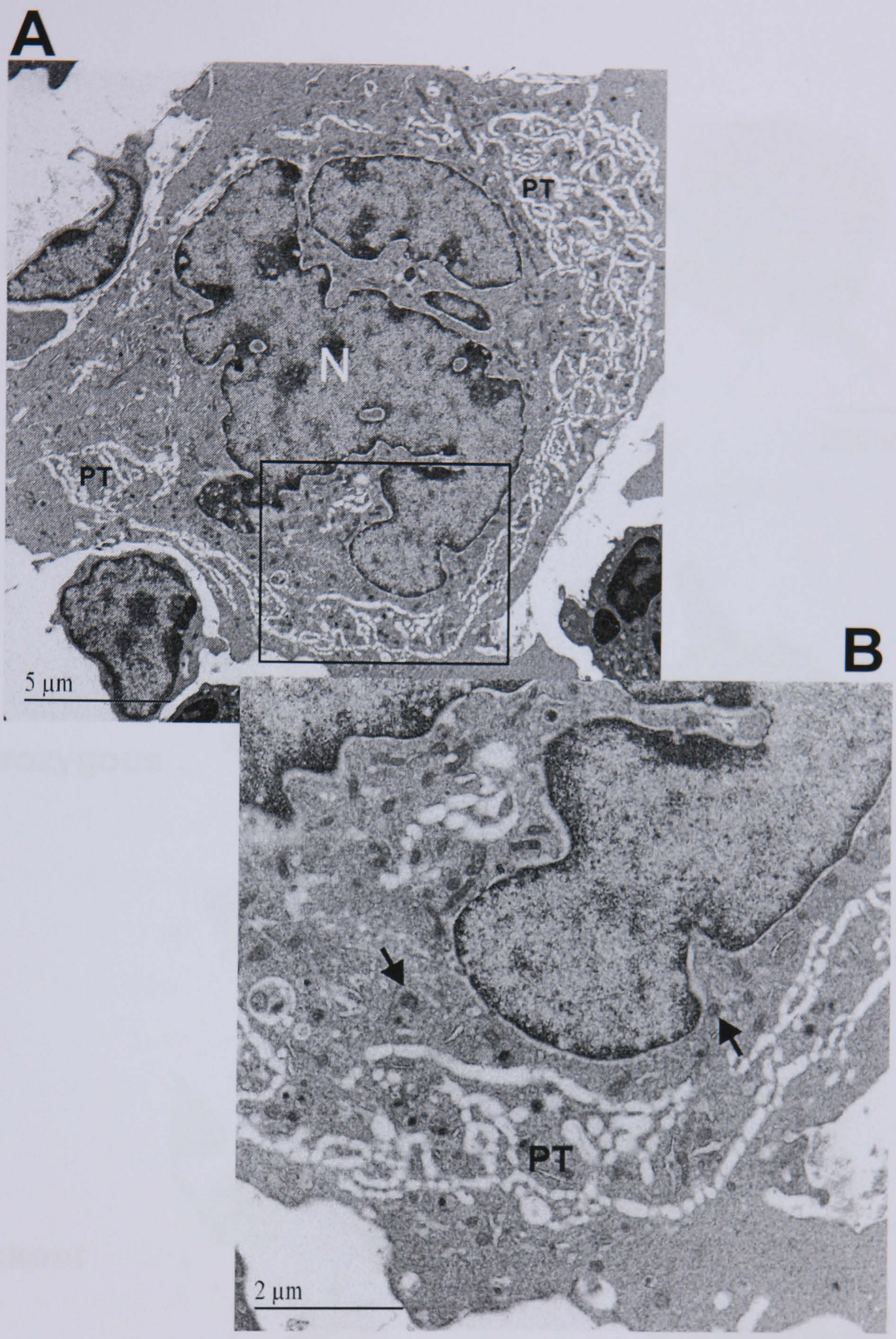


Figure 6.3.6. Ultrastructure of NR1$^{neo\ -/-}$ bone marrow megakaryocytes.
Ultrastructural determination of NR1$^{neo\ -/-}$ bone marrow megakaryocyte morphology by TEM. Megakaryocytes appeared to be of similar size to those found in wildtype bone marrow and exhibited multi-lobed, indented nuclei (N). However, the number of platelet-terratories (PT), formed by demarcation membrane, was less than wildtype controls (A). Higher magnification images demonstrated larger PTs although α-granule formation (B, arrows) appears normal.

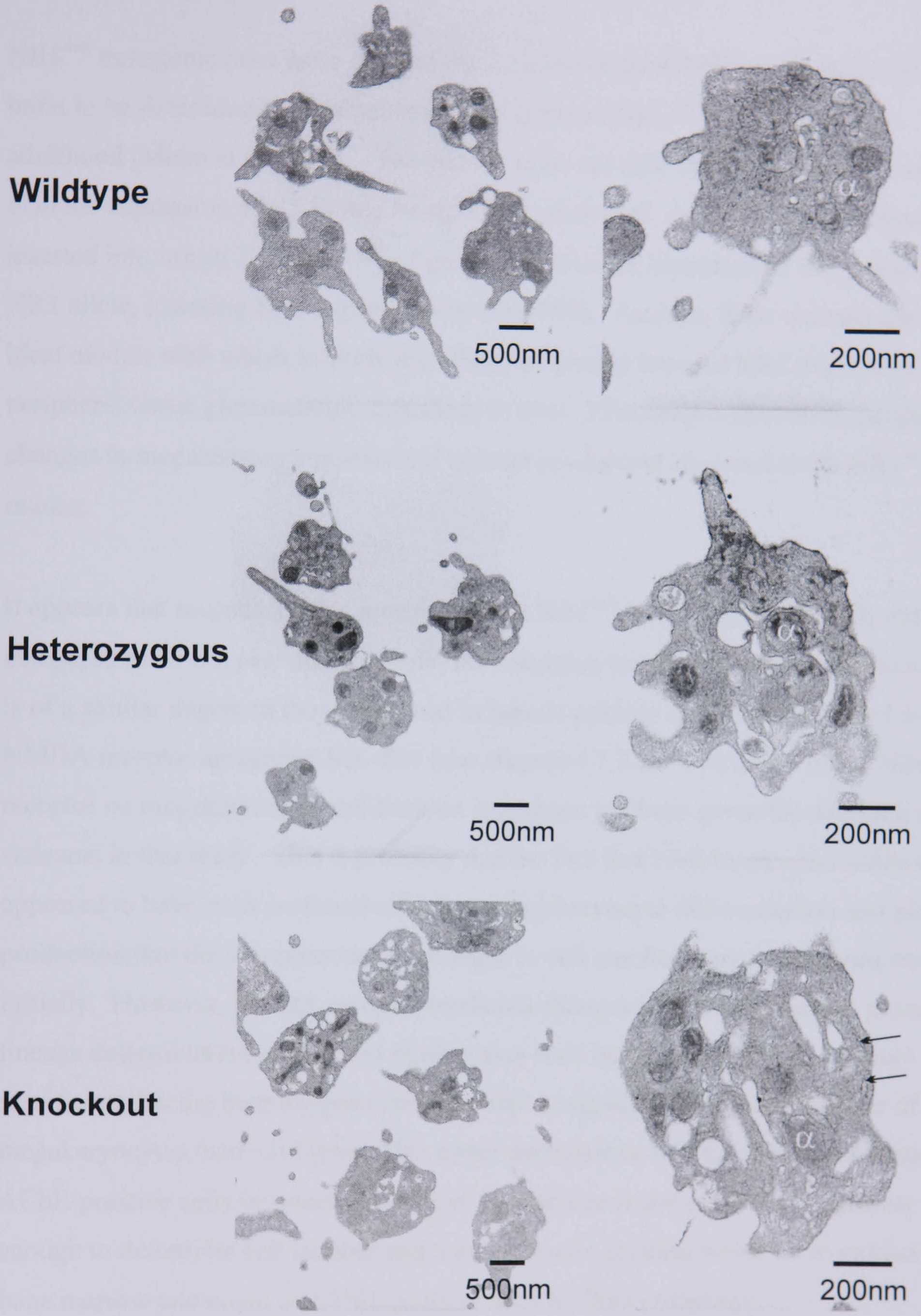


Figure 6.3.7. Circulating platelet ultrastructure from NR1^{neo} mice. Circulating platelets taken from NR1neo wildtype, heterozygote and knockout mice displayed similar cytoplasmic constituents, including α-granules (α) and microtubule formation (arrows).

