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An Investigation into $GRIN2A$ Mutations in Human Melanoma

Stacey Ann Nicole D’mello

A thesis submitted in complete fulfillment of the requirements for the degree of Doctor of Philosophy in Molecular Medicine, The University of Auckland, 2016.
Abstract

Previous exome sequencing studies have reported that the GRIN2A gene is frequently mutated in melanoma. GRIN2A encodes the regulatory GluN2A subunit of the N-methyl-D-aspartate receptor (NMDAR), a glutamate gated calcium ion channel. This thesis investigated the prevalence and significance of GRIN2A mutations in melanoma cell lines developed at the Auckland Cancer Society Research Centre. It also investigated other aspects of NMDAR activity in melanoma proliferation and invasion.

Sanger sequencing revealed that amongst 20 cell lines, 20% carried five non-synonymous mutations in GRIN2A. Patients from whom GRIN2A-mutant cell lines were developed showed faster disease spread to lymph nodes and distant organs and shorter overall survival. All non-synonymous GRIN2A substitutions were computationally predicted to disrupt GluN2A function. The G762E mutation, located in the hinge region of the glutamate-binding domain was modelled into the crystal structure of GluN2A, which predicted that the G762E mutation could alter NMDAR subunit interactions.

Transcripts of genes encoding GluN1, the structural subunit of the NMDAR and five of six possible regulatory subunits were present in all melanoma cell lines, with some heterogeneity in expression. Western blotting detected the GluN1 protein in all melanoma cell lines; in contrast, GluN2A expression was detected in only one cell line. NMDAR functionality as a calcium ion channel was suggested from studies that monitored cytosolic calcium fluxes in melanoma cells in response to NMDAR agonists. NMDAR inhibitors exerted anti-proliferative and anti-invasive effects on melanoma cells. However, no association was found between the presence of GRIN2A mutations and melanoma cell proliferation or invasion. Lastly, melanoma cells were found to release glutamate when cultured in medium containing less glutamate, and cells carrying GRIN2A mutations expressed the transcriptional repressor Slug.

We present a model in which glutamate is required for melanoma progression and is secreted by the cells themselves. We conclude that cells escape glutamate-mediated cytotoxicity by down regulating NMDAR expression or accumulating mutations in GRIN2A, indicating a tumour suppressor role of this subunit. Under low glutamate concentrations encountered in vivo, GRIN2A mutations may confer an oncogenic effect that facilitates melanoma cell
survival, proliferation and invasion. Thus, *GRIN2A* can act as an oncogene or a tumour suppressor, in a context dependent manner dictated by extracellular glutamate levels.
Dedication

Terence and Linda D’mello

Joy and Alishia Rane

Janice Aish

The melanoma patients who make these studies possible
Acknowledgements

“Trust in the Lord with all your heart, and lean not on your own understanding; in all your ways acknowledge Him, and He shall direct your paths.” Proverbs 3:5-6. Firstly, thank You Jesus for the opportunity and the ability to complete this study. May this thesis and the skills learned be used to serve You always.

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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>α-MEM</td>
<td>alpha-minimum essential medium</td>
</tr>
<tr>
<td>µg</td>
<td>micrograms</td>
</tr>
<tr>
<td>µm</td>
<td>micrometers</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>H</td>
<td>tritiated thymidine</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>ACSRC</td>
<td>Auckland Cancer Society Research Centre</td>
</tr>
<tr>
<td>AP5</td>
<td>(2R)-amino-5-phosphonovaleric acid; (2R)-amino-5-phosphonopentanoate</td>
</tr>
<tr>
<td>ATD</td>
<td>amino terminal domain</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaMK</td>
<td>calcium/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>coding deoxyribose nucleic acid</td>
</tr>
<tr>
<td>cm</td>
<td>centimeters</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CTD</td>
<td>carboxy terminal domain</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovines Serum</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>HEMa-LP</td>
<td>human epidermal melanocytes</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
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<td>L</td>
<td>liters</td>
</tr>
<tr>
<td>M</td>
<td>moles per liter</td>
</tr>
<tr>
<td>MDM</td>
<td>mouse double minute</td>
</tr>
<tr>
<td>mg</td>
<td>milligrams</td>
</tr>
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</table>
| MK-801       | [5R,10S]-[+] 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-
5,10-imine
mRNA messenger ribonucleic acid
Mut mutant
NCBI National Centre for Biotechnology Information
nM nanomolar
NMDA N-methyl-D-Aspartate
NMDAR N-methyl-D-Aspartate receptor
NTD amino terminal domain
PBS phosphate buffer saline
PCR polymerase chain reaction
PDZ PSD-95, discs large, zonula occludens-1
PI3-K phosphatidylinositol 3-kinases
PKA protein kinase A
PKC protein kinase C
PLC phospholipase C
PSD-95 Postsynaptic density protein-95
RNA ribose nucleic acid
RPMI Roswell Park Memorial Institute medium
RT-PCR real time polymerase chain reaction
SIFT sorting tolerant from intolerant
SNP single nucleotide polymorphism
UV ultraviolet
WT Wildtype
Co-authorship form

This form is to accompany the submission of any PhD that contains research reported in published or unpublished co-authored work. Please include one copy of this form for each co-authored work. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements.

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Chapter Three: Evidence that GRIN2A mutations in melanoma correlate with decreased survival

<table>
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<tr>
<th>Nature of contribution by PhD candidate</th>
<th>Stacey Ann N. D'mello conducted experimental work, analysed data, and wrote the manuscript</th>
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<td>Extent of contribution by PhD candidate (%)</td>
<td>80%</td>
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**CO-AUTHORS**

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of Contribution</th>
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<tbody>
<tr>
<td>Maggie L. Kalev-Zylinska</td>
<td>Designed the study, helped obtain and analyse clinical data, provided supervision, and helped write the manuscript</td>
</tr>
<tr>
<td>Bruce C. Baguley</td>
<td>Helped design the study, provided supervision and advice</td>
</tr>
<tr>
<td>Graeme J. Finlay</td>
<td>Provided supervision and edited the manuscript</td>
</tr>
<tr>
<td>Jack U. Flanagan</td>
<td>Supervised structural modeling of mutation impact</td>
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<tr>
<td>Taryn N. Green</td>
<td>Provided advice and assisted experimental procedures</td>
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<tr>
<td>Euphemia Y. Leung</td>
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<td>Marjan E. Askanan-Amir</td>
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</tr>
<tr>
<td>Wayne Joseph</td>
<td>Established melanoma cell lines from tumours, assisted experimental procedures</td>
</tr>
<tr>
<td>Michael R. McCrystal</td>
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<td>Richard J. Isaacs</td>
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<tr>
<td>James H. F. Shaw</td>
<td>Contributed patient samples</td>
</tr>
<tr>
<td>Christopher E. Furneaux</td>
<td>Contributed patient samples</td>
</tr>
<tr>
<td>Matthew J. During</td>
<td>Provided mentorship and advice</td>
</tr>
</tbody>
</table>

**Certification by Co-Authors**

The undersigned hereby certify that:
- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

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<td>Matthew J. During</td>
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CHAPTER 1 Literature review

1.1. Introduction

Cancer is one of the leading causes of death worldwide. Melanoma is a type of skin cancer and New Zealand has the highest rate of melanoma incidence and mortality in the world (Globocan 2015, Schadendorf, Fisher et al. 2015) (Figure 1.1). Melanoma is considered one of the most aggressive forms of skin cancer due to its highly invasive and metastatic phenotype (Gaggioli and Sahai 2007, Sanchez and Aplin 2014). Despite decades of public prevention campaigns there is still a continuing increase in melanoma incidence rates worldwide and the highest incidence rates have been observed for individuals with high socioeconomic status (Schadendorf, Fisher et al. 2015). Recent statistics have shown that rates of melanoma risk and incidence in New Zealand have risen over the past ten decades, currently reporting over 2000 diagnoses and 300 deaths per year (Figure 1.2) (Ministry Of Health 2014, Ministry Of Health 2014). These reports indicate that melanoma undoubtedly poses a significant threat to the health of New Zealanders. Further investigations into its pathology and treatment are warranted.

Melanoma has been studied at a physiological, cellular and molecular level in an attempt to decipher the causes and mechanisms by which it progresses, with the ultimate goal of establishing treatment and preventative measures. This thesis focuses on studying a proposed molecular and cellular mechanism of action by which melanoma may progress. This chapter describes melanoma in light of current published literature and describes possible associations with a glutamate receptor family, the N-methyl-D-aspartate receptors (NMDARs). The NMDAR family and its signalling pathways have been well characterised in the central nervous system (CNS) but very little is known about their biological relevance in melanoma.
Figure 1.1: Incidence and mortality of cutaneous melanoma worldwide
Comparative bar graphs showing the incidence (blue) and mortality (red) rates of cutaneous melanoma worldwide. Adapted with permission from Globocan, 2015; Schadendorf, Fisher et al., 2015. ASR: Age Specific Rate (world).
Figure 1.2: Incidence and mortality rate of melanoma in New Zealand from 1948 to 2012

The incidence and mortality rate of melanoma in New Zealand from 1948 to 2012.
1.2. Melanoma

1.2.1. Melanoma, a cancer of melanocytes

Melanoma is a form of cancer that begins in melanocytes, dendritic cells of the neuroectoderm that produce the melanin pigment (Tadokoro, Yamaguchi et al. 2005, Bonaventure, Domingues et al. 2013, NCI 2015, Schadendorf, Fisher et al. 2015). Melanoblasts, the precursor cells of melanocytes, are unpigmented and originate from embryonic neural crest cells (Sviderskaya, Hill et al. 2001, Lei, Virador et al. 2002) (Figure 1.3A). Melanoblasts are able to migrate to various regions of the body to develop into cells other than melanocytes such as those of the peripheral nervous system, bone and cartilage of the head and the choroid of the eye. Melanoblast survival and migration depend on a number of signalling systems and transcription factors including the tyrosine kinase receptor - KIT, and its ligand, stem cell factor (SCF), as well as the microphthalmia-associated transcription factor, MITF (Hou, Panthier et al. 2000). These pathways are frequently studied and have been reported to be defective in melanoma (Chin, Garraway et al. 2006, Hodis, Watson et al. 2012, Tsao, Chin et al. 2012) (Figure 1.3B).

Melanocytes are predominantly found in the basal layer of skin epidermis (Gibbs, Murli et al. 2000), and can be identified by the expression of melanocyte specific markers such as tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), DOPAchrome tautomerase or tyrosinase-related protein-2 (DCT/TYRP2), premelanosome protein 17 (Pmel17/gp1000), melan-A or melanoma antigen recognised by T cells 1 (MART-1) and MITF (Passeron, Coelho et al. 2007). The primary function of melanocytes is the production of the melanin pigment. The molecular structure of melanin is well suited to absorbing ultraviolet (UV) and visible light and thus it serves as protection against UV radiation from sunlight (Anderson and Parrish 1981, Haider, Cho et al. 2014). Melanocytes are also found in other tissues of the body such as the CNS, cardiovascular, cochlear and even adipose tissue (Plonka, Passeron et al. 2009, Bastian 2014). Melanocytes are surrounded by a number of keratinocytes (one melanocyte is surrounded by approximately 36 keratinocytes) (Seiberg 2001, Lin and Fisher 2007) to which they transfer their melanin pigment (Lin and Fisher 2007, Delevoye 2014) (Figure 1.3B).
1.2.1.1. Melanocytes and pigmentation

Melanosomes are subcellular lysosome-like organelles of melanocytes within which melanin pigments are synthesised and stored (Marks and Seabra 2001) before distribution to keratinocytes (Figure 1.3C). The number of melanosomes in keratinocytes contributes to the differences seen in human skin pigmentation (Lin and Fisher 2007). UV radiation can stimulate pigmentation by influencing melanin production (via α-melanocyte-stimulating hormone, α-MSH) and melanocyte distribution in the skin (Tadokoro, Yamaguchi et al. 2005, Plonka, Passeron et al. 2009). The pigmentation renders the skin resistant to subsequent sunburn (Plonka, Passeron et al. 2009).

There are two types of melanin (from dopaquinone precursors) in mammals: the brownish black eumelanin and the reddish yellow phaeomelanin (Thody, Higgins et al. 1991, Lamoreux, Wakamatsu et al. 2001). A higher overall melanin density results in darker skin (Anderson and Parrish 1981, Haider, Cho et al. 2014), but the eumelanin to phaeomelanin ratio also contributes to the differences seen in human skin pigmentation (Lin and Fisher 2007). Individuals with melanocytes that make more phaeomelanin than eumelanin tend to have lighter skin and are more prone to blistering and burning (Gupta 2014, Wu, Han et al. 2014). Skin that has phaeomelanin also produces more reactive oxygen species which can accelerate carcinogenesis, compared with skin that produces eumelanin or has no melanin (Chedekel, Agin et al. 1980, Okazaki, Funasaka et al. 2015).

Melanocytes express the melanocortin 1 receptor (MC1R). MC1R regulates melanin production and is controlled by the α-MSH and adrenocorticotropic hormone (ACTH) ligands (Millington 2006). α-MSH is cleaved from a precursor protein called pro-opiomelanocortin (POMC) produced by epidermal keratinocytes (Tsatmali, Ancans et al. 2002). Eumelanin synthesis is stimulated via α-MSH and ACTH while phaeomelanin synthesis is simulated via agonist stimulating protein (ASP) (Le Pape, Passeron et al. 2009). α-MSH regulates phaeomelanin and eumelanin proportions via the MC1R (Valverde, Healy et al. 1995) (Figure 1.3B). The pH within a melanosome can also determine the rate of melanin production and eumelanin to phaeomelanin ratios (Ancans, Tobin et al. 2001) (Figure 1.3C).

The KIT ligand–KIT receptor tyrosine kinase and the α-MSH-MCR1 signalling pathways are involved in melanocyte pigmentation and development via the activation of MITF transcription factor (the M-MITF isoform is specific to the melanocyte lineage) (Flaherty, Hodi et al. 2012, Bonaventure, Domingues et al. 2013). MITF-target genes regulate
melanocyte pigmentation (such as TYR tyrosinase), but also migration, differentiation, proliferation and survival. Details of this the MITF are described in section 1.2.7 (Flaherty, Hodi et al. 2012, Bonaventure, Domingues et al. 2013) (Figure 1.3).

Melanosomes translocate from melanocytes to keratinocytes takes place by phagocytosis via the Protease Activated Receptor – 2 (PAR2), which is activated by UVB radiation and regulated by $\alpha$-MSH (Seiberg 2001, Virador, Muller et al. 2002). Pigment filled melanosomes move from the perinuclear region to the tips of the melanocyte dendrites along microtubules. From here they translocate and disperse into keratinocyte cytoplasm eventually capping keratinocyte nuclei (Seiberg 2001) (Figure 1.3C).
(A) Melanocyte precursors, melanoblasts originate from embryonic neural crest cells. (B) Melanoblast survival and migration depends on signalling systems and transcription factors including tyrosine kinase receptor KIT and its ligand, melanocortin 1 receptor (MC1R) and its ligands, α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH). Pathways activated downstream of KIT and MC1R include cyclic adenosine monophosphate (cAMP) and mitogen activated protein kinase (MAPK) respectively. (C) Melanocytes are found in the basal layer of skin epidermis where they distribute melanin pigment containing melanosomes to keratinocytes. Panels A and C were drawn using Servier Medical Art.
1.2.1.2. Melanocytes and the inflammatory response

Melanocytes are also phagocytic cells that play a role in the inflammatory response. They respond to inflammatory events in the epidermis by producing more or less melanin (hyperpigmentation or hypopigmentation, respectively) (Plonka, Passeron et al. 2009). α-MSH downregulates the immune response following damage preventing autoimmunity but it also induces DNA damage repair. In addition to α-MSH, melanocytes produce other substances that regulate and enable crosstalk between different cell types in the epidermis. α-MSH and ACTH peptides produced in the epidermis induce nitric oxide production in melanocytes. Moreover, melanocortins may regulate the release of cytokines, catecholamines (CA), and serotonin (5HT) from melanocytes (Tsatmali, Ancans et al. 2002). Melanin has also been found to counteract the effects of damage caused by reactive oxygen species in apoptotic tissue (Plonka, Passeron et al. 2009).

1.2.2. Melanoma classification and staging

Melanoma develops from melanocytes in the epidermal layer of the skin, but can originate from any anatomical area occupied by melanocytes (Tsao, Chin et al. 2012). Benign neoplastic cells that arise from a melanocytic lineage are called naevi and those that become malignant are called melanoma. The malignant melanoma can in some cases arise from a precursor naevus that is outgrown by the melanoma, but in most cases no benign precursor state can be traced (Bastian 2014).

The AJCC (American Joint Committee on Cancer) system of staging melanoma separates patients into stages of disease progression based on the tumour-node-metastases (TNM) classification system. The TNM system describes the size, nature and spread of tumours (Table 1.1). It is based on the evolution of primary tumours (T) to their regional lymphatic (N) or distant metastatic (M) spread (Balch, Buzaid et al. 2001, Mohr, Eggermont et al. 2009).

The T category thresholds define primary melanoma thickness (T1-T4 representing sizes from < 1 mm to > 4 mm). T subcategories, Ta and Tb, are defined by the absence or presence of ulceration respectively. N categories describe tumours that have spread to the lymph node. N1-N3 reflects the number of lymph node metastases, “a” and “b” subcategories of N represent microscopic and macroscopic lymph node involvement, respectively. Satellite metastases or metastases in transit also fall under the N category (N2c and N3). The M category describes tumours that have metastasised to distant regions. M is split into three subcategories based on
the metastasis location and elevated levels of lactate dehydrogenase (LDH) (Ma, M1b and M1c) (Table 1.1) (Balch, Buzaid et al. 2001, Mohr, Eggermont et al. 2009).

Clinical and pathological staging of tumours is based on the TNM categorisation described above. Stages range from stage I, comprising those patients at lowest risk, to Stage IV, comprising those patients at highest risk of mortality (Figure 1.4). A schematic representation of the pathological features associated with the four main stages of melanoma progression is presented in Figure 1.5. Each stage is divided into sub-stages based on tumour size, ulceration and the extent of metastases as specified by TNM categorisation thresholds (Tables 1 and 2). Pathologic staging includes microstaging of the primary melanoma along with pathological information about the regional lymph nodes after partial or complete lymphadenectomy. Techniques such as ultrasound, X-Ray, MRI, PET/CT as well as measurement of serum S100B and LDH are used for staging (Mohr, Eggermont et al. 2009). Clinical staging is conducted after complete excision and microstaging of the primary melanoma with an assessment for regional and distant metastases (Balch, Buzaid et al. 2001).
### TNM Classification

<table>
<thead>
<tr>
<th>TX</th>
<th>Primary tumour cannot be assessed (shave biopsy, regressed primary)</th>
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<tbody>
<tr>
<td>Tis</td>
<td>Melanoma in situ</td>
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#### T classification

<table>
<thead>
<tr>
<th>Thickness</th>
<th>Ulceration status</th>
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<tr>
<td>( \leq 1.0 \text{ mm} )</td>
<td>a: without ulceration and level II/III</td>
</tr>
<tr>
<td>1.01-2.0 mm</td>
<td>b: with ulceration or level IV/V</td>
</tr>
<tr>
<td>2.01-4.0 mm</td>
<td>a: without ulceration b: with ulceration</td>
</tr>
<tr>
<td>&gt; 4.0 mm</td>
<td>a: without ulceration b: with ulceration</td>
</tr>
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#### N classification

<table>
<thead>
<tr>
<th>No. of metastatic nodes</th>
<th>Nodal metastatic mass</th>
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</thead>
<tbody>
<tr>
<td>1 node</td>
<td>a: micrometastasis*</td>
</tr>
<tr>
<td>2-3 nodes</td>
<td>a: micrometastasis*</td>
</tr>
<tr>
<td>4 or more metastatic nodes, or matted nodes, or in transit met(s)/satellite(s) with metastatic node(s)</td>
<td>b: macrometastasis† c: in transit met(s)/satellite(s) without metastatic nodes</td>
</tr>
</tbody>
</table>

#### M classification

<table>
<thead>
<tr>
<th>Site</th>
<th>Serum lactate dehydrogenase level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distant skin, subcutaneous, or nodal mets</td>
<td>Normal</td>
</tr>
<tr>
<td>Lung metastases</td>
<td>Normal</td>
</tr>
<tr>
<td>All other visceral metastases</td>
<td>Normal</td>
</tr>
<tr>
<td>Any distant metastasis</td>
<td>Elevated</td>
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*Micrometastases are diagnosed after sentinel or elective lymphadenectomy.†Macrometastases are defined as clinically detectable nodal metastases confirmed by therapeutic lymphadenectomy or when nodal metastasis exhibits gross extracapsular extension.

**Table 1.1: TNM classification of melanoma**

TNM classification of melanoma extracted with permission from (Balch, Buzaid et al. 2001, Mohr, Eggermont et al. 2009)
Figure 1.4: Survival curves for stage groupings of patients with melanoma

Fifteen-year survival curves for the stage groupings of patients with localized melanoma. Numbers of patients from the AJCC melanoma staging database are shown in brackets. Differences between the survival curves are significant (P < .0001). Adapted with permission from Globocan, 2015; Schadendorf, Fisher et al., 2015.
Stage 0 and I

Patients with melanoma-in-situ are categorised as Tis and considered to be in stage 0 (clinical and pathological staging). Tumours at Stage I are those detected to be primary melanoma of up to 2 mm thickness that have not undergone regional or distant metastases (Figure 1.5). Stage I is subdivided into stage IA (without ulceration, T1a) and IB (with ulceration, T1b, T2a) (Balch, Buzaid et al. 2001, Mohr, Eggermont et al. 2009) (Table 2).

Stage II

Patients in Stage II (clinical and pathological staging) have tumours > 1.01 mm thick without evidence of regional lymphatic or distant metastases (Figure 1.5). Stage II is subdivided into Stage IIA, Stage IIB and Stage IIC depending on tumour size and the presence of ulceration (range from T2b, T3a, T3b, T4a and T4b) (detailed in Table 1 and 2) (Balch, Buzaid et al. 2001, Mohr, Eggermont et al. 2009).

Stage III

Patients in Stage III (clinical staging) have lesions of any size with pathological documentation of regional lymphatic spread or metastases in transit (Any T and N1-N3) (Figure 1.5). Stage III is not subdivided for clinical staging. Pathological Stage III grouping is subdivided into Stage IIIA, Stage IIIB and Stage IIIC depending on the number of types (micro or macroscopic) of lymph node or transit metastases, primary tumour size and the presence of ulceration in primary or metastatic lesions (Table 2).

Stage IV

Patients with Stage IV disease (clinical and pathological staging) have tumours with evidence of distant metastases (Figure 1.5) that fall under any of the previously defined (Table 1) M1 categories (Mohr, Eggermont et al. 2009). These are based on metastatic location such as skin, subcutaneous tissue, lymph nodes, lung or other visceral or distant sites and elevated levels of LDH. The diagnosis of metastatic melanoma is generally associated with poor prognosis. By stage IV the expected median patient survival is 6 to 9 months with a 10% to 20% 5-year survival rate (Agarwala, Kirkwood et al. 2004).
Figure 1.5: Stages of melanoma progression

At Stages I and II melanoma tumours spread into the epidermis and dermis of the skin, respectively. At Stage III the tumours reach the subcutaneous layer and metastasise to the lymph node. By Stage IV tumours have grown deep into the subcutaneous layer and metastasise to distant organs. Adapted with permission from Melanoma Foundation (New Zealand, 2011).
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<th>Pathologic staging†</th>
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<td>0</td>
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<td>Tis N0 M0</td>
</tr>
<tr>
<td>IA</td>
<td>T1a N0 M0</td>
<td>T1a N0 M0</td>
</tr>
<tr>
<td>IB</td>
<td>T2a N0 M0</td>
<td>T2a N0 M0</td>
</tr>
<tr>
<td>IIA</td>
<td>T2b N0 M0</td>
<td>T2b N0 M0</td>
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<tr>
<td>IIB</td>
<td>T3b N0 M0</td>
<td>T3b N0 M0</td>
</tr>
<tr>
<td>IIC</td>
<td>T4b N0 M0</td>
<td>T4b N0 M0</td>
</tr>
<tr>
<td>III‡</td>
<td>Any T N1 M0</td>
<td>N2 N3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIA</td>
<td>T1-4a N1a M0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1-4a N2a M0</td>
<td></td>
</tr>
<tr>
<td>IIB</td>
<td>T1-4b N1a M0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1-4b N2a M0</td>
<td></td>
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<td></td>
<td>T1-4a N1b M0</td>
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<td></td>
<td>T1-4a N2b M0</td>
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</tr>
<tr>
<td></td>
<td>T1-4a/b N2c M0</td>
<td></td>
</tr>
<tr>
<td>IIC</td>
<td>T1-4b N1b M0</td>
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</tr>
<tr>
<td></td>
<td>T1-4b N2b M0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Any T N3 M0</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Any T Any N Any M1</td>
<td>Any T Any N Any M1</td>
</tr>
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*Clinical staging includes microstaging of the primary melanoma and clinical/radiologic evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases.

†Pathologic staging includes microstaging of the primary melanoma and pathologic information about the regional lymph nodes after partial or complete lymphadenectomy. Pathologic stage 0 or stage 1A patients are the exception; they do not require pathologic evaluation of their lymph nodes.

‡There are no stage III subgroups for clinical staging.

Table 1.2: Stage grouping for cutaneous melanoma

Stage grouping for cutaneous melanoma adapted from (Balch, Buzaid et al. 2001, Mohr, Eggermont et al. 2009).
1.2.3. Melanoma types

The World Health Organisation classifies melanoma into histopathological subtypes based on the Clark et al., 1986 convention which takes into account the morphological aspects and anatomical location of the primary tumour. This distinguishes four main types of melanoma: superficial spreading melanoma (SSM), lentigo maligna melanoma (LMM), nodular melanoma (NM), and acral lentiginous melanoma (ALM) (Clark Jr, Elder et al. 1986, Bastian 2014).

Superficial spreading melanoma is characterized by a radial or horizontal growth phase wherein melanocytes are arranged in localised solitary units in an upward moving or pagetoid pattern (Schadendorf, Fisher et al. 2015).

Nodular melanoma occurs exclusively in the vertical growth phase (Schadendorf, Fisher et al. 2015) that is, no melanoma in situ or melanoma in situ confined to no more than three adjacent rete ridges beyond the margins of the tumour nodule.

Lentigo maligna melanoma cells are characteristically singly dispersed along the dermal–epidermal junction and skin appendages. Signs of chronic UV radiation damage are prominent in this type of melanoma (Bastian 2014, Schadendorf, Fisher et al. 2015).

Acral lentiginous melanoma has cells present as single units along the dermal–epidermal junction and as confluent foci. This type of melanoma arises most commonly at acral sites but occasionally occurs at mucosal sites (Bastian 2014, Schadendorf, Fisher et al. 2015). Any of the major histopathologic subtypes or variants can be classified as amelanotic. Amelanotic melanoma lacks clinically evident pigment and often appears pink in colour.

Aside from the major subtypes, other well-defined histopathological variants of melanoma have been defined. These include naevoid melanoma which shows histopathological features of a banal naevus (or ‘small-cell melanoma’), spitzoid melanoma has histopathological features of a Spitz naevus, desmoplastic melanoma that has ‘spindle-shaped’ cells which are morphologically similar to fibroblasts found in scar tissue, ocular melanoma that arises within the uvea of the eye and mucosal melanoma which originates at a mucosal site such as the mouth, nasopharynx, larynx, conjunctiva, vagina or anus (Schadendorf, Fisher et al. 2015).
Although this method successfully describes clinical and histopathological aspects of the cancer it is limited in its impact on patient care particularly when patients are diagnosed at a later stage of disease progression (Clark Jr, Elder et al. 1986, Bastian 2014).

### 1.2.4. Melanoma associated risk factors

Some of the most common risk factors associated with melanoma include chronic exposure to UV light (Leiter and Garbe 2008), the occurrence of moles (naevi), age, gender (males are at higher risk), personal and family history of melanoma and fair skin colour that tans poorly (often dictated by ethnicity) (Gloster Jr and Neal 2006). There is substantial evidence for the mutagenic role of UV light and its characteristic genetic nucleotide substitution signature (G to T for UVA, C to T for UVB) in melanoma pathogenesis (Berger, Hodis et al. 2012, Hodis, Watson et al. 2012, Alexandrov, Nik-Zainal et al. 2013, Schadendorf, Fisher et al. 2015). However, some reports have shown that melanoma occurs on non-sun-exposed sites and UV radiation may not be the only vital etiologic factor (Gloster Jr and Neal 2006, Pleasance, Cheetham et al. 2010).

### 1.2.5. Melanoma in New Zealand

Europeans (generally fair skinned individuals who tan poorly) occupy the largest ethnic group in New Zealand (>65%) with other major groups, Maori, Asian and Pacific, each occupying under 20% of the population (Statistics New Zealand 2006, Sneyd and Cox 2009). A 2009 study showed that non-European ethnicities in New Zealand have a higher than expected risk of thick and more advanced melanoma, with poorer prognosis compared to Europeans. It was reported that nodular melanoma occurs more often in Maori (15.9%) and Pacific peoples (17.1%) compared with Asians (8.7%) and New Zealand Europeans (10.5%). The median thickness of melanoma was 0.78 mm in New Zealand Europeans, 1.2 mm in Maori, 2.5 mm in Pacific peoples, and 0.73 mm in Asians. 37% of melanomas in Pacific peoples were > 4 mm thick compared with 7.9% in New Zealand Europeans. In addition, about 13% of Asians and 11% of Pacific peoples, compared with 4% of New Zealand Europeans, were diagnosed with melanoma by histology of metastases rather than the primary lesion (Sneyd and Cox 2009).

### 1.2.6. Heritable risk genetic factors

Certain heritable genetic factors could predispose individuals to the risk of melanoma onset. 10 percent of melanoma patients have a family history of melanoma and this confers an approximately twofold increase in melanoma risk (Gandini, Sera et al. 2005). There have been
a number of recent discoveries from genome wide association studies (GWAS) that have linked genetic mutations to melanoma predisposition and progression. Such mutated genes include \textit{CDKN2A}, \textit{cKIT}, \textit{MITF} (Tsao, Chin et al. 2012), \textit{BAP1}, \textit{POT1}, \textit{CDK4} (Schadendorf, Fisher et al. 2015). Germline polymorphisms have been reported in pigment related genes such as \textit{ASIP} (agouti signalling protein), \textit{OCA2} (oculocutaneous albinism II), \textit{PAX3} (paired box 3), \textit{EDNRB} (endothelin receptor type B), \textit{SLC45A2} (solute carrier family 45 member 2), \textit{SOX10}, \textit{TYRP1} (tyrosinase-related protein 1) and \textit{TYR} (tyrosinase) (Schadendorf, Fisher et al. 2015).

\textbf{1.2.7. Melanoma associated genetic mutations}

Identification of genes associated with melanoma onset and progression has been useful for diagnostic, prognostic and treatment purposes. The most frequently reported mutations are in genes that encode cell surface receptors, intracellular signalling molecules or transcription factors, which are all linked directly or indirectly to cell cycle progression. The dysregulation generally arises from activating or inactivating mutations in one of more components of cell-cycle associated pathways (Clark Jr, Elder et al. 1986, Chin, Garraway et al. 2006, Namkoong, Shin et al. 2007, Ahmed Kausar Begam and Michael 2011, Hodis, Watson et al. 2012, Krauthammer, Kong et al. 2012, Tsao, Chin et al. 2012, Cheng, Zhang et al. 2013, Schadendorf, Fisher et al. 2015).

The two main types of receptors linked with driving melanoma cell proliferation are receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPRCs). Upon activation, these receptors initiate mitogenic signalling transduction pathways such as the mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways. (Tsao, Goel et al. 2004, Ahmed Kausar Begam and Michael 2011, Hodis, Watson et al. 2012, Tsao, Chin et al. 2012, Cheng, Zhang et al. 2013, Schadendorf, Fisher et al. 2015) (Figure 1.6).

One of the most extensively studied pathways in cancer is the MAPK pathway. The MAPK pathway is a key player in melanoma metastasis owing to its role in promoting cell proliferation, survival, invasion, and tumour angiogenesis (Le, Chan et al. 2010, Cheng, Zhang et al. 2013). In melanoma the MAPK pathway is often initiated by the activation of a RTK. Receptor ligands such as hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) signal via the RTK to activate the MAPK pathway (Lito, Rosen et al. 2013). The pathway consists of MAP kinases that sequentially transfer proliferative signals via a series of phosphorylation events from the cell surface to relevant transcription factors in the nucleus. Among the cascading MAPK pathways, the Ras-Raf-
MEK-ERK1 pathway (Figure 1.6) plays the most critical role in cell survival and proliferation (Ahmed Kausar Begam and Michael 2011, Cheng, Zhang et al. 2013). When the RTK is activated, it triggers the activation of Ras by inducing it into a GTP bound state. Ras activation leads to the recruitment of Raf from the cytosol to the cell membrane where it gets activated. Activated Raf phosphorylates MEK1/2 (MAP/ERK kinase) which in turn leads to the phosphorylation ERK1/2. ERK1/2 (extracellular-signal-regulated kinases) re-enters the nucleus to activate several transcription factors involved with cell cycle regulation such as ELK-1, MYC, CREB and FOS (Ahmed Kausar Begam and Michael 2011, Cheng, Zhang et al. 2013) (Figure 1.6).

A number of the aforementioned components involved in the RTK mediated MAPK pathway are found to be mutated in melanoma, disrupting cell cycle regulation. For instance, recurrent activating mutation in the GTPase RAC1 is found in 9.2% of sun-exposed melanomas (Krauthammer, Kong et al. 2012). The RAC1 mutation keeps the GTPase in a GTP-bound state which activates downstream PAK (p21 activated protein kinase) (Hodis, Watson et al. 2012). KIT encodes c-KIT, an RTK for SCF and its locus is found to be amplified or mutated in melanoma (Tsao, Chin et al. 2012). KIT is commonly mutated in acral and mucosal melanoma (Hodis, Watson et al. 2012). The V559A KIT mutation occurs in < 1% of melanomas overall but it occurs in 10-20% of acral and mucosal melanomas (Beadling, Jacobson-Dunlop et al. 2008, Hodi, Friedlander et al. 2008, Griewank, Scolyer et al. 2014). EGFR encodes the epidermal growth factor receptor (EGFR), a RTK and undergoes frequent polysomy (multiple copies) in advanced melanoma (Koprowski, Herlyn et al. 1985, Bastian, LeBoit et al. 1998, Udart, Utikal et al. 2001, Hodis, Watson et al. 2012). Although EGFR activation has been reported to promote metastatic progression in cell lines amplifying focal EGFR, activating mutations in EGFR have not been observed in melanoma. However, in vitro studies have suggested that ectopic expression of this receptor could enhance melanoma growth (Hodis, Watson et al. 2012).

BRAF is a well-studied gene in melanoma that encodes one of the Raf family cytoplasmic serine/threonine kinases, B-Raf. The B-Raf V600E driver mutation is one of the most common mutations and has been reported in 50% of melanomas (Chapman, Hauschild et al. 2011, Tsao, Chin et al. 2012, Jang and Atkins 2013, Wilson and Nathanson 2015). The B-Raf V600E results in a 500-fold increase in B-Raf activation. This confers the constitutive activation of downstream signalling through the MAPK pathway (Davies, Bignell et al. 2002, Curtin, Fridlyand et al. 2005). Melanomas have been found to retain high levels of MAPK
activation despite B-Raf inhibition through constitutively active mutants MEK1 or NRAS. Pathway restoration can occur through the amplification of BRAF copy number, the generation of alternative splice variants, B-Raf dimerization to mutant C-Raf or the upregulation of RTKs (Schadendorf, Fisher et al. 2015) (Figure 1.6).

Genetic evidence from studies in melanoma have also shown that the MAPK pathway co-operates with the PI3K pathway (Tsao, Goel et al. 2004, Dankort, Curley et al. 2009, Tsao, Chin et al. 2012, Schadendorf, Fisher et al. 2015). The PI3K pathway is often found to be hyper-activated in melanoma (Tsao, Chin et al. 2012, Martini, De Santis et al. 2014, Rosenberg, Niglio et al. 2015). PI3K can transduce signals received by RTKs, GPCRs and activated Ras (Tsao, Chin et al. 2012, Martini, De Santis et al. 2014, Schadendorf, Fisher et al. 2015) (Figure 1.6). PI3K phosphorylates inner membrane leaflet second messenger PIP2 to PIP3 (Martini, De Santis et al. 2014, Schadendorf, Fisher et al. 2015) (Figure 1.6). The PTEN tumour suppressor gene encodes a lipid and protein phosphatase and can antagonise the PI3K by negatively regulating PIP3 mediated signal transduction (Tsao, Chin et al. 2012, Martini, De Santis et al. 2014, Schadendorf, Fisher et al. 2015). Increased intracellular PIP3 levels trigger downstream events that lead to the phosphorylation of the serine/threonine kinase AKT by phosphoinositide-dependent kinase-1 (PDK1). AKT is an oncogene product capable of activating downstream signals that promote cell growth and survival (Tsao, Chin et al. 2012, Martini, De Santis et al. 2014). AKT phosphorylation is sufficient to activate the mammalian target of rapamycin complex 1 (mTORC1). Activation of mTORC1 results in increased protein synthesis and cell survival by direct phosphorylation of its effectors (S6 kinases and 4E-BPs) to relieve the block on protein translation (Tsao, Chin et al. 2012, Vanhaesebroeck, Stephens et al. 2012, Martini, De Santis et al. 2014). In melanoma, elevated phospho-AKT levels are inversely correlated with patient survival (Tsao, Chin et al. 2012) (Figure 1.6).

**CDKN2A** mutations are found in 40% of melanoma patients who have a family history of melanoma (Schadendorf, Fisher et al. 2015). **CDKN2A** encodes two different tumour suppressor genes; p16INK4A and p14ARF, through the use of alternative promoters and protein translation in different reading frames. p16INK4A regulates cell cycle progression by interacting with cyclin dependent kinases (CDKs). p16INK4A binds and inhibits CDK4/6 which prevents them from phosphorylating the retinoblastoma protein (RB). Hyper-phosphorylated RB drives G1-S transition by initiating the release of E2F1 transcription factor, an inducer of S-phase genes. Therefore, loss of p16INK4a, and the consequent loss of
CDK4/6 mediated RB inhibition, encourages G1–S transition and re-entry into the cell cycle (Tsao, Chin et al. 2012) (Figure 1.6).

p14ARF indirectly regulates tumour suppressor p53 via mouse double minute 2 homolog (MDM2). p53 promotes the rapid degradation of MDM2 (Figure 1.6). MDM2 is an E3 ubiquitin-protein ligase and targets p53 for destruction. The net effect of p14ARF loss is a destabilisation of p53 and the consequent impediment of its tumour suppressive function (Tsao, Chin et al. 2012, Schadendorf, Fisher et al. 2015) (Figure 1.6).

A recurrent mutation in TRRAP, a multiprotein coactivator complex that possesses histone acetyltransferase activity, has also been reported (Wei, Walia et al. 2011). This is known to play a central role in the transcriptional activities of p53, c-Myc and E2F1, all of which play key roles in generating cancer related phenotypes (McMahon, Van Buskirk et al. 1998, Barlev, Liu et al. 2001).

Mutations found in melanoma can occur as a consequence of UV induced damage from sunlight. For example, p16INK4A and p14ARF harbour UV-induced inactivating mutations and TP53 which encodes the tumour suppressor p53 has the highest number of UV-induced mutations among those reported in melanoma (Hodis, Watson et al. 2012). UVB mediated damage has been suggested to confer a survival advantage upon melanoma cells. (Schadendorf, Fisher et al. 2015). Mutagenic signatures that arise as a consequence of UVA and UVB have been detected in mutational background noise (or passenger mutations) and in 46% of driver mutations such as those found in RAC1, STK19 (which encodes serine/threonine kinase 19), FBXW7 and IDH1 (which encodes isocitrate dehydrogenase 1) (Hodis, Watson et al. 2012, Schadendorf, Fisher et al. 2015). PPP6C, encoding a serine/threonine phosphatase, was discovered to be mutated in melanoma. These mutations were found to cluster in its active site domain in 12% of sun-exposed melanomas that already had mutations in BRAF or NRAS. Other reported melanoma associated gene candidates include SNX31, TACC1 and ARID2, (Hodis, Watson et al. 2012).