6.4 Discussion

NR1^{neo} transgenic mice have enabled the function of the NMDA receptor in the adult brain to be determined as, unlike other NR1 global knockouts, they survive to adulthood (Mohn et al., 1998). The NR1^{neo} mice are able to survive because, rather than the expression of NR1 gene being totally abolished, the neomycin-resistance gene inserted into intron 20 of the *Grin 1* gene, results in the formation of a hypomorphic NR1 allele, lowering NR1 expression by over 90%. As such, these animals are the ideal models with which to study the effects of greatly lowered NR1 expression on peripheral tissue glutamatergic signalling *in vivo*. This chapter describes changes the changes in megakaryocytopoiesis and platelet production observed in the NR1$^{neo-/-}$ mouse.

It appears that megakaryocyte proliferation in NR1$^{neo-/-}$ mice is significantly reduced compared to wild types. Interestingly, the reduction in splenic megakaryocyte number is of a similar degree to those observed in human primary cell cultures treated with the NMDA receptor antagonist MK-801 (see chapter 4.3.3.2). The effect of the NMDA receptor on megakaryocyte proliferation is perhaps not been given the attention it warrants in this study. This is probably due the fact that NMDA receptor inhibition appeared to have such profound effects on megakaryocyte differentiation and platelet production that the less pronounced changes in cell number were not focused on initially. However, NMDA receptor-mediated changes in proliferation and possibly lineage determination *in vivo* and *in vitro* may well be an attractive line of study. It is significant that the heterozygous animals display significantly greater number of megakaryocytes than wild type. This could demonstrate that the method of counting AChE-positive cells in spleen sections of similar size is not sufficiently accurate enough to determine cell number and a more precise method would be to extract the bone marrow and count all CD61-positive cells by flow cytometry.

To complement the *in vitro* data described in chapter 4, it was demonstrated that bleeding times displayed by NR1$^{neo-/-}$ mice significantly increased compared to heterozygotes and wild type animals. Indeed, the increased clotting time of NR1$^{neo-/-}$ compared to wildtype are comparable to that observed in c-Mpl- and TPO-knockout

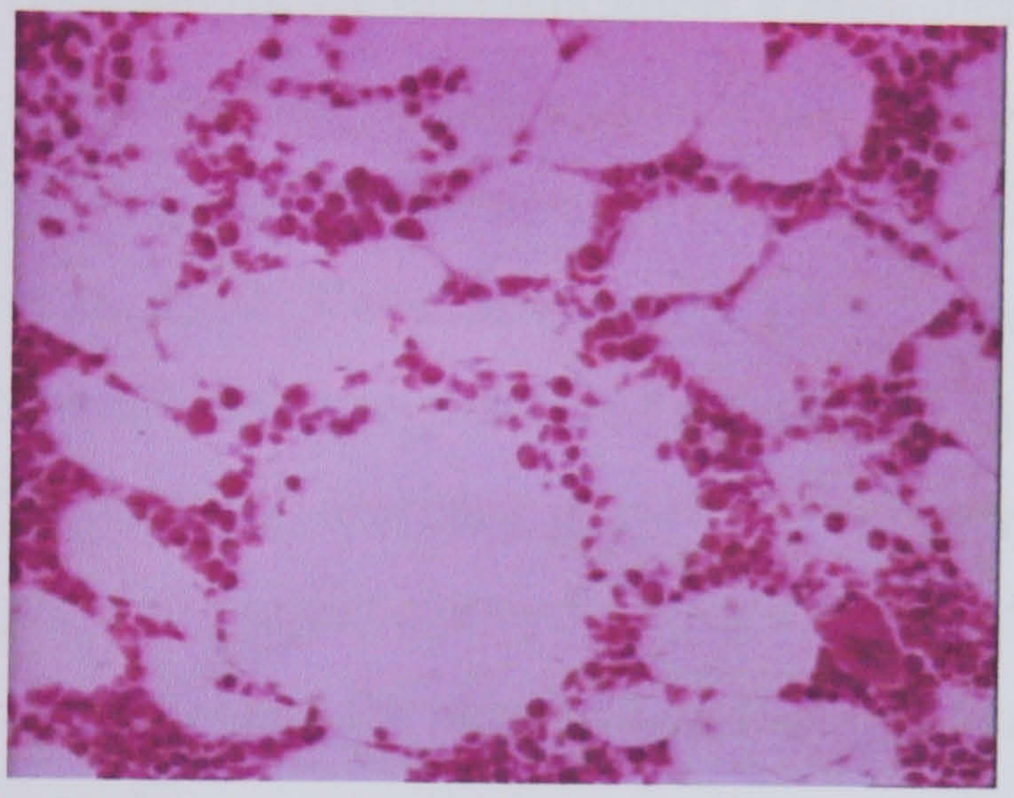
animals, indicating the importance of the NMDA receptor in thrombopoiesis (Gurney et al., 1994; Bunting et al., 1997). However, increased clotting time can be indicative of several conditions in addition to decreased platelet production. One possibility that must be considered, following the work by Franconi and co-workers on the platelet NMDA receptor, is platelet function (Franconi et al., 1996). Unfortunately, the data shown in this chapter cannot provide a tangible explanation as to why clotting was inhibited by NR1 under-expression. The ultrastructure of bone marrow megakaryocytes in NR1$^{neo-/-}$ compared to wild type imply that the demarcation membrane system has not developed throughout the cytoplasm, therefore reducing the number of platelets being released. Demarcation membrane abnormalities were also observed in the ultrastructure of human primary megakaryocytes following MK-801 treatment (chapter 4.3.2.2), which appears to confirm the role of the NMDA receptor in megakaryocyte cytoplasmic fragmentation. However, a reduction in the number of circulating platelets was not demonstrated by automated whole blood counts, a finding that favours platelet dysfunction rather than reduced platelet production. Platelet abnormalities, for example in platelet diseases such as GPS and von Willebrand disease, can often be identified from ultrastructural characteristics (Smith et al., 1997). The platelets extracted from the peripheral blood of NR1$^{neo-/-}$ mice however, appear to be morphologically similar to those from wildtype and heterozygotes animals, suggesting that the observed clotting abnormalities may not be a result of platelet dysfunction.