Melanomas can also develop from skin not subjected to sun damage, as seen by the lack of UV-induced nucleotide transition signatures (Bastian 2014). However, this does not fully exclude the possibility of UV involvement. Free radicals resulting from the biochemical interaction of UVA with melanin can act as secondary mutagens and can indirectly cause genetic aberrations (Bastian 2014, Schadendorf, Fisher et al. 2015). Mutations not attributed to
UV damage occur in the previously described MAPK pathways, which are of the highest oncogenic and therapeutic relevance in melanoma (Hodis, Watson et al. 2012, Schadendorf, Fisher et al. 2015). Mutations that do not carry a UV signature include \textit{BRAF} (V600E), \textit{NRAS} (Q61L/R), \textit{HRAS, KRAS, KIT} (V559A) and \textit{GNA11} (Q209L) (Schadendorf, Fisher et al. 2015).

\textit{CDKN2A} mutations, amplifications and alterations in \textit{MITF, PTEN} disruption and \textit{AKT/PI3K} activation are common genetic abnormalities reported in melanoma that offer potential therapeutic insights (Chin, Garraway et al. 2006, Hodis, Watson et al. 2012).

Population studies have reported that inactivating variants of the highly polymorphic melanocortin 1 receptor (MC1R) have been associated with red hair, poor tanning ability and increased melanoma risk. MC1R is a GPCR that signals through adenylate cyclase to induce expression of \textit{MITF}. \textit{MITF} is amplified in 4-21\% of melanomas. \textit{MITF} regulates pigmentation in response to α-MSH (Flaherty, Hodi et al. 2012, Schadendorf, Fisher et al. 2015). \textit{MITF} is phosphorylated by MAPK–ERK signalling, as well as by ribosomal S6 kinases downstream of KIT or MC1R activation. Signalling downstream of MC1R involves the activation of cAMP and CREB transcription factor which induces the expression of \textit{MITF}. The WNT pathway can also contribute to \textit{MITF} expression (Flaherty, Hodi et al. 2012) (Figure 1.3).

More recently, evidence for the involvement of glutamate signalling pathways in melanoma has been uncovered. Glutamate pathways occur through metabotropic G-protein coupled receptors and ionotropic receptors that are calcium channels (Rzeski, Turski et al. 2001, Wei, Walia et al. 2011, Prickett and Samuels 2012, Song, He et al. 2012, Prickett, Zerlanko et al. 2014, Rosenberg, Niglio et al. 2015). Overexpression of the GRM1 gene encoding metabotropic glutamate receptor GRM1 occurs frequently in melanoma and its ectopic expression in melanocytes was sufficient for neoplastic transformation \textit{in vitro} (Wen, Li et al. 2014). Genes that encode subunits of glutamate receptors have been found to be mutated in melanoma. These include activating mutations in GRM3 (Prickett, Wei et al. 2011) and mutations with unconfirmed functions in \textit{GRIN2A} (Wei, Walia et al. 2011). The \textit{GRIN2A} gene encodes subunit 2 of the N-methyl-D-aspartate receptor (NMDAR), found to be mutated in 33\% of human melanomas.

The NMDAR is a glutamate-gated calcium ion channel that is well studied in the CNS but little is known about its role in non-neuronal and cancerous tissues such as melanoma. The
relatively high frequency of mutations observed in *GRIN2A* suggests that this receptor may have a role in melanoma and hence has been investigated further in this thesis (Wei, Walia et al. 2011).
Figure 1.6: Signalling pathways commonly deregulated in melanoma

The most commonly deregulated pathways in melanoma include the Mitogen Activated Protein Kinase (MAPK) (purple) and Phosphoinositol-3-kinase (PI3K) (green) pathways linked to signalling via the G-protein coupled receptors (GPCRs) and Receptor Tyrosine kinases (RTK). Signalling associated directly with cell cycle progression include the p14 (light blue) and p16 (dark blue) pathways initiated CDKN2A expression.
1.2.8. Melanoma treatment

The treatment regimen for early stage (I and II) melanoma is surgical resection with adjuvant IFN-α-2b therapy. However this has poor efficacy in melanomas detected at later stages (III and IV) of progression (Siegel, Naishadham et al. 2012, Hao, Song et al. 2015).

Dacarbazine, a DNA alkylating agent, is one of the most common standard systemic treatments of melanoma (Serrone, Zeuli et al. 2000, Schindler and Postow 2014), but recent reports have shown that it has not achieved adequate response rates for metastatic lesions (Hao, Song et al. 2015). Temozolomide, an oral analogue of dacarbazine is a lipophilic DNA alkylating agent (Agarwala, Kirkwood et al. 2004, Zhang, Stevens et al. 2012, Schindler and Postow 2014) and is one of the most effective systemic single chemotherapy agents against metastatic melanoma, second to dacarbazine (Augustine, Yoo et al. 2009). Its effectiveness is attributed to its small size and lipophilic nature that allows its passage across the blood brain barrier (Agarwala and Kirkwood 2000). This is useful because metastatic melanomas often spread to the brain. Aside from the neurotoxic effects, dacarbazine and temozolomide have objective response rates of about 20% (Serrone, Zeuli et al. 2000) and 13% respectively (Augustine, Yoo et al. 2009).

Combination therapy such as procarbazine, vincristine and lomustine (POC) has also been used on melanoma patients but seldom improves response rates compared with other chemotherapy regimens (Repetto, Grimaldi et al. 1987). Procarbazine is a mutagenic and carcinogenic methylating cytostatic drug (Kyrtopoulos 1995), vincristine is a vinca alkaloid microtubule destabilising agent and lomustine is a nitrosurea DNA alkylating agent (Weiss and Issell 1982).

Ipilimumab (Bristol Myers-Squibb) is a human monoclonal antibody that targets the CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) protein involved in anti-tumour immunity. Ipilimumab allows T-cell activation and the subsequent targeting of cancerous cells. However, the FDA approval of this therapy indicates a risk of immune over activation that could cause further tissue damage (Eggermont and Robert, Dienstmann and Tabernero 2011, Ribas 2012). Similarly, nivolumab and pembrolizumab are monoclonal antibodies recently approved for the treatment of metastatic melanoma. Nivolumab and pembrolizumab block the programmed death-1 receptor (PD-1, CD279), which releases the inhibition of tumour-specific immune responses. (Burki 2015, Wolchok 2015)
Inhibition of the components of glutamate signalling systems have been shown to be effective in melanoma (Frati, Marchese et al. 2000, Pollock, Cohen-Solal et al. 2003, Marín and Chen 2004). For example, inhibitors such as LY367385 or BAY36-7620 that target G-protein coupled metabotropic glutamate receptors reduce melanoma cell proliferation in vitro (Namkoong, Shin et al. 2007, Shin, Namkoong et al. 2008). Even greater inhibition of melanoma growth was achieved using agents like riluzole that limit glutamate release (Namkoong, Shin et al. 2007, Le, Chan et al. 2010). The clinical availability of riluzole (Miller 2009) facilitated clinical trials with its unlicensed use for melanoma patients. Early trials (phase 0, involving treatment of a few patients with low doses of the drug) reported efficacy of riluzole in patients with advanced melanoma (Yip, Le et al. 2009).

Newer promising agents against metastatic melanoma are those targeted to specific proteins that drive cell cycle progression. These include B-Raf inhibitors: vemurafenib (Roche) and dabrafenib (GlaxoSmithKline) (Dienstmann and Tabernero 2011, Wellbrock 2014). Clinical trials with vemurafenib alone, or in combination with other therapeutic agents, have proven to be successful in patients carrying the BRAF mutation (Ravnan and Matalka 2012, Larkin, Ascierto et al. 2014, Wilson and Nathanson 2015). Pharmacological blockage of EGFR with small molecule inhibitors or monoclonal antibodies was found to suppress melanoma growth alone or in combination with other targeted therapies. Gefitinib, an EGFR inhibitor was later found to be useful in cases of de novo resistance to B-Raf inhibitors. Pharmacological blockade of EGFR with gefitinib was able to achieve a synergistic effect with vemurafenib (Hodis, Watson et al. 2012). Imatinib is an FDA approved small molecule inhibitor that showed responses in KIT mutated melanomas. MEK inhibitors trametinib and MEK162 have recently been shown to be the most active, but only in B-Raf mutated melanoma (Sullivan and Flaherty 2013). MEK1/2 inhibitor selumetinib also reached phase II trials but progression free survival was not found to be significantly different to that achieved with temozolomide (Tsao, Chin et al. 2012, Sullivan and Flaherty 2013, Wilson and Nathanson 2015).

Clinical resistance to B-Raf inhibition has been linked with events such as PTEN loss, elevated levels of C-Raf, cyclin D1 overexpression and CDK4-activating mutations. Palbociclib, an inhibitor of CDK4 and CDK6 is in clinical development (Hodis, Watson et al. 2012). While initial studies of first-generation CDK inhibitors, such as flavopiridol, failed to demonstrate efficacy in preclinical studies, second-generation CDK inhibitors such as dinaciclib have shown more promising activity in mouse xenograft models. This effect is
potentiated when CDK inhibitors are combined with the microtubule destabilising agent paclitaxel (Chakraborty, Wieland et al. 2013).

<table>
<thead>
<tr>
<th>Type of therapy</th>
<th>Name of agent</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systemic</td>
<td>Dacarbazine,</td>
<td>DNA (alkylating agent)</td>
</tr>
<tr>
<td></td>
<td>Temozolomide</td>
<td>DNA (alkylating agent)</td>
</tr>
<tr>
<td>POC</td>
<td>-Procarbazine,</td>
<td>-DNA (alkylating agent)</td>
</tr>
<tr>
<td></td>
<td>-Vincristine (Oncovin)</td>
<td>-Microtubules</td>
</tr>
<tr>
<td></td>
<td>-Lomustine</td>
<td>-DNA (alkylating agent)</td>
</tr>
<tr>
<td></td>
<td>Paclitaxel</td>
<td>Microtubules</td>
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<td></td>
<td>Riluzole</td>
<td>Glutamate release</td>
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<tr>
<td>Immunotherapy</td>
<td>Ipilimumab</td>
<td>CTLA-4</td>
</tr>
<tr>
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<td>Nivolumab</td>
<td>Programmed death-1 receptor (PD-1, CD279)</td>
</tr>
<tr>
<td></td>
<td>Pembrolizumab</td>
<td>Programmed death-1 receptor (PD-1, CD279)</td>
</tr>
<tr>
<td>Targeted</td>
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<td>B-Raf</td>
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<td>Gefitinib</td>
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<td>MEK</td>
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<td></td>
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<td>MEK</td>
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<tr>
<td></td>
<td>Selumetinib</td>
<td>MEK</td>
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<tr>
<td></td>
<td>Palbociclib</td>
<td>Cyclin dependent kinase (CDK) 4/6</td>
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<td></td>
<td>Flavopiridol</td>
<td>CDK</td>
</tr>
<tr>
<td></td>
<td>Dinaciclib</td>
<td>CDK</td>
</tr>
</tbody>
</table>

**Table 1.3: Summary of current targets for melanoma therapy**

CTLA-4: cytotoxic T-lymphocyte-associated protein 4, MEK: MAPK/ERK pathway, CDK: cyclin dependent kinase
1.2.9. The search for new targets

Access to targeted therapies in New Zealand is still limited and the effectiveness of B-Raf inhibitors is confined to patients whose tumours carry BRAF mutations (Chapman, Hauschild et al. 2011) and have not developed resistance to B-Raf inhibitors (Wilson and Nathanson 2015). The quest for better molecular targets is ongoing and is often preceded by the discovery of genes that are frequently mutated in melanoma. As with many of the examples provided above the mutated protein products of these genes are often useful therapeutic targets. Small molecule inhibitors can be designed to these mutated proteins.

One such mutated gene that has been reported to be frequently mutated in melanoma is the aforementioned GRIN2A gene. GRIN2A encodes the regulatory subunit of a glutamate receptor ion channel called the NMDAR (Wei, Walia et al. 2011). In vitro studies have suggested that mutated GRIN2A results in increased melanoma cell survival and a loss of ion channel function (Prickett, Zerlanko et al. 2014). The focus of this thesis is on the role of NMDARs in human melanoma. The following sections will describe the NMDAR and report on relevant evidence from the current literature for the role of glutamate signalling in melanoma.

1.3. The N-methyl-D-aspartate receptor (NMDAR)

NMDARs are prominent ligand-gated calcium ion channels located in the plasma membrane (Hardingham and Bading 2003, Zhawar, Kaur et al. 2010, Stepulak, Luksch et al. 2011). NMDARs can be selectively activated by a synthetic NMDA in addition to endogenous glutamate (Paoletti and Neyton 2007) or the neurotoxin, quinolinic acid (Perkins and Stone 1983).

1.3.1. Classification of glutamate receptors

Systems that involve the use of glutamate are called glutamatergic and consist of glutamate receptors (GluRs) and/or glutamate transporters (GluTs). Two main families of GluRs have been identified: ionotropic (iGluRs) and metabotropic glutamate receptors (mGluRs) (Kalariti, Pissimissis et al. 2005) (Figure 1.7).

mGluRs are G-protein coupled receptors with seven-transmembrane domains, an extracellular N-terminal and an intracellular C-terminal domain. The mGluRs are further divided into three groups, Group I, Group II and Group III, based on their sequence homology, pharmacology
and intracellular signalling mechanisms. Eight of these receptors have been identified and they are encoded by eight different genes (Table 1.4). Group I consists of mGlu1 and -5, Group II consists of mGlu2 and -3, and Group III consists of mGlu4, -6, -7 and -8 (Kalariti, Pissimissis et al. 2005).

The NMDAR is classified as an iGluR. The various forms of this receptor are made up of an assembly of four subunits and have an excitatory function. Other non-NMDA iGluRs include the kainate (KA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Figure 1.7). Each family is named after a distinct synthetic agonist that activates them. AMPA and kainate receptors are made up of subunits GluR1-4 and GluR5-7, respectively. Kainate receptors also complex with subunits KA-1/2. NMDARs are made up of three types of subunits, GluN1, GluN2 A-D and GluN3 A-B (Figure 1.8) (Kalariti, Pissimissis et al. 2005).

Table 1.4 lists the genes that encode the receptors or subunits of the glutamate receptors. Multiple isoforms of the receptors and subunits have been identified.
Glutamate receptors are classified into ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). The NMDAR is an iGluR. Other iGluRs include the kainate (KA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. mGluRs are further divided into three groups, Group I, Group II and Group III.
<table>
<thead>
<tr>
<th>Receptor family</th>
<th>Subunit</th>
<th>Gene</th>
<th>Group</th>
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<th>Gene</th>
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<td>AMPA</td>
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<td>GRIA1</td>
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<td>mGluR1</td>
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<td>mGluR5</td>
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<td>GRIK5</td>
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<td>GluN1</td>
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<td>GluN3B</td>
<td>GRIN3B</td>
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</tbody>
</table>

Table 1.4: Glutamate receptor families

Names of glutamate receptors and the genes that encode their subunits
1.3.2. NMDAR subunits

NMDARs are heterotetrameric structures that form calcium ion channel pores by the complexing of multiple subunits. The channel pore is also permeable to sodium and potassium ions (Xin, Kwan et al. 2005, Blanke ML 2009). NMDAR subunits are categorised into three types: GluN1, GluN2 and GluN3. NMDARs always contain two structural GluN1 subunits which combine with two regulatory GluN2 subunits (of which there are four types A to D) or GluN3 subunits (A and B), or one of each type (GluN2/3) (Laube, Hirai et al. 1997, Anson, Chen et al. 1998, Dingledine, Borges et al. 1999) (Figure 1.8).

The structural NMDAR subunit, GluN1, binds glycine while the GluN2 type regulatory subunits bind glutamate. The GluN3 type regulatory subunits also bind glycine (Paoletti and Neyton 2007). The GluN3 type subunits are characterised under the NMDAR family despite their low homology to other NMDAR and non-NMDAR subunits. This is because the C-terminal domain (CTD) and the regions upstream of M1 are structurally related to other NMDARs (Low and Wee 2010).

The seven subunits of NMDARs are each encoded by a separate gene and have been identified in the brain as GRIN1, GRIN2A to GRIN2D, and GRIN3A and GRIN3B (Laube, Hirai et al. 1997, Anson, Chen et al. 1998, Dingledine, Borges et al. 1999) (Table 1.4). GRIN genes have a number of splice variants (Hardingham and Bading 2003, Paoletti, Bellone et al. 2013). The GRIN1 gene for example has eight spatially and temporally regulated isoforms (1a-4a and 1b-4b). The isoforms result from alternative RNA splicing of exons 5, 21 and 22 (Sugihara, Moriyoshi et al. 1992, Laube, Hirai et al. 1997, Dingledine, Borges et al. 1999).

The subunit composition, biophysical and pharmacological properties, interacting partners and subcellular localisation of NMDARs can be variable. Variation in subunit composition can be further extended during development and at diseased states, and the subunit composition can change (Paoletti, Bellone et al. 2013). The structure of NMDARs in peripheral tissues is yet to be characterised.
Figure 1.8: Types of NMDAR subunits

The three types of NMDAR subunits: GluN1, GluN2 and GluN3. The obligatory GluN1 subunits combine with two regulatory GluN2 subunits (A to D) or GluN3 subunits (A and B), or one of each type (GluN2/3). Gly: glycine, Glu: glutamate.
1.3.2.1. Structure of the GluN subunits that make up the NMDAR

NMDAR subunits (GluN) comprise four separate structural regions that coincide with their corresponding gene sequences. The coding sequence of GRIN2A, for example, can be broken down into sections, each corresponding to the various sub-domains of the receptor subunit (Figure 1.9) and this general structure is shared between all GluN proteins. The channel assembles as a hetero-tetramer associating two structural GluN1 subunits with two regulatory subunits (a dimer of dimers quaternary structure). Each subunit has membrane spanning domains (M1-M4), an extracellular agonist binding domain (ABD), an extracellular N-terminal domain (NTD) and an intracellular C-terminal domain (CTD) (Figure 1.9). Individual domains are described below. GluN1 proteins are retained in intracellular regions before being assembled into the receptor channel pore that is trafficked to the cell membrane (Pabba, Wong et al. 2014).

N-terminal domain

The NMDAR subunits have a large NTD (of approximately 127 amino acids). The NTD functions in subunit oligomerisation and forms the binding sites for the endogenous inhibitor, zinc (Furukawa, Singh et al. 2005, Gielen, Le Goff et al. 2008).

Transmembrane domains

Subunits also have three membrane spanning domains (M1, M3 and M4) and a re-entrant loop (M2) which form the channel pore (Furukawa, Singh et al. 2005, Chang and Kuo 2008). The M1 domain forms a collar-like structure around the extracellular region of the M3 subunit. It then descends into the membrane and connects with the pore lining region of the M3 domain on the same subunit and M4 domain on the adjacent subunit (Figure 1.9) (Lee, Lu et al. 2014). The channel pore has binding sites for magnesium ions and small molecule blockers (Figure 1.9) (Lee, Lu et al. 2014).

Agonist binding domain

The extracellular bilobate domain formed by the NTD and the ABD, can bind glutamate or glycine, depending on the type of the subunit. Binding of the agonist to the agonist binding site induces channel opening (Paoletti and Neyton 2007) (Figure 1.9).
C-terminal domain

The CTD comprises about a third of the length of the peptide (Figure 1.9). Although it is not required for the expression of a functional channel the CTD plays a major role in interacting with intracellular scaffolding proteins (Puddifoot, Chen et al. 2009).
Figure 1.9: Assembly of the NMDAR

NMDARs assemble as hetero-tetramer. Two structural GluN1 subunits complex with two regulatory subunits. Each subunit has four transmembrane domains (TMD): M1, M2, M3 and M4, an extracellular agonist binding domain (ABD), an extracellular N-terminal domain (NTD) and an intracellular C-terminal domain (CTD). Topographical/spatial orientation of the subunits shown below the structure indicates positioning of GluN1 (blue) and GluN2 (red). Linear arrangement of domains according to the amino acid sequence of GRIN2A encoding GluN2A is shown on the right as an example representing domain sizes. Adapted and edited with permission from Wilding, Lopez et al., 2014 and D'Mello, Flanagan et al., 2014.
1.3.3. NMDAR activation

The activation of the NMDAR is both voltage gated and ligand dependent. At a resting state, NMDAR channel pores are blocked tightly by magnesium ions that enter from the extracellular environment (Hinoi, Takarada et al. 2004, Fan, Jin et al. 2014). The blockage of excess ion flow protects neurons from excitotoxicity (Chahal, D'Souza et al. 1998). When the NMDAR ligand binding sites are bound to glutamate and glycine, the cell membrane is depolarised and its cation-permeable channel opens to allow for the flow of calcium ions (Lee, Lu et al. 2014). A membrane depolarisation of sufficient amplitude and duration is required to dislodge and repel the magnesium ion from the channel pore (Seeburg, Burnashev et al. 1995, Blanke ML 2009). In neurons, this amplitude of depolarisation is achieved through the reception of presynaptic signals (Blanke ML 2009) and potassium or sodium ions influx from other ion channels on the membrane. The mechanism of the NMDAR channel opening in non-neuronal cells is not currently understood.

NMDAR activity is greatly influenced by its subunit composition (Anson, Chen et al. 1998, Laube, Kuhse et al. 1998, Anson, Schoepfer et al. 2000, Cull-Candy, Brickley et al. 2001). For example, receptors that contain GluN2A subunits, have the fastest deactivation kinetics (Cull-Candy, Brickley et al. 2001). GluN2A-containing receptors are also more potently blocked by magnesium ions than GluN2D-containing receptors. Chimaeric subunits (GluN1-GluN2/3) can alter the pharmacology and biophysical behaviour of the channels (O'Leary and Wyllie 2009). The GluN3-type subunits confer a dominant negative effect to the NMDAR (Nishi, Hinds et al. 2001, Fukumori, Takarada et al. 2010).

1.3.4. NMDAR regulation

NMDARs may be potentiated by an increase in the level of receptor subunit expression or redistribution of intracellular stores to the plasma membrane (Pabba, Wong et al. 2014). The presence or absence of the regulatory subunits may be a limiting factor for the assembly of NMDARs. A majority of the GluN1 subunits exists in an intracellular pool where they are not associated with regulatory subunits (Huh and Wenthold 1999, Pabba, Wong et al. 2014). The assembled receptor complex is trafficked to the cell membrane only when GluN1 associates with the regulatory subunits (McIlhinney, Molnar et al. 1996, Huh and Wenthold 1999, Perez-Otano, Schulteis et al. 2001).
Extracellular factors such as agonist and ion concentrations can influence the expression of NMDAR components. In mice, chronic treatment with NMDA antagonists selectively upregulates the NMDAR mRNAs and polypeptides (Follesa and Ticku 1996). In rodent cultured cerebellar granule cells, potassium induced membrane depolarisation and NMDA treatment selectively up-regulated GluN mRNA transcription (Bessho, Nawa et al. 1994). Transcripts encoding GluN2B and GluN2C proteins in mouse cerebellar cells are down- and up-regulated, respectively, following exposure to potassium ions (Iijima, Abe et al. 2008).

Sigma receptors (σ1R and σ2R) are ligand gated ion channel chaperon proteins of the endoplasmic reticulum (Kerchner and Nicoll 2008, Pabba, Wong et al. 2014). Treatment with σ1R agonists were shown to correlate with increased protein synthesis of GluN2A, GluN2B and NMDAR associated postsynaptic density protein-95 (PSD-95). It also increased interaction of the GluN2 and GluN1 subunit with σ1R subunits and increased cell surface levels of NMDARs (Pabba, Wong et al. 2014).

1.3.5. NMDAR antagonists

NMDAR activity can be inhibited by molecules that block the agonist binding sites or the channel pore. Inhibitors that block the agonist binding sites are called competitive antagonists. This includes (2R)-amino-5-phosphonovaleric acid or (2R)-amino-5-phosphonopentanoate (AP5) that binds to the glutamate binding site of the receptor subunit. Inhibitors that block the channel are called “non-competitive” antagonists. (Bolshakov, Gmiro et al. 2003, Johnson and Kotermanski 2006) (Figure 1.10).

Non-competitive inhibitors block the receptor channel when it is open but can be trapped when the channel closes and the agonist unbinds. These are called “trapping channel blockers” and include memantine, MK-801 (dizocilpine), phencyclidine, amantadine and ketamine (Bolshakov, Gmiro et al. 2003) (Figure 1.10). Non-trapping inhibitors are able to block the channel pore and prevent channel closure. These are called “sequential” or “foot in the door” blockers. The effects of sequential channel blockers can be voltage dependent (Bolshakov, Gmiro et al. 2003).
Figure 1.10: Classification of NMDAR antagonists

NMDAR antagonists are classified into competitive and non-competitive types. Competitive antagonists bind to the receptor’s agonist binding domain. Non-competitive antagonists bind within the ion channel. Non-competitive antagonists can block the ion channel while the agonists binding site is occupied or not occupied. Non-trapping blockers can bind within the ion channel while still allowing it to open and close. Trapping antagonists bind within the ion channel and do not allow channel reopening.
1.3.5.1. Memantine

Memantine is a NMDAR channel blocker that was first synthesised in the 1960s and is a derivative of amantadine, an anti-influenza agent (Figure 1.11). In the 1970s memantine was found to affect the CNS and by 1989 it was found to inhibit the NMDAR with IC50 value of approximately 1 µM. At this concentration memantine is therapeutically active and is used to treat Alzheimer’s disease (Chen, Pellegrini et al. 1992, Lipton 2005, Johnson and Kotermanski 2006).

Memantine is a non-competitive NMDAR antagonist. It has a three-ring structure with a bridgehead amine (–NH2) that is protonated under physiological conditions to carry a positive charge (–NH3+) (Figure 1.11). Memantine binds at or near the magnesium site of the NMDAR ion. Memantine has two methyl (–CH3) side groups that prolong its binding duration in the channel (Lipton 2006) (Figure 1.11).

Memantine enters the NMDAR ion channel and blocks calcium flow only when the channel has been opened and is thus classified as an open channel blocker. Memantine can be trapped inside the channel when it closes and the agonists unbind and its blockade can be decreased by magnesium. Memantine has rapid kinetics for inhibition at high agonist concentrations. Ketamine, another NMDAR agonist, has a very similar mechanism of action but the IC50 of ketamine is half that of memantine and in addition, memantine exhibits much weaker neurotoxicity making it clinically favourable. Memantine is also eliminated slower from serum with a half-life of 60-80 hours compared with that of ketamine, ~2.5 hours. This longer half-life is thought to be clinically advantageous for treatment of neurological disorders (Chen, Pellegrini et al. 1992, Lipton 2005, Johnson and Kotermanski 2006).

1.3.5.2. MK-801

Dizocilpine, also known as MK-801 is a potent, selective, high-affinity NMDAR antagonist, (Wong, Kemp et al. 1986, Do Couto, Aguilar et al. 2004) but compared to memantine, MK-801 has slower kinetics of action with less rapid channel blocking and unblocking rates (Frankiewicz, Potier et al. 1996, Dilmore and Johnson 1998, Do Couto, Aguilar et al. 2004).

MK-801 is a non-competitive NMDAR antagonist. (Wegener, Nagel et al. 2011, Chang, Huang et al. 2012). MK-801 has a 1-methylnortropane skeleton and represents the heterocycle bearing two fused benzene rings (Figure 1.11). MK-801 is widely used in preclinical research and it has been extensively studied for the treatment of neurodegenerative diseases (Chang,

NMDAR inhibition by MK-801 is voltage dependent (Horvath, Czopf et al. 1997, Kovacic and Somanathan 2010). MK-801 blocks the NMDAR within the ion channel (Wong, Kemp et al. 1986, Huettner and Bean 1988). MK-801 blockade is highly stable and regarded as “irreversible”. Magnesium inhibits the binding of MK-801 which suggests a shared binding site (McKay, Bengtson et al. 2013). MK-801 produces a long-lasting blockade when applied in conjunction with NMDA (Wong, Kemp et al. 1986, Huettner and Bean 1988).

1.3.5.3. AP5

AP5 is a competitive NMDAR antagonist at the agonist (glutamate) binding site and unlike memantine and MK-801 it does not cross the blood brain barrier (Halliwell, Peters et al. 1989) (Figure 1.11). In Drosophila melanogaster, AP5 was reported to be less effective at blocking NMDAR opening compared with other antagonists (Xia S 2009). However, very little is known about the mechanism of action of AP5 in human cells aside from its nature of being targeted to the agonist binding site of the receptor (Laube, Hirai et al. 1997, Gascón, Deogracias et al. 2005, Watanabe, Kanno et al. 2008).

1.3.5.4. Allosteric inhibitors

NMDARs have allosteric domains next to the glutamate binding site that are modulated by additional ligands (Schrattenholz and Soskic 2006). Allosteric modulators include naturally occurring zinc ions and ifenprodil-like compounds (Paoletti and Neyton 2007). Allosteric modulators can show preference for certain subunits, for example, zinc ions bind to the NTD region of the GluN2A subunits while ifenprodil-like compounds bind the NTD region of GluN2B (Paoletti and Neyton 2007).

1.3.5.5. Riluzole

Riluzole interferes with NMDARs and other glutamate receptors by inhibiting glutamate release. Riluzole’s direct action on the NMDAR is less well defined (Kretschmer, Kratzer et al. 1998, Yip, Le et al. 2009, Dolfi, Medina et al. 2015). Proposed targets of riluzole include blockade of excitatory amino acid receptors and G-protein coupled receptors, inhibition of
voltage-dependent sodium channels, inhibition of voltage-dependent calcium channels and activation of intracellular calcium-buffering processes (Doble 1996).

1.3.6. NMDAR agonists

NMDA and glutamic acid are NMDAR agonists that bind to the same site on the receptor’s agonist binding domain. Glutamic acid is the endogenous agonist that can bind and activate NMDAR as well as other glutamate receptors, while NMDA is the synthetic amino acid derivative that binds specifically to the NMDAR (Paoletti, Bellone et al. 2013).

Glutamic acid is a proteinogenic non-essential amino acid. Glutamate is the carboxylate anion and salt of glutamic acid. Glutamate is an important excitatory neurotransmitter in the CNS (Figure 1.11d) (Betts and Russell 2003). NMDA is an amino acid derivative that mimics the action of glutamate (Figure 1.11) (Dingledine, Borges et al. 1999, Liu and Zhang 2000, Cull-Candy, Brickley et al. 2001, Paoletti and Neyton 2007).
Figure 1.11: Chemical structures of NMDAR modulators

Chemical structures of non-competitive NMDAR antagonists memantine, and MK-801, competitive antagonist AP5 and agonists NMDA and glutamic acid.
1.3.7. NMDARs in melanoma and other cancers

Of particular interest to the field of cancer biology is signalling downstream of NMDARs that influence processes of cell survival and cell death (Riccio and Ginty 2002, Hardingham and Bading 2010). The presence of NMDARs has been reported in a number of cancer cell types and in normal tissues including melanocytes (Hoogduijn, Hitchcock et al. 2006). Pro-survival pathways shown to operate downstream of NMDARs include the PI3K and MAPK (ERK) pathways, and those activating CREB transcription factor, a downstream target of cyclic AMP-activated protein kinase cAMPK (Stepulak, Sifringer et al. 2005, Stepulak, Luksch et al. 2009, North, Gao et al. 2010, North, Gao et al. 2010).

There is substantial evidence for the expression of the NMDAR genes in different cancer cells and tissues. For example, mRNA and proteins of various subunits of functional NMDARs have been detected in human MG-63 and SaOS-2 osteosarcoma cells (Kalariti, Pissimissis et al. 2005). GluN1 is a suggested biomarker for determining the prognosis of oral squamous cell carcinoma (OSCC). Overexpression of GluN1 in OSCC correlates with tumour size, lymph node metastasis and cancer stage (Choi, Park et al. 2004). The anti-proliferative effect of the NMDA antagonist MK-801 was reproduced by two other NMDA antagonists, memantine and ketamine, suggesting receptor-ion channel specificity (Rzeski, Turski et al. 2001).

As indicated in section 1.1, exome sequencing revealed that 33% of human melanoma tumours carry mutations in \textit{GRIN2A}, the gene that encodes the GluN2A subunit of the NMDAR (Wei, Walia et al. 2011). NMDARs play an invasive role in pancreatic neuroendocrine tumours among other cancer types. A model has been proposed to suggest that interstitial pressure from within tumours causes cells on the peripheral region to release glutamate, which is taken up again by the NMDAR. Signalling downstream of the receptor results in the activation of signal transduction pathways (MEK-ERK, CamK) through which the transcription of invasive genes is initiated (Li and Hanahan 2013). The expression of invasive genes suggests that the involved gene(s) have an oncogenic rather than a tumour suppressor role to play in this system. However, another study has more recently shown that the \textit{GRIN2A} gene is likely to have a tumour suppressor function (Prickett, Zerlanko et al. 2014). Depletion of endogenous GluN2A in melanoma cells expressing wild-type \textit{GRIN2A} resulted in increased proliferation. Here, transfection of HEK293 cells with vectors expressing either wild-type or mutant \textit{GRIN2A} resulted in a loss of subunit complex formation. Increased migration and anchorage-independent cell growth were seen in cells expressing mutated \textit{GRIN2A}. Somatic mutations of
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GRIN2A in melanoma resulted in a dominant negative effect that inhibits the tumour-suppressive phenotype of the wild-type gene (Prickett, Zerlanko et al. 2014). The three studies mentioned above point specifically to the suggestive role of NMDAR mediated roles in melanoma warranting a need for further investigation.

1.4. Glutamate and cancer

Glutamate is an amino acid that functions as a neurotransmitter received by glutamate receptors in the CNS, but it is also an essential substrate in metabolic pathways (Rzeski, Turski et al. 2001, Zacharias, Lima et al. 2003).

1.4.1. Glutamate as a metabolic substrate

In the CNS, glutamate is predominantly synthesised by astrocytes and converted to glutamine before it is released into the extracellular space (Nedergaard, Takano et al. 2002, Vlassenko, Rundle et al. 2006). Astrocytes are responsible for the replenishment of brain glutamate because they are the only neural cell type that expresses pyruvate carboxylase, a key enzyme that allows for the synthesis of glutamate from glucose (Magistretti, 2008). The citric acid cycle reduces α-ketoglutarate to glutamate by transamination. This is catalysed by glutamate dehydrogenase (Figure 1.12). Glutamate is converted to glutamine by glutamine synthetase through the incorporation of an ammonium ion. Glutamine is then taken up by neurons and converted back to glutamate before being packaged into synaptic vesicles where it can be reused as neurotransmitter. Glutamate is recycled into the transmitter pool after uptake and converted into glutamine again by astrocytes (Nedergaard, Takano et al. 2002) (Figure 1.12).

Brain tissue shows high energy consumption. Though the brain represents only 2% of the human body mass, it consumes 20% of the oxygen and 25% of the supplied glucose. The brain has little energy reserves and is therefore highly dependent upon the uninterrupted supply of energy substrates from the circulation. It was initially postulated that neurometabolism was a strictly oxidative (oxygen-depending) process as opposed to a non-oxidative lactate producing process. However, it was later found that oxidative and non-oxidative processes are involved in neurometabolism to meet the increased metabolic requirements. Brain cells can efficiently utilise various energy substrates in addition to glucose, including lactate, pyruvate, glutamate, and glutamine. Glutamate-mediated neurotransmission is responsible for most (~80%) of the energy expended in the brain’s grey matter (Bélanger, Allaman et al. 2011).
Like brain cells, tumour cells have a high rate of energy consumption probably owing to their proliferative, invasive and inflammatory nature (Diaz-Ruiz, Rigoulet et al. 2011, Hanahan and Weinberg 2011). Cutaneous melanoma grows at a very fast rate and infiltrates relatively quickly into subcutaneous tissue consuming high amounts of adenosine triphosphate (ATP) in order to cope with energy demands (Zacharias, Lima et al. 2003). The main energy sources used by tumours are glucose and glutamine (Kovacevic and McGivan 1983, Zacharias, Lima et al. 2003), and their metabolic pathways are known to be altered in cancerous tissue (Budczies, Pfitzner et al. 2015). In the 1920s, Otto Warburg observed that cancer cells exhibit elevated glycolysis. He also observed that the pyruvate, generated in the first step of glycolysis, fermented to lactate (non-oxidative) rather than being fed into the citric cycle for oxidative phosphorylation and ATP production (Warburg, Wind et al. 1927, Budczies, Pfitzner et al. 2015).

The emerging role of glutamate in cancer has become exceedingly apparent in recent years (Rzeski, Turski et al. 2001). Instead of glucose, glutamine can be used to replenish the citric acid cycle (Figure 1.13). Cancer cells have even been found to prefer glutamine over glucose. Glutamine can feed in the carbon and nitrogen needed for nucleotide, amino acid and glutathione synthesis (Budczies, Pfitzner et al. 2015). It was recently shown that glutamine-driven oxidative phosphorylation was the main source of ATP generated from transformed (Ras activated) cultured cells in both hypoxic and non-hypoxic conditions (Fan, Kamphorst et al. 2013).

Glutamine needs to be converted to glutamate before it enters the citric acid cycle. Many cancer cells also overexpress glutaminase, an enzyme that converts glutamine to glutamate. In addition, cancer cell survival has been found to be dependent on external glutamine which has recently been shown to contribute to invasiveness of ovarian cancer and the growth of pancreatic cancer (Budczies, Pfitzner et al. 2015).
Figure 1.12: Synthesis and subcellular concentrations of glutamate

In the CNS glutamate (Glu) is synthesised by astrocytes and converted to glutamine before it is released into the extracellular space. Gln is released into the extracellular space, taken up by neurons and converted to Glu before being packaged into synaptic vesicles. Glu is recycled into the transmitter pool after uptake and converted into Gln by astrocytes. In the astrocytes the citric acid cycle reduces alpha-ketoglutarate to glutamate catalysed by glutamate dehydrogenase (GDH). The concentration of glutamate in the cerebrospinal fluid and other subcellular components has also been indicated. Adapted with permission from Nedergaard, Takano et al., 2002.
Figure 1.13: Elevated glycolysis in cancer cells

Pyruvate generated in the first step of glycolysis is fermented to lactate (non-oxidative). Instead of glucose, glutamine is used to replenish the citric acid cycle. Glutamine needs to be converted to glutamate before it enters the cycle. Glutaminase is an enzyme that converts glutamine to glutamate.
1.4.2. Evidence for glutamate receptors in non-neuronal tissue and cancer

Many components of the glutamate system exist in non-neuronal tissues. However, the mechanisms and consequences of glutamate signalling are not well understood in non-neuronal and tumour cells. These mechanisms are not likely to be identical to those in neuronal cells because non-neuronal cells are not specialised to transmit electrical signals. The rapid membrane currents that are evoked in neuronal cells by glutamate may have less of a functional long-term impact than neuronal intracellular messengers (Nedergaard, Takano et al. 2002). Nevertheless, there is substantial evidence for the expression and function of glutamate receptors in non-neuronal normal and cancerous tissues.

Ionotrophic and metabotropic glutamate receptors have been identified in a number of non-excitatory tissues such as heart, spleen, testis, ovary, kidney, bone, bone marrow, pancreatic β-cells, intestine, oesophagus, hepatocytes, lung, and keratinocytes. These receptors have been cloned and their sequences were found to be identical to those of neuronal glutamate receptors (Rzeski, Turski et al. 2001). In the skin, human keratinocytes express functional NMDARs, AMPARs and mGluRs (Kalariti, Pissimissis et al. 2005).

1.4.2.1. Evidence for mGluRs in non-neuronal and cancerous cells

Substantial evidence is available for the role of mGluRs in non-neuronal cells particularly in neoplastic diseases. Expression of mGluRs -2, -3 and -5 has been reported in non-neuronal and cancerous tissues (Rzeski, Turski et al. 2001). Human keratinocytes in the skin express mGluR1, -2, and -3 (Kalariti, Pissimissis et al. 2005).

Glutamate receptor antagonists influence the cell cycle of tumour cells (Rzeski, Turski et al. 2001, Nedergaard, Takano et al. 2002). The inhibitor of glutamate release and uptake, riluzole, blocked the growth and invasion of mGluR1 positive melanoma cells by disrupting the glutamatergic pathway leading to G2/M arrest followed by apoptosis (Namkoong, Shin et al. 2007, Le, Chan et al. 2010, Rosenberg, Niglio et al. 2015).

*In vitro* studies have shown that the mGluR4 antagonist MAP4 acts synergistically with 5-fluorouracil (5-FU), a chemotherapeutic agent used for the treatment of advanced colorectal cancer. The antagonist enhanced death in 5-FU-resistant human colon cancer cells SNU-769A, that expresses mGluR4 and interestingly 5-FU resistance, has been associated with an overexpression of mGluR4 (Sobrero, Aschele et al. 1997).
In melanocytes, pharmacological activation of mGluR5 administers a mitotic signal for proliferation but impairs cell viability (Kalariti, Pissimissis et al. 2005). mGluR5 antagonists have been shown to inhibit proliferation of laryngeal cancer cells (Stepulak, Luksch et al. 2011). The inhibitory effect of glutamate antagonists on cancer cell migration is achievable even at lower concentrations and is thus of considerable clinical interest (Kalariti, Pissimissis et al. 2005). Evidence for mGluRs in cancer has been particularly clinically relevant. mGluR4 was associated with poor prognosis of colorectal carcinoma. Overexpression of mGluR4 positively associated with recurrence and poor disease-free survival. (Chang, Yoo et al. 2005).

1.4.2.2. Evidence for iGluRs in non-neuronal and cancerous cells

A number of studies have reported evidence for the role of iGluRs in non-neuronal and cancer cells. In the skin, human keratinocytes express functional NMDARs and AMPARs (Kalariti, Pissimissis et al. 2005). Expression of AMPARs, KA and NMDARs have been confirmed in the mammalian pineal gland and the islets of Langerhans of the pancreas (Nedergaard, Takano et al. 2002). The expression of NMDAR subunits have been documented in rat marrow, human megakaryocytes and Meg-01 megakaryoblastic leukaemia cells (Nedergaard, Takano et al. 2002, Kamal, Green et al. 2015). Many solid tumours are immunopositive for NMDARs and AMPARs (Rzeski, Turski et al. 2001).

Glutamate receptor subunits in the pancreas have been shown to modulate the stimulation of insulin and glucagon secretion in the pancreatic β- and α- cells, respectively. Moreover, electrical properties of AMPA receptors in pancreatic cells have been found to be comparable to those of neuronal AMPA receptors. (Nedergaard, Takano et al. 2002).

iGluRs are involved in megakaryocyte adhesion, proliferation and differentiation (Nedergaard, Takano et al. 2002). Many tumour cell lines show preferential susceptibility to the anti-proliferative action of glutamate antagonists. Tumours of peripheral origin responded favourably to either NMDAR or AMPAR antagonists, whereas those derived from neuronal and glial tissues were less sensitive to the same antagonists (Rzeski, Turski et al. 2001). AMPAR antagonists were found to exert a concentration-dependent anti-proliferative effect in human thyroid carcinoma, lung carcinoma, colon adenocarcinoma and breast carcinoma, an effect attributed to both decreased cell division and increased cell death (Kalariti, Pissimissis et al. 2005).
iGluR channel complexes are permeable to calcium ions and this is a key mediator of the anti-proliferative action of glutamate antagonists. Elevation of extracellular calcium ions as well as stimulation of calcium entry into tumour cells has stimulatory effects on tumour growth. (Rzeski, Turski et al. 2001). iGluR ion channel complexes on tumour cells may differ from those of neuronal receptor ion channel complexes. This may be useful in the design of novel drugs with fewer side effects. Moreover, NMDAR and AMPAR antagonists, which do not penetrate the blood brain barrier and therefore do not cause neurological side effects, may be suitable for therapy of peripheral cancers (Rzeski, Turski et al. 2001).

1.4.3. Non-NMDA glutamate receptors and melanoma


Expression of Group I metabotropic glutamate receptors has been reported in melanocytes and expression of Group I and Group III types of receptors have been reported in melanoma (Kalariti, Pissimisis et al. 2005). Ectopic expression of mGluR1 is sufficient to transform murine melanocytes to melanoma (Yip, Le et al. 2009, Wen, Li et al. 2014). In contrast to parental melanocytes, GRM1-clones lose their dependence for TPA supplement (a phorbol ester, 12-O-tetradecanoylphorbol-13-acetate) that induces neural crest cell differentiation (Akslen, Puntervoll et al. 2008) and melanocyte proliferation (Shin, Namkoong et al. 2008). These clonal melanocytes with ectopic expression of mGluR1 have the ability to form colonies in semi-solid medium and their xenografts form robust tumours, in both immune-deficient nude and syngeneic mice, with a short latency. In addition, siRNA targeted for GRM1 (encodes mGluR1) reduced proliferating and viable cells in vitro and suppressed in vivo tumour growth (Shin, Namkoong et al. 2008).
Studies in transgenic mice that spontaneously develop melanoma showed that the aberrant expression of mGluR1 in melanocytes played a critical role in the onset of melanoma (Namkoong, Shin et al. 2007). Over 60% of human melanoma samples ectopically express mGluR1 and stimulation of the mGluR1 in vitro results in up-regulation of activated ERK (Yip, Le et al. 2009), a key component of the MAPK pathway frequently activated in melanoma (Schadendorf, Fisher et al. 2015). Human melanoma cells were shown to release elevated levels of glutamate, implying an autocrine loop. Treatment of GRM1-expressing human melanoma cells with GRM1 antagonists (LY367385 or BAY36-7620) or an inhibitor of glutamate release (riluzole) led to suppression of cell proliferation. Moreover, treatment of human melanoma cell xenografts with riluzole led to 50% inhibition of tumour growth (Namkoong, Shin et al. 2007). In human mGluR1-positive melanoma cell lines, riluzole was able to enhance the lethal effects of ionizing radiation whereas no difference was seen in the mGluR1-negative cell line (Khan, Wall et al. 2011).

A more recent study has found that treatment of melanoma cell lines both in vitro and in vivo, with drugs that target glutamate signalling and mTOR, decreased anchorage-independent growth and xenograft tumour progression. Melanoma cell lines harbouring activating mutations in the PI3K pathway were particularly susceptible to this combinational treatment independent of BRAF mutational status (He, Li et al. 2010, Rosenberg, Niglio et al. 2015).

The clinical availability of riluzole due to approved use for the treatment of motor neuron disease (Miller 2009) facilitated clinical trials with its unlicensed use for melanoma patients. The early phase 0 trials reported efficacy of riluzole in patients with advanced melanoma (Yip, Le et al. 2009). The trial involved 12 patients all expressing mGluR1. Glutamate blockade with riluzole inhibited the MAPK and PI3/AKT signalling pathways and suppressed metabolic activity of melanoma (Yip, Le et al. 2009).

### 1.4.4. Glutamate concentrations in tissue

The concentration of glutamate in blood plasma of peripheral tissue is between 30 to 80 µM. This is high compared to that in cerebrospinal fluid (<1 µM). The blood brain barrier has a very low permeability to glutamate. It is crucial that the concentrations of glutamate and other neurotransmitters remain low in the extracellular regions of the brain (Nedergaard, Takano et al. 2002) (Figure 1.12).
By comparison, glutamate concentrations within intracellular regions are considerably different. Glutamate is present at millimolar concentrations in the neuronal (5 to 10 mM) and astrocyte (2 mM) cytosol, but storage glutamate in the synaptic vesicles (100 mM) is the main source of extracellular glutamate (Nedergaard, Takano et al. 2002) (Figure 1.12). The concentration of glutamate in the cerebrospinal fluid can increase from ~1 µM to 20 µM under pathological conditions and high glutamate levels can cause cellular damage. This type of neurotoxic event has been linked with the pathophysiology of hypoxic injury, stroke, hypoglycaemia and epilepsy (Nedergaard, Takano et al. 2002).

Neuronal excitotoxicity occurs when glutamate receptors are exposed to high concentrations of glutamate (Nedergaard, Takano et al. 2002). The resulting influx of cations can cause a collapse of mitochondrial function leading to necrosis. Prolonged activation of glutamate receptors results in a delayed increase of intracellular calcium to excessively high, toxic concentrations. This influx of calcium activates intracellular enzymes like phospholipases A2 (PLA2), xanthine oxidase and nitric oxide synthase, which in turn trigger a cascade of reactions that lead to cell death (Stepulak, Luksch et al. 2011).

In non-neuronal tissue plasma glutamate concentrations are chronically high (20 to 50 µM) relative to its receptor affinity. Post-translational modifications, such as glycosylation or phosphorylation may reduce the affinity of glutamate receptors to glutamate in peripheral tissue compared with that in the CNS (Nedergaard, Takano et al. 2002, Julio-Pieper, Flor et al. 2011). Little is known about the affinity, electrical properties, intracellular signalling mechanisms or distinct functions of glutamate receptors in non-neuronal tissue (Rzeski, Turski et al. 2001).

1.5. Thesis objectives

This introductory chapter has described the main molecular mechanisms involved in the pathogenesis of metastatic melanoma, in addition to describing current treatments and challenges. The study of NMDARs in melanoma conducted in this thesis was initiated in response to the finding that the \textit{GRIN2A} gene is frequently mutated in metastatic melanoma. We also investigate the role that glutamate may play in melanoma and postulate mechanisms by which it may contribute to carcinogenesis.

This thesis specifically examined the role of NMDARs in human melanoma with a particular focus on the \textit{GRIN2A} gene. The work was carried out using melanoma cell lines previously
established at the Auckland Cancer Society Research Centre (ACSRC). Cell lines were derived from metastatic melanoma tumours resected from New Zealand patients. The primary objectives of this thesis were to determine whether the \textit{GRIN2A} gene was indeed mutated in the melanoma cell lines from the ACSRC collection and whether \textit{GRIN2A} mutations might be clinically relevant. We then examined whether NMDAR function contributes to melanoma cell biology.

**Specific aims**

Specific aims of this study were to:

1. Investigate the existence and relevance of \textit{GRIN2A} mutations in melanoma cell lines;
2. Characterise the expression of NMDAR subunits in melanoma cell lines both at the mRNA and protein levels;
3. Investigate whether NMDARs function as calcium ion channels in melanoma cells and whether this functionality differs in cell lines with mutated \textit{GRIN2A};
4. Determine effects of NMDAR agonists and antagonists on the proliferation of melanoma cells \textit{in vitro}, and whether sensitivity to modulators is influenced by the presence of \textit{GRIN2A} mutations;
5. Determine effects of NMDAR modulators on the invasiveness of melanoma cells \textit{in vitro};
6. Investigate if melanoma cells release glutamate into culture medium and whether glutamate levels in media affect melanoma cell proliferation.
## Materials and methods

### 2.1. Materials

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**Table 2.1: Material, sources and catalogue numbers**
2.2. Cell culture

All tissue culture work was carried out in a laminar flow hood using sterile techniques.

Reagents and materials
Alpha-modified minimal essential medium (α-MEM)
RPMI1640
0.05% Trypsin (5 mL trypsin in 45 mL 1X PBS)
Foetal Bovine Serum (FBS)
Penicillin and Streptomycin (Penstrep)
Plasticware (flasks, test tubes)
Glass and plastic pipettes
1X Phosphate Buffer Saline (PBS)
Haemocytometer

Medium preparation: α-MEM or RPMI
Foetal calf serum (5% v/v)
Penicillin (60 µg/mL) and Streptomycin (100 µg/mL)
Insulin-Transferrin-Selenium (ITS)

1X Phosphate Buffered Saline (PBS)
Na₂HPO₄ 10.9 g
NaH₂PO₄ 3.2 g
NaCl 90 g
Milli-Q water up to 1000 mL final volume

0.05% Trypsin
Trypsin EDTA - 5 mL
1 X PBS – 45 mL

2X Freezing medium
α-MEM 10 mL
Foetal Bovine serum (FBS) 6 mL
DMSO – 4 mL

2.2.1. Melanoma cell lines

Melanoma cell lines were derived from patients with metastatic melanoma who were treated at two independent national sites (Auckland City Hospital and Palmerston North). The cell lines were developed from tumours that were pathologically confirmed to be metastatic malignant melanoma. Written informed consent was obtained from all participants prior to enrolment and all study procedures were approved by the Northern Health and Disability Ethics Committee. Patients underwent skin, lymph node or distant organ biopsies for diagnosis, staging or
treatment, as required clinically. Excess tissue was used to establish melanoma cell lines by
the method described in Marshall, Matthews et al. 1994. When they could be regularly and
reproducibly subcultured, the cultures were classified as cell lines, and designated the name
NZM (New Zealand Melanoma cell lines).

NZM cell lines for DNA, RNA and protein analysis were chosen randomly in this thesis, and
all were passaged less than 30 times during the period of experimental use. Cells were
maintained in 25 or 75 cm\(^2\) flasks to 75% confluency in a low-oxygen humidified incubator
(37°C, 5% O\(_2\) and 5% CO\(_2\)). Cultures were maintained in Alpha-modified Minimal Essential
Medium containing 5% foetal bovine serum (FBS), penicillin (100 units/ mL), streptomycin
(100 μg/ mL), insulin (5 μg/ mL), transferrin (5 μg/ mL) and sodium selenite (5 ng/ mL). Melanoma cells were sub-cultured weekly to maintain them in a proliferative state.

### 2.2.2. Melanocytes

Medium254
Human Melanocyte Growth Supplement

Normal human epidermal melanocytes were purchased (HEMa-LP) and cultured in
Medium254 (M-254-500) supplemented with Human Melanocyte Growth Supplement (Life
Technologies). Cultures were maintained in a humidified incubator (5% CO\(_2\) in air) at 37°C.
Melanocytes were sub-cultured every 4 weeks. Every five days, 60% of the medium was
replaced with fresh medium.

### 2.2.3. Primary cultures of rat hippocampal neurons

Primary hippocampal cultures were obtained from the Birch lab at the School of Biological
Sciences, University of Auckland. The cells were cultured from embryos of Wistar rats on day
18 of development and maintained for 21 days in Neurobasal medium containing B27
supplement and 2 mM GlutaMax. Preparation and maintenance of the culture is described in
(Borges, Lee et al. 2010).

### 2.3. Experimental methods

#### 2.3.1. DNA isolation

PureLink Genomic DNA kit
100% Ethanol
DNA was isolated using a PureLink Genomic DNA kit (Life Technologies), according to the manufacturer’s instructions. Concentrations and purity of DNA were determined using a ND-1000 nanodrop spectrophotometer (ThermoFisher Scientific, Rockford, IL).

2.3.2. **GRIN2A sequencing**

- Ladder 1X PCR buffer
- 5 U Expand High Fidelity Enzyme mix
- deoxynucleoside triphosphates
- MgCl₂
- Bovine serum albumin
- Agarose gel
- GelRed DNA intercalating agent
- Low-Mass
- ABI Prism 3730xl Genetic Analyzer

Twelve coding exons of *GRIN2A* (numbered 3 to 14), including their flanking intronic regions, were sequenced using the Sanger method. The sequencing primers were as previously reported (Wei, Walia et al. 2011) except for 7 Reverse and 8 Forward (Table 2.2) that were designed using Primer3web version 4.0.0 software (http://primer3.wi.mit.edu/) to reduce interference from poly-A sequences located in the intron between exons 7 and 8. Primer sequences and PCR conditions for all primers are provided in Table 2.2. PCR reactions were performed in a final volume of 25 µl 1X PCR buffer containing 50-100 ng DNA, 0.3 µM forward and reverse primers each, 5 U Expand High Fidelity Enzyme mix (Roche Applied Science), 0.2 mM deoxynucleoside triphosphates and 1.5 mM MgCl₂. Bovine serum albumin (BSA; 3 ng/µl; Life Technologies) was used to counteract PCR inhibition, where required. The correct sizes of the amplicons were confirmed using 2% agarose gels with DNA visualised using GelRed (Biotium, Hayward, CA). DNA concentrations were estimated against Low-Mass Ladder (Life technologies). Sequencing was performed in both directions using an ABI Prism 3730xl Genetic Analyzer with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit, version 3.1 (Applied Biosystems, Foster City, CA).
## Table 2.2  PCR cycling conditions used to amplify GRIN2A sequences

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</tr>
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<td>GRIN2A_12</td>
<td>F: AAGTGTTGGATGTTTCTGCTG R: ATGCAGAGATACCCACCTGGAAG</td>
<td>12</td>
<td>373</td>
<td>94°C, 30 s</td>
<td>60°C, 30 s</td>
<td>72°C, 30 s</td>
<td></td>
</tr>
<tr>
<td>GRIN2A_13</td>
<td>F: GTCTGTTTCAAAACGCCAACAGC R: TGAGACACTCAAGAAGCCAACC</td>
<td>13</td>
<td>381</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIN2A_14</td>
<td>F: CCCTATGCTTTGCAACTTGTTC R: GAAACCATGATGCTAGAGG</td>
<td>14</td>
<td>397</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIN2A_15 and 16</td>
<td>F: CAGGCATCTACAGCTGATTC R: TGGTGACTGGTGCTCTCTGG</td>
<td>14</td>
<td>633</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIN2A_17 and 18</td>
<td>F: ATTCATACGCGGAGAGTTCAC R: GCCCAGTGTGCTGGTAGAC</td>
<td>14</td>
<td>759</td>
<td>94°C, 30 s</td>
<td>60°C, 30 s</td>
<td>72°C, 45 s</td>
<td></td>
</tr>
<tr>
<td>GRIN2A_19 and 20</td>
<td>F: GTGAGAGCGCGAGGATAC R: TTTACCCCTCAGACATTTGCG</td>
<td>14</td>
<td>758</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3.3. BRAF sequencing

The BRAF V600E mutation status had been determined previously for most cell lines (Kim, Stones et al. 2012). For the remaining samples, Cobas® 4800 BRAF Mutation Test (Roche Molecular Systems, Pleasanton) was used.

2.3.4. Sequence analysis and prediction of mutation impact

Exon sequences of GRIN2A were analysed by reference to human GRIN2A (NCBI; NG_011812) using Geneious Pro 5.6.4 software (Biomatters, Auckland, New Zealand). To help predict if amino-acid substitutions would affect protein function, Sorting Intolerant from Tolerant (SIFT) analysis of mutations was performed (http://sift.jcvi.org/) [18], relying on the UniProt SWISS-PROT 57.15 database. Catalogue of somatic mutations in cancer (COSMIC) (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) and MelanomaDB [19] databases were interrogated to search for GRIN2A mutations previously found in melanoma. Common germline single nucleotide polymorphisms (SNPs) in GRIN2A were eliminated using the NCBI database of SNPs (http://www.ncbi.nlm.nih.gov/projects/SNP).

2.3.5. Three-dimensional modelling of the mutated GluN1-GluN2A dimer

The G762E mutation was modelled into the X-ray crystal structure of the GluN1-GluN2A S1S2 heterodimer using Modeller9.11 [21] and the Protein Data Bank entry 2A5T [22] as the template. Ten models were constructed using two rounds of optimisation with the slow autoschedule and molecular dynamics refinement options, all other settings were kept at default values. The models were superimposed on the 2A5T heterodimer using PyMol [23], and inspected visually.

2.3.6. Collection of clinical data and statistical analysis

Data on antecedent primary melanoma, disease progression and treatment were obtained retrospectively from medical notes and electronic records. Quantitative data are presented as the mean ± standard error of the mean (SEM) or median (range). Statistical analysis was conducted using IBM SPSS Statistics software package for Windows, version 19.0 (Chicago, IL). To compare groups, cross-tabulations with significance tests were performed for data in categories. Analysis for mean differences between groups was performed using one-way ANOVA. Kaplan–Meier curves were produced for patients with and without GRIN2A and BRAF mutations, with differences between groups tested using Log-rank test. P values less than 0.05 were considered statistically significant.
2.3.7. RNA extraction

Barrier tips
Trizol
Chloroform
Cold centrifuge
Sterile RNase/DNase free tubes to collect lysates and RNA
Isopropanol
70% ethanol in sterile RNase free water
100% ethanol
Nanodrop spectrophotometer

Cells were grown to 95% confluency in a T75 flask and lysed with 3 mL Trizol reagent at room temperature. Cells were scraped off the surface of the flask thoroughly before removing the lysate. One mL of the lysate was collected into each 1.5 mL RNAs-free microfuge tube (the samples were stored at -80°C until ready for RNA purification). The samples were thawed on ice and 0.2 mL of chloroform was added to each tube. Each tube was vortexed for 2-3 minutes until the solution was light pink in colour. The homogenate was centrifuged at 17,000 rpm for 15 minutes at 4°C. The top aqueous layer containing RNA was removed and added to 0.6 mL of isopropanol.

The RNA in isopropanol was incubated at room temperature for 15 minutes to allow the nucleic acid to precipitate. The tubes were flicked and inverted a few times during the incubation. The sample was centrifuged at 17,000 rpm for 30 minutes at 4°C to pellet the RNA. The supernatant was discarded and the pellet was resuspended in 70% ethanol made up in ultrapure water. The sample was centrifuged at 17,000 rpm for 10 minutes at 4°C to pellet the RNA again and resuspended in 70% ethanol. Finally the supernatant was discarded and the pellet was air dried before dissolving the RNA in ~4 µL Milli-Q water for ~10 minutes. RNA concentration and purity (defined by the 260/280 and 260/230 ratio) was determined using a ND-1000 nanodrop spectrophotometer (ThermoFisher Scientific, Rockford, IL).
2.3.8. Re-precipitating RNA

High speed refrigerated centrifuge
sodium acetate
100% ethanol
70% v/v ethanol
Sterile RNase-free water

Chemically contaminated RNA was re-precipitated in 10% volume of sodium acetate (3 M, pH 5.2) and 2X volume of 100% ethanol. The tubes were incubated overnight at -20 °C. Following incubation the samples were centrifuged at 18,000 rpm for 40 minutes at 4°C. The supernatant was decanted and the RNA pellet was resuspended in 70% ethanol in RNase free water by gently flicking or vortexing the tube. The tubes were centrifuged again at 18,000 x g for 15 minutes at 4°C. The supernatant was decanted and the pellet resuspended in 100% ethanol and centrifuged at 18,000 x g for 40 minutes at 4°C. The supernatant was decanted and the pellet resuspended in 27 μl RNase-free water and incubated for 10 minutes at room temperature. The removal of the contaminants was assessed by nanodrop analysis.

2.3.9. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

0.2 mL sterile PCR tubes
1.5 mL sterile PCR tubes
RNase H
10X RT buffer
MgCl₂, 25 mM
RNase Out
RT enzyme
RNase free water
Sterile Milli-Q water
PCR cycler machine
Reverse transcription reaction

The Superscript III First Strand Synthesis Kit (Life Technologies) was used to synthesise cDNA from purified whole cell RNA. All tubes and reagents were handled on ice, except where specified.
2.3.9.1. Preparing the RNA extracts for reverse transcription

After determining the amount of RNA to be used for the reaction, the volumes of stock RNA to be used was calculated as shown below. Ideally, 2000 ng was used where possible but less was used when necessary:

Volume of RNA to be used = \[ \frac{2000 \text{ ng}}{\text{concentration of stock RNA}} \]

Volume of RNase free Water: 6 µl – volume of RNA

2.3.9.2. Mastermix for cDNA synthesis

A master mix of Oligo dT, random primers and dNTPs was prepared at a 1:1:2 ratio. 3 µL of the master mix was added to each tube containing the RNA and water (Table 2.3). The tubes were incubated at 65°C for 5 minutes and then on ice for at least 1 minute. Tubes were flicked to mix reagents and briefly centrifuged before and after incubation.

For “RT+” samples (reverse transcriptase added) the master mix was prepared by multiplying volume per tube by the number of “RT+” samples, allowing an extra 0.2 µL to account for pipetting error (Table 2.3). 10 µl of cDNA mix was added to each tube containing the RNA and incubated at 25°C for 10 minutes, 50°C for 60 minutes. Samples were heated to 85°C for 5 minutes to stop the reaction “RT-” (no reverse transcriptase added) tubes were prepared separately with volumes of each reagent.
### RNA and primer preparation

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume in µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo (dT)20 (50 µM)</td>
<td>1</td>
</tr>
<tr>
<td>Random primers (3 µg/µl)</td>
<td>1</td>
</tr>
<tr>
<td>10mM dNTP mix</td>
<td>2</td>
</tr>
<tr>
<td>Template RNA (2 µg)</td>
<td>1</td>
</tr>
<tr>
<td>DEPC-treated water (up to 10 µl)</td>
<td>5</td>
</tr>
<tr>
<td>Total volume</td>
<td>10</td>
</tr>
</tbody>
</table>

### cDNA mix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume in µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x RT buffer</td>
<td>2</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>4</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>2</td>
</tr>
<tr>
<td>RNase OUT (40 U/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Superscript III (200 U/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
</tbody>
</table>

**Table 2.3: Volumes of reagents need for cDNA synthesis**

1 µl of RNase H was added to each tube after the reaction was completed to degrade the template RNA leaving only the cDNA. Tubes were heated to 37 °C for 20 minutes to activate the RNase H and stored on ice thereafter. The synthesised cDNA was stored at -20°C.

### 2.3.10. PCR

10X PCR buffer for Taq from Fraser lab (University of Auckland)

500 mM KCl (10 mL of 1 M)

100 mM Tris HCl pH 9 (2 mL of 1 M)

1%v/v Triton-X 100 (200 µL)

Milli-Q water 7.8 mL

Stored at -20°C

**PCR reagents**

25 mM stock MgCl₂ (6-hydrate)
10 mM dNTPs
Forward and reverse primers
Taq polymerase
0.2 mL PCR tubes sterile
1.3 mL sterile tubes
High speed centrifuge
TAE buffer 10X stock
48.8 g Tris
11.43 mL glacial acetic acid
20 mL 0.5 M EDTA
Made up in Milli-Q water to 1 L
DNA loading Dye
50% aqueous glycerol
0.15 mg bromophenol blue
Dissolved in Milli-Q water

**DNA gel: 2% agarose**
Agarose was dissolved in TAE buffer.

### 2.3.10.1. Reconstituting primers

The primers were centrifuged at 14,000 rpm for 20 seconds and reconstituted in sterile water. The molar mass of each primer was noted and the volume of ultrapure water was added accordingly to give a 100 μM final stock concentration.
2.3.10.2. PCR reactions

Tubes for primer-specific PCR were prepared as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL) per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>up to 25</td>
</tr>
<tr>
<td>PCR Buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>2.5</td>
</tr>
<tr>
<td>Forward Primer 10 µM</td>
<td>0.75</td>
</tr>
<tr>
<td>Reverse Primer 10 µM</td>
<td>0.75</td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.5</td>
</tr>
<tr>
<td>BSA</td>
<td>If needed 2 µL (adjust with water)</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.2 µL</td>
</tr>
<tr>
<td>DNA template or water control</td>
<td>0.5 - 2 µL (adjust with water)</td>
</tr>
</tbody>
</table>

*Table 2.4: Volumes of reagents needed for polymerase chain reaction*

The master mix of the PCR reagents was prepared by multiplying each volume of reagents presented in table 2.4 by the number of tubes and taking into account pipetting error (20%). The master mix was aliquoted at 25 µL per PCR tube. Undiluted cDNA (0.5 - 2 µL) or negative controls was added to each tube. The RT- negative control contained no Reverse Transcriptase during cDNA synthesis and hence was not expected to contain cDNA. The water control consisted of ultrapure water without any template or reagent. The tubes were flicked to mix contents and briefly centrifuged before commencing the PCR cycle. PCR cycling conditions varied depending on the primer set used (Table 2.5 and 2.6).

Primers were tested at a range of annealing and extension temperatures in order to determine their optimum parameters for gene-specific PCR. For gradient PCR the columns on the PCR machine were set up with a range of temperatures assigned to each tube.

2.3.10.3. PCR troubleshooting

If the afore-mentioned PCR conditions were not successful, the following optimisation methods were used in order to troubleshoot the PCR using positive control DNA. This
included using a range of MgCl₂ (1.5-2.5 mM), BSA (0.2-0.8 µg/L) and DMSO (2-10%) concentrations and volumes of Taq polymerase (0.1–0.2 µL).

After optimisation, 2.5 mM MgCl₂, 0.2 µg/L BSA, no DMSO and 0.2 µL Taq polymerase were used per PCR reaction.

### 2.3.10.4. DNA gel electrophoresis

After the PCR, the tubes were briefly centrifuged at high speed and stored on ice. 2 µL of 6X DNA loading dye was added to 10 µL of post PCR product and run on a 2% agarose gel for ~50 minutes at 70 V until the dye bands ran two-thirds the length of the gel. The loading dye contained DNA intercalating agent GelRed (1:50 GelRed to loading dye). The amplified cDNA was visualised under UV light using the GelDoc system (Bio-Rad). A 100 bp DNA ladder was run alongside the samples to ascertain that the amplification products were of the expected size.
### Table 2.5: Sequences and cycling temperatures for primers designed against transcripts of GRIN1.

(F) Forward primer, (R) Reverse primer

Exon number refers to the position that the primer was targeted to the GRIN1 gene (see Figure 1).

All initial denaturation and final extension steps were run at 94°C for 2 minutes, and 72°C for 7 minutes, respectively.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence 5' to 3'</th>
<th>Amplicon size (bp)</th>
<th>Variant</th>
<th>Exon number</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>No of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRIN1-1 (F)</td>
<td>CAAGTATGCAGGATGCGGTGAC</td>
<td>211</td>
<td>all</td>
<td>8</td>
<td>94°C, 30 s</td>
<td>62°C, 30 s</td>
<td>72°C, 30 s</td>
<td>40</td>
</tr>
<tr>
<td>GRIN1-1 (R)</td>
<td>CAGTCTGGTGAGACATTGCGGTAC</td>
<td>all</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIN1-2 (F)</td>
<td>GGGTACCGAGATGCTCCAGCAG</td>
<td>227</td>
<td>all</td>
<td>9</td>
<td>94°C, 30 s</td>
<td>62°C, 30 s</td>
<td>72°C, 30 s</td>
<td>40</td>
</tr>
<tr>
<td>GRIN1-2 (R)</td>
<td>AGCTTGATGACCCATTGCGGAT</td>
<td>all</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIN1-3 (F)</td>
<td>CGACCACCAAGATGCTCAACTATG</td>
<td>768, 831</td>
<td>1-1, 1-2, 1-3</td>
<td>1</td>
<td>94°C, 15 s</td>
<td>60°C, 30 s</td>
<td>72°C, 60 s</td>
<td>40</td>
</tr>
<tr>
<td>GRIN1-3 (R)</td>
<td>GACTCGTTCTGCGTCCAGTCGAGC</td>
<td>3b, 1-4, 4b, 5</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIN1-4 (F)</td>
<td>GTCTCCCTGTCCATCCTGAAGTCC</td>
<td>410, 773</td>
<td>1-1, 3b, 5</td>
<td>17, 18</td>
<td>94°C, 15 s</td>
<td>60°C, 30 s</td>
<td>72°C, 60 s</td>
<td>40</td>
</tr>
<tr>
<td>GRIN1-4 (R)</td>
<td>CGAGCAATGACAGCCTCCATCAGTG</td>
<td>1-2</td>
<td>21a/b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRIN1-5 (F)</td>
<td>CAAGGATGACAGCGAGGAG</td>
<td>241</td>
<td>all</td>
<td>14</td>
<td>94°C, 30 s</td>
<td>62°C, 30 s</td>
<td>72°C, 30 s</td>
<td>40</td>
</tr>
<tr>
<td>GRIN1-5 (R)</td>
<td>GTCGGATGACCCCGATG</td>
<td>all</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 2.6: Sequences and PCR cycling conditions for primers designed to amplify GRIN2 and GRIN3 genes.

Primers cover all known variants of respective genes.

All initial denaturation and final extension steps were run at 94°C for 2 minutes, and 72°C for 7 minutes, respectively.
2.3.11. Western Blotting

**Phosphate Buffered Saline Tween 0.05% (PBS-T)**
10x Phosphate Buffered Saline 100 mL
Tween-20 0.5 mL
Milli-Q water 900 mL

**Running buffer**
10X BioRad running buffer

**Transfer buffer**
Glycine 14.4 g
Tris Base 3 g
Methanol 200 mL
Milli-Q water up to 1 L final volume

**BCA Reagent**
1 part CuSO₄ (4 g/100 mL Milli-Q water):50 parts BCA
(BCA= Bicinchoninic Acid)

**BSA stock solution 4mg/mL**
1 M NaOH
BSA 40 mg
1 M NaOH to 10 mL final volume

**Loading dye**
2M Tris pH 6.8
6% w/v SDS
50% v/v glycerol
50% v/v Milli-Q water
0.1% w/v Bromophenol blue

**Blocking buffer (5%)**
0.5 g milk powder
PBS-T to 10 mL final volume
RIPA buffer
Distilled water – 75 mL
Tris base – 790 mg
NaCl – 900 mg
pH adjusted to 7.4 with 6 M HCl
10% Nonidet P40 – 10 mL
10% Na deoxycholate – 2.5 mL
100 nM EDTA – 1 mL
Milli-Q water up to 100 mL final volume

2.3.11.1. Protein extraction

Cell lines for protein quantification were grown in 175 cm² (T175) flasks to about 90% confluency. Cells were washed with ice-cold PBS twice to remove medium and lysed with 1 mL cold RIPA lysis buffer. A cell scraper was used to scrape the cells off the surface of the flask. The lysate was then collected in a 1.5 mL microfuge tube and incubated on ice for 30 minutes. The tube was centrifuged at 14,000 rpm for 5 minutes to pellet cellular debris.

2.3.11.2. Protein quantification (BCA assay)

Protein quantification was carried out either immediately after protein extraction, or if tubes were frozen at -80 °C, after they were thawed on ice. This quantification was done to determine the volume of each cell lysate to be added to the Western blot, to ensure an equal amount of protein was added from all samples. Serial dilutions of stock BSA solution in 1 M NaOH were prepared to the following concentrations; 0, 31.25, 62.5, 125, 250, 500, 1000, 2000 µg/mL. In a 96-well plate, 50 µl was added from each tube in the dilution series. A 1:10 dilution of each sample (5 µl of sample and 45 µl of 1 M NaOH) was also added to the 96-well plate. BCA reagent (100 µl) was added to each well, the samples were mixed, incubated (37°C for 30 minutes) and absorbencies determined using ELX808 spectrophotometer (at 625 nm). The protein concentration of the cell lysates and the volume of each required was determined using the KC4 programme to generate a standard curve. Volumes of each sample needed were calculated using the equation generated from the standard curve.
Figure 2.1: Serial dilution and plate setup for BCA assay
2.3.11.3. Protein electrophoresis

Protein samples were prepared by thawing aliquots on ice and diluting in PBS according to the calculation from the BCA assay. Loading dye (6X) was also added according to the amount of protein being loaded. Samples were incubated at 95°C for 5 minutes and then kept at room temperature until loading commenced.

The gel was placed in an electrophoresis gasket (TGX Mini Stain-free, Bio-Rad). The samples and molecular weight markers were loaded in the gel and run at 80 V for 20 minutes (until samples have run past the stacking gel), then 120 V for 1 hour or until the dye had reached the bottom of the plate.

2.3.11.4. Protein transfer from gel to membrane

To transfer the protein from the gel, nitrocellulose membranes were pre-soaked in transfer buffer for at least 5 minutes. The plates were removed from the gel and the transfer cassette prepared in the order of gauze mat, 2X filter papers, nitrocellulose membrane, electrophoresis gel, 2X filter papers and gauze mat. The cassette which was kept wet at all times with transfer buffer was placed in the transfer tank with an ice coolant block and remaining transfer buffer. The transfer was run at 100 V for 1 hour.

2.3.11.5. Membrane processing

After the protein transfer, the nitrocellulose membrane was removed and blocked in blocking buffer for 30 minutes. Blocking buffer was decanted and the membrane washed (5 minutes) in 0.1 % PBS-T or TBS-T. The primary antibody was added and incubated overnight at 4°C on a shaker providing gentle agitation.

The primary antibody was decanted and the membrane washed in PBS-T (3 x 5 minutes). The Horseradish peroxidase (HRP) conjugated secondary antibody was added for 1 hour at room temperature on a shaker platform, then decanted and the membrane was washed in PBS-T or TBS-T (3 x 5 minutes).

Chemiluminescent substrate (ECL clarity) was added and incubated for 5 minutes at room temperature and the membrane was placed between plastic overhead transparency sheets. Air bubbles were removed with a tissue and the membrane was developed using the Fujifilm LAS 3000 imager, with an exposure time of 30 s intervals for approximately 5 minutes.
Settings for the photodeveloper

- Tray to position 3
- For Proteins: Type: Chemiluminescent
  Exposure: Increment
  Time: 30 s intervals
  Save photos as: 8 or 16 bit colour Tiff files
- For ladder: Type: Digitize, Epi
  Exposure: Precision, 1/1000s
  Save photos as: 8 or 16 bit colour Tiff file

2.3.11.6. Stripping of the membrane

Stripping and re-probing of the membrane was done to visualise the proteins used as loading controls. Following membrane development, the blot was stripped with 2 mL stripping buffer (15 minutes), washed in PBS-T and blocked in blocking buffer (30 minutes). The next primary antibody was then added at the required concentration and subsequent steps carried out as described in 1.3.12.2.
### 2.3.11.7. Dilutions of antibodies

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primary antibody</th>
<th>Supplier</th>
<th>Source</th>
<th>Reactivity</th>
<th>Isotype control</th>
<th>Concentration and diluent</th>
<th>Secondary antibody</th>
<th>Supplier</th>
<th>Concentration and diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluN1</td>
<td>NMDAR1 (D65B7)</td>
<td>Cell Signalling 5704</td>
<td>Rabbit monoclonal</td>
<td>Hu, Ms, Rt</td>
<td>IgG</td>
<td>1:1000 in 3% BSA</td>
<td>Goat anti-rabbit HRP</td>
<td>Santa Cruz</td>
<td>1:5000 in 5% milk</td>
</tr>
<tr>
<td>GluN1</td>
<td>NMDAR1</td>
<td>BD 556308</td>
<td>Mouse monoclonal</td>
<td>Hu, Mk, Rt</td>
<td>IgG2a</td>
<td>1:500 in 5% milk</td>
<td>Goat anti-mouse HRP</td>
<td>Santa Cruz</td>
<td>1:5000 in 5% milk</td>
</tr>
<tr>
<td>GluN2A</td>
<td>NMDAR2A EPR7063</td>
<td>Abcam Ab133265</td>
<td>Rabbit monoclonal</td>
<td>Hu, Ms, Rt</td>
<td>IgG</td>
<td>1:1000 in 3% BSA</td>
<td>Goat anti-rabbit HRP</td>
<td>Santa Cruz</td>
<td>1:2000 in 5% milk</td>
</tr>
<tr>
<td>GluN2A</td>
<td>NMDAR2A</td>
<td>Millipore MAB5216</td>
<td>Mouse monoclonal</td>
<td>Hu, Rt</td>
<td>IgG1</td>
<td>1:500 in 5% BSA</td>
<td>Goat anti-mouse HRP</td>
<td>Santa Cruz</td>
<td>1:2000 in 5% milk</td>
</tr>
<tr>
<td>GluN2A</td>
<td>NMDAε1 (H-54)</td>
<td>Santa Cruz Sc-9056</td>
<td>Rabbit polyclonal</td>
<td>Hu, Ms</td>
<td>IgG</td>
<td>1:500 in 5% BSA</td>
<td></td>
<td></td>
<td>1:2000 in 5% milk</td>
</tr>
<tr>
<td>Slug</td>
<td>Slug</td>
<td>Cell Signalling</td>
<td>Rabbit monoclonal</td>
<td>Hu, Ms</td>
<td>IgG</td>
<td>1:1000 in 5% BSA</td>
<td>Goat anti-rabbit HRP</td>
<td>Santa Cruz</td>
<td>1:2000 in 5% milk</td>
</tr>
<tr>
<td>B-Actin</td>
<td>Actin clone C4</td>
<td>Millipore MAB1501</td>
<td>Mouse monoclonal</td>
<td>Hu, Ms, Rt</td>
<td>IgG2bk</td>
<td>IgG</td>
<td>Goat anti-mouse HRP</td>
<td>Santa Cruz</td>
<td>1:10,000 in 5% milk</td>
</tr>
</tbody>
</table>

**Table 2.7: Antibodies**

Hu, Human; Ms, Mouse; Rt, Rat; Mk, Monkey.
2.3.12. Thymidine incorporation assay

Tritiated (3H) thymidine (1 mCi/mL)
800 nM Fluorodeoxyuridine (FUdR)
Non-tritiated thymidine (TdR)
4 mM Na₄EDTA in PBS
Pronase
Cell culture medium
96 well culture plates
Plate harvester
Membrane
Scintillation fluid with clear plastic pockets
Cell counting instrument

2.3.12.1. Plating cells

Cells used for the thymidine incorporation assay were plated at a density of 1000 cells/well in 96-well plates. Cultured cells in log phase growth were diluted to a concentration of 8000 cells/mL. 125 µL was added to each well of a 96 well plate (1000 cells/ well). Four cell lines were plated per 96 well plate as shown in Figure 2.2. Cells in the first two columns of the plate (1 and 2) were untreated and only medium and no drug was added to these wells. Cells in wells corresponding to columns 3 - 12 were treated with descending concentrations of the drug or drugs to be tested. Cells were incubated at 37°C, 5% O₂, 5% CO₂ until the drug was prepared and added to the plate.
 CHAPTER 2

Figure 2.2: Cell plating set up for the thymine incorporation assay

<table>
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</table>

Cells treated from highest to lowest concentration (1 drug well 3-12) or (2 drugs: well 3-7 and 8 to 12)
2.3.12.2. Setting up drug working dilutions

All drug stocks used (memantine, MK-801, riluzole, AP5, NMDA and glutamic acid) were made up to 100 mM and diluted down as working stocks to 6X the final desired concentration before they were added to the plate containing cells. In order to set up the working drug concentrations that differ by a factor of ten, 200 µL of medium was added in duplicates to the wells 1, 2, 3 and 8 and 225 µL of medium was added to wells 4-7 and 9-12 of a 96 well plate (Figure 2.3 (a)). Two drugs were set up per row. Wells in column 3 had the highest drug concentration and this was diluted 10X in a series up to well 7 by adding 25 µl from each consecutive well to another (Figure 2.3). The same was done from well 8-12 with well 8 having the highest and well 12 having the lowest drug concentration.