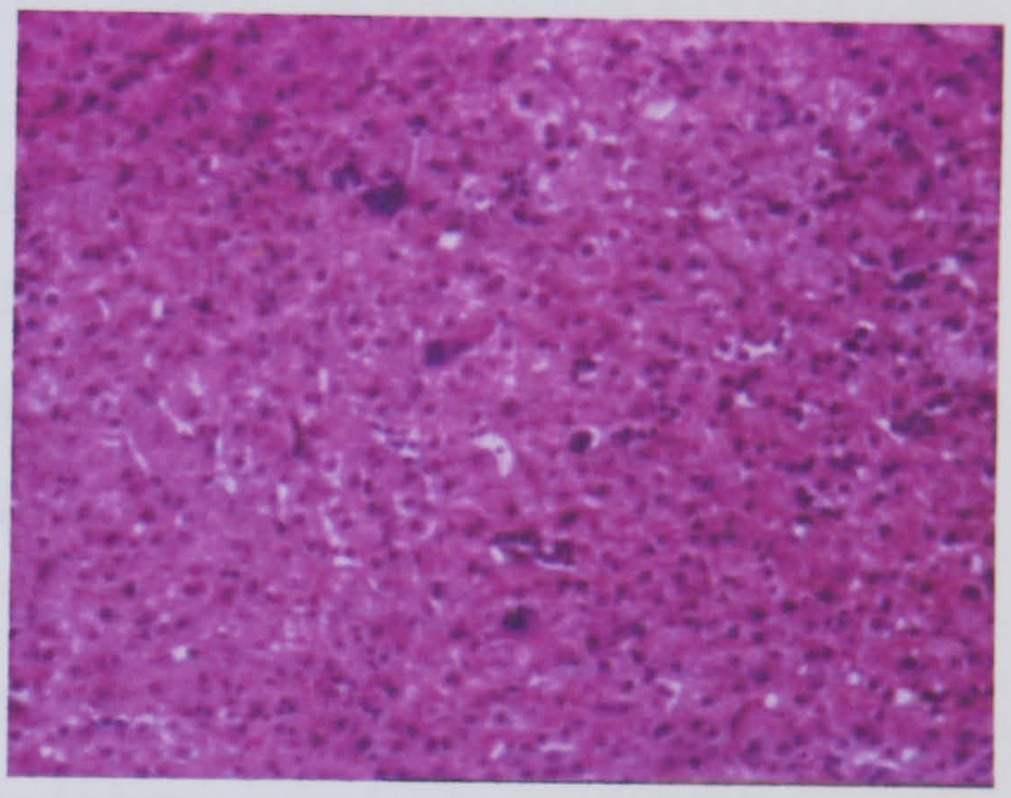
The findings from the NR1^{neo} transgenic studies underline the complexity of this newly-identified signalling mechanism. However, the data presented in this chapter was the result of experiments performed over an extremely short period of time. It is of integral importance that further experiments are performed on these animals, focusing on determining whether the clotting abnormalities are as a result of lowered platelet production, or platelet dysfunction. For example, platelet function can be determined by platelet adhesion assay (Bunting et al., 1997) or assays determining platelet activation (Newman and Chong, 2000; Valles et al., 2002) from peripheral blood. It would also be possible to culture bone marrow megakaryocytes by immunoselecting CD61-positive cells and determining the extent of proplatelet production in response to TPO and plasma (Rojnuckarin et al., 1999). It would also be

of interest to record the concentration of circulating TPO and other cytokines in the $NR1^{neo-/-}$ mouse, to determine whether the haematopoietic system compensates for the lack of NMDA receptor-mediated effects. It may also be the case that the low levels of NR1 expression displayed in $NR1^{neo-/-}$ mice, remain high enough to not affect certain aspects of the NMDA receptor-mediated megakaryocytopoiesis, or that unlike neuronal cells, the role of the NR1 subunit is not essential to receptor function.

From these encouraging preliminary studies it is possible to state that mice exhibiting decreased levels of the NR1 subunit display significant bleeding abnormalities. Unfortunately, it was not possible to determine the role of the NMDA receptor in this condition because of the contradictions presented with regards to platelet production and platelet function. By performing the further experiments described above, it would be possible to clarify the cause of the bleeding abnormality and also the role of the megakaryocytic NMDA receptor *in vivo*.

Chapter 7





General Discussion

Comparison of human bone marrow trephines from a normal patient (left) and a patient suffering from myelfibrosis (right), a condition in which increased numbers of megakaryocytes leads to excessive fibrosis of the bone marrow (magnification x40)
(©Bristol Biomedical Image Archive, University of Bristol)

Chapter 7
General Discussion

Haematopoiesis is tightly regulated by numerous cytokines. Megakaryocytopoiesis is no exception, with cell survival, proliferation and differentiation all rigidly modulated by a large number of growth factors and cytokines (reviewed in chapter 1.3) ensuring that the number of platelets released into the circulation is within physiological limits. However, since the characterisation of TPO, the key positive regulator of megakaryocyte differentiation, a great deal of research has focused on increasing the volume of knowledge on the effects of this particular cytokine. Indeed, since the cloning and characterisation of TPO and c-Mpl in 1994 (de Sauvage et al., 1994; Lok et al., 1994; Kaushansky et al.; 1994; Wendling et al., 1994), studies attempting to identify novel regulators of megakaryocytopoiesis have been scarce. This seems particularly surprising in view of the findings by Bunting and colleagues that normal platelets and megakaryocytes are produced *in vivo* in the absence of TPO (Bunting et al., 1997) and the acknowledgment by Kaushansky that mouse primary megakaryocytes require a component of blood plasma in addition to TPO, to produce platelets *in vivo* (K. Kaushansky, personal communication). The data presented in this thesis not only characterises a novel signalling system that regulates megakaryocyte differentiation and platelet production, but also enhances the field of peripheral glutamatergic signalling.

The principal aim of this work was to advance the preliminary findings regarding the expression of the NMDA receptor by megakaryocytes and its role in the differentiation of megakaryocytic cell lines (Genever et al., 1999). The findings described in this thesis clarifies the expression of NMDA receptor expression and its role both in megakaryocytic primary cells and cell lines, in addition to determining the function of the receptor in primary cells, identification of NMDA receptor-mediated intracellular signalling pathways and the effect of lowering NR1 gene expression on megakaryocytopoiesis and platelet production *in vivo*. However, it was beyond the capacity of this study to concisely determine the action of the receptor in every aspect of megakaryocyte differentiation and function. Indeed, the apparent multifaceted

action of the megakaryocytic NMDA receptor has lead to numerous lines of study undergoing preliminary investigation, many of which warrant concise analysis.

To continue the advances made during this study, it is imperative that certain aspects attain further investigation. The selective expression of NMDA receptor subunits by megakaryocytes during differentiation suggests that certain adaptations in receptor function may alter the effect they have on the cell (Fig. 7.1). It is of key importance to determine the full-length sequence of the NMDA receptor subunits NR1, NR2A, NR2C, NR2D and NR3 expressed by cell lines and primary cells. Once this is determined, it would be possible to compare the sequence with that of human neuronal NMDA receptor subunits and this may reveal the expression of splice variants or novel megakaryocyte-specific isoforms that modulate the conductivity of the receptor. It would then be possible to co-transfect MEG-01 and HEL cell lines with different combinations of the full-length cloned NMDA receptor subunits. This would result in megakaryoblastic cell lines stably transfected with diheteromeric (for example NR1/NR2A) and triheteromeric (for example NR1/NR2A/NR3) receptors. In conjunction with these studies, it would also be possible to transfect the megakaryoblastic cells with small interfering RNAs (siRNAs), which would result in the knocking down of endogenous NMDA receptor subunit expression. These molecular techniques would allow the manipulation of NMDA receptor subunit expression in megakaryoblastic cell lines and by performing functional experiments, comparable to those described in chapter 4, it would be possible to characterise the cellular effects of specific NMDA receptor subunit expression.