Example of drug dilution values: 12 µL of 100 mM stock drug added to wells 3 and/or 8 respectively to give a working highest concentration of 6 mM. This is then diluted 10X to 600 µM, 60 µM, 6 µM and 0.6 µM. 25 µL of the working drug or control medium was then added to the plated cells in 125 µL medium (Figure 2.2). This gave a final volume of 150 µL (cells + drug) in the culture plate and a final highest drug concentration of 1 mM, 100 µM, 10 µM, 1 µM and 0.1 µM from wells 3 to 7 (or 9 to 12) respectively (Figure 2.3a.). The plate was set up similarly for a 3X drug dilution (Figure 2.3b) with appropriate modifications.
Figure 2.3: Treating cells for the thymine incorporation assay

Modulators diluted at a (A) 2X and (B) 3X dilution factor
2.3.12.3. Harvesting cells

Following drug treatment the cells were exposed to tritiated thymidine and harvested onto a membrane using a TomTec harvester.

A mastermix containing 2.06 mL complete medium, 4.2 µL tritiated thymidine, 16.8 µL of 100 µM FUdR and 16.8 µL of 100 µM TdR (dissolved in medium) was prepared (Table 2) and 20 µL was added to each well. The plates were incubated with these reagents at 37°C for six hours. Protease (pronase) was dissolved (in 4 mM Na4EdTA in PBS) to a final concentration of 2 mg/mL. 150 µL was added to each well of the plate and incubated at a 37°C for 20-30 minutes or until cells were rounded. The cells were harvested onto a membrane using the TomTec harvester. The membrane was dried overnight and then sealed into a plastic pocket with scintillation fluid. The membrane was then placed into cassettes which were loaded into the Trilux for counting, cell counts were analysed using the GraphPad Prism software.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume for 1 96 well plate</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tritiated (3H) thymidine (1m Ci/mL)</td>
<td>4.2 µL</td>
<td>0.25 mCi/µL</td>
</tr>
<tr>
<td>100 µM Fluorodeoxyuridine (FUdR)</td>
<td>16.8 µL</td>
<td>10 nM</td>
</tr>
<tr>
<td>100 µM Non-tritiated thymidine (TdR)</td>
<td>16.8 µL</td>
<td>10 nM</td>
</tr>
<tr>
<td>Cell culture medium</td>
<td>2.06 mL</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.8: Reagent preparation for thymidine incorporation

2.3.13. Modulators

Memantine, MK-801 and riluzole
Stock solutions were made to 100 mM in DMSO and stored at -20°C.
AP5, NMDA and glutamic acid
Stock solutions were made to 100 mM in water and stored at -20°C.

2.3.14. Glutamate release assay

Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit
α-MEM medium as diluent
NZM cell lines were cultured in duplicates in six well plates in α-MEM and RPMI 1640 supplemented with 5% FBS at 37°C for nine days at 5% CO₂ and 5% O₂. NZM 3, NZM 6, NZM 7 and NZM 40 were seeded in 2 mLs of medium at a density of 5 x 10³ cells/mL. NZM 11, NZM 61 and NZM 100 were seeded in 2 mLs of medium at a density of 10 x 10³ cells/mL. Preliminary cell culture trials were carried out in order to determine the seeding density needed to achieve 90-100% confluency over a nine day period.

Each type of medium (α-MEM and RPMI with 5% FBS, ITS and Penstrep) was also plated in a six well plate with no cells in order to determine the level of spontaneous degradation of glutamine in the medium to glutamate. Samples of each type of medium were collected in 1.5 mL microfuge tubes on day 0 (the day the cells were plated) and on day nine. Tubes were stored at -80°C. These samples of medium were analysed for their concentration of glutamate using the Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit as per manufacturer’s instructions. The reaction volume recommended was 100 µL in a black 96 well plate but the reaction volume used was halved for the assay as it was run in a black 384 well plate with appropriate positive and negative controls. The reaction involves the oxidation of L-glutamic acid by glutamate oxidase to produce α-ketoglutarate, NH₃ and H₂O₂. L-Alanine and L-glutamate-pyruvate transaminase regenerate L-glutamic acid by transamination of α-ketoglutarate, resulting in multiple cycles of the initial reaction and amplification of the H₂O₂ produced. The H₂O₂ reacts with 10-acetyl-3,7-dihydroxy phenoxazine (Amplex® Red reagent) in a 1:1 stoichiometry in the reaction catalysed by horseradish peroxidase (HRP) to generate the highly fluorescent product, resorufin.1,2 because resorufin has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively (Figure 2.4). 10 µM H₂O₂ was used as a positive technical control for the reaction. The samples of medium were diluted 40X before being assayed for glutamate concentration. The fluorescence signal was read from the top of the plate using an EnSpire 2300 Multimode Plate Reader (Perkin-Elmer) at excitation 571 nm and emission 585 nm (25°C).
Figure 2.4: Mechanism behind the Amplex® Red Glutamic Acid/Glutamate Oxidase Assay Kit.
2.3.15. Invasion assay

Wright Giemsa stain
100% methanol
Milli-Q water
Sterile tweezers
Matrigel coated Boyden chamber inserts
Companion plate
Cell culture medium
Cotton swabs
Brightfield microscope

The invasion assay was conducted using the BD Biocoat™ Matrigel™ Invasion system. The system includes a 24-well companion plate containing transwell inserts with a porous (8 µm pore) Matrigel coated membranes. Cells were seeded in duplicates at a density of 80 x 10³ cells/mL in 500 µL of serum free α-MEM on the apical side of the membrane. 700 µL of α-MEM containing 10% FBS as a stimulant was added to the basal side of the membrane. The cells were incubated at 37°C with 5% CO₂ and 5% O₂ for 30 minutes. Drugs were added to the medium on both the apical and basal sides of the membrane to a final concentration of 100 µM before incubating the plate for another 20 hours. During the incubation period the cells were allowed to invade from the apical to the basal side of the membrane towards the chemoattractant (10% FBS). The Matrigel (on the apical side of the membrane) was removed with a cotton swab according to manufacturer’s instructions (Figure 2.5). The cells that had invaded through the Matrigel to the basal region of the membrane were fixed in methanol and stained (Wright-Giemsa nuclear and cytoplasmic stain). Bright field microscopic images of these cells were taken using a Nikon TE2000E inverted light microscope equipped with a Nikon Digital Sight colour camera. Four images per membrane were captured using a Plan Fluor 10x/0.45 NA objective lens so that the maximum portion of the membrane was covered. The images were analysed using Image J software (version 1.49) to quantify the number of cells that had invaded through to the basal side of the membrane. The method involved determining the invasiveness of the cells by quantifying the confluency of cells in the images captured (measured by the area occupied by the cells per field of view). The images were separated into their component red, green and blue channels and the analysis was conducted on the green channel. Thresholds were set to include cells but exclude the 8 µM membrane...
pore (Figure 2.6). A macro was created that used the ImageJ software to automatically analyse all images using the same set parameters. The numerical output generated was a percentage value representing cell density based on surface area of the image occupied by stained objects. These numerical values were statistically analysed and graphed using Prism.
Figure 2.5: Method for setting up and conducting cell invasion assay

Cells were seeded on Matrigel coated porous (8 µm pores) membranes. Medium containing FBS as a stimulant was added to the basal side of the membrane. Modulators were added to the medium on both the apical and basal side of the membrane. Cells invade through the Matrigel from the apical to the basal side of the membrane. The invaded cells are fixed in methanol and stained.
Figure 2.6: Method for quantifying cell invasiveness

Bright-field microscopic images of fixed and stained cells were taken at 10x magnification. The method of analysis involved quantifying the confluency of cells in the images captured. The confluency of the area was measured by the percent area occupied by the cells.
CHAPTER 2

2.3.15.1. MACROS created for image analysis

dir1 = getDirectory("Choose Source Directory ");{
dir2 = getDirectory("Choose Destination Directory ");
list = getFilePath(dir1);
setBatchMode(true);
for (i=0; i<list.length; i++) {
    showProgress(i+1, list.length);
    open(dir1+list[i]);
    run("Set Scale...", "distance=1026 known=500 pixel=1 unit=um");
    imgName = getTitle();
    run("Split Channels");
    selectWindow(imgName + " (blue)");
    close();
    selectWindow(imgName + " (green)");
    selectWindow(imgName + " (red)");
    close();
    selectWindow(imgName + " (green)");
    setAutoThreshold("Default");
    run("Threshold...");
    setAutoThreshold("Default");
    run("Set Measurements...", "area standard area_fraction limit display redirect=None decimal=3");
    run("Analyze Particles...", "size=110-Infinity show=Outlines summarize");
    selectWindow(imgName + " (green)");
    run("Create Selection");
    run("Add Selection...");
    saveAs("Tiff", dir2+imgName);
    close();
    selectWindow("Drawing of " + imgName + " (green)");
    newtitle = imgName + "-Overlay-";
    saveAs("TIFF", dir2 + newtitle);
    close();
}
selectWindow("Summary");
saveAs("Results", dir2 + "Summary.xls");
}
2.3.16. Calcium flux

Fluo-4 acetoxymethyl ester (AM)
Foetal Bovine Serum (FBS)
Fluorescence microscope

Locke’s imaging buffer
8.6 mM HEPES
5.6 mM KCl
154 mM NaCl
5.6 mM glucose
1 mM MgCl₂
2.3 mM CaCl₂

2.3.17. Fluorescence microscopy

Melanoma cells and melanocytes were plated in four-well Falcon™ Chamber Culture Slides (BD Biosciences, USA) at a density of 50 × 10³ cells/mL in 800 µL of α-MEM medium. Cells were cultured for 24 hours and washed once with modified Locke's buffer. Melanoma cells were loaded with 5 µM Fluo-4 acetoxymethyl ester (AM) for 15 minutes at 37°C. They were washed and incubated in Locke's buffer for another 15 minutes in the dark at room temperature to allow for completion of the de-esterification by intracellular esterases. The buffer was replaced with buffer containing 1% FBS in which the cells were imaged. Melanocytes were stained in the same manner with the exception of the Fluo-4 AM concentration being 2 µM and the final imaging buffer containing 5% FBS.

Culture slides were placed in a custom-built Solent incubation chamber attached to a Nikon TE2000E inverted fluorescence microscope (Tokyo, Japan). Cells were imaged using a Plan Fluor 10 ×/0.45 NA objective lens (Nikon). Fluorescence images were taken using a Photometrics Evolve camera and FITC filter cube (IDEX, Lake Forest, IL), with excitation at 457–487 nm and long-pass band emission filter at 520 nm. Glutamate (100 and 500 µM), NMDA and glycine (100 and 200 µM) were applied to melanoma cells to determine whether they induce calcium fluxes in these cells. Ionomycin (1 µg/mL) was added as a positive control and buffer was used for the negative control. Baseline fluorescence was recorded for 10 s, after which the activator (NMDAR agonist:NMDA, glutamate or ionomycin) or buffer was added and imaging continued for a further 90 seconds. Fluorescence intensity of each cell was
tracked and analysed over time using Image-Pro Plus 7.0 software (Media Cybernetics, Rockville, MD).

**2.3.17.1. EnSpire plate reader**

Fluo-4 NW Calcium Assay kit  
Foetal Bovine Serum (FBS)  
Fluorescence plate reader  
Multichannel pipettes  
Black clear bottom 96 well plate

Melanoma cells, melanocytes and rat embryonic hippocampus cells were plated on a black clear bottom 96well plate overnight. Cells were loaded with Fluo-4 AM with 0.06% pluronic acid and 2.5 mM probenecid using the Fluo-4 NW Calcium Assay kit for 15 minutes at 37°C, then washed with buffer and incubated for another 15 minutes at room temperature. The fluorescence signal was read from the bottom of the plate at 1 s intervals (3 wells per s) using an EnSpire 2300 Multimode Plate Reader (Perkin-Elmer) at excitation 494 nm and emission 506 nm (25°C, 3 mm measurement height, 50 flashes). Activators were applied to melanoma cells to determine whether they induced calcium fluxes. Ionomycin (1µg/mL) was added as a positive control and buffer as a negative control. Baseline fluorescence was recorded for 10 seconds, after which the activator or buffer was added and imaging continued for a further 90 seconds.
CHAPTER 3

GRIN2A mutations in melanoma correlate with decreased survival

This chapter is a reproduction of a published paper:


3.1. Introduction

The genomic revolution of recent years has led to substantial advances in the cataloguing of mutations in melanoma, most notable of which have been activating mutations in BRAF, NRAS and KIT genes (Hodis, Watson et al. 2012) (described in Section 1.1.). The presence of these mutations helps guide treatment with RAF, MEK and KIT inhibitors (Section 1.1.), but they do not predict disease progression or survival of patients (Jang and Atkins 2013). In general, the usefulness of molecular biomarkers in determining melanoma prognosis remains limited.

Mutations in GRIN2A have been reported in up to a third of melanoma samples (Wei, Walia et al. 2011), although with wide variation between studies, and no data on their clinical relevance. The GRIN2A gene, located on chromosome 16p13.2, encodes the GluN2A protein, a regulatory subunit of the glutamate-gated N-methyl-D-aspartate receptor (NMDAR) (Collingridge, Olsen et al. 2009, Traynelis, Wollmuth et al. 2010). The finding of GRIN2A mutations in melanoma had been unexpected because NMDARs are best known for their roles in the brain. Nevertheless, NMDARs have attracted attention for their potential contribution in cancer due to effects on cell death, survival and migration (North, Gao et al. 2010, Li and Hanahan 2013). Current knowledge on the NMDARs in the context of melanoma is limited,
although expression of GluN2A in both normal and malignant melanocytes has been demonstrated (Hoogduijn, Hitchcock et al. 2006, Song, He et al. 2012).

In response to the previously published exome sequencing data (Wei, Walia et al. 2011), an investigation was carried out in order to determine the prevalence of \textit{GRIN2A} mutations in 19 low-passage metastatic melanoma cell lines. The melanoma cell lines were developed at the Auckland Cancer Society Research Centre, and retrospectively correlated the presence of \textit{GRIN2A} mutations with patient outcome.

\textbf{3.2. Results}

Nineteen low-passage melanoma cell lines, derived from 19 patients with metastatic melanoma were used to sequence 12 coding exons of \textit{GRIN2A} together with their flanking intronic regions. These were exons 3 to 14. Exons 1 and 2 are untranslated and were not sequenced. Cell lines for sequencing were chosen randomly; all were passaged less than 30 times.

\textbf{3.2.1. Clinical associations of \textit{GRIN2A} mutations}

Sequencing of \textit{GRIN2A} was conducted using cell lines derived from tumours spread to lymph nodes in 12 patients (63%), distal organs (brain, small bowel, ascites or lung) (five patients; 26%), and locoregional metastases (two patients; 11%). Patient characteristics at enrolment into the study and the treatment they received are shown in Tables 3.1. Apart from two patients who presented with either bulky or disseminated disease and entered this study on presentation, other patients had a prior history of skin melanoma dating back a median of 34 (1–211) months. Seventeen patients had primary skin lesions in sun-exposed areas; in the other two patients, primary skin lesions remained occult. At the time of enrolment (between 1989 and 2010), 12 patients (63%) had stage IV disease, with the remainder in stage III (table 3.2a). All patients were managed surgically and with radiotherapy. Five patients received chemotherapy – three had POC (procarbazine, vincristine and lomustine), one temozolomide and one DTIC (dacarbazine), with the number of cycles ranging from one to six. Adjuvant immunotherapy was used in two patients and one patient also received an experimental agent within a stage I clinical trial. No patients were treated with \textit{BRAF} inhibitors. One patient remains free of melanoma 12 years after nodal recurrence. The other 18 patients have all died of their disease. The median survival time was 36 (4–229) months (Table 3.2A).
There was no difference in age and gender between patients whose melanoma lines carried \textit{GRIN2A} mutations and those lines that did not (Table 3.2A). Two patients in this study presented with disseminated melanoma and both were found to carry nonsynonymous mutations in \textit{GRIN2A}. The other two patients with the nonsynonymous \textit{GRIN2A} mutations presented with skin lesions that spread to lymph nodes within 9 and 27 months, compared with a median of 37 (0–140) months for patients with non-mutated \textit{GRIN2A} (P = 0.041; Table 3.2a).

Patients with \textit{GRIN2A} mutations (both nonsynonymous and synonymous) had faster progression of melanoma from skin lesions to the involvement of lymph nodes (P = 0.041) and distant organs (P = 0.016), compared with patients with non-mutated \textit{GRIN2A} (Table 3.2A, Figure 3.1 and 3.3). The overall survival of patients with \textit{GRIN2A} mutations was shorter than in patients without \textit{GRIN2A} mutations (P = 0.02; Figure 3.2A, Table 3.2A). A summary of disease progression events for patients with no mutations, synonymous and nonsynonymous mutations in \textit{GRIN2A} is presented in Figure 3.3. The \textit{BRAF} V600E mutation was found in 11 of 19 (58%) tumour samples but in contrast to \textit{GRIN2A}, there was no correlation with overall survival (P = 0.963) (Figure 3.2B). Table 3.2B shows that patient treatments had no impact on the differences in survival and tumour spread. Table 3.3 shows all the NZM cell lines tested, their mutation status and systemic therapy received by the corresponding patients prior to their establishment.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Patient Therapy</th>
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<tbody>
<tr>
<td>NZM 1 and NZM 2</td>
<td>Surgery and Radiotherapy</td>
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<tr>
<td>NZM 4</td>
<td>POC chemotherapy</td>
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<tr>
<td></td>
<td>2 cycles</td>
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<td></td>
<td>Commenced after tissue collection</td>
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<tr>
<td>NZM 6</td>
<td>Surgery and Radiotherapy</td>
</tr>
<tr>
<td>NZM 9</td>
<td>Surgery and Radiotherapy</td>
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<tr>
<td>NZM 11</td>
<td>POC chemotherapy</td>
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<tr>
<td></td>
<td>6 cycles</td>
</tr>
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<td>Treatment commenced after tissue collection</td>
</tr>
<tr>
<td>NZM 17</td>
<td>Received vaccination before tissue collection</td>
</tr>
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<td>NZM 20</td>
<td>Surgery and Radiotherapy</td>
</tr>
<tr>
<td>NZM 30</td>
<td>Surgery and Radiotherapy</td>
</tr>
<tr>
<td>NZM 34</td>
<td>Received vaccination after tissue collection</td>
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<tr>
<td>NZM 46</td>
<td>No information</td>
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<td>NZM 63</td>
<td>DTIC chemotherapy</td>
</tr>
<tr>
<td></td>
<td>4 cycles</td>
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<tr>
<td></td>
<td>Commenced after tissue collection</td>
</tr>
<tr>
<td>NZM 3</td>
<td>Surgery and Radiotherapy</td>
</tr>
<tr>
<td>NZM 7</td>
<td>Surgery and Radiotherapy</td>
</tr>
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<td>NZM 61</td>
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<td>NZM 100</td>
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<td>NZM 40</td>
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<td>Received before tissue collection DMXAA</td>
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<td>IFN</td>
</tr>
<tr>
<td></td>
<td>Received vaccine</td>
</tr>
<tr>
<td></td>
<td>Commenced after tissue collection</td>
</tr>
<tr>
<td>NZM 86</td>
<td>Surgery and Radiotherapy</td>
</tr>
</tbody>
</table>

Table 3.1: Treatments received by melanoma patients
<table>
<thead>
<tr>
<th>Age: mean (SEM) years</th>
<th>All patients n = 19</th>
<th>Non-synonymous GRIN2A mutations n = 4</th>
<th>Synonymous GRIN2A mutations only n = 3</th>
<th>Non-mutated GRIN2A n = 12</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males n (%)</td>
<td>13 (68)</td>
<td>4 (21)</td>
<td>1 (5)</td>
<td>8 (42)</td>
<td>0.168²</td>
</tr>
<tr>
<td>Patients with stage IV at enrolment n (%)³</td>
<td>12 (63)</td>
<td>1 (25)</td>
<td>2 (67)</td>
<td>9 (75)</td>
<td>0.198²</td>
</tr>
<tr>
<td>Duration of known melanoma prior to enrolment mean (SEM) months</td>
<td>62 (17)</td>
<td>14 (8)</td>
<td>22 (17)</td>
<td>90 (24)</td>
<td>0.110⁹</td>
</tr>
<tr>
<td>Progression from first-ever melanoma to stage III median (range) months</td>
<td>9 (0–140)</td>
<td>0 (0–27)</td>
<td>6 (0–17)</td>
<td>37 (0–140)</td>
<td>0.041⁴</td>
</tr>
<tr>
<td>Progression from first-ever melanoma to stage IV median (range) months</td>
<td>34 (0–205)</td>
<td>2 (0–34)</td>
<td>14 (4–35)</td>
<td>108 (1–205)</td>
<td>0.016⁴</td>
</tr>
<tr>
<td>Overall survival median (range) months</td>
<td>36 (4–229)</td>
<td>5 (4–36)</td>
<td>15 (5–61)</td>
<td>114 (4–229)</td>
<td>0.020⁴</td>
</tr>
</tbody>
</table>

Table 3.2A: Clinical characteristics for all patients according to the presence or absence of GRIN2A mutations in tumour derived cell lines

¹One-way ANOVA; ²\( \chi^2 \) test; ³Other patients were enrolled while in stage III; ⁴Log-rank test
### Table 3.2B: Summary of disease progression showing that patient treatments had no impact on the differences in survival and tumour spread

<table>
<thead>
<tr>
<th>Treatment effects</th>
<th>Surgery and Radiotherapy n=11</th>
<th>Chemotherapy or vaccination n=7</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis to stage III (Lymph node metastasis) median (range) months</td>
<td>27 (0-140)</td>
<td>9 (0-140)</td>
<td>0.849</td>
</tr>
<tr>
<td>Diagnosis to stage IV (Distant Metastasis) median (range) months</td>
<td>34 (0-205)</td>
<td>35 (2-152)</td>
<td>0.499</td>
</tr>
<tr>
<td>Diagnosis to death (Overall survival) median (range) months</td>
<td>36 (4-205)</td>
<td>61 (5-229)</td>
<td>0.843</td>
</tr>
</tbody>
</table>
### Table 3.3: Summary of NZM cell lines tested in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Non-synonymous mutations in <em>GRIN2A</em></th>
<th>Synonymous mutations in <em>GRIN2A</em></th>
<th>BRAF V600E mutation</th>
<th>Treatment received prior to tumour resection</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZM 3</td>
<td>P1133S</td>
<td>F1344F</td>
<td>present</td>
<td>treatment naïve</td>
</tr>
<tr>
<td>NZM 7</td>
<td>G889E</td>
<td></td>
<td>present</td>
<td>treatment naïve</td>
</tr>
<tr>
<td>NZM 40</td>
<td></td>
<td>F186F</td>
<td>absent</td>
<td>Two cycles of POC chemotherapy completed 8 months prior to tumour resection. This was followed by a vascular-disrupting agent (6 months before) and Interferon α (2 months before resection)</td>
</tr>
<tr>
<td>NZM 55</td>
<td>L794L</td>
<td>present</td>
<td>treatment naïve</td>
<td></td>
</tr>
<tr>
<td>NZM 61</td>
<td>S349F; G762E</td>
<td>F177F</td>
<td>absent</td>
<td>treatment naïve</td>
</tr>
<tr>
<td>NZM 86</td>
<td>A1409A</td>
<td>absent</td>
<td>treatment naïve</td>
<td></td>
</tr>
<tr>
<td>NZM 100</td>
<td>P1132L</td>
<td></td>
<td>present</td>
<td>treatment naïve</td>
</tr>
<tr>
<td>NZM 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZM 11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZM 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZM 30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZM 34</td>
<td>absent</td>
<td>absent</td>
<td>present</td>
<td>treatment naïve</td>
</tr>
<tr>
<td>NZM 76</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZM 1</td>
<td></td>
<td></td>
<td></td>
<td>Patient from whom NZM 017 was established received an autologous tumour vaccine a year before tumour resection. Other cell lines were treatment naïve.</td>
</tr>
<tr>
<td>NZM 9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZM 17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZM 63</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZM 46</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1: Disease progression to lymph node and distant organ metastases

Times of disease progression from diagnosis to (A) lymph node or (B) distant organ metastases for individual patients according to the presence or absence of GRIN2A mutations. Levels of statistical significance are shown. *Progression data for one patient with non-mutated GRIN2A was not available.
Figure 3.2: Overall survival of melanoma patients according to the \textit{GRIN2A} and \textit{BRAF} V600E mutation status

Overall survival according to the \textit{GRIN2A} (\textbf{A}) or \textit{BRAF} V600E (\textbf{B}) mutation status. Levels of statistical significance are shown. *Data for one patient with non-mutated \textit{GRIN2A} (V600E absent) was not available.
Summary of disease progression events for patients with no, synonymous and non-synonymous GRIN2A mutations

Data points for individual patients are shown; horizontal lines mark median values in each group. Levels of statistical difference between groups are shown.

*Disease progression data for one patient with non-mutated GRIN2A was not available.
3.2.2. *GRIN2A* mutations in melanoma

Of 19 tumour samples tested, four (21%) carried five nonsynonymous mutations in *GRIN2A* (Table 3.4). Five synonymous mutations were also detected (Figure 3.4, Table 3.5), as well as four SNPs (Table 3.6). The SNPs were excluded from further analysis. All nonsynonymous mutations were missense: three clustered in exon 14 (G889E, P1132L, P1133S), the other two in exon 5 (S349F) and exon 12 (G762E) (Figure 3.5A). These locations corresponded with the evolutionarily conserved domains in the GluN2A protein: C-terminal, N-terminal and the S2 segment, respectively (Figure 3.5B and C). The S2 segment forms the ligand-binding domain, and the intracytoplasmic C-terminus is involved in intracellular signalling and interactions with the cytoskeleton. Catalogue of somatic mutations in cancer (COSMIC) and MelanomaDB (Trevarton, Mann et al. 2013) databases were interrogated to search for *GRIN2A* mutations previously found in melanoma. Two mutations, G889E and P1132L were previously reported (Wei, Walia et al. 2011) and (Dahlman, Xia et al. 2012), respectively.
Figure 3.4: Sanger sequencing output of non-synonymous mutations in GRIN2A

Each panel represents a non-synonymous mutation detected. Nucleotides are labelled below their respective peaks. Corresponding amino acid sequences are indicated.
Figure 3.5: Schematic of the GRIN2A gene and corresponding GluN2A protein showing the locations of detected mutations

Schematic of the GluN2A protein together with the nonsynonymous (in red) and synonymous (in grey) mutations in GRIN2A.

(A) GRIN2A cDNA is 4,392 nucleotides long and can be divided into sections encoding evolutionarily conserved domains in the GluN2A protein. The first and last amino acid residues of the (B) SP, S1, S2, and M1-M4 domains are numbered. Symbols and mark mutations that coincided in the same tumour samples; * marks mutations reported previously. Abbreviations: SP, signal peptide; NTD, N-terminal domain; S1 and S2 segments form the glutamate-binding domain; M1-4 transmembrane segments form the ion channel pore; CTD, C-terminal domain. A schematic depicting the locations of the membrane bound domains M1 to M4, S1 and S2 loops and the Amino terminal domain (ATD) to the Carboxy terminal domain (CTD). (C) A schematic of GluN2A with conserved functional domains. The somatic mutations have been indicated with arrows. SP, signal peptide; PBP1_iGluR_NMDA_GluN2, N-terminal leucine/isoleucine/valine-binding protein LIVBP-like domain of the NR2 subunit of NMDA receptor family; PBPb, bacterial periplasmic substrate-binding protein; Lig_chan, ligand-gated ion channel; NMDAR2_C, N-methyl D-aspartate receptor 2B3 C terminus. Modified from Wei, Walia et al., 2011.
3.2.3. Predicting the effects of mutations on protein function

In order to predict whether amino acid substitutions would affect protein function, Sorting Intolerant from Tolerant (SIFT) analysis of mutations was performed (Ng and Henikoff 2006), applying UniProt SWISS-PROT 57.15 database. SIFT analysis predicted that S349F, G762E and P1133S would deleteriously affect protein function (Table 3.4).

We modelled the G762E substitution into the 3-dimensional X-ray crystal structure of the GluN1-GluN2A heterodimer, 2A5T (Furukawa et al., 2005). This revealed that G762E was located in the distal ‘hinge’ of the glutamate-binding clam-shell-like region of GluN2A corresponding to the S2 loop (Figure 3.6). In this location, the mutated glutamate residue was seen to interact with K531 in the GluN1 protein interfacing GluN2A in this region (Figure 3.7). While K531 formed a hydrogen bond with the backbone carbonyl of F524 in GluN2A, its proximity to the mutated glutamate side-chain (G762E) indicated the potential for new electrostatic interactions between G762E (in GluN2A) and K531 (in GluN1) (Figure 3.7) with the ability to alter interactions between GluN2A and GluN1 subunits and consequently, impact NMDAR functionality. Conformational changes that developed to accommodate G762E could also affect ligand binding, as the residue preceding G762E (Y761) was part of the glutamate-binding site (Figure 3.7).
<table>
<thead>
<tr>
<th>NZM cell line</th>
<th>Substitution and nucleotide number</th>
<th>Amino acid change</th>
<th>Exon</th>
<th>Zygosity</th>
<th>SIFT score</th>
<th>SIFT median</th>
</tr>
</thead>
<tbody>
<tr>
<td>061</td>
<td>C&gt;T c.1046 g.291693</td>
<td>S349F</td>
<td>5</td>
<td>Hetero-</td>
<td>0.00</td>
<td>3.33</td>
</tr>
<tr>
<td>061</td>
<td>G&gt;A c.2285 g.384407</td>
<td>G762E</td>
<td>12</td>
<td>Hetero-</td>
<td>0.00</td>
<td>3.08</td>
</tr>
<tr>
<td>007</td>
<td>G&gt;A c.2666 g.417877</td>
<td>G889E</td>
<td>14</td>
<td>Hetero-</td>
<td>0.29</td>
<td>3.24</td>
</tr>
<tr>
<td>100</td>
<td>C&gt;T c.3395 g.418606</td>
<td>P1132L</td>
<td>14</td>
<td>Hetero-</td>
<td>0.20</td>
<td>3.32</td>
</tr>
<tr>
<td>003</td>
<td>C&gt;T c.3397 g.418608</td>
<td>P1133S</td>
<td>14</td>
<td>Hetero-</td>
<td>0.00</td>
<td>3.32</td>
</tr>
</tbody>
</table>

**Table 3.4: Non-synonymous mutations in **GRIN2A**

Mutations are listed in the order of location along the sequence. Deleterious substitutions were predicted from SIFT scores ≤ 0.05. Abbreviations: c = cDNA; g = genomic DNA; hetero- = heterozygous; SIFT = Sorting Intolerant from Tolerant analysis.
<table>
<thead>
<tr>
<th>NZM cell line</th>
<th>Substitution and nucleotide number</th>
<th>Amino acid</th>
<th>Exon</th>
<th>Zygosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>061</td>
<td>C&gt;T c.531 g.244320</td>
<td>F177F</td>
<td>4</td>
<td>Hetero-</td>
</tr>
<tr>
<td>040</td>
<td>C&gt;T c.558 g.244347</td>
<td>F186F</td>
<td>4</td>
<td>Hetero-</td>
</tr>
<tr>
<td>055</td>
<td>C&gt;T c.2380 g.413689</td>
<td>L794L</td>
<td>13</td>
<td>Homo-</td>
</tr>
<tr>
<td>003</td>
<td>C&gt;T c.4032 g.419243</td>
<td>F1344F</td>
<td>14</td>
<td>Hetero-</td>
</tr>
<tr>
<td>086</td>
<td>A&gt;C c.4227 g.419438</td>
<td>A1409A</td>
<td>14</td>
<td>Hetero-</td>
</tr>
</tbody>
</table>

Table 3.5: Synonymous mutations in GRINZA

Mutations are listed in the order of location along the sequence. Abbreviations: c = cDNA; g = genomic DNA; hetero- = heterozygous; homo- = homozygous.
<table>
<thead>
<tr>
<th>NZM cell line</th>
<th>Substitution and nucleotide number</th>
<th>Amino-acid</th>
<th>Exon</th>
<th>Zygosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>011, 007, 055</td>
<td>G&gt;A c.1275 g.332946</td>
<td>L425L</td>
<td>6</td>
<td>Homo-</td>
</tr>
<tr>
<td>001, 002, 034, 061</td>
<td>G&gt;A c.1275 g.332946</td>
<td>L425L</td>
<td>6</td>
<td>Hetero-</td>
</tr>
<tr>
<td>011, 007, 055</td>
<td>G&gt;C c.2085 g.360408</td>
<td>R695R</td>
<td>11</td>
<td>Homo-</td>
</tr>
<tr>
<td>001, 002, 061</td>
<td>G&gt;C c.2085 g.360408</td>
<td>R695R</td>
<td>11</td>
<td>Hetero-</td>
</tr>
<tr>
<td>006</td>
<td>C&gt;T c.2190 g.384312</td>
<td>W730W</td>
<td>12</td>
<td>Homo-</td>
</tr>
<tr>
<td>003</td>
<td>C&gt;A c.3228 g.418439</td>
<td>N1076K</td>
<td>14</td>
<td>Hetero-</td>
</tr>
</tbody>
</table>

**Table 3.6: GRIN2A SNPs in tumour derived cell lines**

Abbreviations: c, cDNA; g, genomic DNA; homo-, homozygous; hetero-, heterozygous.

3D modelling of the G762E substitution mutation
The G762E mutation in GluN2A located in the distal ‘hinge’ of the glutamate-binding clam-shell-like region corresponds to the S2 domain.
Figure 3.7: Model of G762E within the GluN1–GluN2A X-ray crystal structure

The S1S2 loop of GluN2A (in green) is shown interfacing with GluN1 (in cyan). A portion of the GluN2A agonist-binding site is on the left together with bound glutamate (Glu). G762E and F524 residues of GluN2A are 2.7 and 3.9 Å away from K531 in GluN1, respectively; potential new electrostatic interactions are indicated as dashed lines. Nitrogen atoms are in blue, and oxygen in red.
3.3. Discussion

Cell lines derived from four of 19 (21%) patients with metastatic melanoma carried five missense mutations in \textit{GRIN2A}. They occurred in three of the four evolutionarily conserved domains of the GluN2A subunit of the NMDAR: the N-terminal, glutamate-binding and C-terminal domains. The S349F, G762E and P1133S substitutions were predicted by SIFT to disrupt protein function. When modelled into the crystal structure of GluN2A, the G762E substitution was predicted to alter GluN1-GluN2A interactions and potentially affect ligand binding, implying disruption of NMDAR functionality. Patients whose tumours carried mutations in \textit{GRIN2A} had faster disease progression and shorter overall survival (Figure 3.1, 3.2 and 3.3, Table 3.2a). The findings indicate that \textit{GRIN2A} mutations may drive melanoma progression.

3.3.1. The clinical relevance of \textit{GRIN2A} mutations

The detection of \textit{GRIN2A} mutations in melanoma cell lines is in agreement with seminal whole-exome sequencing work, where non-synonymous mutations in \textit{GRIN2A} were found in 26% of melanoma samples (Wei, Walia et al. 2011). Although other exome-wide sequencing projects detected \textit{GRIN2A} mutations at lower frequencies (Berger, Hodis et al. 2012, Hodis, Watson et al. 2012, Krauthammer, Kong et al. 2012, Nikolaev, Rimoldi et al. 2012, Stark, Woods et al. 2012), it is possible that the stage of tumours examined influenced the differences between studies. The detection of these mutations reinforces previously reported high prevalence of \textit{GRIN2A} mutations in metastatic melanoma and demonstrates that patients with \textit{GRIN2A} mutations may have more aggressive disease. There is currently no reliable genetic biomarker that predicts melanoma progression. \textit{GRIN2A} mutation testing may offer valuable prognostic information. \textit{GRIN2A} mutation testing could be incorporated into larger prospective studies for further evaluation.

3.3.2. Molecular implications of \textit{GRIN2A} mutations in tumour progression

The results have strong implications for basic research. NMDAR pathways in melanoma are unknown and should be elucidated. The mechanisms through which G762E and other \textit{GRIN2A} mutations interfere with the NMDAR can be hypothesised to include reduced NMDAR channel function and disturbed intracellular signalling downstream. Such effects would be most relevant under conditions of NMDAR over activation, under which excessive calcium uptake induces cell toxicity. The lack of NMDAR-mediated cell death could facilitate tumour progression. Our hypothesis is consistent with the previously suggested role for the NMDAR
as a tumour suppressor (Prickett and Samuels 2012). Other GluN subunits (if expressed in melanoma cells) could compensate for the GluN2A disruption or contribute additional functionality. NMDAR-mediated pro-cell-survival signalling could also provide oncogenic effects, in keeping with the functional dichotomy of the NMDAR (North, Gao et al. 2010). Normal NMDAR activity promotes cell survival through the phosphatidylinositol 3-kinase (PI3-K) and extracellular signal-regulated kinase (ERK) signalling pathways (Perkinton, Ip et al. 2002). In addition, NMDAR effects on cell migration may affect tumour spread in tissue (Li and Hanahan 2013). NMDAR inhibitors reduce migration and proliferation of melanoma cells \textit{in vitro} (Song, He et al. 2012, Prickett, Zerlanko et al. 2014). The effect of NMDARs on cell phenotype is further investigated in Chapter 6.

\textbf{3.3.3. Predicting impact of the mutations based on amino acid properties}

\textbf{3.3.3.1. Agonist binding domain}

The non-synonymous mutation 762 lies within the S2 loop of the receptor and causes a glycine to glutamate substitution. Glutamate is a comparatively large negatively charged amino acid that provides rigidity within the protein structure and frequently forms salt bridges, while glycine is a very small molecule with a flexible conformation (Betts and Russell 2003). The mutation at residue 762 lies within this hinge and may be potentially destabilising or could affect the mobility of the hinge, either by hindering or prolonging agonist binding (Yuan, Hansen et al. 2009).

\textbf{3.3.3.2. Amino terminal domain}

A mutation found at residue 349 is located on the large extracellular amino-terminal domain (NTD) that leads the agonist-binding domain (ABD). The NTD functions in subunit oligomerization, and receptor deactivation kinetics (Yuan, Hansen et al. 2009), and also contains binding sites for zinc ions, the endogenous NMDAR inhibitor (Gielen, Le Goff et al. 2008). The mutation involves a serine to phenylalanine substitution which could potentially alter this site. Serine is a very small and reactive polar nucleophilic molecule and when present in an extracellular environment, as is here, it can be O-glycosylated. Phenylalanine in comparison is a fairly non-reactive non-polar aromatic molecule (Betts and Russell 2003).
3.3.3.3. Carboxy terminal domain

GluN2A’s long intracellular C-terminal domain (CTD) comprises about a third of the length of the peptide (Puddifoot, Chen et al. 2009) and four mutations were detected here. The first mutation detected in this region was at residues 889 and involves a glycine to glutamate substitution. As described above glutamate is a comparatively larger negatively charged salt-bridge forming molecule in comparison to glycine which is a very small neutral molecule (Betts and Russell 2003). These distinct properties could potentially confer an effect on the reactivity or conformation of this domain.

The mutation at residue 1076 gave rise to an asparagine to lysine substitution. Asparagine is a small, polar, neutral molecule while lysine is a hydrophobic polar alkaline positively charged amphipathic molecule that can interact with phosphate groups and form salt (Betts and Russell 2003). The last two mutations were detected at residues 1132 and 1332 and resulted in a proline to leucine, and proline to serine substitution respectively. These appear to be notable changes as proline is a small non-reactive molecule that causes sharp turns in the protein structure. Leucine is non-reactive but it is a flexible hydrophobic molecule in comparison (Table 3.7). Serine, like proline is a very small molecule but unlike proline it is a reactive polar nucleophile that can be phosphorylated in an intracellular environment (as is the case with this domain). This region is not required for expression of the functional channel but it interacts with intracellular scaffolding proteins and trafficking of the NMDAR signalling complex (Ayush et al., 2013; Puddifoot et al., 2009). PSD-95 (postsynaptic density protein) for example binds at residues ESDV and PSDPYK of this domain (Cousins & Stephenson, 2012). Interactions like these could lead to the initiation of downstream signalling pathways. For example, the cyclin-dependent kinase 5 (cdk5)-induced phosphorylation of the CTD can enhance the current passed through the receptor channel (Ayush, Lee et al. 2013, Kunz, Dannemann et al. 2013) while other sites have been shown to be phosphorylated by protein kinase C (PKC) as well as by tyrosine kinases (Fyn and Src) (Pleasance, Cheetham et al. 2010, Ayush, Lee et al. 2013). Other NMDAR subunits (like GluN1 and GluN2B) have been shown to have serine resides that can be phosphorylated by various scaffold proteins (Lin, Skeberdis et al. 2004). Pro-survival and anti-apoptotic pathways often found to be dysregulated in cancer have been linked to NMDAR activation and may be initiated by one or more of these CTD interactions (Voglis and Tavernarakis 2006, Hardingham and Bading 2010). Furthermore, the CTD regulates receptor activation and function by increasing the period of time for which the channel remains open (Rossi, Sola et al. 2002).
CHAPTER 3

Table 3.7: Summary of amino acid changes brought about by mutations in GRIN2A mutations

<table>
<thead>
<tr>
<th>Cell line</th>
<th>AA change</th>
<th>Side chain feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZM 3</td>
<td>Asn1076Lys</td>
<td>hydrophilic to hydrophilic basic</td>
</tr>
<tr>
<td>NZM 3</td>
<td>Pro1133Ser</td>
<td>hydrophobic to nucleophilic</td>
</tr>
<tr>
<td>NZM 7</td>
<td>Gly889Glu</td>
<td>small amphoteric to large acidic</td>
</tr>
<tr>
<td>NZM 100</td>
<td>Pro1132Leu</td>
<td>hydrophobic to hydrophobic (aliphatic)</td>
</tr>
<tr>
<td>NZM 61</td>
<td>Gly762Glu</td>
<td>small amphoteric to large acidic</td>
</tr>
<tr>
<td>NZM 61</td>
<td>Ser349Phe</td>
<td>nucleophilic to hydrophobic (aromatic)</td>
</tr>
</tbody>
</table>

3.3.4. Limitations

The numbers of patients involved in this study were small and outcome was assessed retrospectively. It was not possible to access the DNA of non-diseased tissue from patients in order to exclude germline polymorphisms and hence SNPs were excluded using online databases. The finding that patients with synonymous GRIN2A mutations have shorter survival is unexpected but intriguing. Recent studies indicate that synonymous mutations may be important in cancer, primarily through mechanisms that affect RNA processing and protein translation (Sauna and Kimchi-Sarfaty 2011, Gartner, Parker et al. 2013). Future work will need to test larger cohorts of melanoma patients to confirm the associations we found.

3.4. Conclusions

In conclusion, the results demonstrate that mutations in GRIN2A are present in approximately a quarter of patients with metastatic melanoma and associate with faster disease progression and shorter overall survival. Hence, this is the first study to suggest that mutated NMDARs impact melanoma progression. The most direct clinical implication is that GRIN2A mutation status may be of use as a prognostic tool in melanoma. This will require confirmation in larger studies. The data also imply that the NMDARs may be a novel molecular modifier in melanoma; hence further studies into its biological role should be pursued.
CHAPTER 4
Expression of NMDAR subunits in human melanoma cell lines

4.1. Introduction
Mutations in genes that encode NMDAR subunits have been reported but there is minimal evidence that functional NMDARs are present in melanoma, or that the subunits that comprise them are expressed in melanoma cells.

The NMDAR can be assembled using seven known subunits (Chapter 1, section 1.3.2). NMDAR subunits include an obligate GluN1 structural component and six possible regulatory subunits (GluN2 A-D and GluN3 A or B). The GluN2 subunits bind glutamate while GluN1 and GluN3 bind glycine. The NMDAR is a heterotetramer formed by the complexing of GluN1 with GluN2 or GluN3 dimers. The subunits assemble to form a calcium ion channel. A mixture of GluN2 and GluN3 subunits in one receptor assembly is also possible, which further impacts receptor functionality. Each NMDAR subunit can confer a different functional property to the receptor as a whole, and as such, determining the composition of the NMDARs can provide some insight into the nature of its expected activity in a given cell type.

4.2. Results
This chapter will describe the types of NMDAR subunits expressed in melanoma cell lines and in a strain of cultured human epidermal melanocytes (HEMa-LP, Cascade Biologics Gibco C-024-5C). Transcripts for receptor subunits were identified using RT-PCR and attempts were made to demonstrate the expression of selected NMDAR proteins by immunoblotting. These assays were carried out under the assumption that the NMDAR proteins and transcripts were similar to those expressed in neuronal tissue. Hence, primers designed for PCR and antibodies used for immunoblotting were selected for this work based on prior knowledge of these subunits in the nervous system.
4.2.1. Assay development for mRNA detection

RNA was extracted from melanoma cells and melanocytes using the method described in section 2.2. The cDNA was synthesised from 2 µg of the extracted RNA and primer specific PCR was conducted in order to detect mRNA transcripts from each of the GRIN genes: *GRIN1, GRIN2A, GRIN2B, GRIN2C, GRIN2D, GRIN3A* and *GRIN3B* (section 2.2). Details of the primers used for PCR are presented in Tables 2.2 and 2.3. Figure 4.1 is a schematic that depicts the position of each primer on its respective gene.

Human cerebellum RNA (1µg) was used to synthesise the cDNA to be used as a positive control because NMDARs are expressed at high levels in this region of the brain. cDNA synthesis was conducted using the RT enzyme along with standard reagents such as oligo-dTs that prime polyadenylated mRNA transcripts for reverse transcription. BSA was added to some reactions in order to sequester melanin that can inhibit PCR.
A  \textit{GRIN1}

B  \textit{GRIN2A}  \textit{GRIN2B}  \textit{GRIN2C}  \textit{GRIN2D}
Figure 4.1: Exons encoding NMDAR subunits

Schematic of exons encoding NMDAR subunits (purple) showing the position of each primer set tested. Each of five different primer sets used to detect (A) \textit{GRIN1} transcripts have been numbered and shown in colours that indicate their pairing. Schematic of the (B) \textit{GRIN2A}, \textit{GRIN2B}, \textit{GRIN2C}, \textit{GRIN2D}, (C) \textit{GRIN3A} and \textit{GRIN3B} exons shown separately with the position of each primer pair shown in yellow.
4.2.2. Controls for genomic DNA

The RNA used in these reactions was not DNase treated hence, negative controls were prepared alongside each of the samples during the cDNA synthesis. No RT was added to these reactions. The no-RT controls identified bands that arise from the amplification of genomic DNA rather than RNA transcripts.

4.2.3. Primary screening for NMDAR subunit transcripts

The GRIN2A and GRIN2D primers were first tested on cDNA from 15 and 16 melanoma cell lines, respectively with products detected in about a third of these cell lines (Supplementary Figures 4.1 and 4.2). The cDNA used in these reactions was reverse transcribed using oligo-dTs only, with no random primers. These assays provided an indication of whether the selected primer pairs could be used to detect NMDAR subunit transcripts in melanoma cell lines. The nine melanoma cell lines chosen for further analysis of GRIN expression comprised a subset of those in which GRIN2A was previously sequenced (Chapter 3). Four of these carried GRIN2A mutations (NZM 3, NZM 7, NZM 61 and NZM 100) and five did not (NZM 1, NZM 2, NZM 6, NZM 11 and NZM 40).

4.2.4. Detection of GRIN1 transcripts encoding the structural and obligate GluN1 subunit

Five primer sets were used to detect GRIN1 transcripts in human melanoma cell lines. (Table 2.2 and Figure 4.1). The outcomes of these studies are described in sections 1.2.4.1 and 1.2.4.2 below.

4.2.4.1. Detection of GRIN1 transcripts using primer set encompassing exon 15

A set of primers GRIN1-5 (Table 2.2, Figure 4.1A) were designed around the binding site of the GluN1-specific antibody used for Western blotting (Cell Signalling D65B7). This antibody was produced using a peptide encompassing the proline 660 residue of GluN1. This residue is predicted to be located at amino acid position 660 that corresponds to exon 15 of the GRIN1 4a isoform (with no exon 4) (Figure 4.1A). The GRIN1-5 primer pair was designed around this residue. The forward and reverse primers of GRIN1-5 annealed within GRIN1 exons 15 and 16, respectively.

GRIN1 mRNA was detected at the expected size of 241 bp in melanocytes and all the melanoma lines tested (Figure 4.2A). An additional band between the 300 and 400 bp markers
was also detected. This was consistent with an expected genomic product (315 bp) resulting from the small intron positioned between exons 15 and 16. The genomic product was confirmed in no-RT controls (Figure 4.2B).
**Figure 4.2: GRIN1 transcripts in melanoma cell lines and melanocytes**

**(A)** Detection of GRIN1 transcripts encoding the obligatory subunits of the NMDAR in melanoma cell lines and melanocytes. β-ACTIN was used as a positive control. **(B)** The no-reverse transcriptase (RT) controls used to identify bands that resulted from amplification of genomic DNA (gDNA) rather than RNA transcripts by the same GRIN1 targeting primers. Cell lines in which GRIN2A mutations were detected are shown in red. n = 2, representative gel shown.
4.2.4.2. Detection of GRIN1 transcripts using primer sets outside of exon 15

GRIN1 mRNA was not detected in the melanoma cell lines using primers designed to bind outside of exon 15 (Table 4.2: GRIN1-1, GRIN1-2, GRIN1-3 and GRIN1-4).

GRIN1-1 and GRIN1-2 were designed to cover all eight isoforms of GRIN1, encompassing regions from exon 8 to exon 11 (Figure 4.1A). GRIN1 transcripts were detected in the melanoma cell lines NZM 30 and NZM 76 (two of 16 cell lines screened) using GRIN1-1 primers (Supplementary Figure 9.3). However, no GRIN1 transcripts were detected in the 16 melanoma cell lines tested using GRIN1-2 primers (Supplementary Figure 9.4).

Primers GRIN1-3 and GRIN1-4 were designed to target the regions between exon 1 and 7, and exon 17 and 21, respectively (Figure 4.1A). Details for these primers and the expected isoforms targeted are shown in Table 2.2. These primer sets were expected to detect isoform-specific transcripts. No GRIN1 transcripts were detected in the 16 melanoma cell lines tested using GRIN1-3 and GRIN1-4 primers (Supplementary Figures 9.5 and 9.6).

4.2.5. Detection of GRIN2 and GRIN3 transcripts encoding the regulatory subunits of the NMDAR

The forward and reverse primers used to detect GRIN2A mRNA were designed to target exons 13 and 14 respectively (Figure 4.1B), and were designed to cover all known splice variants in human neuronal tissue. GRIN2A transcripts were detected in eight of nine cell lines (NZM 1, NZM 2, NZM 3, NZM 6, NZM 7, NZM 40, NZM 61 and NZM 100) and in human melanocytes. No GRIN2A transcripts were detected in NZM 11 (Figure 4.3A). GRIN2A transcripts were detected in NZM 7 only when BSA was used during cDNA synthesis (Figure 4.3A).

The forward and reverse primers used to detect GRIN2B mRNA were targeted against exons 12 and 13 respectively (Figure 4.1B), and were designed to cover all known splice variants in human neuronal tissue. GRIN2B transcripts were detected in human melanocytes and six of nine melanoma cell lines tested: NZM 1, NZM 2, NZM 3, NZM 7, NZM 11 and NZM 100. GRIN2B transcripts were not detected in NZM 6, NZM 40 and NZM 61 (Figure 4.3B).

The forward and reverse primers used to detect GRIN2C mRNA were targeted against exons 10 and 11 (Figure 4.1B), respectively, and were designed to cover all known splice variants in human neuronal tissue. GRIN2C transcripts were detected in human melanocytes and two of
nine melanoma cell lines tested: NZM 3, NZM 61 and NZM 100. No GRIN2C transcripts were detected in NZM 1, NZM 2, NZM 3, NZM 7, NZM 11, NZM 40 and NZM 100 (Figure 4.3C).

The forward primer used to detect GRIN2D transcripts was targeted against the junction of exons 9 and 10 (Figure 4.1B), and the reverse primer was targeted against exon 11. GRIN2D transcripts were detected in human melanocytes and in all nine melanoma cell lines tested (Figure 4.3D).

The forward and reverse primers used to detect GRIN3B transcripts were targeted against exons 3 and 5, respectively (Figure 4.1C). GRIN3B was detected in NZM 3, NZM 40, NZM 61 and human melanocytes (Figure 4.3E). Results obtained from primers optimised to detect GRIN3A mRNA were inconclusive and will be discussed in section 4.3.
Figure 4.3: Transcripts encoding regulatory subunits of NMDARs

(A-E) Detection of GRIN2A, GRIN2B, GRIN2C, GRIN2D and GRIN3B transcripts encoding the regulatory subunits of the NMDAR in melanoma cell lines and melanocytes. (F) Transcripts of β-ACTIN were used as a positive control. Cell lines in which GRIN2A mutations were detected are shown in red. n = 2, representative gel shown.
4.2.6. Cell lines carrying GRIN2A mutations

Previous sections have shown that NMDAR transcripts were detected in all melanoma cell lines and this finding was regardless of whether they carried GRIN2A mutations or not (Figure 4.3). No associations were seen with cell lines in which NMDAR transcripts were detected or with the presence of GRIN2A mutations (Chapter 3).

4.2.7. Negative controls

Results of the no-RT controls are shown in Figure 4.4. No genomic DNA products were seen for no-RT negative PCR controls of GRIN2A, GRIN2B and GRIN3B (Figure 4.4A, B and C). An expected (716 bp) genomic DNA product was detected in all melanoma samples following the use of primers targeted against GRIN2C (Figure 4.4C). Unexpected products were detected between 200 and 300 bp for NZM 6 and NZM 7 samples following the use of primers targeted against GRIN2C and GRIN2D (Figure 4.4C and 4.4D).
Figure 4.4: Negative controls for detection of NMDAR subunit transcripts  
(A-E) The no-reverse transcriptase (RT-) controls used to identify bands that reflect the amplification of genomic DNA (gDNA) rather than RNA transcripts. Primers for GRIN2A, GRIN2B, GRIN2C, GRIN2D and GRIN3B were used. n = 2, representative gel shown.
4.2.8. Sequence verification of GRIN1 and GRIN2A transcripts

The GRIN1 and GRIN2A cDNA products amplified from the NZM 40 melanoma cell line were sequenced in order to validate their identity. The amplicons were cut out of the agarose gel following electrophoresis and purified using the method described in section 2.2. The sequence reads were as follows:

**GRIN1:**
GATGATCTTCTGTCATTAGGATGACGTGGGTGCCATTGTAGATGCCCACCTTGCA
CCAGCTTGCGGTTCTGCAGGTTCATGATGCTGTAGTTGGCGAACTTCCGGTCCCCA
TCCTCATTTGAACCTCCACGCGACMAGTCACCCCCCATCCGCATACTTGA

**GRIN2A:**
TTTTGGCTGACCGGAGGAGTTTTAACATGTTGCTCTGGGATCCCGTCAGATTGAAG
TCTGGAGACTTCTTCTTTTCTTCAATGTGCACTCCATGAATGCGACTCCATGAATGCGCTGATAGCATGCC
CCTGCTGATGGGAGAAGAGCAAC

An image displaying the raw data including the quality of signals (peaks) generated from Sanger sequencing is presented in Figure 4.5A and 4.5B.

The BLAST (Basic Local Alignment Search Tool) option on NCBI (National Canter for Biotechnology Information) database detects regions of similarity between sequences. BLAST was used to align the GRIN1 and GRIN2A sequences against all known human transcript sequences (Zhang, Schwartz et al. 2000, Morgulis, Coulouris et al. 2008).

For the GRIN1 segment amplified, maximum similarity (99% identity, 98% coverage and an E value of 4e-74) was found when aligned with human GRIN1 transcripts (Figure 4.5C). For the GRIN2A segment amplified, maximum similarity (100% identity, 100% coverage and an E value of 4e-61) was found against human GRIN2A transcripts (Figure 4.5D).
Sequence of amplified (A) GRIN1 and (B) GRIN2A cDNA segment from NZM 40
Checking the identity of the amplified sequenced DNA product using *GRIN1* primers.


**Figure 4.5**: DNA sequences and verification of the *GRIN1* and *GRIN2A* transcripts in melanoma cells

Sequence of amplified cDNA segment from NZM 40 using primers targeted to (A) *GRIN1* and (B) *GRIN2A*. DNA was sequenced in both forward and reverse directions. Verification of the (C) *GRIN1* and (D) *GRIN2A* transcript sequences.
CHAPTER 4

4.2.9. Summary of mRNA transcripts in melanoma cell lines

The results presented in this section provide evidence for the expression of NMDAR subunit transcripts in melanoma cells. Table 4.1 summarises the detection of NMDAR subunit transcripts in melanoma cell lines from two independent biological repeats (RNA extracted from cell lines at two separate passages). Repeats for most subunits (GRIN1, GRIN2A, GRIN2B, GRIN2D, GRIN3A and GRIN3B) matched for at least 7 of 9 cell lines. GRIN2C was the only subunit for which only three of nine biological replicates matched. Repeats matched for all subunits in melanocytes except for GRIN2C, for which no transcripts were detected in the second biological replicate.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>GRIN1</th>
<th>GRIN2A</th>
<th>GRIN2B</th>
<th>GRIN2C</th>
<th>GRIN2D</th>
<th>GRIN3B</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZM 1</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+/-</td>
<td>++</td>
<td>-/-</td>
</tr>
<tr>
<td>NZM 2</td>
<td>++</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>++</td>
<td>-/-</td>
</tr>
<tr>
<td>NZM 3 + SYN</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>NZM 6</td>
<td>++</td>
<td>+/+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/+</td>
<td>-/-</td>
</tr>
<tr>
<td>NZM 7</td>
<td>++</td>
<td>+/+</td>
<td>++</td>
<td>+/-</td>
<td>+/+</td>
<td>-/-</td>
</tr>
<tr>
<td>NZM 11</td>
<td>+/-</td>
<td>+/-</td>
<td>++</td>
<td>+/-</td>
<td>+/+</td>
<td>+/-</td>
</tr>
<tr>
<td>NZM 40 SYN only</td>
<td>++</td>
<td>+/+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/+</td>
<td>+/-</td>
</tr>
<tr>
<td>NZM 61 + SYN</td>
<td>++</td>
<td>+/+</td>
<td>+/-</td>
<td>++</td>
<td>+/-</td>
<td>-/-</td>
</tr>
<tr>
<td>NZM 100</td>
<td>++</td>
<td>+/+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-/-</td>
</tr>
<tr>
<td>Melanocytes</td>
<td>++</td>
<td>+/+</td>
<td>++</td>
<td>+/-</td>
<td>+/+</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 4.1: Summary of the presence of NMDAR subunit transcripts from two independent biological repeats.

Grey: cell lines that harbour GRIN2A non-synonymous mutations

SYN - cell lines that harbour GRIN2A synonymous mutations,

+ transcript detected; - transcript not detected; / separation of two independent biological repeats
4.2.10. Optimisation of Western blotting to detect the GluN1 protein in melanoma cells

It was hypothesised that melanoma cells should have the highest probability of expressing GluN1 subunits at a protein level. Although all three antibodies detected GluN1 protein in the rat brain lysate used as positive control, only one antibody (D65B7 from Cell Signalling®) detected GluN1 in the melanoma cell lysates. Consequently, all Western blots that tested melanoma cell lines were probed with D65B7.

Western blotting was also used to examine expression of the GluN2A protein. The assay was attempted using two antibodies raised against GluN2A (Santa Cruz Biotechnology(H54) sc-9056 and Abcam 133265). Cells were lysed before separating the proteins according to size on an SDS PAGE gel for detection of protein by Western blotting (see section 2.3.11). The assay was optimised by testing varying concentrations of antibodies against lysates from rat brain hippocampus and a non-neuronal cell line. NZM 40 cell lysates were used as the non-neuronal sample because transcripts of \textit{GRIN2A} were detected first in this cell line.

4.2.11. Expression of GluN1 in melanoma cell lines

An initial screen of 17 randomly selected NZM cell lysates revealed the presence of GluN1 protein in 16 cell lines (Figure 4.6). Nine cell lines were chosen for further analysis. These included the four cell lines with and five without \textit{GRIN2A} mutations (Chapter 3).

The GluN1 protein size is 120 kDa and a band of this size was detected with the D65B7 antibody in eight of nine melanoma lysates tested in two independent biological replicates. The GluN1 band of 120 kDa was not detected in NZM 2 of the first biological replicate but was detected in the second replicate. Conversely, NZM 1 (Figure 4.7) showed GluN1 expression in the first biological assay but not in the repeat. GluN1 expression was not detected in melanocytes in either of the two biological replicates (Figure 4.7 and Table 4.2).
Figure 4.6: Screening melanoma cell lines for GluN1 expression

Immunoblot detection of GluN1 protein expression in 17 randomly selected melanoma cell lysates. Cell lines in which GRIN2A mutations were detected are shown in red. n = 2, representative gel shown.
Figure 4.7: Validating GluN1 expression in melanoma cell lines

Representative immunoblot detection of GluN1 protein expression in nine melanoma cell lines and melanocyte cell lysates. Cell lines in which GRIN2A mutations were detected are shown in red. Rat hippocampus lysate was used as a positive control. n = 2 or 3, representative gel shown.
4.2.12. Validating the identity of the GluN1 band

Bands corresponding to the GluN1 protein in rat hippocampus appeared to run slightly faster than the adjacent bands in some of the melanoma cell lines (Figure 4.8A). To help verify the protein identity, GluN1 was also probed with the same antibody in HCT116 and HepG2 cell lysates, in which GluN1 expression has been previously reported (Yamaguchi, Hirata et al. 2013). Bands detected in lysates of HCT116 and HepG2 were of indistinguishable mobility to those detected in the selected melanoma cell line NZM 40. In order to determine whether the relative spatial positions of samples on a gel affected the rate at which the proteins migrated, lysates were run twice in different regions of the gel. No differences in band migration were observed (Figure 4.8A).

A number of additional higher and lower molecular weight bands were also detected on the blots probed with the D65B7 antibody. D65B7 is a polyclonal antibody (rabbit IgG). Therefore, it was important to examine if these additional bands were a result of non-specific antibody binding. Blots were probed with an isotype control rabbit IgG at a matched antibody concentration (5 µg/µL). This approach detected higher and lower molecular weight bands, similar to those seen with D65B7, consistent with non-specific antibody binding at these positions (Figure 4.8B). However, no signal was observed between 100 and 130 kDa where full-length GluN1 was detected, supporting specificity at this position. Specificity of the secondary antibody was also checked and confirmed independently (Figure 4.9).
Figure 4.8: Isotype and positive controls for validating GluN1 expression

(A) Immunoblot detection of GluN1 protein in NZM 40, HCT116 and HepG2 cell lysates. Rat hippocampus lysate was used as a positive control. (B) Rabbit IgG isotype control at a matched antibody concentration that was used for GluN1 detection. Higher and lower molecular weight bands were detected indicating non-specific antibody binding. No signal was observed between 100 and 130 kDa where GluN1 was expected (120 kDa), providing evidence for specificity at this position.
Figure 4.9: Secondary antibody control for GluN1 protein detection

Immunoblot probed with goat anti-rabbit immunoglobulin antibody at matched concentrations used for GluN1 detection. Blot appears clean verifying that the secondary antibody is not responsible for any non-specific binding. β-Actin was used as a positive control.
4.2.13. Expression of GluN2A in melanoma cell lines

The GluN2A protein is 165 kDa in size and a co-migrating band was detected in lysates from one (NZM 40) of nine melanoma cell lines tested, each probed in two independent biological replicates. Although this result was repeatable, the signal (band) differed from that of the positive control (rat hippocampus). Compared to a single band in the rat brain hippocampus lysate, three separate bands positioned in close proximity around 165 kDa marker were seen in NZM 40 lysates. No GluN2A protein was detected in human melanocytes (Figure 4.9).
**Figure 4.10: Expression of GluN2A in melanoma cell lines**

Representative immunoblot detection of GluN2A protein expression in nine NZM cell lysates and melanocytes. Cell lines in which *GRIN2A* mutations were detected are shown in red. Rat hippocampus lysate was used as a positive control. n = 3, representative gel shown.
4.2.14. Summary of GluN1 and GluN2A expression in melanoma cell lines

Table 4.2 summarises data on GluN1 and GluN2A protein expression in melanoma cell lines from two independent biological repeats corresponding to separate passage numbers. In addition Table 4.2 displays whether or not protein and transcript expression for each subunit were 'concordant' in each cell line; RNA and protein were extracted from the same cultures in these experiments. GluN1 protein and transcript expression was concordant in all cell lines but a variation was seen between passages in two cell lines (NZM 1 and NZM 2). Conversely, the observations were different for GluN2, where no GluN2A protein was detected in eight of nine cell lines carrying its transcripts (Table 4.2).
# Protein expression

<table>
<thead>
<tr>
<th>Cell line</th>
<th>GluN1</th>
<th>Transcript match?</th>
<th>GluN2A</th>
<th>Transcript match?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanocytes</td>
<td>+/-</td>
<td>X</td>
<td>+/-</td>
<td>X</td>
</tr>
<tr>
<td>NZM 1</td>
<td>+/-</td>
<td>X</td>
<td>+/-</td>
<td>√</td>
</tr>
<tr>
<td>NZM 2</td>
<td>+/-</td>
<td>X</td>
<td>+/-</td>
<td>X</td>
</tr>
<tr>
<td>NZM 3 + SYN</td>
<td>+/-</td>
<td>√</td>
<td>+/-</td>
<td>X</td>
</tr>
<tr>
<td>NZM 6</td>
<td>+/-</td>
<td>√</td>
<td>+/-</td>
<td>X</td>
</tr>
<tr>
<td>NZM 7</td>
<td>+/-</td>
<td>√</td>
<td>+/-</td>
<td>X</td>
</tr>
<tr>
<td>NZM 11</td>
<td>+/-</td>
<td>√</td>
<td>+/-</td>
<td>X</td>
</tr>
<tr>
<td>NZM 40 SYN</td>
<td>+/-</td>
<td>√</td>
<td>+/-</td>
<td>√</td>
</tr>
<tr>
<td>NZM 61 + SYN</td>
<td>+/-</td>
<td>√</td>
<td>+/-</td>
<td>X</td>
</tr>
<tr>
<td>NZM 100</td>
<td>+/-</td>
<td>√</td>
<td>+/-</td>
<td>X</td>
</tr>
<tr>
<td>Melanocytes</td>
<td>+/-</td>
<td>√</td>
<td>+/-</td>
<td>√</td>
</tr>
</tbody>
</table>

**Table 4.2: Summary of the presence or absence of GluN1 and GluN2A proteins and their corresponding transcripts.**

- **grey blocks:** cell lines carrying *GRIN2A* non-synonymous mutations
- **SYN** cell lines carrying *GRIN2A* synonymous mutations
- + protein detected; - protein not detected; / separation of two independent biological repeats
- √ matches transcript detection
- X does not match transcript detection
4.3. Discussion

This chapter provides evidence for transcription of *GRIN* genes in human melanoma cell lines and cultured melanocytes that encode the obligate and regulatory subunits of the NMDAR. Transcripts for the NMDAR’s obligate subunit and six of seven known regulatory subunits were detected among all the nine melanoma cell lines and melanocytes (Table 4.1, Figures 4.2 and 4.3). Expression of the GluN1 protein (obligate subunit of the NMDAR) was detected by immunoblotting in eight of nine melanoma cell lines while expression of the GluN2A protein (regulatory subunit of the NMDAR) may have been detected in one of the nine melanoma cell lines. Results presented in this chapter are representative of two to three independent determinations.

4.3.1. Consideration of technical difficulties during transcript detection

There were a number of technical difficulties that needed to be addressed in order to reliably demonstrate the expression of NMDAR subunits in melanocytes and melanoma cell lines. Melanin encapsulates the nucleus of a number of skin cells (Mercer et al., 2009). Melanocytes and melanoma cells often secrete melanin even in culture and this can interfere with the polymerase chain reaction (PCR) (Price and Linge 1999, Rådström, Knutsson et al. 2004). From observations during cell culture it became apparent that NZM 7 secreted a relatively higher level of melanin compared with the other cell lines. The melanin from NZM 7 was suspected to interfere with the process of reverse transcription and PCR. BSA was added (Rådström, Knutsson et al. 2004) to the reaction to relieve this interference with a successful outcome (Figure 4.3A). It has been suggested that BSA helps to overcome PCR inhibition attributed to melanin due to its ability to bind phenolics. If PCR inhibition by melanin can be resolved by the addition of BSA, perhaps it could be important to use BSA to validate other samples such as NZM 11 in which transcripts have not been detected.

Most difficulties arose when attempting to detect transcripts for *GRIN1*. Four of five sets of previously validated *GRIN1* primers were not able to generate a PCR product (Supplementary Figures 4.3, 4.4, 4.5 and 4.6). There may be a number of reasons why most of the primers did not generate a PCR product. Amplicons were successfully detected using human brain RNA with all five sets of primers. Therefore it is likely that PCR conditions were optimal and that cDNA was successfully reverse transcribed. However, the existence of full-length cDNA will need to be checked using either a RNA sequencing or Northern blot method. We found that we
had more success with detecting transcripts if cDNA was reverse transcribed using random primers in addition to Oligo dTs suggesting that transcripts may be circular (Memczak, Jens et al. 2013) or not polyadenylated. The successful amplification of GRIN1 transcripts in the positive control sample also rules out the possibility of GC rich regions interfering with priming or the possibility of PCR products being too large for amplification.

The formation of mRNA secondary structure can also inhibit PCR. The formation of GRIN1 pre-mRNA secondary structures has been reported previously (Zimmer, Fink et al. 1995). DMSO prevents secondary structure formation. It has been suggested that DMSO affects the thermal stability of the primers and the thermal activity profile of the DNA polymerase, thereby increasing the specificity of amplification. The effect on thermal stability seems to be caused by the general capability of organic solvents to destabilise DNA in solution (Rådström, Knutsson et al. 2004). DMSO was added to the PCR buffer when using GRIN1 primer sets that were not successful in amplifying transcripts in melanoma cells. However, this did not prove to be a successful strategy and it is possible that the DMSO should rather have been used in the reverse transcriptase reaction to suppress the formation of any existing pre-mRNA structures before cDNA synthesis. Further epigenetic effects that may influence detection of mRNA are discussed in subsequent sections.

Technical difficulties were also encountered when immunoblotting for expression of GluN1 and GluN2A. Additional bands of lower and higher molecular weight were detected with the Cell Signalling antibodies. This was likely a consequence of non-specific binding attributed to the antibody isotype (Figure 4.8) and not the secondary antibody (Figure 4.9). Mass spectroscopic analysis of the use of an antibody blocking peptide may be needed to verify that these are not partial protein products. Protein representing GluN1 was not detected with the Becton Dickinson or Millipore antibodies suggesting a lack of sensitivity, particularly in light of evidence for functional NMDARs shown in chapter five.

It was also unusual that immunoblotting was not able to convincingly detect any protein representing GluN2A despite the detection of abundant GRIN2A transcripts. In both cases (GluN1 and GluN2A) additional antibodies may be needed to detect protein. Thirty-forty 30-40 µg of protein was needed to detect GluN1 in melanoma cell lysates where only 5 µg of rat hippocampal cell lysate was required to achieve the same. It is possible that the GluN proteins are expressed at a much lower level in non-neuronal cells or that they are tightly
regulated and undergo post-translational modification. Perhaps a specialised method to analyse membrane fractions of cell lysates would also be a useful method to employ for detecting expression of NMDAR subunits. In addition, the use of glycosylases or glycosylase inhibitors could also be useful to investigate the effects of these post-translational modifications.

4.3.2. Epigenetic mechanisms behind NMDAR transcriptional and translational regulation

Epigenetics is a broad term that refers to the heritable modifications of chromatin and DNA without changing the DNA sequence. Major epigenetic mechanisms include DNA methylation, histone methylation and acetylation and chromatin remodelling, and noncoding RNA (Bai and Hoffman 2009).

4.3.2.1. Post-transcriptional modification

Results from this chapter have shown that GRIN2A transcripts were present without protein in eight of nine melanoma cell lines plus melanocytes (Figure 4.3 and 4.10). This suggests that GluN2A expression is post-transcriptionally regulated. Conversely, GRIN1 transcripts were almost always accompanied by the presence of GluN1 protein barring one biological replicate of NZM 11 (Figures 4.3 and 4.7). The phenomenon of detecting mRNA encoding NMDAR gene transcripts without the corresponding protein is not an unusual occurrence. One study found that the pineal gland expresses GRIN2C mRNA but found no evidence for the presence of NMDAR glutamate binding sites suggesting that GRIN2C mRNA may not be translated in the pineal gland (Buller, Larson et al. 1994). Another study found a lack of data related to protein levels of GluN3 and GluN1 and have suggested a difficulty with identifying NMDAR proteins by conventional immunodetection methods (Low and Wee 2010).

4.3.2.2. DNA methylation

Clusters of CpG sequences often reside in the promoter regions of genes or their proximal regions to form CpG islands that may become methylated to negatively regulate transcription. Cytosine in a CpG dinucleotide is the major target of DNA methyltransferases (Bai and Hoffman 2009). CpG islands have been found within the promoter or proximal regions of the GRIN1, GRIN2A, GRIN2B, and GRIN2C genes. However, in this instance mRNA transcripts have been observed in most melanoma cell lines and therefore DNA methylation is not a plausible explanation behind a lack of GluN2A protein expression for these cell lines.
Although GluN2B containing NMDARs have been previously found to be linked to cancer biology (Li and Hanahan 2013), *GRIN2B* was not detected in three of nine melanoma cell lines (Figure 4.3) for which promoter methylation could be responsible. The *GRIN2B* promoter was found to be hypermethylated in primary esophageal squamous cell carcinoma. Demethylation by 5-aza-2’-deoxycytidine released the promoter from inhibition and led to transcription of *GRIN2B*. (Bai and Hoffman 2009).

### 4.3.2.3. Histone modification

The concept of whether histones and other nuclear proteins regulate NMDAR gene transcription via chromatin remodelling is largely unexplored. However, many transcription factors such as REST and CREB found to directly interact with NMDAR promoters are subject to regulation by chromatin remodelling. It has been hypothesised that this mechanism is also utilised to regulate NMDAR transcription, but detailed direct evidence does not exist. (Bai and Hoffman 2009).

### 4.3.2.4. Alternative splicing

It has been suggested that alternative splicing of *GRIN1* is controlled by secondary structures of the pre-mRNA or other cell-specific factors (Zimmer, Fink et al. 1995) but the functional significance of the differential expression of GluN1 isoforms is not clear (Sanz-Clemente, Nicoll et al. 2013). Alternative mRNA splicing may account for the difficulty we encountered in detecting *GRIN1* transcripts in melanoma. Transcription is the initial step of gene expression and is generally the most sensitive to cellular needs and environmental cues (Wray, Hahn et al. 2003, Bai and Hoffman 2009). It serves as a major mechanism controlling gene expression (Levine and Tjian 2003). For example, transcription of each NMDAR subunit gene in a given neuron or cell must be co-ordinately controlled but differentially responsive to cell type, developmental stage and environmental signals (Bai and Hoffman 2009).

### 4.3.2.5. micro RNA

Pleiotropic miRNAs can control gene expression by binding to complementary sequences in the 3’ untranslated region of mRNA transcripts targeting them for degradation (Kocerha, Faghihi et al. 2009). No studies have directly addressed the involvement of miRNAs in NMDAR gene expression (Bai and Hoffman 2009) though one miRNA has been reported to effect activity downstream of NMDARs (Kocerha, Faghihi et al. 2009).
**4.3.2.6. Long non-coding RNA**

Long non-coding RNAs can regulate gene expression at the mRNA level by binding to targeted complementary transcripts (He, Vogelstein et al. 2008, Mercer, Dinger et al. 2009) or to the ribosome itself (Carrieri, Cimatti et al. 2012). In order to silence gene expression, antisense ncRNA can negatively regulate expression by targeting protein effector complexes towards their protein-coding sense mRNA transcripts. Many tumour suppressor genes that are frequently silenced by epigenetic mechanisms in cancer also have antisense partners. For example antisense ncRNA transcribed from the p15 tumour suppressor locus induces changes to local heterochromatin and DNA methylation status which regulates p15 expression, and is potentially involved in oncogenesis in leukaemia since the antisense ncRNA and protein have inverse expression profiles. (Carnero, Hudson et al. 2000, Yu, Gius et al. 2008, Kotake, Nakagawa et al. 2011, McCarthy 2011). This mechanism could be further investigated in melanoma although to date no ncRNA has been directly linked to NMDAR genes.

**4.3.2.7. Post-translational regulation**

Protein degradation or post-translational modification is another plausible explanation for the lack of GluN2A detection. Proteolysis mechanisms like ubiquitination or proteasomal degradation following translation are examples of some well-defined mechanisms (Ratovitski, Patturajan et al. 2001, Burger and Seth 2004). Post-translational modifications such as glycosylation could be investigated by subjecting cell extracts to glycosylases or treating with glycosylase inhibitors (Spiro 2002) before immunoblotting.

**4.3.3. Evidence for an unidentified isoform of GRIN1**

The human GRIN1 gene is made up of 21 exons and spans approximately 31 kb (Zimmer, Fink et al. 1995) and eight isoforms of GRIN1 are produced by alternative splicing of GRIN1 mRNA (Tolle, Berthele et al. 1995, Zukin and Bennett 1995, Prybylowski, Grossman et al. 2001). The complex composition of different subunits and splicing variants forms the primary basis of NMDAR functional diversity. Results from the detection of GRIN1 transcripts in melanoma cell lines indicate the possibility of a new gene isoform that has not previously been identified. Of the five primers pairs targeted to different regions of the GRIN1 exome, only one set was able to detect transcripts of GRIN1. No transcripts corresponding to the amino terminal domain (ATD) or carboxy terminal domain (CTD) of the GluN1 subunit were detected.
No *GRIN1* transcripts were detected using primer sets GRIN1-1, GRIN1-2 or GRIN1-3, indicating a lack of evidence for transcripts corresponding to the GluN1 ATD in melanoma cells. Exons 1 to 11 encode the ATD of the GluN1 receptor subunit (Zimmer, Fink et al. 1995). Primer sets GRIN1-1 and GRIN1-2 were designed to amplify regions from exons 8 to 9, and 9 to 11 respectively corresponding to the 3’ end of the GluN1 ATD (Figure 4.1). The sequence of these exonic regions is shared among the eight identified *GRIN1* isoforms (Vrajová, Šťastný et al. 2010) and thus these primers were expected to detect transcripts that represented any of these isoforms. (Zimmer, Fink et al. 1995, Wilding, Lopez et al. 2014). Primer set GRIN1-3 was designed to amplify *GRIN1* transcripts from exon 1 to exon 7. This region corresponds to the beginning (or 5’ end) of the ATD (Figure 4.1). The sizes of transcripts generated with the primer set GRIN1-3 were expected to reflect particular alternatively spliced *GRIN1* isoforms (Winkler, Mahal et al. 1999). The exonic region targeted for the GRIN1-3 primer set also corresponds to the ATD of the GluN1 subunit (Wilding, Lopez et al. 2014).