The megakaryocytic NMDA receptor has a number of roles during megakaryocytopoiesis and platelet production (Fig. 7.2). Several of these aspects warrant fuller examination to accumulate a greater understanding of how the receptor imposes such changes on the megakaryocyte. One such experiment would be to determine the effect of activation and antagonism of the megakaryocytic NMDA receptor on the activity of the transcription factors GATA-1 and NF-E2 (for review see chapter 1.3.4), both of which have specific key roles in megakaryocytopoiesis and platelet production. By establishing whether there are changes in the action of these transcription factors, for example by electromobility shift assay (EMSA), which

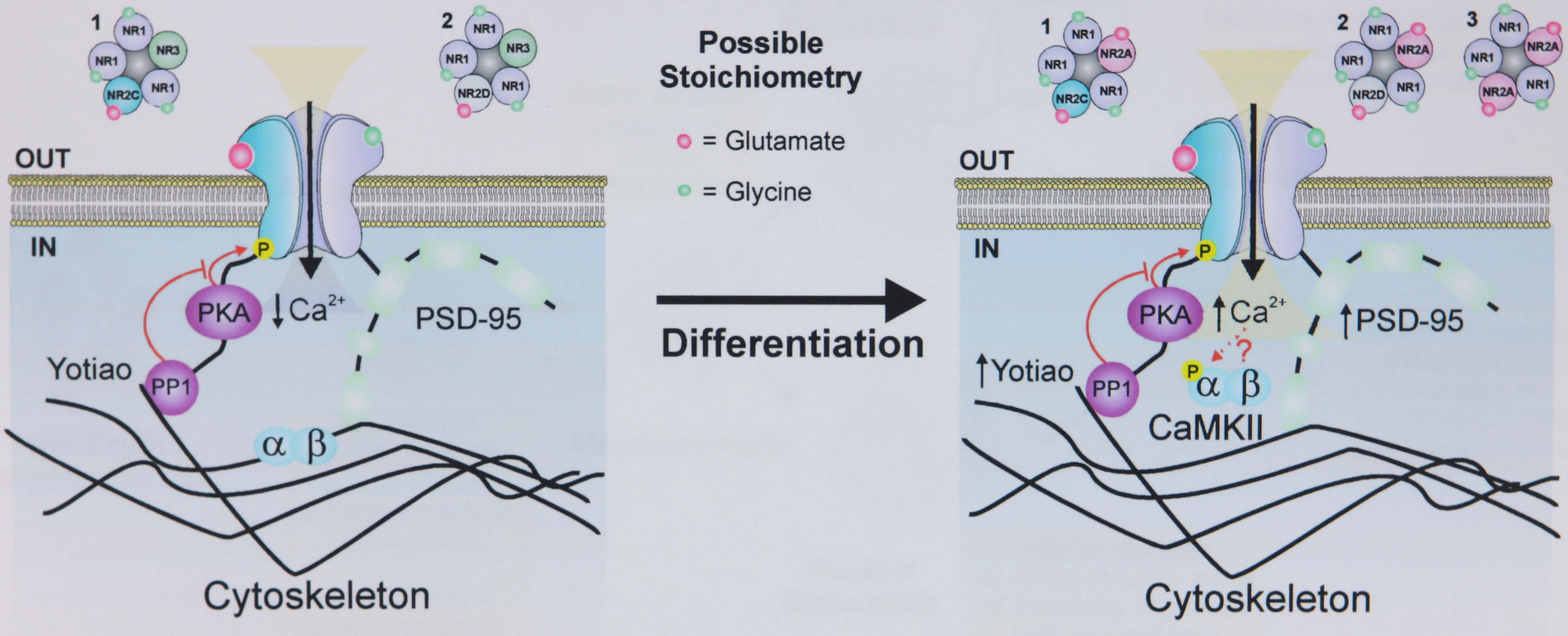


Figure 7.1. Schematic diagram representing the expression of NMDA receptor subunit and associated proteins by megakaryocytes and their possible interactions. Megakaryocytes express several different subunit types, of which NR2A and NR2D increase and NR3 decreases in expression following differentiation, moderating the conductance of the receptor. NMDA receptor associated proteins may participate in the receptor/cytoskeletal interactions and signal transduction

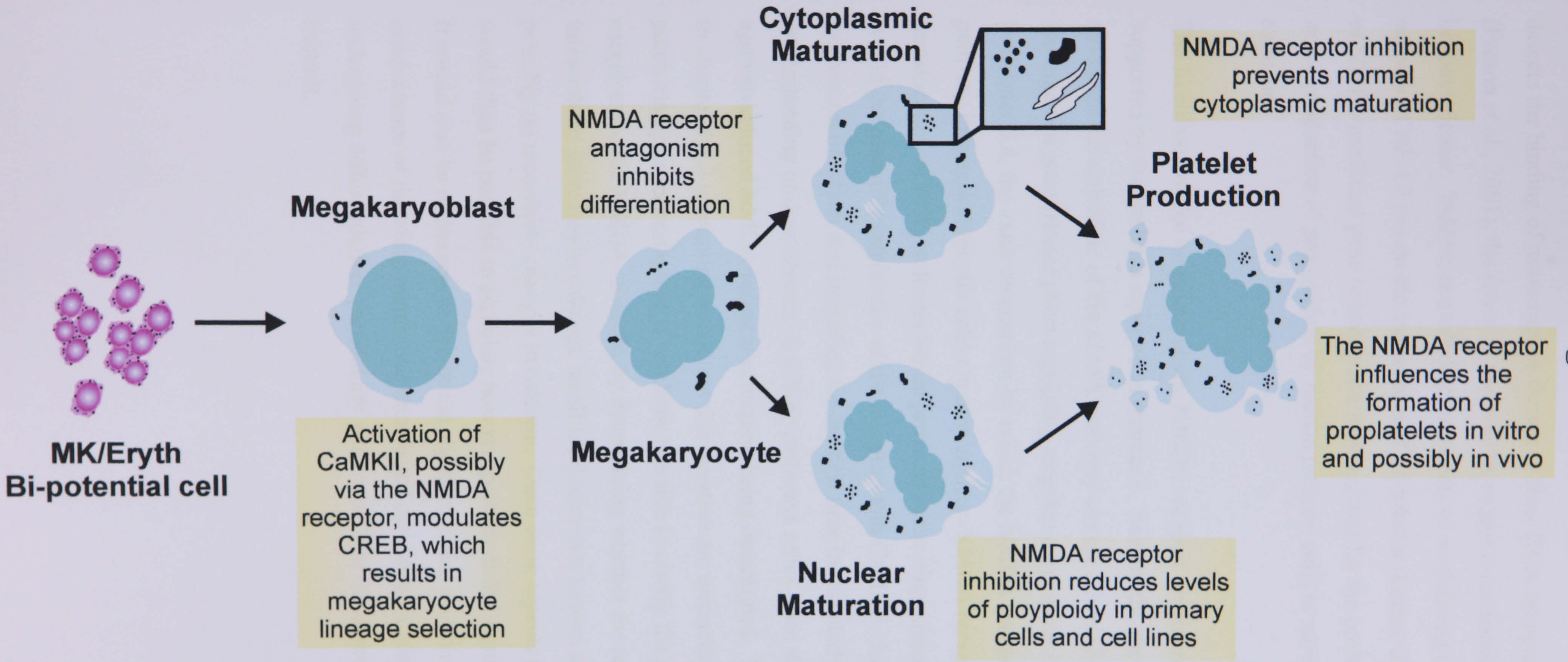


Figure 7.2. The role of the NMDA receptor in megakaryocyte differentiation and platelet production. Schematic representing the multi-faceted functions of the megakaryocytic NMDA receptor, regulating the many stages of differentiation from the bi-potential progenitor cell to platelet production

detects the binding of transcription factors to their DNA recognition sequences (Freson et al., 2001), the cause of the NMDA receptor-mediated effects would then become clearer. Indeed, considering the effects of modulating the transcriptional activity of NF-E2 has on the expression of β1-tubulin (Lecine et al., 2000), a reduction of NF-E2-mediated gene transcription may account for the profound deficits observed in the production of proplatelets by human primary cells *in vitro* following MK-801 exposure.

The likelihood of the megakaryocytic NMDA receptor mediating gene transcription is supported by the findings reported in chapter 5. Seemingly, the NMDA receptor can modulate the activation of the MAPK pathway among others (Fig. 7.3), which directly impinges on gene transcription regulating megakaryocytopoiesis. However, as stated in chapter 5.4, the exact mechanism by which the NMDA receptor activates this pathway in megakaryocytic cells remains unclear. NMDA receptor–mediated translocation of PKC isoforms would prove an interesting subject of further study, as would the plausible interaction of the receptor with CaMKII, the megakaryocytic expression of which was identified for the first time in this study. However, a greater understanding of downstream signalling pathways relies upon the immediate effects of agonist-induced NMDA receptor activation being determined. The results presented in chapter 4.3.3 attempting to address this question are inconclusive. By performing patch-clamping experiments, it would be possible to clarify the conductance of the megakaryocytic NMDA receptor, by determining whether the receptor causes large intracellular increases in calcium, small local increases known as "calcium sparks" or possibly no recordable changes in calcium. Once these aspects have been resolved, it would then be possible to postulate how receptor-mediated signal transduction occurs. It would also be of interest to determine, again by patch clamping, the receptor conductance of the afore mentioned transfected cell lines, in addition to that of cells undergoing differentiation, particularly with regards to the expression of the NR3 subunit.

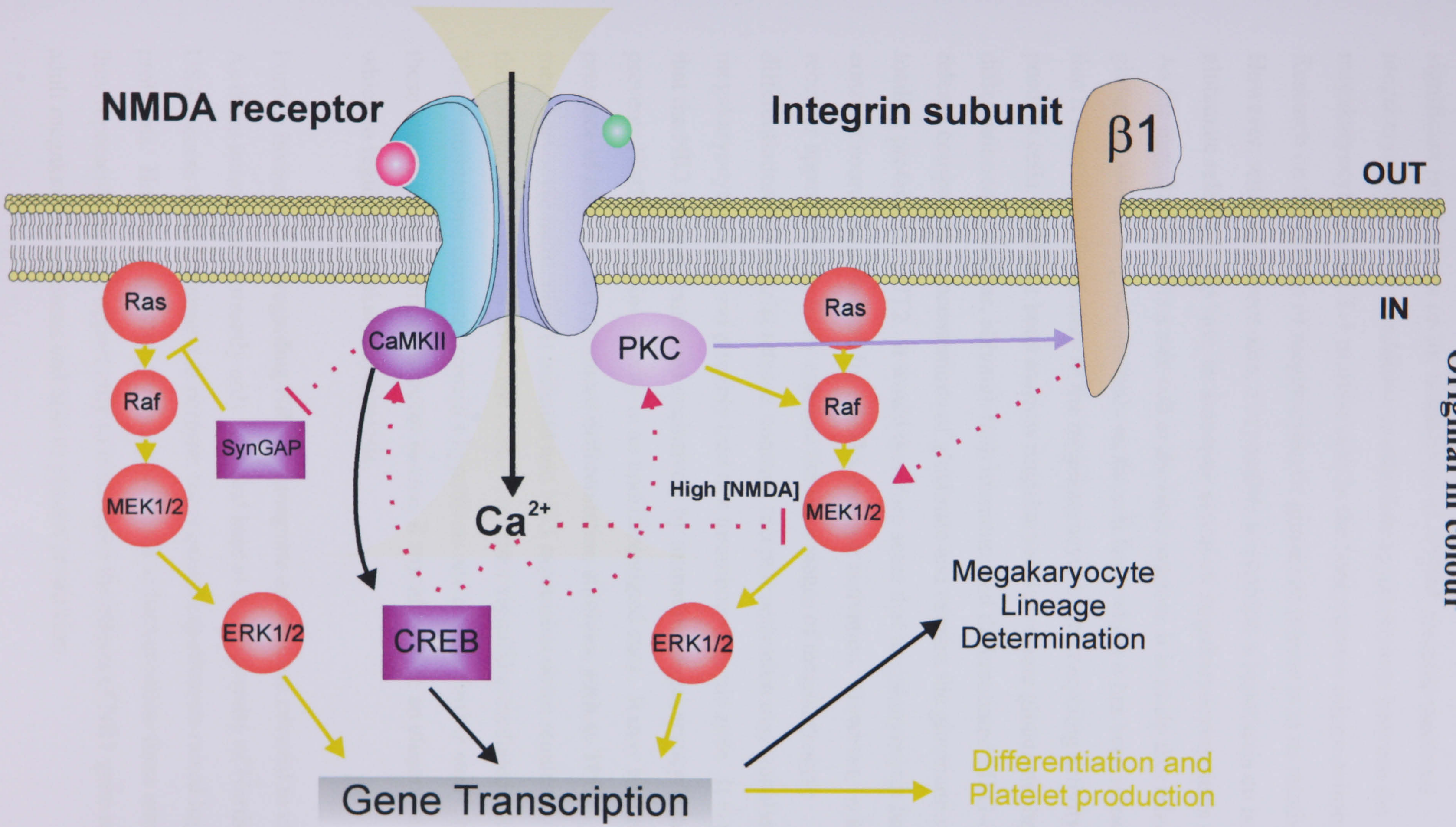
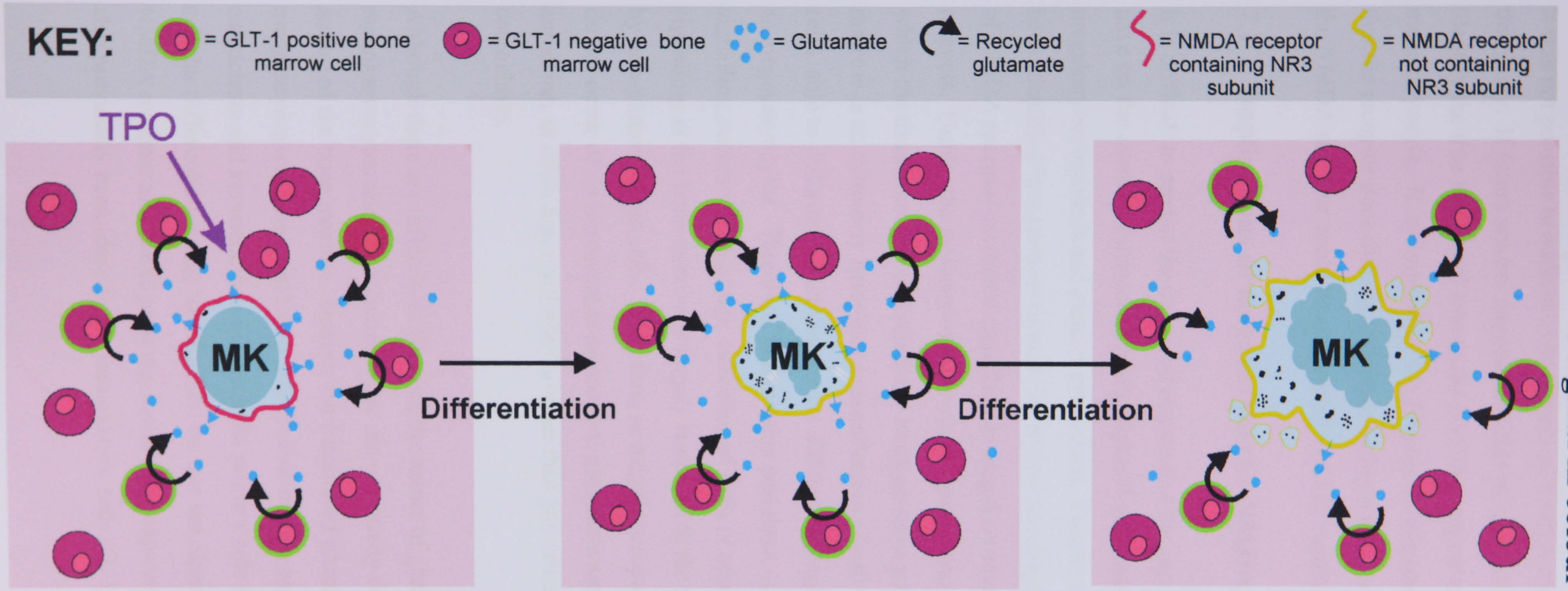