*GRIN1* transcripts were not detected in melanoma cells with primer set GRIN1-4. The exonic region targeted by the GRIN1-4 primer set corresponds to the CTD of GluN1, which is encoded for by *GRIN1* exons 20 and 21 (Zimmer, Fink et al. 1995, Wilding, Lopez et al. 2014). The sizes of transcripts that were expected corresponded to particular *GRIN1* isoforms that would have arisen from alternate splicing mechanisms.

Although we were not able to provide evidence for transcripts of the ATD and CTD of *GRIN1*, we were able to detect transcripts corresponding to the membrane spanning domains. Exons 12-19 of *GRIN1* encode the glutamate binding site and membrane spanning domains of GluN1 (Zimmer, Fink et al. 1995). Primer set *GRIN1*-5 was designed to amplify *GRIN1* transcripts from exon 14 to exon 15. The exonic region targeted by the *GRIN1*-5 primer set corresponds to two membrane spanning domains of the GluN1 subunit, M2 and M3, along with their linker regions (Wilding, Lopez et al. 2014). *GRIN1* transcripts were detected using only primer set *GRIN1*-5 indicating evidence for the presence of transcripts that correspond to the M2 and M3 structural domains. Parts of the M2 and M3 domain line the calcium channel of the NMDAR. GluN1 protein was detected using an anti-human-GluN1 rabbit polyclonal antibody targeted to a recombinant peptide surrounding the GluN1 amino acid proline 660. The proline 660 residue resides in the linker region between the M3 domain and the S1 loop that forms part of the ligand binding site (Furukawa, Singh et al. 2005, Wilding, Lopez et al. 2014) and is included
in the part of the *GRIN1* gene amplified by the *GRIN1*-5 primer set. Although we were not able to provide evidence for transcripts of the ATD and CTD of *GRIN1* we cannot hypothesise the existence of a truncated form of GluN1 because a full size (120 kDa) protein was detected by immunoblotting. Thus the existence of a new GluN1 variant is more likely.

### 4.3.4. A calcium channel with different signalling mechanisms

From the *GRIN1* transcript results it might be considered that the NMDAR ion channel has a different ATD and CTD that was not detectable using the primer sets designed. In this instance, the receptor as a whole would retain its functionality as a calcium ion pore while altering the domains that are involved with receiving and sending intra- and extracellular signals respectively. Non-neuronal cells such as melanoma would not be expected to receive and transmit signals in the same manner as neuronal cells (Nedergaard, Takano et al. 2002). Thus it is possible that the ATD and CTD are different in non-neuronal cells.

### 4.3.5. Composition and expected characteristics of NMDARs in melanoma

mRNA for more than one type of NMDAR was detected in each melanoma cell line suggesting the potential for the presence of multiple NMDAR subunits and thereby multiple receptor types. Transcripts for GluN2 subunit types were detected in all of the melanoma cell lines and melanocytes. GRIN3 type (GluN3B) transcripts were detected in three melanoma cell lines, NZM 3, NZM 40 and NZM 61 (Figure 4.3). GluN3A transcripts were detected in 13 of 16 initially screened melanoma cell lines (supplementary Figure 9.7).

The subunit composition of the NMDAR can influence its functionality. Each type of subunit confers a different property to the NMDAR (Cull-Candy, Brickley et al. 2001, Paoletti and Neyton 2007). All NMDARs have a GluN1 subunit that binds glycine and the GluN2 subunits bind glutamate molecules with high affinity. The type of the GluN2 subunits that makes up the receptor strongly influences the receptor’s affinity for glutamate (Cull-Candy, Brickley et al. 2001). The GluN3 subunit binds glycine and acts to reduce the effects of NMDAR activity conferred by the GluN2 subunits (Pachernegg, Strutz-Seebohm et al. 2012). Incorporation of the GluN3 subunit decreases single-channel conductance, calcium permeability and magnesium block in contrast with GluN2 subunit incorporation (Paoletti and Neyton 2007).

The subtypes of GluN2 (A to D) and GluN3 (A and B) regulatory subunits can also influence channel functionality. It is possible for one receptor to incorporate two types of GluN2
subunits. Each combination can confer a different aspect of functionality to the receptor (Paoletti and Neyton 2007). For example, zinc ions are highly potent to the GluN1/GluN2A responses compared with other GluN1/2 combinations (Neyton and Paoletti 2006). NMDAR deactivation times for diheteromeric GluN1/GluN2 receptors can vary by a 50-fold range. For example the deactivation time for GluN2A<GluN2C = GluN2B<<GluN2D (Cull-Candy, Brickley et al. 2001). Almost 100% of the melanoma cell lines that were screened in this chapter expressed transcripts of \textit{GRIN2A} and \textit{GRIN2D}, as did the melanocytes. Fewer cells expressed transcripts of GluN2B and GluN2C (66% and 33%, respectively). Most melanoma cells seem to express transcripts at either extremes of this spectrum.

Transcripts for GluN2D were always detected in all biological replicates of melanoma cell lines and GluN2A in almost all melanoma cell lines. Interestingly, not only are GluN2A-containing NMDARs more potently blocked by magnesium than those containing GluN2D but 20 to 50 fold differences in the duration of activation (or deactivation rates) are seen between GluN2A and GluN2D containing receptors. Furthermore, agonist potencies at GluN2D-containing NMDARs are the highest compared with all other subtypes (O'Leary and Wyllie 2009). GluN2D expression is highest during early rat brain development and diminishes in adult rat brain (Monyer, Burnashev et al. 1994, Wyllie, Livesey et al. 2013). This suggests the receptor's relatively active state during early stages of development when cells are less differentiated. The effects of subunit composition on the NMDAR functionality in the melanoma cell lines are further discussed in Chapter five.

**4.3.6. Downstream effects of NMDAR subunit composition**

NMDAR subunit composition can also effect downstream signalling. For example CaMK (calcium/calmodulin-dependent protein kinase) is a class of enzymes that are activated by increases in intracellular calcium concentrations and protein phosphorylation. GluN2B containing NMDARs bind CaMKII at their CTD with higher affinity than GluN2A containing NMDARs (Barria and Malinow 2005, Gambrill and Barria 2011).

Expression of NMDAR subunits is spatiotemporally regulated within tissue. For example, different regions of the brain express different subunits at each developmental stage and specific subunits have been found to be expressed in the peripheral cells of tumours (Bai and Hoffman 2009, Li and Hanahan 2013). There are also important differences in the subcellular expression of the NMDAR subunits. For example, GluN1 exists in two pools: a population in
the plasma membrane, assembled with GluN2 or GluN3 subunits, and another pool retained in the ER with a short half-life (Huh and Wenthold 1999). GluN1 retention in the ER is modulated by alternative splicing and PKC phosphorylation (Scott and others 2001). In contrast, GluN2 subunits are mainly localised at the plasma membrane (Bai and Hoffman 2009).

Receptor subunit expression is regulated by several posttranslational mechanisms such as phosphorylation, palmitoylation, ubiquitination and nitrosylation. These modulations influence a number of regulatory processes including targeting and trafficking of protein to subcellular regions, influencing downstream interactions with other proteins and signalling outcomes (Bai and Hoffman 2009, Barki-Harrington, Elkobi et al. 2009, Qiu, Li et al. 2011). For example, it has been shown that the phosphorylation of the GluN2A C-terminus by cyclin-dependent kinase 5 (cdk5) increases NMDAR currents (Wang and others 2003). However, no cdk5-mediated phosphorylation has been reported for GluN2B. Conversely, CK2 (casein kinase 2) and CaMKII phosphorylate GluN2B within its PDZ binding domain and the CaMKII binding site, respectively, but not GluN2A (Omkumar and others 1996; Sanz-Clemente and others 2010). Other kinases, such as PKA, PKC and several protein tyrosine kinases (Fyn and Src), phosphorylate both GluN2A and GluN2B subunits, although the precise residues and the consequences of their phosphorylation differ (Bai and Hoffman 2009).

4.3.7. Unmatched biological replicates

Although obligatory and regulatory subunits of the NMDAR were detected in melanoma cells, biological replicates for each cell line did not always match. Passage number or variations in cell culture may influence the initiation of NMDAR gene transcription. NMDARs are regulated by stimulus-dependent gene expression and protein synthesis. The most likely external candidate molecule to effect expression of these genes is the receptor agonist itself, glutamate. Intracellular levels of the messenger ion, calcium, may also effect expression of the NMDAR genes at the transcriptional and translational levels (Qiu, Li et al. 2011).

Excessive stimulation or blockade of the renal NMDAR has been shown to cause cell death (Leung, Ragland et al. 2008). Cells expressing functional NMDARs do not always grow under standard culture conditions due to the receptor’s excitotoxic activity. Culturing cells in media that contain agonists like glutamate could thus result in the selection of cells that have very little or no NMDAR expression. Glutamate in culture medium also degrades over time and
when heated (by undergoing deamination to alphaKG). In addition, glutamate from FBS also adds to the glutamate pool already present in culture medium. Glutamine in medium spontaneously degrades to glutamate and is also degraded by cells in the culture medium further adding to this pool. All of these factors add variability to the levels of glutamate in culture medium containing melanoma cells. This variability may be reflected in the discrepancies seen between NMDAR subunit expressions between biological replication and this has been further discussed in chapter eight. Future experiments where cells are cultured in increasing concentrations of glutamate and where their NMDAR expression and activity is tracked would be useful in testing this hypothesis.

Cell density in culture may also have affected NMDAR expression. Cell-to-cell contact promotes NMDA receptor expression. Increasing cell-to-cell contact (confluency) in cultured keratinocytes was associated with greater levels of NMDA receptor expression, particularly at the cell surface (Genever, Maxfield et al. 1999).

4.4. Conclusions

Genes encoding the NMDAR subunits are transcribed in melanoma. This is evident by the detection of transcripts representing six of the seven known NMDAR subunits that make up the receptor. There is little evidence that these transcripts are translated as proteins, because although protein representing the GluN1 obligatory subunit was detected by immunoblotting, evidence for expression of the regulatory GluN2A protein subunit was not convincing. This could be the result of post-transcriptional and post-translational modifications. Expression of protein representing the obligatory GluN1 subunit was detected in all nine melanoma cell lines but not in melanocytes. GluN1 is known to be sequestered in the ER when not trafficked to the cell membrane to be complexed with regulatory subunits to be assembled into a function channel. Extracellular stimuli such as agonists in culture may dictate the expression and assembly of NMDAR components in melanoma cells and also explain discrepancies in expression observed between biological replicates.
CHAPTER 5
Evidence for NMDAR ion channel functionality in melanoma cells

5.1. Introduction

The previous Chapters have provided evidence for the expression of the NMDAR subunits in melanoma cells. This Chapter describes the use of functional assays to investigate whether functional NMDARs are present in melanoma cells. The NMDAR is a ligand gated calcium ion channel well characterised through studies on the central nervous system (CNS). The activation of the NMDAR is both voltage gated and ligand dependent (Hinoi, Takarada et al. 2004, Fan, Jin et al. 2014). Following sufficient membrane depolarization the magnesium ion blocking the NMDAR channel is dislodged (Seeburg, Burnashev et al. 1995, Blanke ML 2009). When agonists like NMDA, glutamate and glycine are bound, the ion channel opens to allow for the flow of calcium ions (Lee, Lu et al. 2014).

In addition to its excitatory function glutamate-induced calcium influx through the NMDARs has been linked to the activation and regulation of a number of pro-survival, proliferation and apoptotic signal transduction pathways, including those known to be deregulated in cancer (Hardingham and Bading 2003, Hardingham and Bading 2010, Deutsch, Tang et al. 2014). Cytoplasmic calcium levels are very tightly controlled and systems that regulate calcium concentration are often modified in cancerous cells (Roderick and Cook 2008).

Here, NZM cells were exposed to NMDAR agonists to investigate whether NMDAR activation contributes to calcium responses in melanoma cells.

5.2. Results

5.2.1. Monitoring of calcium responses using the plate reader method

Melanoma cells and melanocytes were cultured in 96-well plates and loaded with Fluo-4 AM using the Fluo-4 NW Calcium assay kit (as described in section 2.2). NMDAR agonists were
applied to melanoma cells to determine whether they induced calcium fluxes. Ionomycin (1 µg/mL) was used as a positive control and buffer containing 1% FBS as a negative control. Ionomycin is a calcium ionophore that increases cytosolic calcium levels primarily by allowing calcium influx across the extracellular plasma membrane (Beeler, Jona et al. 1979, Morgan and Jacob 1994, Kao, Li et al. 2010).

Baseline fluorescence was recorded for 10 s, after which the activator or buffer was added and imaging continued for a further 90 s. Mean fluorescence values from the 20 to 30 s interval for each well were calculated in response to each activator. The mean values were used to quantify the fold change response to activators relative to the negative control.

5.2.2. NMDAR agonists elicited calcium responses in embryonic rat hippocampal neurons

Embryonic rat hippocampal neurons were derived from embryos of Wistar rats and cultured as described in Section 2.2 (Monyer, Burnashev et al. 1994, Ewald RC 2009).

Baseline fluorescence in rat embryonic cells was measured for 10 s, after which NMDA (50 and 500 µM) or glutamate (100 and 500 µM) was added. In response to the addition of the NMDAR agonists, fluorescence increased compared with buffer controls (Figure 5.1A), indicating an increase in the cytosolic calcium in the neuronal cells. NMDA at 50 µM increased cytosolic calcium levels up to 3.4-fold and 100 µM glutamate increased cytosolic calcium levels up to 4.6 fold (Figure 5.1B). The cytosolic calcium responses elicited using NMDAR agonists in neuronal cells validated the plate reader method to examine NMDAR functionality in the melanoma lines.
Figure 5.1: Calcium responses to NMDAR agonists in embryonic rat hippocampal cells

(A) Relative cytosolic calcium levels (indicated by fluorescence units) over 100 seconds in embryonic rat hippocampal cells in response to NMDA (50 and 500 µM) or glutamic acid (100 and 500 µM). Ionomycin (1 µg/mL) was used as a positive control and buffer containing 1% FBS as the negative control. Baseline values were collected for 10 seconds after which the activator or controls were added. Each point on the lines represents an average of three values recorded from triplicate wells per second. (B) Fold change in calcium levels in response to NMDA or glutamic acid relative to the buffer control. Fold change was calculated from average fluorescence values between the 20 and 30 second time points. Colours of the bars in B correspond to their respective lines in A. Statistical differences against the buffer control were tested using Dunnett’s multiple comparisons test, *p<0.05 **p<0.001 ***p<0.0001.
5.2.3. Calcium responses elicited in the melanoma cell lines

A panel of six melanoma cell lines was chosen to investigate the effects of NMDAR agonists on the cytosolic calcium levels. Four of these cell lines carried mutations in \textit{GRIN2A} (NZM 3, 7, 61, 100) and two lacked \textit{GRIN2A} mutations (NZM 6, 40). NZM 61 carried the G726E substitution predicted to potentially disrupt the NMDAR assembly. This mutation was considered to most likely affect NMDAR calcium channel functionality but all mutated cell lines were included in these assays.

All cell lines were tested for responses to NMDAR agonists two to three times independently. The magnitude of responses varied between replicates, which will be discussed in section 5.3. Here, the best calcium responses for each cell line observed in independent experiments will be presented (Figures 5.1-5.8). The range of responses seen in all experiments performed is summarised in Table 5.1.

Increases in cytosolic calcium levels were detected for both NZM lines without \textit{GRIN2A} mutations (NZM 6 and NZM 40; Figure 5.2 and 5.3, respectively). Maximum calcium responses reached 1.8-fold in NZM 6 and 4.3-fold in NZM 40. The curve responses to glutamate peaked and declined within 100 s. Increases in calcium levels were not seen in all replicate experiments (Table 5.1). The significance of this variability is unclear but is thought to be mostly influenced by technical reasons discussed in section 5.3. Cells responded differently to loading with Fluo-4-AM, showing morphological changes suggestive of toxicity. Further optimisation of this assay will be required to address the inter-experimental variability.

Out of the NZM lines that carried \textit{GRIN2A} mutations, best calcium responses were recorded for NZM 100 and NZM 3 cell lines (Table 5.1; Figures 5.4 and 5.5, respectively), both carrying mutations in the \textit{GRIN2A} C-terminus.

For NZM 100, up to 4.1-fold increases in cytosolic calcium levels were detected with the strongest effect observed in the presence of 50 µM NMDA (Figure 5.4). For NZM 3, rises in cytosolic calcium were smaller, up to 1.8-fold and similar for NMDA and glutamate across all concentrations (Figure 5.5A and B). For NZM 100 and NZM 3, calcium responses were reproducible between replicates (Table 5.1).
The NZM 7 cell line carried a G889E mutation in the proximal C-terminus. No significant increases in cytosolic calcium levels were detected in these cells in all experiments performed (n=2-3) (Figure 5.6 and Table 5.1).

The NZM 61 cell line carried two *GRIN2A* mutations, one in the N-terminal domain (S349F) and one in the vicinity of the agonist-binding domain (G762E). Computational modelling suggested that mutations were likely to impact NMDAR calcium channel functionality. Calcium responses were detected in these cells in one of the two independent replicates performed for this cell line (Figure 5.7). Cytosolic calcium increased up to 2.4-fold compared with baseline. Calcium responses in this cell line were highest in the presence of NMDA at 50 µM and responses to glutamate were weaker (Figure 5.7).
Figure 5.2: Calcium responses to NMDAR agonists in NZM 6

(A) Relative cytosolic calcium levels (indicated by fluorescence units) over 100 seconds in NZM 6 in response to NMDA (50, 100 and 500 µM) or glutamic acid (100, 200 and 500 µM). Ionomycin (1 µg/mL) was used as a positive control and buffer containing 1% FBS as the negative control. Baseline values were collected for 10 seconds after which the activator or controls were added. Each point on the lines represents an average of three values recorded from triplicate wells per second. (B) Fold change in calcium levels in response to NMDA or glutamic acid relative to the buffer control. Fold change was calculated from average fluorescence values between the 20 and 30 second time points. Colours of the bars in B correspond to their respective lines in A. Statistical differences against the buffer control were tested using Dunnett’s multiple comparisons test, ***p<0.0001.
Figure 5.3: Calcium responses to NMDAR agonists in NZM 40

(A) Relative cytosolic calcium levels (indicated by fluorescence units) over 100 seconds in NZM 40 in response to NMDA (50, 100 and 500 µM) or glutamic acid (100, 200 and 500 µM). Ionomycin (1 µg/mL) was used as a positive control and buffer containing 1% FBS as the negative control. Baseline values were collected for 10 seconds after which the activator or controls were added. Each point on the lines represents an average of three values recorded from triplicate wells per second. (B) Fold change in calcium levels in response to NMDA or glutamic acid relative to the buffer control. Fold change was calculated from average fluorescence values between the 20 and 30 second time points. Colours of the bars in B correspond to their respective lines in A. Statistical differences against the buffer control were tested using Dunnett’s multiple comparisons test, **p<0.001 ***p<0.0001.
Figure 5.4: Calcium responses to NMDAR agonists in NZM 100

(A) Relative cytosolic calcium levels (indicated by fluorescence units) over 100 seconds in NZM 100 in response to NMDA (50, 100 and 500 µM) or glutamic acid (100, 200 and 500 µM). Ionomycin (1 µg/mL) was used as a positive control and buffer containing 1% FBS as the negative control. Baseline values were collected for 10 seconds after which the activator or controls were added. Each point on the lines represents an average of three values recorded from triplicate wells per second. (B) Fold change in calcium levels in response to NMDA or glutamic acid relative to the buffer control. Fold change was calculated from average fluorescence values between the 20 and 30 second time points. Colours of the bars in B correspond to their respective lines in A. Statistical differences against the buffer control were tested using Dunnett’s multiple comparisons test, **p<0.001 ***p<0.0001.
(A) Relative cytosolic calcium levels (indicated by fluorescence units) over 100 seconds in NZM 3 in response to NMDA (50, 100 and 500 µM) or glutamic acid (100, 200 and 500 µM). Ionomycin (1 µg/mL) was used as a positive control and buffer containing 1% FBS as the negative control. Baseline values were collected for 10 seconds after which the activator or controls were added. Each point on the lines represents an average of three values recorded from triplicate wells per second. (B) Fold change in calcium levels in response to NMDA or glutamic acid relative to the buffer control. Fold change was calculated from average fluorescence values between the 20 and 30 second time points. Colours of the bars in B correspond to their respective lines in A. Statistical differences against the buffer control were tested using Dunnett’s multiple comparisons test, ***p<0.0001.
**Figure 5.6: Calcium responses to NMDAR agonists in NZM 7**

(A) Relative cytosolic calcium levels (indicated by fluorescence units) over 100 seconds in NZM 7 in response to NMDA (50, 100 and 500 µM) or glutamic acid (100, 200 and 500 µM). Ionomycin (1 µg/mL) was used as a positive control and buffer containing 1% FBS as the negative control. Baseline values were collected for 10 seconds after which the activator or controls were added. Each point on the lines represents an average of three values recorded from triplicate wells per second. (B) Fold change in calcium levels in response to NMDA or glutamic acid relative to the buffer control. Fold change was calculated from average fluorescence values between the 20 and 30 second time points. Colours of the bars in B correspond to their respective lines in A. Statistical differences against the buffer control were tested using Dunnett’s multiple comparisons test, ***p<0.0001.
Figure 5.7: Calcium responses to NMDAR agonists in NZM 61

(A) Relative cytosolic calcium levels (indicated by fluorescence units) over 100 seconds in NZM 61 in response to NMDA (50, 100 and 500 µM) or glutamic acid (100, 200 and 500 µM). Ionomycin (1 µg/mL) was used as a positive control and buffer containing 1% FBS as the negative control. Baseline values were collected for 10 seconds after which the activator or controls were added. Each point on the lines represents an average of three values recorded from triplicate wells per second. (B) Fold change in calcium levels in response to NMDA or glutamic acid relative to the buffer control. Fold change was calculated from average fluorescence values between the 20 and 30 second time points. Colours of the bars in B correspond to their respective lines in A. Statistical differences against the buffer control were tested using Dunnett’s multiple comparisons test, *p<0.05 **p<0.001 ***p<0.0001.
5.2.4. Summary of plate reader results

Cytosolic calcium responses to glutamate and NMDA were elicited for most NZM cell lines tested here, both with wild-type and mutated GRIN2A. This assay was technically difficult to conduct and significant inter-experimental variability was observed. Therefore, firm conclusions cannot be drawn. However, these results support NMDAR functionality as a calcium ion channel in melanoma cells. Technical challenges of this assay will need to be addressed in the future using better platforms to allow further characterisation of calcium responses in melanoma cells. In these experiments, S349F and G762E GRIN2A mutations in NZM 61 did not abrogate NMDAR calcium channel function and neither did P1133S and P1132L mutations in NZM 3 and NZM 100. Stronger responses to NMDA over glutamate in some cell lines may be of biological significance but will need to be confirmed in future assays. No calcium responses in NZM 7 cell line was seen, therefore effects of the G889E mutation on the NMDAR function will warrant investigation.

5.2.5. Calcium responses in normal melanocytes

Melanocytes are slow growing cells that are difficult to maintain. Hence, they were only used once for calcium flux testing. Experimentation was further complicated because these cells displayed toxicity in response to Fluo-4AM loading reflected by changes in morphology (rounding membranes and loss of adherence). In a single experiment performed on melanocytes, increases in cytosolic calcium to NMDA levels were detected only at 200 µM (Figure 5.8). Further experiments will need to be performed in the future to characterise calcium responses to NMDAR agonists in normal melanocytes.
5.2.6. Monitoring of calcium responses at a single cell level

Using the plate reader approach described above, fluorescence read-outs represented the average fluorescence from one well of a 96 well plate. In order to achieve a more detailed understanding of calcium flux in cells exposed to NMDAR agonists, melanoma cells were imaged using a fluorescence microscope, which allowed examination of cytosolic calcium responses at a single cell level.

Melanoma cells were plated in four-well culture slides and loaded with calcium indicator Fluo-4 AM (method described in Section 2.2). NMDA (50 and 100 μM) was applied to melanoma cells to determine whether they induced increases in cytosolic calcium concentrations. Ionomycin (1 μg/mL) was added as a positive control and buffer was used for the negative control. All modulators to be added during the assay were prepared in a modified Locke's buffer containing 1% FBS. Baseline fluorescence was recorded for 10 seconds, after which the activator (NMDA or ionomycin) or buffer was added and imaging continued for a further 90 seconds. In some cases, the positive control was added to the same chamber directly after the agonist tested and monitoring continued for an additional 90 seconds. The average fluorescence intensity of each cell was tracked and analysed over time using Image-Pro Plus 7.0 software.

Due to the comparatively low throughput nature of this system relative to the plate reader method, three melanoma cell lines were selected for testing by this fluorescence microscopy method: NZM 40 (with wild-type GRIN2A), NZM 3 (carrying P1133S GRIN2A mutation) and NZM 61 (carrying S349F and G762E mutations). Calcium fluxes were detected in these cell lines by the plate reader method, as described above.

Assays at a single cell level added more insight but also further complexity to the observations. Unfortunately, the inter-experimental variability persisted, further emphasising that the process of loading with Fluo-4-AM will require optimisation.

Most responses at a single cell level were seen for the NZM 40 cell line, followed by NZM 3 and weakest in NZM 61 (Figures 5.9, 5.10 and 5.11). All responses were much weaker compared with those to the ionomycin positive control. Further, calcium rises were recorded only in some cells, showing fluctuations over time and a lack of synchronisation between cells (Figure 5.9). The additional difficulty has been that the exposure to buffer containing 1% FBS
also evoked fluctuations in calcium level in some cells. It was very difficult to compare single cell responses between replicates and against the buffer control.
Figure 5.8: Calcium responses to NMDAR agonists in melanocytes

(A) Relative cytosolic calcium levels (indicated by fluorescence units) over 100 seconds in human melanocytes in response to NMDA (50, 100 and 500 µM) or glutamic acid (100, 200 and 500 µM). Ionomycin (1 µg/mL) was used as a positive control and buffer containing 1% FBS as the negative control. Baseline values were collected for 10 seconds after which the activator or controls were added. Each point on the lines represents an average of three values recorded from triplicate wells per second. (B) Fold change in calcium levels in response to NMDA or glutamic acid relative to the buffer control. Fold change was calculated from average fluorescence values between the 20 and 30 second time points. Colours of the bars in B correspond to their respective lines in A. Statistical differences against the buffer control were tested using Dunnett’s multiple comparisons test, ***p<0.0001.
Figure 5.9: Single cell tracking of calcium response in NZM 40 in response to NMDAR receptor stimulation

Calcium uptake (indicated by fluorescence units) over 150 seconds in NZM 40 cells. Baseline values were collected for 10 seconds after which the activator or controls were introduced. Each line represents the fluorescence output over time from one cell in response to addition of (A) buffer or (B) 50 µM NMDA for 90 seconds. Ionomycin (1 µg/mL) was used as a positive control and was added at the 110 second time point.
Figure 5.10: Single cell tracking of calcium uptake in NZM 3 in response to NMDAR receptor stimulation

Calcium uptake (indicated by fluorescence units) over 210 seconds in NZM 3. Baseline values were collected for 10 seconds after which the activator or controls were introduced. Each line represents the fluorescence output over time from one cell in response to addition of (A) buffer, (B) 50 µM NMDA or (C) 100 µM NMDA for 90 seconds. Ionomycin (1 µg/mL) was used as a positive control and was added at the 110 second time point.
Figure 5.11: Single cell tracking of calcium uptake in NZM 61 in response to NMDAR receptor stimulation

Calcium uptake (indicated by fluorescence units) over 150 seconds in NZM 61. Baseline values were collected for 10 seconds after which the activator or controls were introduced. Each line represents the fluorescence output over time from one cell in response to addition of (A) buffer, (B) 50 µM NMDA or (C) 100 µM NMDA for 90 seconds. Ionomycin (1 µg/mL) was used as a positive control and was added at the 100 second time point.
5.2.7. Overall summary

Results in this chapter demonstrated that cytosolic calcium levels increase in melanoma cells following exposure to NMDA and glutamic acid. Responses measured in populations of cells using a plate reader method ranged from 1.3 to 4.1 fold between cell lines, with the weakest shown in NZM 6 and strongest in NZM 100. The use of a fluoresce microscope to monitor cytosolic calcium responses in individual cells proved to be technically difficult but still revealed some additional insight into the complexity of calcium responses in melanoma cells. The microscopy method will require further optimisation taking into account the inherent extreme heterogeneity of cancer cells. Nevertheless, insights gained from this method include the surprising finding that not all cells responded to the NMDAR activators, and that responses were highly asynchronous and to a varying level of magnitude.
<table>
<thead>
<tr>
<th>Cell lines</th>
<th>NMDA</th>
<th></th>
<th></th>
<th>Glutamate</th>
<th></th>
<th></th>
<th>GRIN2A mutation</th>
<th>Domain mutated</th>
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<tr>
<td></td>
<td>50 µM</td>
<td>100 µM</td>
<td>200 µM</td>
<td>100 µM</td>
<td>200 µM</td>
<td>500 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZM 3+ SYN</td>
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<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
<td>+/+/+</td>
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<td>C-terminal domain</td>
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<td>-/-</td>
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<td>-/-</td>
<td>-/-</td>
<td>G889E</td>
<td>C-terminal domain</td>
</tr>
<tr>
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<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>S349F</td>
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<tr>
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<td>+/-</td>
<td>+/+</td>
<td>+/-</td>
<td>P1132L</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>NZM 6</td>
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<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZM 40 SYN only</td>
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<td>-/-</td>
<td>+/-</td>
<td>+/-</td>
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<td>+</td>
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</tbody>
</table>

**Table 5.1: NMDA and glutamate-stimulated calcium uptake in melanoma cell lines.** The table provides a summary of independent biological assays in which a response was (+) or was not (-) detected.

Grey: cell lines that harbour *GRIN2A* non-synonymous mutations

SYN: cell lines that harbour *GRIN2A* synonymous mutations.
5.3. Discussion

Results in this section provide evidence for NMDAR calcium ion channel functionality in melanoma cells. We show increases in cytosolic calcium levels in melanoma cells in response to NMDAR agonists, NMDA and glutamic acid. Responses were most reproducible in the NZM 3, 7 and 100 cell lines. Responses of the greatest magnitude were seen in NZM 3 and NZM 100. Interestingly, NZM 3 and NZM 100 were the only two melanoma cell lines in which transcripts for all subunits of the NMDAR were detected (Chapter four). Both NZM 3 and NZM 100 carry \textit{GRIN2A} mutations located in regions corresponding to the intracellular C-terminus of GluN2A. No response to NMDA or glutamate was detected in NZM 7. Calcium responses were seen in NZM 61 cells in one experiment but not the other. NZM 61 carries two \textit{GRIN2A} mutations, one of which is located in the vicinity of glutamate-binding site. Although responses to NMDA and glutamic acid were detected in NZM 6 cells (wild type for \textit{GRIN2A}), these responses were not recorded in all replicates. Responses to NMDAR agonists did not obviously correlate with \textit{GRIN2A} mutation status.

Single cell analysis of cytosolic calcium had poor reproducibility but indicated that cells responded to the NMDAR agonist in an asynchronous manner and at varying levels of magnitude. In melanocytes response to NMDAR agonists were detected with 200 µM NMDA only, even though like NZM 3 and NZM 100, transcripts for all subunits of the NMDAR were detected.

5.3.1. NMDAR activation in neuronal cells and melanoma cells

In order to understand and discuss the functionality of NMDARs in melanoma, it is first useful to consider the CNS where the functionality of this receptor has been very well characterised. It is interesting to consider whether the mechanism of NMDAR-induced increase in cytosolic calcium concentration in melanoma is comparable to mechanisms associated with NMDARs in the CNS. Small amounts of the glutamate agonist are not enough to reverse the magnesium blockade of the NMDAR in cells of the CNS but when ion channels such as AMPARs are activated by high concentrations of glutamate, the resulting influx of sodium ions reverses the magnesium blockade by electrostatic repulsion. The prolonged influx of calcium activates signal transduction cascades and transcription factors which increases the expression of a number of genes (section 1.5) (Zhang, Ehlers et al. 1998, Cull-Candy, Brickley et al. 2001).
During synaptic transmission, NMDAR activation generates a current with a slow rise and slow decay time. NMDAR channels first open about 10 milliseconds after glutamate is released into the synaptic cleft, and continue to open and close for hundreds of milliseconds until glutamate dissociates from the receptor (Cull-Candy, Brickley et al. 2001). The slow rise in current is not reflected by the cytosolic calcium profiles generated with the plate reader method, although in some cases, the single cell analysis assays also reflect this slow rise in calcium concentrations. The repeated open and close profiles may be reflected in the plate reader time course graphs (Figures 5.2-5.8A) where a slight fluctuation in signal is apparent from one second to the next. Interestingly, this is also observed in some of the single cell assays (Figure 5.11A and C) but not usually seen in response to the calcium ionophore, ionomycin.

Evidence for the presence of functional NMDARs in melanoma cells and melanocytes has been reported, though evidence for NMDAR functionality in melanocytes remains conflicting (Zacharias, Lima et al. 2003, Hoogduijn, Hitchcock et al. 2006, Prickett, Zerlanko et al. 2014). Evidence of NMDAR functionality in other skin cells such as keratinocytes has also been reported (Fuziwara, Inoue et al. 2003, Nahm, Philpot et al. 2004, Fischer, Glanz et al. 2009). There are reports of NMDAR activation in response to agonists in skin cells (Fuziwara, Inoue et al. 2003). Many of these have been at concentrations comparable to those used in our experiments (10 -100 µM), but some reports indicate the use of millimolar concentrations of NMDAR agonists (Nahm, Philpot et al. 2004). If functionality of NMDARs is tissue-specific, in the future it may be necessary to include high millimolar concentrations of agonists in these assays in order to detect responses. Moreover, higher concentrations of agonists may also be needed to compensate for the lower expression levels of the receptor and to compete with existing endogenous concentrations of glutamate.

Results from the single cell analysis raise the possibility that only a proportion of cells being imaged responded to NMDAR agonists and that responses varied in magnitude (Figure 5.9B and 5.10B). Surprisingly, this was not an unusual occurrence because Hoogduijn, Hitchcock et al., 2006 also reported a response only in subpopulations of melanocytes, and in these the amplitude of responses varied considerably between individual cells. The authors report a lack of understanding for this outcome but suggested that the non-responding melanocytes may not express sufficient glutamate receptors to induce a calcium response due to differential expression of the receptors specific to cell cycle phase. Alternatively, they suggest that the
receptors become temporarily de-sensitised and incapable of increasing cytosolic calcium. (Hoogduijn, Hitchcock et al. 2006). This phenomenon was reported again in keratinocytes by another group (Fuziwara, Inoue et al. 2003).

5.3.2. Factors that can influence NMDAR channel opening

NMDAR channel opening is influenced by a number of factors and physiological processes, which makes it difficult to study its exact mechanism. These include phosphorylation or dephosphorylation of its subunits, polymerisation of the actin cytoskeleton and receptor interactions with calcium-calmodulin (Chen, Luo et al. 1999). The N-terminal domain of the NMDAR and the linker connecting it to the ligand binding domain regulate the receptor’s open-state probability (O’Leary and Wyllie 2009). Postsynaptic density protein-95 (PSD-95) is a multivalent synaptic scaffolding protein that co-associates with NMDARs to modulate channel gating. PSD-95 increases channel opening rates and the number of functional channels at the cell surface via the PDZ (PSD-95, discs large, zonula occludens-1) binding motif at the C-terminal domain of GluN2-type subunit (Lin, Skeberdis et al. 2004).

Endogenous modulators including protons are known to play a part in regulating NMDAR function (Low, Lyuboslavsky et al. 2003). An acidic pH reduces the receptor’s open probability (Kovacic and Somanathan 2010). An extracellular site has been reported to be particularly pH-sensitive in that it is able to completely inhibit NMDAR activation (Giffard, Monyer et al. 1990). This may be particularly relevant to the experiments of this chapter where melanoma cells were cultured in and further subjected to glutamic acid.

NMDAR functionality can be influenced by extracellular factors. These include reagents that reduce or oxidise the disulphide bonds of the receptor (Chen, Luo et al. 1999). Magnesium ions inhibit more than 90% of the NMDAR-mediated current at typical neuronal resting potentials in neurons (Johnson and Kotermanski 2006). The concentrations of magnesium in the imaging buffer and culture medium were 1 mM and 0.8 mM respectively. Perhaps this needs to be taken into account and magnesium ions chelated when studying NMDAR functionality in the future.

Calcium influx induced by the NMDAR itself has been shown to promote rapid receptor inactivation. NMDARs can also undergo indirect calcium-dependent inactivation and become inhibited by the calcium/calmodulin-dependent protein phosphatase calcineurin (Zhang, Ehlers
et al. 1998). Imaging buffer used in this chapter for both assay types had 2.3 mM calcium chloride.

NMDARs are assembled and delivered to the cell surface through the endoplasmic reticulum (ER) -Golgi secretory pathway. Perinuclear staining in one study in keratinocytes suggested that NMDARs reside in pre-packaged form in the ER-Golgi apparatus. The study hypothesised that NMDARs contain an ER retention signal, as has been observed in neuronal NMDARs (Scott et al., 2001). Cell-to-cell contact was shown to cause delivery and stabilisation of NMDARs on keratinocyte surfaces, again not unlike that observed with neuronal synaptic maturation (Genever, Maxfield et al. 1999). The variability in cell density and cell surface contact of the melanoma cells we tested may have influenced the detection of calcium responses and the inconsistencies observed within biological replicates. In the future it may necessary to account for cell density and cell surface contact of melanoma cells when studying NMDAR functionality.

5.3.3. Composition and expected functionality of NMDARs in melanoma

In this chapter calcium responses of varying magnitudes were detected among melanoma cell lines. A number of factors, such as those described in the previous sections can account for this variability. However, it must be noted that NMDARs can be made up of several distinct subunits that can confer different functional characteristics to the receptor (Cull-Candy, Brickley et al. 2001, Paoletti and Neyton 2007). In chapter four, mRNA species for several types of NMDAR subunits were detected in each melanoma cell line suggesting the presence of NMDARs that constitute multiple subunit compositions.

GluN2 type subunits strongly influence the receptor’s affinity for glutamate (Cull-Candy, Brickley et al. 2001) while GluN3 subunit also binds glycine and acts to reduce the effects of NMDAR activity conferred by the GluN2 subunits (Pachernegg, Strutz-Seebohm et al. 2012) by impairing single-channel conductance, calcium permeability and magnesium block (Paoletti and Neyton 2007). Transcripts for both the GluN2 and GluN3 -types of subunits have been found in cell lines that we tested for calcium response.

Channel functionality can also be influenced by subtypes of GluN2 (A to D) and GluN3 (A and B). For example the deactivation time for GluN2A<GluN2C = GluN2B<<GluN2D and differences occur over a 50-fold range, with GluN2A and GluN2B containing receptors having
NMDARs containing GluN2A or Glu2B subunits generate ‘high-conductance’ channel opening with a high sensitivity to magnesium block whereas GluN2C- or GluN2D-containing receptors give rise to ‘low-conductance’ openings with a lower sensitivity to magnesium (Cull-Candy, Brickley et al. 2001). The melanoma cell lines and melanocytes that were used in this chapter expressed transcripts of GRIN2A and GRIN2D which are at either extremes of this spectrum suggesting the potential for both greater and smaller deactivation times.

The greatest and most reliable responses were seen in GRIN2A mutated cell lines NZM 3 and NZM 100 in which all GluN2-type transcripts were detected (chapter four). No responses were detected in GRIN2A mutated NZM 7 cells in which all GluN2 subunit types were detected except GluN2B. All GluN2 subunit types except GluN2B were also detected in NZM 61 for which calcium responses were detected in one of two replicate experiments (Chen, Luo et al. 1999).

It is interesting that almost all the NZM cell lines and melanocytes have GRIN2A and GRIN2D transcripts. Not only are GluN2A-containing NMDARs more potently blocked by magnesium than those containing GluN2D but 20 to 50 fold differences in the duration of activation (or deactivation rates) have been seen between GluN2A and GluN2D containing receptors. Furthermore, agonist potencies at GluN2D-containing NMDARs are the highest compared with all other subtypes (O’Leary and Wyllie 2009). It is difficult to make any further assumptions on functionality without evidence that these subunits are expressed as proteins in melanoma cells. In order to fully associate NMDAR composition with functionality it will be necessary to determine whether transcripts of the subunits are being expressed, assembled into functional channels and trafficked to the cell membrane.

5.3.4. Limitations of this study

The Fluo-4-AM dye in combination with probenecid and pluronic acid were used to label cytosolic calcium in both the plate reader assay and for single cell calcium monitoring by fluorescence microscopy. Over time, toxicity of the dye tended to affect the viability of melanoma cells and to a greater extent, melanocytes.