Figure 7.3. Putative NMDA receptor-mediated signal transduction in megakaryocytes. Simplified representation of signal transduction cascades in megakaryocytes modulated by the NMDA receptor. Identified pathways include CREB (black arrows) and ERK1/2 (gold arrows), which have previously been shown as key regulators of lineage determination and differentiation respectively. How these pathways are modulated by the NMDA receptor remains unclear (red broken arrows).

Original in colour

As implied in chapter 4.4, the fact that megakaryocytes release glutamate could have significant implications on the modulation of NMDA receptor-mediated megakaryocytopoiesis, in addition to establishing interactions between the megakaryocyte and GLT-1 positive cells in the bone marrow microenvironment. Research on the subject of megakaryocytic glutamate release is in its relative infancy. However, with the current data, it is possible to establish a hypothesis on how glutamate release could have an autocrine action on megakaryocytopoiesis (Fig. 7.4). As the megakaryocyte is a rare cell in the bone marrow, it is likely that released glutamate would act predominantly on the cell from which it has been released and that it remains in the vicinity of the megakaryocyte by the recycling activity of GLT-1 positive cells. Whether bone marrow megakaryocytes release glutamate throughout differentiation is unclear, although both immature and differentiated MEG-01 cells release comparable concentrations of glutamate and express the glutamate vesicular loading protein, VGLUT2. It would therefore seem that megakaryocytes have a constant source of glutamate for NMDA receptor activation. However, as the NMDA receptor appears to positively regulate the latter stages of megakaryocyte differentiation and platelet release, constant receptor activation may unbalance megakaryocytopoiesis and possibly lead to a thrombocythaemic state. It is plausible that the NR3 subunit, expressed exclusively by immature megakaryocytic cell lines, prevents NMDA receptor activity in an undifferentiated state. It may be that in the presence of an externally produced differentiation stimulus, such as TPO, megakaryocyte maturation is initiated and NR3 expression down regulated, leading to the restoration of normal autocrine NMDA receptor signalling-mediated events. These interactions currently remain a hypothesis and require further study, however, if these events were confirmed to occur *in vivo*, it would provide an elegant means by which to regulate megakaryocytopoiesis.

Further investigations regarding $NR1^{neo}$ transgenic animals are covered in chapter 6.4. As these animals are currently only bred and kept at the University of North Carolina, USA, access to these animals to perform the required experiments raised logistical problems. However, all attempts should be made to further utilise these animals, as they represent a unique opportunity to investigate the effects of NR1 gene reduction in adult megakaryocytopoiesis and also in platelet production.



Original in colour

Figure 7.4. Hypothesised role of megakaryocytic glutamate release in the bone marrow microenvironment. Possible interactions between glutamate-releasing megakaryoctyes and GLT-1 positive bone marrow cells regulating megakaryocyte differentiation.

It was not possible to address every aspect of NMDA receptor-mediated signalling in this study. If future studies suggested in this thesis were to be performed, that may identify the true functional role of the NMDA receptor and its role in the megakaryocyte. If, as implied by the findings in this study, these experiments confirm that the receptor does directly influence multiple aspects of megakaryocyte behaviour, the NMDA receptor could be considered as a key regulator of megakaryocytopoiesis.

7.1 Implications of megakaryocytic NMDA receptor signalling

It is difficult to predict the degree of impact that the data presented in this thesis will have on the current understanding of peripheral glutamatergic signalling, megakaryocytopoiesis and platelet production. Functional glutamate signalling has been identified in numerous tissues (for review see chapter 1.6.), however the process of NMDA receptor-mediated signalling in megakaryocytes is unique in several respects. Unlike the majority of other sites of peripheral glutamatergic signalling, in which the tissues consist of a relatively homogeneous population of cells, the bone marrow is an extremely heterogeneous collection of cell types. These range from stem cells, able to differentiate into numerous different lineages, to mature cell types, such as megakaryocytes. Therefore, unlike other peripheral tissues in which glutamate signalling occurs within a large collection of cells or even an entire tissue, glutamate signalling in the bone marrow is largely restricted to the megakaryocyte, surrounded by non-glutamatergic cells. Therefore, as megakaryocyte/megakaryocyte interactions do not occur often in normal bone marrow, glutamate signalling is likely to be autocrine, as often discussed above, rather than paracrine, as described for example in osteoblasts.

Although there are differences between the nature of glutamate signalling in megakaryocytes and other tissues, these are essentially due to the heterogeneous nature of the bone marrow. Indeed, the function of glutamate signalling in megakaryocytes is comparable to that in other tissues, especially osteoblasts, osteoclasts and keratinocytes, in which the primary role for glutamate signalling is in cellular differentiation and function (for review see Skerry and Genever, 2001). The NMDA receptor associated proteins, found to be expressed by megakaryocytes have also previously been identified in osteoblastic cells (G. J. Spencer, personal

communication), although whether these proteins are expressed in other tissues that utilise glutamate signalling has currently not been defined. However the α and β CaMKII isoforms, identified in megakaryocytes, are also expressed in peripheral NMDA receptor expressing tissues (Inagaki et al., 1995; Krizbai et al., 1998).