In order to compensate for the toxicity of Fluo-AM it was necessary to supplement the imaging buffer with 1% FBS. This resulted in a final concentration of about 10 µM glutamate.
in the imaging buffer (Ye and Sontheimer 1998). FBS supplementation is a source of variability in assays for NMDAR functionality because glutamate is a NMDAR agonist, and is present in varying concentrations in FBS batches. Glutamate concentrations in culture medium also vary with age and are affected by the number of cultured cells releasing glutamate as a metabolic product (hydrolysis) of glutamine. Thus the cells may be in an underlying state of pre-activation which masks the responses to exogenous agonists.

The concentration of glutamate in cell culture medium can dictate the presence of functional receptors in cells. From studies on cytotoxicity in neuronal cell culture it is apparent that culturing cells that express NMDARs in glutamate-reduced or glutamate-free medium reduced cytotoxicity, supporting cell survival and eliminating the selection for glutamate resistant cells (Ye and Sontheimer 1998, Ye, Rothstein et al. 1999, Ye and Sontheimer 1999). It may be beneficial to culture melanoma cells and melanocytes in this type of low glutamate medium so that cells expressing functional NMDARs are not subject to negative selection. The melanoma cells used for these experiments were cultured in α-MEM supplemented with 5% FBS and they were also established from primary tumours in the same medium. α-MEM is reported to contain 500 µM glutamate without FBS supplementation. This high glutamate concentration may have had long term effects in altering the expression of functional NMDARs. The high glutamate concentration in the medium may have selected against cells expressing functional NMDARs to escape glutamate induced cytotoxicity.

Inherent heterogeneity between cell populations and variations brought about by passaging of cultured cells further adds to the inconsistencies in the magnitude of responses between one independent assay and the next. Moreover, membranes depolarise when cells start to divide (Yang and Brackenbury 2013) and cell cycle phase may also dictate the ability to document a calcium response to NMDA. Existing voltage-gated calcium channels may not be affected by agonists if cells already have a high baseline membrane depolarisation that has facilitated calcium flux. Considering that the melanoma cells have been exposed to excess glutamate in culture medium it may be unlikely that further glutamate exposure would generate a detectable calcium influx via NMDAR channels. In light of this it is interesting that GRIN2A mutated NZM 3 and NZM 100 cells showed the best responses to NMDAR agonists and begs the consideration that mutated forms of the receptor may surpass this effect of receptor desensitisation. Measuring voltage across cell membranes in the future may provide better insight into the working of a normal and mutated NMDAR in melanoma cells.
Cell culture density can affect the expression of functional NMDARs (Nahm, Philpot et al. 2004, Li and Hanahan 2013). For example, one group reported that confluent but not semiconfluent keratinocytes demonstrated a robust calcium response after application of NMDA, which was blocked in the presence of MK-801 (Nahm, Philpot et al. 2004).

5.3.5. Technical difficulties with using the fluorescence plate reader and microscope methods for tracking cytosolic calcium

The high throughput fluorescent plate reader method of monitoring cytosolic calcium provided a time- and cost- effective strategy for analysing multiple melanoma cell lines. However, this method of detection involved the generation of one fluorescent value per culture well containing a population of cells. Though this system was very sensitive in measuring fluorescence intensity, the fluorescence output was an average value that represented a number of cells. The method is not able to consider potentially asynchronous and heterogeneous responses of cells, which can however be achieved by the single cell tracking method using a fluorescence microscope (Figure 5.9– 5.12). This limitation was partially compensated for by reading each well 50 times and also assaying three wells per second per experimental condition.

A small amount of data was also lost when the modulator was added after acquisition of the baseline fluorescence readings. In order to add the modulator the plate containing cells was expelled from the plate reader for ~8 seconds during which the fluorescence output was not immediately recorded. Thus certain aspects of the response after immediate exposure to the agonist may have been lost.

The reliability of using the microscopy method to track relative changes in cytosolic calcium was largely influenced by the sensitivity of the system. The sensitivity of this method was dependent on the quality and ability of the camera to capture relatively large numbers of high quality images. The sensitivity of the camera is also affected by the opacity and thickness of the culture slide through which the images are captured. We feel that this method is usable in detecting relative changes in cytosolic calcium but a more reliable method requires further optimisation. In addition to being relatively low throughput, this method was not as cost effective as using the plate reader in terms of time and economy. However, the fluorescence microscopy method provided interesting and useful insights into the complexities of cytosolic calcium measurements at a single cell level.
5.4. Conclusions

This chapter used functional assays in order to find evidence for NMDAR ion channel functionality in melanoma cells. Although the assays used proved to be technically challenging and in need of further optimisation, we were successful in providing evidence for increases in cytosolic calcium levels in response to NMDA and glutamic acid. We can conclude that melanoma cells may possess functional NMDARs through which calcium enters into the cytosol. This conclusion will need further investigation and validation in order to be considered reliable. Future experiments may aim to culture and assay cells in glutamate-reduced or -depleted medium to avoid secondary desensitisation of down-regulation of receptor expression. Lower glutamate levels in routine culture may not only reduce variability between experiments but also provide insight into precise functioning of the receptor. A key finding in this work is that GRIN2A mutations did not correlate with channel functions in vitro. They may still have an effect in an in vivo environment, where their role in cell invasion has been previously reported (Li and Hanahan 2013).
CHAPTER 6  Effects of NMDAR modulators on melanoma cell proliferation and invasion

6.1. Introduction

The role of NMDARs in melanoma biology is yet to be defined but their involvement in the progression of neoplastic diseases has been previously suggested. The **GRIN2A** gene that encodes a regulatory subunit (GluN2A) of the NMDAR is frequently mutated in melanoma (Wei, Walia et al. 2011). It has been suggested that **GRIN2A** plays a tumour suppressor role in melanoma cell proliferation and survival (Song, He et al. 2012, Prickett, Zerlanko et al. 2014). Another regulatory subunit of the NMDAR, GluN2B, was also shown to play a role in facilitating tumour cell invasiveness (Li and Hanahan 2013).

This thesis found a high frequency of **GRIN2A** mutations in cell lines derived from metastatic melanoma tumours and evidence was obtained for their clinical relevance (Chapter Three). Evidence for the expression of protein corresponding to the obligatory NMDAR subunit and transcription of the regulatory subunits was found in melanoma cell lines (Chapter Four). Signs of potential NMDAR functionality as a calcium ion channel were also observed in some cell lines (Chapter Five).

NMDAR antagonists have been previously reported to have inhibitory effects on cell proliferation and cell death (Stepulak, Sifringen et al. 2005, Malsy, Gebhardt et al. 2015). In this chapter we use NMDAR agonists and antagonists to examine effects on proliferation of the melanoma cell lines. The effect of NMDAR modulators on cell proliferation was quantified by considering the concentrations of modulator required to inhibit cell proliferation by 50% relative to untreated cells (IC$_{50}$ values). Finally, cell lines were screened for
invasiveness and NMDAR contribution towards the invasive phenotype was determined using NMDAR antagonists.

6.2. Results

6.2.1. Effects of NMDAR modulators on melanoma cell proliferation

Cell proliferation was measured by employing a tritiated (3H) thymidine incorporation assay (described in section 2.2). 3H incorporation allows for the quantification of newly synthesised chromosomal DNA which reflects the number of dividing (S-phase) cells. NZM cells were cultured in an α-MEM-based growth medium and exposed to five modulators, three receptor antagonists (memantine, MK-801 and AP5) and two agonists (NMDA and glutamic acid), for five days. The effect on proliferation was determined by the concentration of modulator required to inhibit cell proliferation by fifty percent (IC\textsubscript{50} values). Cells were incubated with \textsuperscript{3}H thymidine to determine their relative DNA-synthetic activity. The IC\textsubscript{50} values were determined by interpolating the concentration of a modulator which reduced \textsuperscript{3}H thymidine uptake to 50\% of untreated cells.

6.2.2. Non-competitive NMDAR antagonists

Memantine and MK-801 are both non-competitive NMDAR open channel blockers. They can enter and block the NMDAR ion channel only when it is opened following activation (Johnson and Kotermanski 2006). Compared to MK-801, memantine has faster kinetics of action with rapid channel blocking and unblocking rates (Frankiewicz, Potier et al. 1996, Dilmore and Johnson 1998, Do Couto, Aguilar et al. 2004). MK-801 is a potent, selective, high-affinity NMDAR antagonist, with much slower kinetics compared to memantine (Wong, Kemp et al. 1986, Do Couto, Aguilar et al. 2004).

6.2.2.1. Effects of memantine on melanoma cell proliferation

Memantine inhibited proliferation of all 23 NZM cell lines tested with mean IC\textsubscript{50} values that ranged between 28 and 90 µM. A large proportion of melanoma lines (14 of 23) were sensitive to memantine with IC\textsubscript{50} values below 50 µM (Figure 6.1A). Amongst these were NZM 100 and NZM 7 that had \textit{GRIN2A} mutations (Chapter Three). The remaining nine cell lines required higher memantine concentrations to inhibit proliferation with IC\textsubscript{50} values of 50 to 90 µM. Amongst these cell lines were another two with mutated \textit{GRIN2A}, NZM 3 and
NZM 61. Overall, cell lines with mutated and wild-type GRIN2A did not vary in their mean IC₅₀ values when exposed to memantine (p = 0.3948) (Figure 6.1B). Nevertheless, cell lines varied in their sensitivity to memantine, including the subset of GRIN2A mutated lines. Figures 6.2a, b and c demonstrate the anti-proliferative effect of each memantine concentration on melanoma cells. It is notable here that at lower concentrations of 0.1 – 10 µM of memantine, the proportion of proliferating cells increased (Figure 6.2A).
Figure 6.1: Anti-proliferative effect of memantine on melanoma cell lines

(A) Average concentrations of memantine needed to achieve 50% inhibition of cell proliferation (IC$_{50}$) in each melanoma cell line (n = 23). (B) Box plot representing the distribution of memantine IC$_{50}$ values in $GRIN2A$ mutant (Mut) versus wildtype (WT) cell lines exposed to memantine (n = 23).
Figure 6.2: Response profile of the anti-proliferative effect of memantine on melanoma cell lines

The effect of memantine on melanoma cell proliferation, as determined by the thymidine incorporation of drug-treated cells relative to that of control (untreated) cells. Cells were treated with memantine concentrations of (A) 0.1 µM to 1 mM or (B, C) from 200 µM to 12.5 µM. Response profiles for cells carrying GRIN2A mutations are shown in red with symbols corresponding to each respective cell line. Each point represents the mean of one determination conducted in duplicate.
6.2.2.2. Effects of MK-801 on melanoma cell proliferation

MK-801 inhibited proliferation in 19 of 20 NZM cell lines tested with IC₅₀ values ranging from 23 to 176 µM, and these values were higher than those seen following exposure to memantine.

The proliferation of most NZM cell lines was inhibited by MK-801 at mean IC₅₀ values >100 µM except for five cell lines: NZM 9, NZM 4, NZM 76 and NZM 3 that were more sensitive to MK-801, with IC₅₀ values of 50-100 µM (Figure 6.3A). NZM 61 was most sensitive to the anti-proliferative effect of MK-801 with an IC₅₀ value of 23.3 µM. NZM 61 carries two GRIN2A mutations. NZM 17 (with wild-type GRIN2A) was the only melanoma cell line with proliferation not inhibited by MK-801.

Overall, cell lines with mutated GRIN2A tended to be more sensitive to MK-801 with its anti-proliferative effect occurring at mildly lower IC₅₀ values compared with cell lines carrying wild-type GRIN2A (t test p = 0.0769) (Figure 6.3B). Figures 6.4A, B and C demonstrate the anti-proliferative effect of each MK-801 concentration on melanoma cells. At lower concentrations of up to ~ 20 µM the proportion of proliferating cells in many cell lines was increased or unaffected (Figure 6.4A).

Memantine and MK-801 are both NMDAR channel blockers and as such could be expected to have similar effects, on cell proliferation. We therefore compared IC₅₀ values generated from the treatment of melanoma cells with memantine and MK-801. Regression analysis revealed no obvious correlation between the IC₅₀ values of memantine and MK-801 (R² = 0.22 Figure 6.5A). Four cell lines, NZM 6, NZM 76, NZM 3 and NZM 61, were least responsive to memantine but no correlation was seen between the IC₅₀ values of the memantine and MK-801 regardless of whether these four cell lines were included in the analysis (Figure 6.5B).
Figure 6.3: The anti-proliferative effect of MK-801 on melanoma cell lines

(A) Average concentrations of MK-801 needed to achieve 50% inhibition ($IC_{50}$) of cell proliferation in each melanoma cell line ($n = 23$). (B) Box plot representing the distribution of $IC_{50}$ values in $GRIN2A$ mutant (Mut) versus Wildtype (WT) cell lines exposed to MK-801 ($n = 23$).
Figure 6.4: Response profile of the anti-proliferative effect of MK-801 on melanoma cell lines

The effect of MK-801 on melanoma cell proliferation as determined by the percent of treated to untreated cells. Cells were treated with memantine concentrations of (A) 1 mM diluted 3X to 0.05 µM or (B, C) from 200 µM diluted 2X to 12.5 µM. Response profiles for cells carrying GRIN2A mutations are shown in red with symbols corresponding to each respective cell line. Each point represents the mean of one determination conducted in duplicate.
Figure 6.5: Regression analysis of the anti-proliferative effect of memantine and MK-801

Regression analysis of IC₅₀ values generated from the anti-proliferative effect of memantine and MK-801 on (A) all melanoma cell lines (p = 0.033), and (B) excluding those least responsive to memantine.
6.2.3. Effects of AP5 on melanoma cell proliferation

In contrast to memantine and MK-801, AP5 did not inhibit proliferation of any melanoma cells. AP5 is a competitive NMDAR antagonist that binds the NMDAR at the agonist (glutamate) binding site. NZM cells were exposed to concentrations of AP5 ranging from 0.1 µM to 1 mM. At this concentration range, no evidence for inhibition of cell proliferation was detected for any of the cell lines tested (Figures 6.6A and 6.6B). Unexpectedly, a number of cell lines, including NZM 61 responded to AP5 with increased thymidine uptake.
Figure 6.6: Effect of AP5 on melanoma cell lines proliferation

The effect of AP5 on melanoma cell proliferation as determined by the percent of treated to untreated cells. Each line represents one independent determination. Cells were treated with memantine concentrations of (A) 1 mM diluted to 0.1 µM or (B) from 200 µM to 12.5 µM. Response profiles for cells carrying GRIN2A mutations are shown in red with symbols corresponding to each respective cell line. Each point represents the mean of one independent determination conducted in duplicate.
6.2.4. Effects of NMDAR agonists on melanoma cell proliferation

NMDA and glutamic acid are NMDAR agonists that bind to the same site on the receptor’s agonist binding domain, in order to activate the NMDAR and open its ion channel. Glutamic acid is the endogenous receptor agonist that can bind and activate the NMDAR as well as other glutamate receptors, while NMDA is the synthetic amino acid derivative that binds specifically to the NMDAR.

NZM cells were cultured in the presence of NMDA and glutamic acid at concentrations ranging from 0.1 µM to 1 mM; this was in addition to the glutamic acid already present in the media (estimated 500 µM). No inhibition of cell proliferation was detected in the presence of NMDA or additional glutamic acid for any of the cell lines exposed to these agonists (Figure 6.7A and B).

The proportion of proliferating cells exposed to NMDA and glutamic acid increased > 100% (sometimes < 200%) suggesting a stimulatory rather than inhibitory effect on cell proliferation. These increases in cell proliferation were seen at lower concentration ranges of the modulators. Proportions of proliferating cells tended to stabilise at higher concentrations of NMDA (Figure 6.7A) and decrease after exposure to higher concentrations of glutamic acid (Figure 6.7B).
Figure 6.7: Effect of NMDA and glutamic acid on melanoma cell lines proliferation

Representative graphs showing the effect of (A) NMDA and (B) glutamic acid on melanoma cell proliferation as determined by the percent of treated to untreated cells. Cells were treated with NMDA concentrations of up to 200 µM. Each point represents the mean of one determination conducted in duplicates.
6.2.5. Primary melanoma cells and melanoma cell lines respond differently to memantine

Previous work at the ACSRC involved exposing primary cultures of metastatic tumour cells from melanoma patients to memantine. Anti-proliferative effects of memantine on these primary cultures were seen at concentrations with IC$_{50}$ values of $<10$ to $>50$ µM (Figure 6.8).
Figure 6.8: Anti-proliferative effect of memantine on primary melanoma cultures

Average concentrations of memantine needed to achieve 50% inhibition (IC$_{50}$) of cell proliferation (IC$_{50}$) in primary melanoma cultures (data from Wayne Joseph).
6.2.6. Determining the invasiveness of NZM cell lines

In order to determine the effects of NMDAR modulators on melanoma cell invasiveness, melanoma cell lines derived from metastatic tumours were subjected to the Boyden chamber Matrigel invasion assay (described in section 2.3.14, Figure 6.9) using α-MEM as the basal medium. Cell lines screened for invasiveness included NZM 3, NZM 6, NZM 9, NZM 7, NZM 11, NZM 40, NZM 61 and NZM 100. Only one cell line, NZM 40 invaded through the Matrigel from the apical to the basal side of the membrane (data not shown).

6.2.7. Effects of NMDAR modulators on the invasiveness of NZM 40 melanoma cells

NZM 40 cells were used to determine the effect of NMDAR modulators on melanoma cell invasiveness because this was the only melanoma cell line found to be invasive among the eight tested. NZM 40 cells were seeded in duplicate in a 24 well plate containing inserts with Matrigel coated porous (8 µm pores) membranes as described in section 2.2. Cells were seeded on the apical side of the membrane. Medium containing 10% FBS as a stimulant was added to basal side of the membrane. NMDAR modulators (memantine, MK-801, AP5 and NMDA) were added at 100 µM concentrations on both the apical and basal side of the membrane. During the incubation period of 20 hours, cells that invaded through the Matrigel from the apical to the basal side of the membrane were quantified (Figure 2.6). The experiment was repeated three to four times for each treatment.

When compared to control cells, a reduced number of NZM 40 cells exposed to 100 µM of antagonists AP5 (p = 0.001) and MK-801 (p = 0.028), and 100 µM of agonist NMDA (p < 0.0001) invaded through the Matrigel and migrated to the basal side of the filter. In comparison, memantine had no effect on the number of invading and migrating NZM 40 cells (p = 0.2306) (Figure 6.9).
Figure 6.9: The effect of NMDAR modulators on the invasiveness of melanoma cell lines

The effect of NMDAR modulators on the invasiveness of NZM 40 melanoma cells as determined by the percent area of treated to untreated cells. *p<0.01  **p<0.001  ***p<0.0001.
6.3. Discussion

In this chapter the effects of NMDAR modulators on melanoma cell proliferation and invasiveness were investigated. Here, experimental results have indicated that non-competitive NMDAR channel blockers are effective at inhibiting melanoma cell proliferation. The NMDAR channel blockers memantine and MK-801 inhibited melanoma cell proliferation at IC_{50} values that were lower for memantine compared with MK-801. NZM 17 was the only cell line in which no anti-proliferative effect was seen after exposure to MK-801. The competitive antagonist of NMDARs, AP5, did not inhibit cell proliferation even at high millimolar concentrations but was seen to impair invasion of NZM 40. The NMDAR agonists NMDA and glutamic acid also did not inhibit cell proliferation up to high millimolar concentrations but had a stimulatory effect on cell proliferation. NMDA exposure also impaired the invasiveness of NZM 40.

6.3.1. Effects of non-competitive NMDAR antagonists on melanoma cell proliferation

Non-competitive NMDAR channel blockers were effective at inhibiting proliferation of the melanoma cell lines used in this chapter. Memantine inhibited melanoma cell proliferation at IC_{50} values ranging from 28 to 90 µM and MK-801 inhibited melanoma cell proliferation at generally higher IC_{50} values between 23 and 176 µM. Memantine and MK-801 block the NMDAR ion channels that are opened following agonist induced activation. Both memantine and MK-801 are “trapping channel blockers”. After memantine blocks the channel of an NMDAR, the channel can close and agonists can unbind, trapping memantine inside (Johnson and Kotermanski 2006). In the CNS where NMDARs are highly expressed, NMDAR blockade has been shown to have antagonistic effects at a 1-100 µM range (Parsons, Gruner et al. 1993, Frankiewicz, Potier et al. 1996, Johnson and Kotermanski 2006). Anti-proliferative effects of MK-801 and memantine in carcinomas have been previously reported at 1 to 500 µM (Ravnan and Matalka 2012). This falls into a similar range as that seen in the experiments of this thesis. Compared with memantine, the effect of MK-801 was observed as being pronounced with time rather than concentration (Rzeski, Turski et al. 2001). This might explain the reason for the observation of relatively higher IC_{50} values seen in response to MK-801.

Despite being non-competitive, memantine has been shown to be increasingly effective even when subjected to high concentrations of agonists (glutamate or NMDA) (Frankiewicz, Potier
et al. 1996, Dilmore and Johnson 1998, Do Couto, Aguilar et al. 2004). This may explain why its anti-proliferative effect tended to be at lower concentrations compared with the concentration of MK-801 needed to confer the same effect (Figure 6.1A and 6.3A).

Memantine has faster kinetics of action compared to MK-801 with rapid channel blocking and unblocking rates at low micromolar concentrations. In fact, its previously described (Chapter one) clinical tolerance may be attributed to its lower binding affinity (Parsons, Gruner et al. 1993, Frankiewicz, Potier et al. 1996, Dilmore and Johnson 1998, Parsons, Danysz et al. 1999, Danysz, Parsons et al. 2000, Do Couto, Aguilar et al. 2004, Johnson and Kotermanski 2006, Lipton 2006). Conversely, MK-801 is a potent, selective, high-affinity NMDAR antagonist, which has much slower kinetics and weaker voltage dependency compared to memantine (Wong, Kemp et al. 1986, Do Couto, Aguilar et al. 2004, Johnson and Kotermanski 2006). MK-801 also has a shorter half-life than memantine (Vezzani, Serafini et al. 1989, Johnson and Kotermanski 2006). This may also explain the generally higher concentration of MK-801 required to inhibit melanoma cell proliferation compared with the overall lower concentration of memantine required to inhibit cell proliferation to the same extent.

NZM 61 carries two GRIN2A mutations and was most sensitive to MK-801 with an IC50 value of 23 µM. The GRIN2A mutations in NZM 61, G762E and S346F, are present at sites corresponding to the glutamate binding site and the NTD respectively of the GluN2A subunit. Other GRIN2A mutated lines (NZM 3, NZM 7 and NZM 100) were less sensitive to the anti-proliferative effects of MK-801, with NZM 7 cells being least inhibited. The latter is also consistent with calcium flux results in which responses were detected in NZM 7 (Chapter six). The sensitivity of NZM 61 to MK-801 supports the hypothesis that the G762E GRIN2A mutation rendered the NMDAR hyperactive in this cell line. However, it is difficult to derive a conclusive association between functional aspects of the receptor and its sensitivity to channel blockers. This was because the aforementioned relative effects on cell lines seen with MK-801 were not reflected in NZM 7 and NZM 61 responses to memantine.

Regression analysis of memantine and MK-801 IC50 values did not show any correlation between the two channel blockers indicating that one or both may have off-target effects in its ability to inhibit proliferation. Non-specific effects of memantine cannot be excluded. At high concentrations (up to 500 µM), memantine has been shown to affect CNS targets other than the NMDAR. Non-NMDAR targets of memantine include serotonin and dopamine uptake,
nicotinic acetylcholine receptors (nAChRs), serotonin receptors, sigma-1 receptors, and voltage-activated sodium channels. Memantine also inhibits steady-state 5-HT3 receptor responses with an IC$_{50}$ of just 2.3 μM. It has been hypothesised that this off-target action might contribute to its therapeutic effect (Johnson and Kotermanski 2006).

When NMDAR antagonists like MK-801 were used to treat normal cells, such as human skin stromal fibroblasts and bone marrow stromal cells, proliferation was unaffected at concentrations as high as 250 μM (Rzeski, Turski et al. 2001). This indicates that NMDARs may be preferentially expressed by cancerous as opposed to normal cells. However, this concept may not be relevant in all tissues, including the skin, because of evidence that exists for the expression and function of NMDARs in normal cells such as osteocytes, megakaryocytes, platelets, melanocytes and keratinocytes (Franconi, Miceli et al. 1998, Rzeski, Turski et al. 2001, Hoogduijn, Hitchcock et al. 2006, Song, He et al. 2012). It is more likely that NMDARs are regulated in a manner that is specific to respective tissue microenvironments whether normal or cancerous.

NZM 17 was the only melanoma cell line in which proliferation was not inhibited by MK-801. This cell line was derived from a patient who had limited single node disease at biopsy. It was the only patient who received a therapeutic anti-tumour vaccination after tissue collection and is the only patient currently still alive. The lack of MK-801 effect supports a lack of NMDAR activity in these cells. This strengthens clinical correlations of NMDAR activity contributing to faster disease progression.

6.3.2. Melanoma cells in primary culture and melanoma cell lines respond differently to memantine

Previous work at the ACSRC involved exposing primary cultures of metastatic tumour cells from melanoma patients to memantine. Anti-proliferative effects of memantine on these primary cultures were seen at concentrations with IC$_{50}$ values of <10 to >50 μM, therefore lower compared to those reported in this chapter where melanoma cell lines were used (Figure 6.8). The difference in sensitivity to memantine inhibition between melanoma cells in primary culture and cell lines likely reflects adaptation in culture.

NMDAR expression and functionality likely differs in vivo compared to in culture. Adaptation processes may include the spatial arrangement of the cells in both environments driven by the
concentration of glutamate that they have been subjected to. For example, in their pancreatic neuroendocrine model (Li and Hanahan 2013) showed that NMDARs are present on the membranes of peripheral tumour cells. Here Li and Hanahan propose a model wherein the peripheral cells become invasive via the activation of their NMDARs by extracellular glutamate signals. NMDAR activation causes the activation of the signalling pathways that lead to expression of invasive genes. Three-dimensional spatial organisation is lost in two dimensional cell culture models. The gradients of oxygen resulting from blood vessels in tumours generate a distribution of tumour hypoxic regions and this may dictate the use of glutamate over glucose as an energy source. Nahm, Philpot et al. 2004 propose that cells in closer proximity express the NMDAR at higher levels and this hypothesis can also be applied to inconsistencies seen in our results. Cells in a two-dimensional culture system may or may not be in close proximity further adding to the heterogeneity in the expression and presence of functional NMDARs. In addition glutamate concentrations in tissue are lower compared to those in culture medium. Therefore cells expressing the receptor may not be selected for in vitro.

6.3.3. NMDAR modulators targeted to the glutamate binding site

The competitive NMDAR antagonist AP5 did not inhibit cell proliferation of NZM cell lines even at 1 mM concentrations. In Drosophila, AP5 was shown to be less effective at blocking NMDAR opening compared with other antagonists (Xia S 2009). However, very little is known about the mechanism of action of AP5 in human cells, aside from its nature of being targeted to the agonist binding site of the receptor (Laube, Hirai et al. 1997, Gascón, Deogracias et al. 2005, Watanabe, Kanno et al. 2008).

For proliferation assays melanoma cells were cultured for five days in α-MEM containing relatively high concentrations of glutamate. The concentration of glutamate in the medium is quoted at ~ 500 µM in the manufacturer’s specifications. However, this may have fluctuated depending on the spontaneous degradation of glutamate and hydrolysis of glutamine to glutamate by cells in culture (further investigated in Chapter Seven). It could be hypothesised that AP5 was able to protect melanoma cells from the cytotoxic effects induced by high glutamate levels in the medium. Other NMDAR agonists like memantine have been shown to have neuroprotective effects in CNS pathologies (Sobrado, Roda et al. 2004, Kotermanski and Johnson 2009). It is difficult to interpret the effects of adding agonistic modulators to culture mediums in which endogenous agonists of the receptor already exist. However, we can
conclude a lack of anti-proliferative effect in the presence of this receptor. These results do not fit with the aforementioned hypothesis of NMDAR over-activation inducing a cytotoxic effect.

Contrary to the expected anti-proliferative effect of AP5, cell proliferation increased for a number of cell lines (Figure 6.6A). This may also be in keeping with a well described physiological phenomenon where weak antagonists can act as partial agonists, inducing cell survival and proliferation (Maekawa, Namba et al. 2009). Exposure to NMDA (12 – 200 µM) identified two groups of cell lines: those that hyperproliferated in the presence of NMDA (n=8; including NZM 61) and those that did not (n=4; including NZM 3) (Figure 6.7A). In the presence of glutamic acid, the distinction between such proliferative responses was less obvious but the difference in cell proliferation persisted between NZM 61 and NZM 3, where proliferation of NZM 61 was more dependent on the presence of the additional glutamic acid (Figure 6.7B).

It is intriguing that the proliferation of the NZM 61 cell line increased in the presence of AP5 by 50% across all concentrations. This was the highest increase in proliferating cells seen amongst all other cell lines tested. NZM 61 contains mutations in GRIN2A with the highest potential to disrupt NMDAR functionality. The G762E mutation was detected at sites corresponding to the glutamate binding site and N-terminal domain. The results obtained in this chapter support the computational prediction that the G762E mutation impacts NMDAR function and are most consistent with a gain-of-function effect. In support, the NZM 61 cell line was most sensitive to the anti-proliferative effect of MK-801 with the IC50 value of 23 µM. Further, both NMDAR agonists (NMDA and glutamic acid) increased proliferation of these cells. In comparison, the NZM 3 cell line that carried the most distal mutation in the GRIN2A (P1133S at the intracellular carboxy terminal domain) was comparatively less proliferative than NZM 61 when exposed to AP5.

Competitive antagonists of NMDARs such as AP5 have to compete with a relatively high concentration of agonist in culture medium. In order to reliably determine whether AP5 can confer an anti-proliferative effect it may be necessary to culture cells in medium with reduced glutamate concentrations of <100 µM which better reflects physiological conditions. Because AP5 does not cross the blood brain barrier, it could be a good candidate for deciphering the functionality of the NMDAR in vivo (Halliwell, Peters et al. 1989).
6.3.4. Inhibiting melanoma cell invasiveness with NMDAR modulators

Seven of the eight melanoma cell lines tested for invasiveness in Boyden chambers were found to be non-invasive. The only cell line that invaded was NZM 40 which was also previously reported to be motile (He, Li et al. 2010). NZM 40 was also the only cell line in which the expression of both GluN1 and GluN2A subunits was detectable by Western blotting (Chapter Four). The other two cell lines previously reported to be motile (NZM 9 and NZM 11) were found to be non-invasive in this assay (He, Li et al. 2010).

The NMDAR may play a role in invasiveness via activity controlled at its agonist-binding site. The invasiveness of NZM 40 was attenuated by antagonists AP5 and MK-801, and agonist NMDA, suggesting NMDAR contribution towards this phenotype (Figure 6.9). In comparison, memantine showed no significant effect in this context. Further testing will be required to clarify this as memantine at 100 µM and 250 µM was previously shown to have an effect on cell invasive phenotype (Rzeski, Turski et al. 2001, Gill and Pulido 2007).

The greatest effects on invasiveness were seen when cells were exposed to AP5 and NMDA both targeted to the NMDAR glutamate binding site. This result suggests that cell invasion may be facilitated by the NMDAR. A model has been proposed in which pancreatic neuroendocrine tumour cells express NMDARs in cells positioned at the peripheral regions of tumours. The model, based on in vivo and in vitro experimental evidence, proposes that cells on the periphery of the tumour become invasive in response to secretion of glutamate which in turn activates the NMDAR. Activation of the receptor results in a downstream signalling effect which induces the CREB transcription factor to express genes that are involved in producing an invasive phenotype (Li and Hanahan 2013).

Interestingly, the channel blocker memantine that was previously shown to have anti-proliferative effects on NZM 40 cells at IC50 51 µM, was not as effective in inhibiting NZM 40 invasiveness at 100 µM. Conversely, AP5, which blocks the NMDAR agonist binding site, was most effective at inhibiting invasiveness at 100 µM over a 20 hour period even though it did not show any evidence of having an anti-proliferative effect at 1 mM over a five day period. Interestingly, NMDAR agonists can modulate MK-801 binding properties under certain conditions, suggesting a close association between the two sites (Wong, Kemp et al. 1986). Thus, the role that the NMDAR may play in facilitating invasiveness may be regulated at the agonist binding site.
6.3.5. Using α-MEM and RPMI 1640 to culture test NMDAR modulator effects

It may also be relevant that cells were tested for invasion in a basal medium of RPMI 1640 containing less glutamate as opposed to α-MEM (136 vs 500 μM, respectively). Effects on cell proliferation were tested in α-MEM. We have used RPMI 1640 medium in the invasion assay after having realised the difference in glutamate concentrations between the two media (Chapter Seven). It has been a standard practice for many years to culture the NZM lines in a basal medium of α-MEM in the ACSRC laboratory. It is possible that in the RPMI 1640 medium with less glutamate, AP5 effects were easier to elicit.

Future work should investigate the role of glutamate concentrations in media on NZM cells’ proliferation, invasion and inhibitor effects. Glutamate is an almost universal component in culture media which poses an important difficulty in studying its effects on cells. It is also possible that the presence of glutamate has critical consequences for the selection of a particular cell phenotype in culture. This is discussed further in Chapters Seven and Eight. In the future it would be useful to test anti-proliferative effects of NMDAR modulators in RPMI 1640 medium where the modulator effects may be less inhibited by the high glutamate levels in α-MEM. Using medium with lower glutamate concentrations such as RPMI 1640 may help to gain insight into potential associations between the GRIN2A mutated and wildtype cell lines, a trend that may have been masked by high glutamate in the cultures conditioned used for the anti-proliferative assays.

6.3.6. Other factors affecting NMDAR activation

There are a number of factors that affect NMDAR activity. These include components of the extracellular environment, cell density, as well as receptor composition comprising seven possible subunits each consisting of multiple splice variants (Zukin and Bennett 1995, Nahm, Philpot et al. 2004, Stepulak, Luksch et al. 2009, Traynelis, Wollmuth et al. 2010). In the CNS, synaptic and extra-synaptic NMDARs also serve different functions. Moreover, the NMDAR is part of a postsynaptic signalling complex known as the postsynaptic density, composed of PSD-95 and many other PDZ domain-interacting proteins, including kinases and other intracellular signalling and scaffolding proteins. Many regulatory enzymes interact with NMDA receptors in a subunit-selective manner (Kemp and McKernan 2002). These may be useful new sites for targeted modulation of the NMDAR. Ifenprodil is one example of a modulator that binds to a unique NMDAR site. This site is at the interface between the
extracellular N terminal domains of GluN1 and GluN2B subunits (Kemp and McKernan 2002). S-nitrosylation sites are also potential targets that block excitotoxicity. Other modulatory sites also exist, and these include binding sites for zinc and polyamines, as well as a pH-sensitive site (Lipton 2006).

6.3.7. Normal NMDAR activity requires fine adjustment as opposed to pathological inhibition or over-activation

In order for NMDAR modulators to be physiologically tolerable it may be more useful to implement modulators that adjust the intensity of receptor function rather than completely inhibiting or over-activating the receptor. Lipton (2005), uses a television set analogy to describe the modulation of the NMDAR where the agonist binding sites are the ‘on and off’ switch. Drugs that block the agonist binding site inhibit over-activation of NMDARs in pathological conditions but this usually results in clinically unacceptable side effects. Instead of turning off the receptor, Lipton proposes the equivalent of adjusting the ‘volume control’ of the NMDAR. Thus, during excessive calcium flux through the ion channel, ‘volume’ of ion flow is adjusted to ‘normal’.

The magnesium ion blocker is not a sufficient inhibitor on its own as it results only in a ‘flickery’ block. On the other hand, if a channel blocker binds too tightly it will block normal as well as excessive activation and be clinically unacceptable. This is akin to turning the volume on a television all the way down which in essence is the same as blocking normal function of the television, and this is the mechanism used by MK-801. It is a potent excitotoxicity blocker but because its ‘dwell time’ in the ion channel is so long, due to its high affinity for the magnesium site, it also blocks crucial normal functions (Lipton 2005). The same concept might be applied to agonists, like NMDA and glutamic acid, that can restore impaired NMDAR activity but can also leave the receptor in a constitutively activate state resulting in uncontained cell proliferation or cytotoxicity.

6.4. Conclusions

This chapter investigated the effect of NMDAR modulation on melanoma proliferation and invasiveness. Non-competitive NMDAR channel blockers, memantine and MK-801, were effective at inhibiting melanoma cell proliferation at concentrations comparable to those used in other studies. The competitive antagonist of NMDARs (AP5) and agonists (NMDA and
glutamic acid) did not inhibit but had some stimulatory effect on cell proliferation. NZM 61 was most sensitive to the anti-proliferative effect of MK-801, and both NMDAR agonists increased proliferation of these cells. The results support the computational predictions in Chapter Three that \textit{GRIN2A} mutations in NZM 61 impact NMDAR function. Results from this chapter suggest that this mutation may cause a gain-of-function effect.

The invasiveness of melanoma cell line NZM 40 was attenuated by NMDAR antagonists AP5 and agonist NMDA, both of which bind to the NMDAR’s glutamate binding site and both of which failed to inhibit cell proliferation. No effects on invasiveness were seen after exposure to the NMDAR antagonist memantine which had the strongest inhibitory effect on cell proliferation. The channel blocker, MK-801 inhibited invasion but its activity too has been linked to that of the agonist binding site. The NMDAR role in facilitating invasiveness may be regulated at the agonist binding site but its role in promoting proliferation may be facilitated specifically through the opening of the channel.
CHAPTER 7

Do glutamate concentrations in culture affect melanoma cell phenotype?

7.1. Introduction

Previous chapters of this thesis have presented evidence for the expression and function of NMDARs in melanoma cell lines, and for the role of NMDARs in melanoma cell proliferation and invasion. Glutamate is an endogenous NMDAR agonist but it is also a proteinogenic non-essential amino acid and excitatory neurotransmitter.

All melanoma cell lines used for the experiments presented in this thesis were cultured in α-MEM. We questioned whether media such as α-MEM that contains relatively high concentration of glutamate (indicated by the manufacturer to be at 500 µM) may affect the mesenchymal (invasive) phenotype of cultured cells. RPMI 1640 is another medium that can be used to culture melanoma cells. RPMI 1640 has a lower concentration of glutamate (indicated as 136 µM) that better aligns with its concentrations in plasma under normal physiological conditions. This chapter examines whether different media would have an effect on the amount of glutamate secreted by cells in culture and on expression of Slug, which associates with the mesenchymal cell phenotype. Finally, we examined whether riluzole which inhibits glutamate release would affect melanoma cell proliferation.

We hypothesised that levels of glutamate in the tumour microenvironment may contribute to melanoma progression by encouraging proliferation and facilitating invasion.

7.2. Results

7.2.1. The effect of different culture media on melanoma cell phenotype

NZM cells were cultured either in α-MEM or RPMI 1640 (containing high and low glutamate levels, respectively) over a period of nine days after which the concentrations of glutamate in
cultures were determined. On day nine, cells were lysed and the expression of Slug and GluN1 were examined by Western blotting.

NZM cell lines used in these experiments were as described in section 2.2. NZM 3, NZM 6, NZM 7 and NZM 40 were seeded at 5 x 10^3 cells/mL and NZM 11, NZM 61 and NZM 100 at 10 x 10^3 cells/mL, as determined by earlier experiments to produce 90-100% confluency over a nine day period. This seeding adjustment compensated for the differences in cell cycle times between cell lines determined previously. Media were also incubated with no cells to control for the level of spontaneous hydrolysis of glutamine to glutamate and to allow for the change in glutamate levels to be attributed to the contribution by cells in culture. Samples of media were collected on days 0 and nine to measure glutamate concentrations, and cells were lysed on day nine for immunoblot analysis (two independent experiments were performed).

### 7.2.2. Effects of different media on glutamate secretion by melanoma cells in culture

In keeping with the manufacturer’s specifications, we found that glutamate concentrations were lower in RPMI 1640 (99 ± 6.7 µM) compared with α-MEM (321 ± 7.6 µM) and this relative difference between media remained present after nine days in culture (p < 0.0001) (Figure 7.1).

Without cells, the concentration of glutamate in α-MEM increased from 321.2 µM on day 0 to 441.8 µM on day nine, indicating a background of spontaneous glutamine hydrolysis (p < 0.0001) (Figure 7.1). In the presence of cells, further increases in glutamate concentrations were seen for four of seven cell lines: NZM 7, NZM 100 (both with GRIN2A mutations) as well as NZM 11 and NZM 40 (with no GRIN2A mutations) (Table 7.1 and Figure 7.2A).