Peripheral glutamate receptor signalling is rapidly gaining credibility in a number of different fields. A recent report aimed at encouraging research into the role of glutamate signalling in cancer (which will be addressed with regards to megakaryocytes later in this chapter) and also possibly stem cell lineage selection and differentiation (Cavalheiro and Olney, 2001), suggests that studies of peripheral glutamate signalling is set to expand, perhaps focusing on aberrant signalling in tissue diseases. A minority still treat the role of the glutamate receptor outside the CNS with scepticism (Gray et al., 2001). However, the data presented by these authors would appear to be over-interpreted, due to the use of a competitive NMDA receptor antagonist D-APV, on osteoblastic and osteoclastic cells in high glutamate-containing media. The work described in this thesis will add to the escalating evidence demonstrating that glutamate may be considered as a more widely distributed "cytokine".

Furthermore, the findings of this study also have significant implications regarding the current understanding of megakaryocytopoiesis. The action of TPO, although profound on many aspects of megakaryocyte differentiation, does not regulate platelet release. This thesis provides fundamental evidence of a signalling system previously uncharacterised in megakaryocytes, which is of considerable importance to platelet formation and release *in vitro*. Many cytokines that regulate megakaryocytopoiesis are involved in various aspects of cell proliferation and differentiation and also affect several different cell types in addition to megakaryocytes. However, current data suggests that haematopoietic glutamate signalling is restricted only to cells of the megakaryocyte lineage (with exception to osteoclast progenitor cells) and appears to affect distinct stages of differentiation. Exactly where the megakaryocytic NMDA receptor fits into the more established signalling mechanisms remains unclear. Various signal transduction proteins activated by the NMDA receptor are the same as those activated by c-Mpl, but it is unclear as to whether the two signalling systems are able

to directly interact. Although the tyrosine kinase c-Mpl receptor is far removed from the ligand-gated ionotropic NMDA receptor, it is possible that these receptors activate convergent signalling pathways that are important in the normal regulation of megakaryocytopoiesis. As far as the implications of future megakaryocyte research are concerned, the NMDA receptor should at least be recognised as an interesting addition to the complex signalling hierarchy controlling normal megakaryocytopoiesis.

This thesis begins by identifying the continuing need for pharmacological agents with which to prevent thrombus-induced ischaemic disease. As acknowledged in chapter 1, current anti-thrombotic agents, such as the anti-coagulant aspirin and anti-platelet drugs, are to an extent, successful in lowering the incidence of CVD in high-risk patients. However, the direct prevention of platelet function can often have severe bleeding side effects and commonly a number of patients are resistant to these therapies. It is likely that by modulating platelet production rather than function, it would be possible to obtain a greater level of control over the blood clotting potential of high-risk patients. The data presented in this study suggests that, in the future, it may be possible to control platelet production by using therapeutic agents aimed at modulating the megakaryocytic NMDA receptor.

MK-801, the pharmacological antagonist used in these studies, although being a highly specific NMDA receptor antagonist and having marked effects on megakaryocyte differentiation and platelet production, would not be suitable as a therapeutic agent due to its ability to easily pass the blood-brain barrier, preventing normal synaptic neurotransmission and causing severe psychoses (Leppik et al., 1988). However, due to the potentially wide range of therapeutic applications of NMDA receptor antagonists, especially with regards to the treatment of neurodegenerative diseases such as stroke, Parkinson's disease and Alzheimer's disease, has lead to the creation of clinically well-tolerated NMDA receptor antagonists (Parsons et al., 1998). One such drug, now widely used as a neuroprotectant in Parkinson's disease, is memantine (for review see Parsons et al., 1999), a non-competitive NMDA receptor antagonist that displays minimal side effects (Riederer et al., 1991). How this is achieved is unclear, however it is thought that memantine and Mg^{2+} block the same or a similar site on the NMDA receptor channel. At pathological glutamate

concentrations, memantine remains in site and blocks the NMDA receptor ion channel. However at physiological concentrations, memantine dissociates from the receptor and allows the normal activation passage of calcium through the receptor channel (Blanpied et al., 1997; Sobolevsky et al., 1998), unlike MK-801 which can not be dislodged from the channel. Memantine would therefore prove a good candidate as an NMDA receptor antagonist in peripheral tissues, as it would not result in neurological abnormalities, although whether the high physiological concentrations of glutamate in neuronal cells is comparable to that of megakaryocytes is yet to be determined.

A more desirable alternative would be a non-competitive NMDA receptor that does not cross the blood-brain barrier and will therefore not affect CNS receptors. The development of such compounds however is currently restricted as they are specifically designed as neuroprotective drugs with the ability to cross the blood-brain barrier. As a result, it can only be assumed that blood-brain barrier-impermeable drugs do exist, but were not completely developed and therefore are not commercially available. Consequently these "failed" compounds, may be exploited to rapidly advance the clinical relevance of the megakaryocytic NMDA receptor as a therapeutic target to control platelet numbers in high-risk patients. It may also be possible discriminate between glutamate receptor expressing tissues by gene therapy by using tissue-specific promoters to alter receptor function therapeutically without affecting neuronal and peripheral receptors.

Considering the apparent importance of the NMDA receptor in normal megakaryocytopoiesis, it would also be of great interest to address expression of the receptor in myeloproliferative disorders (MPDs). As discussed in chapter 1.5, very few megakaryocytic disorders are as a result of aberrant TPO/c-Mpl signalling, making it increasing likely that such conditions occur as a result of yet unidentified dysfunctional signalling systems. It could be postulated that abnormal NMDA receptor expression and/or receptor function influences these conditions. For example, the considerable effects induced by point mutations of the NR1 subunit, such as those induced in NR1 transgenic (N598Q/R) mutants (Single et al., 2000) which completely prevent NMDA receptor function, may similarly prevent megakaryocytic NMDA receptor signal transduction. Interestingly, I did perform a small-scale study on human

bone marrow aspirates, comparing NR1 expression by megakaryocytes from control and ET patients. Only a few samples were studied, as ET is a relatively rare condition (largely due to the disorder being misdiagnosed or undiagnosed), it did appear that expression of NR1 was reduced in ET samples (data not shown). The implications of this are difficult to interpret, as the data presented here indicates that reduced NMDA receptor signalling should prevent platelet production, whilst the major characteristic of ET is a greatly increased number of circulating platelets. To address NMDA receptor dysfunction with regards to MPDs such as ET, PV and AMM would require extensive retrospective molecular and histological investigations, but may prove to be extremely interesting.

7.2 The megakaryocytic NMDA receptor – Concluding remarks

It is nearly 50 years since L-glutamate was first championed as a major neurotransmitter in the CNS (Hayashi, 1954) although its full importance in neuronal synaptic transmission was not realised until over 20 years later (Watkins and Evans, 1981; Foster and Fagg, 1984). In the last decade, the key importance of glutamatergic signalling in memory and learning, in addition to the implications in neurodegenerative disorders has been realised. With glutamate now universally accepted as the major neurotransmitter in the brain, it seems strange that when the role of glutamate was first being postulated, there was considerable scepticism that a simple amino acid, abundantly available in the brain, would act as a neurotransmitter (for review see Watkins, 2000). However, with time and skilled research, the sceptics were overcome as the fundamental roles of glutamate in the CNS were revealed.

Overall, glutamate signalling in peripheral tissues is at a similar developmental stage as the early days of neuroscience, with the signalling mechanism in megakaryocytes less well characterised than that in other cell types. This thesis significantly advances the understanding of the megakaryocytic NMDA receptor and puts it on a par with some of the better-characterised glutamatergic tissues. One question often posed with regards to peripheral glutamatergic signalling, is why these tissues require a receptor type specifically intended for rapid responses at the neuronal synapse? With reference to the megakaryocyte receptor, the high-speed response of the receptor is almost certainly not as important as the long-term effects of NMDA receptor-mediated

protein phosphorylation and gene transcription. Indeed, with mounting evidence of peripheral glutamate signalling, classifying the glutamate receptor as a "neuroreceptor" may be a misnomer. It appears that each tissue in which glutamate signalling has been identified express certain receptors types, subunits and associated proteins, of which the megakaryocyte is one such example, indicating that the each cell type only express the type of receptor required for its specific function.

Only future work regarding the molecular characterisation and the detailed function of the receptor will truly clarify the importance of the megakaryocytic NMDA receptor. The data presented in this thesis provides a stable grounding on which to continue and expand such analyses. To borrow a line from Winston Churchill when he addressed the Lord Mayor's Luncheon, London in November 1942: "This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning".

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Abbreviations

AA	arachidonic acid (not the first time this is used)
AChE	acetylcholinesterase
ACI	acetylcholine iodide
ADP	adenosine diphosphate
AGM	aorta-gonad-mesonephros
AMM	agnogenic myeloid metaplasia
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ANOVA	analysis of variance
ATCC	American tissue culture collection
ATF-1	associated transcription factor-1
BFU-MK	burst forming unit megakaryocyte
BFU-E/MK	burst forming unit-erythroid / megakaryocyte
BMEC	bone marrow endothelial cells
BMSC	bone marrow stromal cells
BNPI	brain-specific Na^+-dependent inorganic phosphate (Pi) transporter I
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium concentration
CaM	calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
cDNA	complementary DNA
CFM-2	1-(4'Aminophenyl)-3, 5-dihydro-7, 8-dimethoxy-4H-2, 3-benzodiazepin-4-one
CFU-GM	CFU-granulocyte-macrophage
CFU-MK	Colony forming unit megakaryocyte
chapsyn-110	channel associated protein of synapses-110
CHD	coronary heart disease
CHO	Chinese hamster ovary
CML	chronic myeloid leukaemia

CNS	central nervous system
CNTF	ciliary neurotrophic factor
CREB	cAMP response element binding protein
CT-1	cardiotrophin-1
CVD	cardiovascular disease
DAB	3, 3`-diaminobenzidine
DAG	diacylglycerol
D-AP5	D-(-)-2-amino-5-phosphonopentanoic acid
dCTP	deoxycytosine triphosphate
dH_2O	distilled H_2O
Dlg	Disks large
DMS	demarcation membrane system
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNPI	differentiation-associated BNPI
dNTP	deoxyribonucleotide triphosphate
dpm	dissintegrations per minute
DTT	dithiothreitol
EAAT-1	excitatory amino acid transporter
EDTA	ethylenediaminetetraacetic acid
EMSA	electromobility shift assay
EPO	erythropoietin
ERK1/2	extracellular signal regulated kinase 1/2
ESPC	excitatory postsynaptic current
ET	essential thrombocythaemia
FBS	foetal bovine serum
FGFβ	fibroblastic growth factor β
FITC	fluorescein isothiocyanate I
FN	fibronectin
FOG	friend of GATA
GAP	GTPase activating protein
GAP-43	growth associated protein-43

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
GPS	grey platelet syndrome
GTPase	guanosine triphosphatase
HBSS	Hank's buffered salt solution
HEK	human embryonic kidney
HEL	human erythroleukaemia
HSC	haematopoietic stem cell
IgG	immunoglobulins G
IL	interleukin
IMDM	Iscove's Modified Dulbecco's Medium
Indo-1-AM	1H-Indole-6-carboxylic acid, 2-[4-[bis[2-[(acetyloxy)methoxy]-2- oxoethyl]amino]-3-[2-[2-[bis[2- [(acetyloxy)methoxy]-2-oxoetyl]amino]-5- methylphenoxy]ethoxy]phenyl]-, (acetyloxy)methyl ester
IP_3	inositol triphosphate
ITP	idiopathic thrombocytopenic purpura
JAK	Janus kinase
KA	kainate
kainate	[2S-(2a, 3b, 4b)]-2-carboxyl-4-(1-methylethenyl)-3-pyrrolidinaecetic acid
kb	kilobases
kD	kilodalton
KN-62	(S)-5-isoquinolinesulfonic acid, 4-[2-[(5-isoquinolinesulfonyl)]]
KN-92	2-[N-(4-methoxynemzenesulfnoyl)]amino-N-(4-chlorocinnamyl)-N-methyl-benzlamine
KN-93	N-(2-[N- [4-chlorocinnamyl]-N-methylaminmethyl]phenyl)-N-(2-hydroxymethyl)-4-methoxysulfonamide
LIF	leukaemia inhibitory factor

LTD	long-term depression
LTP	long-term potentiation
m	meter
μ	micro
M	molar
MACS	magnetic activated cell sorting
MAGUK	membrane associated guanylate cyclases
MALS	mammalian analogue LIN-7
MAPK	mitogen activated protein kinase
mGluR	metabotropic glutamate receptors
min	minute
MK-801	5-Methyl-10, 11-dihydro-5H-dibenzo-[α,δ] cyclohepten-5, 10-imine
ml	millilitre
MOPS	3-[N-morpholino] propanesulphonic acid
MPD	myeloproliferative disorders
mRNA	messenger-ribonucleic acid
MTT	methylthiotetrazole
n	nano
$NADP^+$	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide hydrogen phosphate
neo	neomycin
NF-E2	nuclear factor-erythrocyte 2
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NR	NMDA receptor
PAF	platelet-activating factor
PBS	phosphate buffered saline
PCP	phencyclidine
PDGF	platelet-derived growth factor
PDZ	PSD-95/Dlg/ZO-1
PF4	platelet factor 4

PI	propidium iodide
PI3-K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKD	protein kinase D
PLC	phospholipase C
PMA	phorbol myristate acetate
PP1	protein phosphatase 1
PPDA	cis-1-[phenanthren-2yl-carbonyl] piperaxzin-2, 3-dicarboxylic acid
PRP	platelet rich plasma
PS	phosphatidyl serine
PSD	postsynaptic density
PSD-95	postsynaptic density-95
PV	polycythaemia vera
RACK-1	receptor for activated C-kinase-1
RANK ligand	receptor activator of NF-kB
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minutes
RT-PCR	reverse transcriptase polymerase chain reaction
SAP-90	synaptic associated protein-90
SCF	stem cell factor
SDS	sodium dodecyl sulphate
SEM	scanning electron microscope
SHANK	SH3 domain ankryin repeat
siRNAs	small interfering RNAs
SNARE	soluble N-ethyl maleimide-sensitive factor attachment protein receptors
(v)-SNARE	vesicle-SNARE protein
(t)-SNARE	target-SNARE protein
SSC	sodium citrate
STATs	signal transducers and activators of transcription

SynGAP	synaptic GTPase activating protein
TBE	Tris-borate EDTA
tbr-1	transcription brain-1
TEM	transmission electron microscopy
TFIIB	transcription factor IIB
TGFβ1	transforming growth factor β 1
TPEN	*N,N,N',N'*-tetrakis-[2-pyridmethyl]-ethylenediamine
TPO	thrombopoietin
TRIS	tri (hydroxy) methyl aminomethane
TRITC	tetramethyl rhodamine isothiocyanate
UCB	umbilical cord blood
U.V.	Ultraviolet
VGLUT1	vesicular glutamate transporter 1
VGLUT2	vesicular glutamate transporter 2
vWf	von-Willebrand factor
ZO-1	zona occludens protein 1

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