Unlike in the α-MEM, the average concentration of glutamate in RPMI 1640 did not increase without cells (p = 0.515). In the presence of cells, glutamate levels increased in α-MEM for the same cell lines as in RPMI 1640 (NZM 7, NZM 100, NZM 11, NZM 40) and also for NZM 61 (that carried GRIN2A mutations) (Table 7.1 and Figure 7.2B). Relative increases in glutamate levels were higher when cells were grown in RPMI.
**Figure 7.1: Glutamic acid concentration in media after nine days**

Mean glutamic acid concentration (µM) in α-MEM or RPMI 1640 after culturing melanoma cells for nine days. Values represent the mean of two independent determinations.
Figure 7.2: Fold change of glutamic acid concentration in medium after nine days

Fold changes of mean glutamic acid concentration (µM) in α-MEM (a) or RPMI 1640 (b) after culturing melanoma cells for nine days. Values represent the mean of two independent determinations. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
Glutamate released by melanoma cell lines over nine days

**α-MEM (321.2 µM on day 0)**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mean glutamate concentration on day nine (µM)</th>
<th>Fold change relative to no cells on day nine</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cells</td>
<td>441.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZM 3</td>
<td>405.8</td>
<td>0.998</td>
<td>0.9593</td>
</tr>
<tr>
<td>NZM 7</td>
<td>590.1</td>
<td>1.340</td>
<td>0.0274</td>
</tr>
<tr>
<td>NZM 61</td>
<td>576.2</td>
<td>1.304</td>
<td>0.0523</td>
</tr>
<tr>
<td>NZM 100</td>
<td>621.9</td>
<td>1.410</td>
<td>0.0057</td>
</tr>
<tr>
<td>NZM 6</td>
<td>445.6</td>
<td>1.010</td>
<td>0.9999</td>
</tr>
<tr>
<td>NZM 11</td>
<td>641.3</td>
<td>1.452</td>
<td>0.0021</td>
</tr>
<tr>
<td>NZM 40</td>
<td>649.8</td>
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</table>

**RPMI 1640 (99 µM on day 0)**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mean glutamate concentration on day nine (µM)</th>
<th>Fold change relative to no cells on day nine</th>
<th>P value</th>
</tr>
</thead>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>NZM 3</td>
<td>67.89</td>
<td>0.592</td>
<td>0.6565</td>
</tr>
<tr>
<td>NZM 7</td>
<td>219.6</td>
<td>1.916</td>
<td>0.0371</td>
</tr>
<tr>
<td>NZM 61</td>
<td>298.0</td>
<td>2.600</td>
<td>0.0002</td>
</tr>
<tr>
<td>NZM 100</td>
<td>323.2</td>
<td>2.820</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>NZM 6</td>
<td>92.11</td>
<td>0.804</td>
<td>0.9827</td>
</tr>
<tr>
<td>NZM 11</td>
<td>384.9</td>
<td>3.359</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>NZM 40</td>
<td>343.5</td>
<td>2.997</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Table 7.1: Mean glutamate concentration and fold change for media in which melanoma cell lines were grown.

Cell lines with GRIN2A mutations are shown in red. Control medium contained no cells. P values represent the significance of differences between glutamate concentration in α-MEM and RPMI 1640.
Figure 7.2: Fold change of glutamic acid concentration in medium after nine days

Fold changes of mean glutamic acid concentration (µM) in (A) α-MEM or (B) RPMI 1640 after culturing melanoma cells for nine days. Values represent the mean of two independent determinations. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
7.2.3. Effects of different media on the expression of Slug in cultured melanoma cells

Cells were harvested and lysed on day nine; expression of Slug and GluN1 were examined by Western blotting as described in section 2.2 (Figure 7.3). Slug was expressed in all cell lines. Densitometry analysis relative to β-Actin revealed no difference in Slug expression between cell lines cultured in α-MEM (Table 7.2, Figures 7.4 and 7.5A) (p = 0.3147). However, significant differences in Slug expression were detected for cell lines cultured in RPMI 1640 (p < 0.0001) (Figure 7.5B). Slug expression was higher for cells cultured in RPMI 1640 compared with α-MEM, in particular for NZM 7, NZM 61 and NZM 100 which all had \textit{GRIN2A} mutations (Figure 7.5B). These were the same cell lines that secreted high amounts of glutamate in RPMI 1640 (Figure 7.2).

GluN1 expression was variable between replicates so no reliable conclusions on the impact of media on GluN1 expression could be drawn; further work will be required to determine this.
Figure 7.3: Slug expression in melanoma cell lines cultured in α-MEM and RPMI 1640

Immunoblot detection of Slug and GluN1 in melanoma cell lines cultured in (blue) α-MEM or (green) RPMI 1640 based media for nine days. Representative blot of two independent determinations.

Figure 7.4: Densitometry ratio of Slug expression in melanoma cell lines cultured in α-MEM and RPMI 1640

Quantification of Slug expression in melanoma cell lines cultured in α-MEM or RPM 1640 media for nine days. Values represent the mean of two independent determinations. *p<0.05 **p<0.01
Figure 7.5: Quantified Slug expression in melanoma cell lines cultured in α-MEM and RPMI 1640

Quantified Slug expression in melanoma cell lines cultured in (A) α-MEM or (B) RPMI 1640 for nine days. Values represent the mean of two independent determinations.
### Table 7.2: Densitometry ratios of Slug to β-Actin expression in melanoma cells cultured in α-MEM or RPMI 1640 for nine days

<table>
<thead>
<tr>
<th>Cell line</th>
<th>α-MEM</th>
<th>RPMI 1640</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZM 3</td>
<td>0.358901</td>
<td>0.4384655</td>
<td>0.708</td>
</tr>
<tr>
<td>NZM 7</td>
<td>0.6640225</td>
<td>1.672057</td>
<td>0.0346</td>
</tr>
<tr>
<td>NZM 61</td>
<td>0.65757</td>
<td>1.255148</td>
<td>0.0104</td>
</tr>
<tr>
<td>NZM 100</td>
<td>0.769024</td>
<td>2.826824</td>
<td>0.0088</td>
</tr>
<tr>
<td>NZM 6</td>
<td>0.795948</td>
<td>1.219735</td>
<td>0.0774</td>
</tr>
<tr>
<td>NZM 11</td>
<td>0.584809</td>
<td>0.7020445</td>
<td>0.7041</td>
</tr>
<tr>
<td>NZM 40</td>
<td>0.497874</td>
<td>0.6199535</td>
<td>0.3920</td>
</tr>
</tbody>
</table>

**GRIN2A** mutant cell lines are shown in red. P values represent the significance of differences between Slug expression in α-MEM and RPMI 1640 grown cells.
7.2.4. Effects of riluzole on melanoma cell proliferation

NZM cell lines were cultured in the presence of riluzole, a postulated inhibitor of glutamate release and effects on cell proliferation were determined as previously described (section 2.2).

Twenty NZM cell lines were cultured for five days in α-MEM without and with riluzole at concentrations from 0.3 to 200 µM; IC₅₀ values were determined to quantify the anti-proliferative effects of riluzole. Riluzole inhibited proliferation of all the 20 NZM cell lines with mean IC₅₀ values from 4.5 µM to 40.5 µM (Figure 7.6A). The cell lines most sensitive to riluzole were NZM 100 and NZM 7 (IC₅₀ values 4.5 and 7.5 µM, respectively); both these lines carried \textit{GRIN2A} mutations (G889E and P1132L, respectively). Overall there was no obvious difference in riluzole effects on cell lines with and without \textit{GRIN2A} mutations (p = 0.1397) (Figure 7.6B and 7.7).
Figure 7.6: The sensitivity of melanoma cells to riluzole

(A) Average concentrations of riluzole needed to achieve 50% inhibition of cell proliferation (IC$_{50}$) in melanoma cell lines. (B) Box plot representing the distribution of riluzole IC$_{50}$ values in GRIN2A mutant (Mut) versus wildtype (WT) cell lines exposed to riluzole. Each value represents the mean of two to three independent determinations.
Figure 7.7: The anti-proliferative effect of riluzole on melanoma cell lines

The effect of riluzole on melanoma cell proliferation as determined by \(^3\)H thymidine incorporation, expressed as the percent of treated to untreated cells. Each line represents one determination. Response profiles for cells carrying GRIN2A mutations are shown in red with symbols corresponding to cell lines as indicated. Cells were treated with riluzole concentrations of (A) 200 µM diluted 3X to 2.4 µM, (B) 200 µM diluted 2X to 12.5 µM or (C) from 25 µM diluted 3X to 0.4 µM
7.2.5. Correlating Slug expression with glutamate in media and clinical data

Among the *GRIN2A* mutated cell lines cultured in RPMI 1640 the highest increases of glutamate concentration and Slug expression (relative to α-MEM) were seen for three of the four *GRIN2A* mutated cell lines NZM 7, NZM 61 and NZM 100. Tumours from which these cells lines were derived had the highest rates of lymph node and distant metastases.

No increases of glutamate or Slug expression were seen in NZM 3 (also *GRIN2A* mutated) (Figure 7.1, 7.2B and 7.5B). The tumour from which NZM 3 was derived had the lowest rate of lymph node and distant metastases (Table 7.3).

Among the cell lines possessing no mutations in *GRIN2A* (NZM 6, NZM 11 and NZM 40) no difference in Slug expression was seen between cells cultured in RPMI 1640 and α-MEM (Figure 7.4). When glutamate concentrations were considered among these cell lines, an increase was detected in NZM 11 and NZM 40 but not in NZM 6. The tumour from which NZM 6 cell line was derived had the lowest rate of lymph node and distant metastases among the cell lines wild-type for *GRIN2A* (Table 7.3).

<table>
<thead>
<tr>
<th></th>
<th>Time from diagnosis to lymph node metastasis (months)</th>
<th>Time from diagnosis to distant organ metastasis (months)</th>
<th>Relative Slug expression (in RPMI)</th>
<th>Fold increase in glutamate concentrations (in RPMI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NZM 3</strong></td>
<td>27</td>
<td>7</td>
<td>0.4385</td>
<td>0.592</td>
</tr>
<tr>
<td><strong>NZM 7</strong></td>
<td>0</td>
<td>2</td>
<td>1.672</td>
<td>1.916</td>
</tr>
<tr>
<td><strong>NZM 61</strong></td>
<td>9</td>
<td>2</td>
<td>1.255</td>
<td>2.600</td>
</tr>
<tr>
<td><strong>NZM 100</strong></td>
<td>0</td>
<td>0</td>
<td>2.826</td>
<td>2.820</td>
</tr>
<tr>
<td><strong>NZM 6</strong></td>
<td>66</td>
<td>93</td>
<td>1.22</td>
<td>0.804</td>
</tr>
<tr>
<td><strong>NZM 11</strong></td>
<td>48</td>
<td>28</td>
<td>0.702</td>
<td>3.359</td>
</tr>
<tr>
<td><strong>NZM 40</strong></td>
<td>17</td>
<td>18</td>
<td>0.6199535</td>
<td>2.997</td>
</tr>
</tbody>
</table>

*Table 7.3: Associations between disease progression, Slug expression and glutamate secretion in melanoma cells.*

*GRIN2A* mutated cell lines shown in red.
7.3. Discussion

This chapter has shown that glutamate concentrations in cell culture media were influenced by the cells cultured in them, implying secretion of glutamate from cells into media. Increases and variations in glutamate release were greater when cells were cultured in RPMI 1640 compared with α-MEM i.e. when media contained less glutamate. Further, we showed that the expression of Slug increased in three of four GRIN2A mutated melanoma cell lines when they were cultured in RPMI 1640. No increase in Slug expression was detected in cell lines that did not have GRIN2A mutations. Riluzole, an inhibitor of glutamate release, reduced proliferation of melanoma cell lines with IC₅₀ values from 4.5 µM to 40.5 µM. The release of glutamate by melanoma cells and its effect on proliferation and expression of mesenchymal markers such as Slug indicates the biological relevance of glutamate in culture medium. This is further discussed in the following sections.

7.3.1. Seeding density did not affect glutamate release and Slug expression

Fluctuating glutamate concentrations in cell culture medium can be accounted for by pre-existing glutamate, hydrolysis of glutamine to glutamate, degradation of glutamate or as a by-product of cellular metabolism. In this chapter, the increases in glutamate concentrations and Slug expression observed in RPMI 1640 could be attributed to melanoma cells because no increase was detected in medium without cells. Although the cells were seeded at different densities to compensate for their varying proliferation rates, there was no relationship between seeding cell densities and the levels of glutamate or Slug expression in media on day nine.

7.3.2. Slug expression and glutamate release may be of clinical significance

Glutamate released by melanoma cells may influence the metastatic nature of melanoma by facilitating a mesenchymal phenotype through the expression of Slug. Slug is a member of the Snail family of zinc finger transcription factors and been associated with epithelial-to-mesenchymal transformation EMT (Shirley, Greene et al. 2012). The influence of glutamate on the mesenchymal phenotype of melanoma cells may be particularly applicable to cells carrying GRIN2A mutations for the reasons described below.

Among the cell lines cultured in RPMI 1640 the highest increases in glutamate concentration and Slug expression were seen in three of the four GRIN2A mutated cell lines (Figure 7.2B, 7.4). Tumours from which these cells lines were derived had the highest rates of lymph node...
and distant metastases (Table 7.3). Among the wild-type cell lines, disease spread was associated with glutamate release but not Slug expression (Table 7.3). Thus we could hypothesise that the poor clinical outcome of patients with \textit{GRIN2A} mutated melanomas is attributed to a Slug driven mesenchymal phenotype that is facilitated by glutamate release. Only seven cell lines were used in these assays and this hypothesis will need to be tested using a larger numbers of cell lines.

Slug is expressed in neural-crest-delaminated melanoblasts, melanocytes, and benign nevi (Gupta, Kuperwasser et al. 2005, Shirley, Greene et al. 2012, Vandamme and Berx 2014). The role of Slug in facilitating an invasive phenotype is not fully elucidated but Slug expression has been linked to EMT-like loss of cell adhesion and increased cell motility exhibited during melanoma progression. Slug also plays a role in EMT facilitated melanocyte migration from the neural crest (Shirley, Greene et al. 2012). The regulation of Slug expression in melanoma is unclear (Fenouille, Tichet et al. 2012) but from the results presented in this chapter it could be suggested that Slug expression is influenced by the concentration of glutamate in the cellular microenvironment.

\textbf{7.3.3. The anti-proliferative effects of riluzole could be NMDAR mediated}

Riluzole exerted anti-proliferative effects on all melanoma cell lines with mean IC\textsubscript{50} values ranging between 4.5 to 40.5 µM. The most potent anti-proliferative effects of riluzole were seen in \textit{GRIN2A} mutated cell lines, NZM 100 and NZM 7 cells with the lowest IC\textsubscript{50} values (4.5 and 7.5 µM respectively).

Riluzole’s exact mechanism of action is not well-defined but it is a postulated inhibitor of glutamate release (Yip, Le et al. 2009, Dolfi, Medina et al. 2015). Proposed targets of riluzole include blockade of excitatory amino acid receptors and G-protein coupled receptors, inhibition of voltage-dependent sodium channels, inhibition of voltage-dependent calcium channels and activation of intracellular calcium-buffering processes (Doble 1996). The hypersensitivity of \textit{GRIN2A} mutated NZM 7 and NZM 100 to riluzole suggests that the NMDAR may well be a player in eliciting a proliferative effect. There is some evidence to suggest that riluzole may act through the NMDAR but its exact site of action on the receptor is unknown (Doble 1996). In prostate cancer riluzole blocks the increase in intracellular calcium evoked by NMDA (Hubert, Delumeau et al. 1994, Kanako, Shibata et al. 2009) and electrophysiological experiments on Xenopus oocytes have shown that riluzole inhibits
currents evoked by NMDA at IC$_{50}$ = 18 µM (Doble, 1996). Furthermore, riluzole blocked calcium mobilisation in response to NMDA in primary cultures of rodent brain neurons (Doble 1996).

Riluzole has been evaluated in pre-clinical melanoma studies and clinical trials showing promising results (Yip, Le et al. 2009). Riluzole has been shown to enhance WNT3A mediated gene expression in vitro which promotes melanoma cell pigmentation, and decrease cell proliferation. Furthermore riluzole treatment, decreases metastases in a mouse melanoma model (Biechele, Camp et al. 2010).

Riluzole affects signalling pathways that are frequently disrupted in neoplastic disease (Chapter One). It was reported to suppress the metabolic activity of melanoma by inhibiting signalling through the MAPK kinase and PI3K/AKT pathways. (Yip, Le et al. 2009). The mechanism proposed as a consequence is the accumulation of cells in G2/M phases of the cell cycle followed by apoptotic cell death via the activation of caspase -3, -4 -8 and -9 (Kanako, Shibata et al. 2009, Khan, Wall et al. 2011). The combination of riluzole with the tyrosine kinase inhibitor sorafenib exhibited enhanced antitumor activities in mGluR1-expressing melanoma regardless of BRAF mutation status (Lee, Wall et al. 2011). This additive effect implies that glutamate has a significant role to play in melanoma progression. Moreover, in vivo studies using xenografts treated with riluzole and radiation fractions have shown tumour growth inhibition and DNA damage further indicating a role of glutamate release in vivo (Khan, Wall et al. 2011).

7.3.4. Limitations with culturing and studying patient derived melanoma cell lines

Other factors that need to be taken into consideration when interpreting the results from this chapter include the variety of driver and passenger mutations already present in melanoma cell lines, the heterogeneity within the cell lines and other differences between the compositions of two media. In order to test our hypotheses it would be helpful to conduct the same experiment by adding glutamate or NMDA to RPMI 1640 instead of using α-MEM.

It is possible that riluzole effects could be greater if cells were cultured in RPMI 1640. The lower glutamate concentration in RPMI 1640 is better representative of physiological conditions. More glutamate was secreted when melanoma cell lines were cultured in RPMI 1640 compared with α-MEM (Figure 7.2). Thus treatment with riluzole in RPMI 1640 may
not only ease the glutamate burden but may also facilitate finding associations with \( GRIN2A \) mutated cell lines that may be masked when cells are cultures in \( \alpha \)-MEM.

### 7.3.5. Glutamate in skin and neoplastic tissue

The involvement of glutamate in components of the skin is not unusual. Glutamate receptors have been described in both keratinocytes and melanocytes (Fischer, Glanz et al. 2009). Keratinocytes are capable of producing and releasing glutamate which acts as a transmitter on epidermal glutamate receptors (Fischer, Glanz et al. 2009). It would be expected that melanocytes may be involved in glutamate signalling (Hoogduijn, Hitchcock et al. 2006, Ogundele, Okunnuga et al. 2014) as their natural location in the epidermis is within a bed of keratinocytes (Seiberg 2001, Lin and Fisher 2007, Delevoye 2014). Glutamate secretion by cancerous cells has been previously reported and suggestive of a survival advantage. For example, glutamate-secreting gliomas expand 15-fold faster than those that do not secrete glutamate. Furthermore, the growth of these gliomas was attenuated by the NMDAR antagonists MK-801 and memantine (Takano, Lin et al. 2001), thus indicating the involvement of NMDARs.

### 7.4. Conclusions

Pre-existing extracellular glutamate concentrations in culture media affect glutamate release in melanoma cells. Our results have shown that cells release glutamate and that more glutamate was released when cells were cultured in RPMI 1640 compared to \( \alpha \)-MEM. We also found that cell lines derived from patients who had faster spread of their disease released more glutamate when grown in RPMI 1640. \( GRIN2A \) mutated cell lines derived from patients who had faster spread of their disease also showed higher expression of Slug when grown in RPMI 1640. Finally, we have shown the anti-proliferative effect of riluzole, an inhibitor of glutamate release, on melanoma cells. We hypothesise that glutamate in the tumour microenvironment contributes to melanoma progression by not only facilitating proliferation but also through its effect influencing mesenchymal phenotype. We can also hypothesise that melanoma cell proliferation and the transition to a mesenchymal phenotype is elicited through glutamate receptors and that NMDARs, particularly those with mutated \( GRIN2A \) may enhance this effect.
CHAPTER 8

Concluding Discussion

This chapter consists of two parts, the first summarising the results and the second introducing a new hypothesis to bring together results on NMDARs and the results on glutamate.

8.1. Summary of results

This study began with an investigation into the prevalence of GRIN2A mutations in melanoma cell lines. Previous high throughput exome sequencing found that GRIN2A was frequently mutated in melanoma (Wei, Walia et al. 2011) but at the time no explanation was suggested for the significance of this frequency. As mentioned in previous chapters, the GRIN2A gene encodes the GluN2A regulatory subunit of the NMDAR. In light of this the role of NMDARs in melanoma proliferation and invasion was investigated with a particular focus on GRIN2A. The sequence of investigations for this study is shown in Figure 8.1. Our Sanger sequencing of GRIN2A in 20 metastatic melanoma cell lines previously collected by the ACSRC demonstrated that 20% carried non-synonymous mutations in GRIN2A, in keeping with previous reports. The presence of GRIN2A mutations in melanoma cell lines was associated with shorter survival and faster spread of cancer to lymph nodes and distant organs in patients from whom these lines were derived. The non-synonymous GRIN2A substitutions detected in this thesis were predicted by computational analysis to disrupt GluN2A function. The G762E mutation located in the hinge region of the receptor's agonist binding domain was modelled into the existing crystal structure of GluN2A. Structural modelling predicted that the G762E mutation could alter GluN2A-GluN1 interactions (Chapter 3).

Comprehensive screening of NMDAR subunits expression in melanoma cell lines was performed at both transcript and protein levels, and provided evidence for the heterogeneous expression of GRIN genes in melanoma (Chapter 4). Transcripts of genes encoding GluN1, the structural subunit of the NMDAR and five of six possible regulatory subunits were detected in melanoma cell lines using RT-PCR. Data on GluN3B expression was difficult to validate.
Immunoblotting detected the GluN1 protein in all melanoma cell lines while GluN2A was detected in only one cell line. Expression of other GluN proteins were not tested due to the lack of antibodies validated for use in non-neuronal tissues. NMDAR functionality was tested in melanoma cells by tracking variations in cytosolic calcium levels in response to the NMDAR agonists. Although further evidence is required, calcium flux results support the presence of functional NMDARs in melanoma cells. Investigations into the NMDAR’s role in melanoma cell proliferation and invasion revealed that cell proliferation may be facilitated though the opening of the ion channel, while invasiveness may be regulated at the receptor’s agonist binding site. NMDAR channel blockers (memantine and MK-801) were the most effective at inhibiting melanoma cell proliferation. Conversely, AP5, a competitive NMDAR inhibitor did not inhibit proliferation. Instead, AP5 had a mild stimulatory effect on cell proliferation in some cell lines. The invasiveness of melanoma cells was attenuated by AP5 and NMDA, which bind to the agonist binding site. Negligible effects on invasiveness were demonstrated when cells were exposed to ion channel blockers that had the strongest inhibitory effect on cell proliferation.

Finally, we reported that pre-existing glutamate concentrations in culture media influenced glutamate release by melanoma cells. Cells released more glutamate when cultured in RPMI 1640 compared to α-MEM, supporting the cells’ requirement for glutamate. Intriguingly, cell lines derived from patients with faster disease progression released more glutamate when grown in RPMI 1640 and showed higher expression of Slug, a transcription factor that promotes EMT. Congruently, inhibition of glutamate release had anti-proliferative effects on melanoma cells.

In the following sections we discuss factors that could have influenced the results presented in this thesis. We propose explanations for the involvement of glutamate and NMDARs in melanoma, and indicate possible future directions for this work.
1. *GRIN2A* found to be frequently mutated in human melanoma

2. *GRIN2A* mutations correlated with decreased patient survival and increased tumour spread

3. Evidence provided for the expression of NMDAR genes in melanoma

4. Evidence provided for the function of NMDARs in melanoma

5. Glutamate concentration in culture medium may effect cell phenotype

Figure 8.1: The sequence in which investigations for the study were carried out
8.2. Why might *GRIN2A* be mutated in melanoma?

Large sequencing studies indicate that somatic mutations in genes encoding regulatory subunits of the NMDAR occur most frequently in melanoma compared with other cancers, present in 25-40% of melanoma samples (Figure 8.2) (Whetzel PL 2011). Further, *GRIN2A* mutations are particularly common at 8-26%, compared with other tumours (Figure 8.3) (Whetzel PL 2011). One of the main questions driving this thesis was the reason for this frequent occurrence of *GRIN2A* mutations in melanoma. Our data suggest that mutations in *GRIN2A* may confer a survival advantage to melanoma cells, at least *in vivo*.

NMDARs activate pathways that regulate cell survival, migration and invasion. Over-activation of these pathways may result in oncogenic effects (North, Gao et al. 2010). Activation of NMDARs may contribute to the maintenance of PI3-K and ERK signalling, through which cell survival and proliferation are promoted. During brain development, NMDAR activity engages these pathways to promote cell survival (Perkinton, Ip et al. 2002) and these pathways are often deregulated in cancer (Hardingham and Bading 2003, Hardingham and Bading 2010, Deutsch, Tang et al. 2014). NMDAR inhibitors reduce proliferation of melanoma cells *in vitro* (Song, He et al. 2012, Prickett, Zerlanko et al. 2014), suggesting NMDAR role in cell cycle progression. One study has shown that decreased expression of GluN1 reduced cancer cell proliferation (Luksch, Uckermann et al. 2011), while others have shown that NMDAR inhibitors reduce melanoma cells migration *in vitro* (Song, He et al. 2012, Prickett, Zerlanko et al. 2014). NMDARs have been hypothesised to play a role in tumour spread by initiating an invasive phenotype through activation of the CREB transcription factor (Li and Hanahan 2013). Chapter Six of this thesis has provided evidence for the anti-proliferative and anti-invasive effects of NMDAR antagonists while results from Chapter Seven indicate that NMDARs may be involved in the maintenance of a mesenchymal or invasive phenotype following glutamate release.

The role of NMDAR associated genes (such as *GRIN2A*) as a tumour suppressor has been previously suggested (Wei, Walia et al. 2011, Prickett, Zerlanko et al. 2014). NMDAR over-activation can cause cell toxicity through excessive calcium influx into the cell (Paoletti, Bellone et al. 2013). Calcium mediated cytotoxicity via the NMDAR may inhibit tumour growth under high glutamate concentrations, suspected to rise in tumour microenvironments. A lack of NMDAR-mediated cell death as a result of mutations has been suggested to facilitate tumour progression (Prickett and Samuels 2012, Prickett, Zerlanko et al. 2014). Loss of
NMDAR mediated cytotoxicity could be accounted for by a number of factors such as mutations in the NMDAR subunits, downregulation of receptor expression, gene deletions, or selection of cells that downregulated receptor expression or attenuated its function.

In the CNS, synaptic NMDAR activation promotes neuronal survival, whereas extra-synaptic NMDAR activation results in cell death (Chen, Lu et al. 2008). NMDARs play a dual role regulating cell survival and cytotoxicity in a context dependent manner. Therefore, it is appropriate to suggest that in cancer, NMDAR can act as an oncogene or a tumour suppressor, depending on the environmental context. Lipton’s television set analogy described in Chapter Five provides a fitting interpretation of NMDAR modulation. Lipton’s model proposes that simply inducing or inhibiting the receptor activation altogether may not be adequate in pathological contexts because the outcome may be clinically unacceptable in normal tissue. He suggests instead that receptor activity in pathological condition (melanoma in this case) may need to be adjusted in a context dependent manner (Lipton 2005). Our results have shown that mutations in GRIN2A may confer a survival and metastatic advantage to melanoma cells.
Figure 8.2: Summary of genetic alterations in genes encoding regulatory NMDAR subunits (GRIN2A-D) detected in 91 studies

Data extracted from BioPortal. Melanoma cancer type indicated with black circles.
Figure 8.3: Summary of genetic alterations in GRIN2A detected in 91 studies

Data extracted from BioPortal. Melanoma cancer type indicated with black circles.
8.3. How might NMDARs function in non-excitable cancerous cells?

Neuronal NMDARs receive glutamate mediated signals as a consequence of previously induced electrical signals. In response to glutamate, cell membranes can become depolarised as a consequence of ion influx through NMDAR channels. Membrane depolarisation results in the activation of various intracellular signalling cascades (Nedergaard, Takano et al. 2002). In non-neuronal cells, including cancerous cells, there is evidence for calcium influx in response to NMDAR agonists such as glutamate and NMDA (Hoogduijn, Hitchcock et al. 2006, Prickett, Zerlanko et al. 2014) indicating the presence of functional NMDARs. It is essential to question why and how NMDARs exist in non-excitable cells that are not specialised for the transmission of electrical signals (Nedergaard, Takano et al. 2002). The resting membrane potential in tumour cells is -30 to -50 mV (Gill and Pulido 2007). In neurons, depolarised membranes like these lose their magnesium block and calcium flux through the ion channel is enabled (Gill and Pulido 2007), suggesting that NMDARs in tumour cells are in a constitutively active state.

The physiological concentration of glutamate in blood plasma is 30-80 µM. In cerebrospinal fluid it is regulated at <1 µM but under pathological conditions such as hypoxic injury this can increase from ~1 µM to 20 µM, leading to cellular damage (Nedergaard, Takano et al. 2002). Glutamate concentrations can rise to almost 100 times higher outside of the brain and this is unusual, considering the NMDAR’s high affinity for glutamate and its propensity to induce cytotoxic effects when over activated (Nedergaard, Takano et al. 2002). Glutamate is tightly regulated in the CNS where transporters maintain a 10,000-fold gradient of intracellular glutamate (3-10 mM) to extracellular glutamate (0.3-1 µM) driven by ionic gradients that are generated by ion-exchanging pumps (such as sodium/potassium-ATPase) (Hinoi, Takarada et al. 2004). It is interesting to consider the mechanism by which cells expressing NMDARs in non-neuronal tissue might escape concentrations of glutamate that would be considered pathological for neurons. One study has shown that millimolar concentrations of glutamate did not induce melanocyte toxicity but that blockade of NMDARs with memantine or MK-801 caused a rapid and reversible change in melanocyte morphology (Hoogduijn, Hitchcock et al. 2006). Melanocytes may not only possess some kind of a coping mechanism used to evade glutamate toxicity but may also depend on glutamate for the maintenance of their normal functions.
A number of regulatory factors may be involved in enabling the NMDAR to tolerate glutamate mediated excitotoxicity. Firstly, the density of NMDAR subunit expression in non-neuronal cells is much lower than in neuronal cells. From Chapter Four of this thesis and from other studies it is apparent that subunits which make up the NMDAR are expressed at low levels in non-neuronal tissues compared with the CNS and this may contribute to tolerance of these tissues to high glutamate concentrations. Post-translational modifications such as glycosylation or phosphorylation of the NMDAR subunits have been reported and this might reduce affinity of the receptor to glutamate (Gill and Pulido 2001). Composition of the subunits that make up the receptor may affect its affinity for glutamate binding (Dickman, Youssef et al. 2004) (discussed in Sections 4.3 and 5.3). For example the GluN3 type subunits bind glycine and not glutamate, and therefore NMDARs containing the GluN3 type subunits would have less glutamate binding sites (Chatterton, Awobuluyi et al. 2002). Glutamate binding to GluN2A or GluN2B containing receptors gives rise to greater levels of toxicity driven by NMDAR overactivation as compared with GluN2C and GluN2D containing receptors (Lynch and Guttmann 2002). Factors that define properties or composition of NMDARs may be tissue-specific and peripheral NMDARs may require a relatively higher concentration of the agonist for receptor activation (Frankiewicz, Potier et al. 1996). A number of studies have reported the presence of functional NMDARs in tissues made up of non-excitable cells, in which effects are similar to those in the CNS. High concentrations of NMDA of around 1 mM can induce excitotoxicity in the lung in the form of an acute pulmonary oedema, which can be prevented by MK-801 (Said, Berisha et al. 1996, Dickman, Youssef et al. 2004). In this context, NMDAR inhibitors attenuate oxidant lung injury (Said, Pakbaz et al. 2000, Dickman, Youssef et al. 2004).

The binding affinities of NMDAR channel blockers are different in various regions of the brain, indicating that NMDARs are heterogeneous (Bresink, Danysz et al. 1995). This raises the question of whether heterogeneity in NMDARs exists in other tissues of the body. Changes in tissue microenvironment such as serum and extracellular glutamate levels may also influence the expression and functionality of NMDARs, making their composition context dependent. The inconsistencies apparent in the experimental results presented in Chapters Four to Six, between NMDAR expression and function indicate that the expression and presence of functional glutamate receptors may depend on the concentration of glutamate in media or tissue microenvironment.
In Chapter Seven we showed that melanoma cells can survive and proliferate in high concentrations of glutamate (321 µM to 650 µM). Cell culture medium is generally supplemented with 5% FBS carrying ~1 mM glutamate (Ye and Sontheimer 1998), adding an additional 50 µM of glutamate to the media. It is likely that melanoma cells develop mechanisms by which they can withstand high glutamate concentrations or perhaps their survival is dependent on the ample availability of glutamate. In light of the heterogeneity often seen in these cell lines it could also be hypothesised that melanoma cells are lost when they do not have a phenotype required for survival in higher glutamate concentrations, while those that survive get selected to repopulate the culture. Alternatively, expression of relevant genes may change, and the consequent phenotypic shift may allow for survival in glutamate rich culture conditions. Genes regulated for this purpose may include glutamate transporters and receptors, such as the NMDAR.

High glutamate concentrations in culture media do not reflect steady state in vivo (Ye and Sontheimer 1998) and this may be rectified by the use of glutamate depleted media. Hippocampal astrocytes can absorb glutamate from media and reduce glutamate concentrations from 90 µM to less than 1 µM. This caused a 10-fold increase in neuronal survival. The death of neurons exposed to untreated media was blocked by the NMDAR antagonist MK-801 (Ye and Sontheimer 1998), suggesting that glutamate toxicity was facilitated by the NMDARs. The use of glutamate depleted media may be advantageous for culturing melanoma cells, which may eliminate the possible selection of glutamate resistant cells.

8.4. The role of calcium influx in neuronal and melanoma cells

Evidence that intracellular calcium in melanoma cells increases in response to NMDAR agonists was presented in Chapter Five. NMDAR activation causes the opening of the receptor ion channel pore and the influx of calcium. Calcium influx can give rise to a number of intracellular signalling pathways with diverse consequences such as apoptotic or necrotic cell death but also trophic effects (such as cell survival and proliferation) that are relevant in cancer (Johnson and Kotermanski 2006). Calcium can stimulate tumour cell growth and survival.

In neurons calcium controls axon extensions and influences migration (Gill and Pulido 2007). Both melanocytes and neurons differentiated from neural crest cells during embryonic
development (Hou, Panthier et al. 2000) and as such it may be worth investigating the role of calcium in melanocyte dendrite formation. In astrocytoma, glutamate increases cell migration via calcium oscillations. Calcium oscillations in turn induce glutamate secretion via focal adhesion kinase (FAK) phosphorylation and focal adhesion disassembly (Hamadi, Giannone et al. 2014), this pathway could also be further investigated in melanoma.

During neuronal excitotoxicity an influx of cations causes a collapse of mitochondrial function leading to necrosis. Prolonged activation of glutamate receptors results in a delayed increase of intracellular calcium to excessively high (toxic) concentrations. This calcium influx activates intracellular enzymes like phospholipases A2 (PLA2), xanthine oxidase and nitric oxide synthase, which in turn trigger a cascade of reactions that lead to cell death (Stepulak, Luksch et al. 2011). This is another pathway that may be worth further investigation in melanoma.

8.5. Evidence for the role of glutamate in melanocytes of the skin

There is little evidence for NMDAR functionality in melanocytes, as compared to the ample evidence available for AMPAR and mGluR functionality (Genever, Maxfield et al. 1999, Skerry and Genever 2001, Hinoi, Takarada et al. 2004, Nahm, Philpot et al. 2004, Hoogduijn, Hitchcock et al. 2006). Melanocytes are sensitive to extracellular glutamate concentrations, and glutamate signalling in melanocytes is involved in their differentiation, proliferation and morphology (Hoogduijn, Hitchcock et al. 2006). Melanocytes express glutamate transporters, but neither produce nor release glutamate. Stimulating melanocytes with 10 or 100 µM NMDA elevated intracellular calcium concentrations, implying that they express functional NMDARs (Hoogduijn, Hitchcock et al. 2006).

In the skin, cells other than melanocytes release glutamate that is recycled in the extracellular regions of the epidermis (Genever, Maxfield et al. 1999, Nahm, Philpot et al. 2004, Hoogduijn, Hitchcock et al. 2006). NMDAR expression (characterised by GluN2 expression) was reported in proliferating cells of the skin such as keratinocytes (Skerry and Genever 2001, Hoogduijn, Hitchcock et al. 2006) where glutamate was found to play a role in epidermal renewal (Genever, Maxfield et al. 1999). It is possible that glutamate affects proliferation of premature melanocytes as it does in neurones (Luk et al., 2003), but has no such effects on fully differentiated normal human melanocytes (Luk, Kennedy et al. 2003, Hoogduijn, Hitchcock et al. 2006).
8.6. Glutamate in tumour progression and wound healing

It is well established that the generation of tumour stroma is akin to a deregulated process of wound healing (Dvorak 1986, Schafer and Werner 2008). Wound sites and tumours have a number of similarities that include processes of tissue building and regeneration (Dvorak 1986). This includes the involvement of tissue infiltrating immune cells that facilitate processes such as proliferation, apoptosis, survival and angiogenesis (Dvorak 1986, Schafer and Werner 2008). Glutamate is produced by monocytes, neutrophils, lymphocytes and macrophages (Curi, De Melo et al. 1997, Pithon-Curi, De Melo et al. 2004, Yawata, Takeuchi et al. 2008), all immune cells that are present in abundance at sites of wound healing (Schafer and Werner 2008). In the CNS, nerve injury results in an upregulation of glutamate receptors following a prolonged activation of the NMDARs in addition to the continuous increases in the levels of glutamate (Suzuki, Matthews et al. 2001). The majority of the high glutamate concentrations in tumours can be attributed to glutamine hydrolysis in tumour cells themselves, through induced mitochondrial glutaminase activity (Magistretti and Allaman 2013). Glutamine transporters are often overexpressed in tumour cells (Ye, Rothstein et al. 1999, Hinoi, Takarada et al. 2004), but glutamine hydrolysis by tumour infiltrating immune cells could also contribute (Pithon-Curi, De Melo et al. 2004).

Wounds, like tumours, are hypoxic. Blood vessels around wounds are damaged and again like tumours, the process of repair leads to poorly vascularised scar tissue (Dvorak 1986). Glutamate signalling mediated through NMDARs may play a role in this type of hypoxia-induced wound repair. Glutamate is released from intracellular stores under neurological ischaemic conditions (Drejer, Benveniste et al. 1985).

NMDAR channels are an important component in the pathogenesis of hypoxic-ischaemic brain injury (Wong, Kemp et al. 1986) and cerebral ischaemia is associated with a massive increase in extracellular glutamate (Ozyurt, Graham et al. 1988). NMDAR blockade was suggested as a therapeutic strategy for hypoxia-associated disorders (McDonald, Silverstein et al. 1987). MK-801 for example is thought to protect the brain from hypoxic-ischaemic injury (Wong, Kemp et al. 1986). Studies on a rat brain model found that MK-801 blocks the necrosis produced by direct injection of NMDA (McDonald, Silverstein et al. 1987). The hypoxic microenvironments of tumours as a consequence of leaky blood vessels may be similar to the microenvironment of injured brain tissue. NMDARs may function similarly in tumours as they do in injured neuronal tissue.
Signalling as a result of NMDAR activation in tumours has been shown to lead to the activation of CREB transcription factor that induces expression of genes that promote invasive and proliferative phenotypes (Li and Hanahan 2013). CREB phosphorylation and subsequent gene expression has also been suggested to play an important role in the acquisition of ischemic tolerance (Mabuchi, Kitagawa et al. 2001).

Glutamate is present in normal skin and may be released at high levels by keratinocytes and other cells during wounding and inflammation (Fischer, Glanz et al. 2009). The expression of NMDARs was altered in keratinocytes involved in re-epithelialisation during wound healing (Nahm, Philpot et al. 2004). In addition, calcium entry through NMDARs might influence the cycle of keratinocyte proliferation, differentiation and migration during epithelialisation (Nahm, Philpot et al. 2004). As previously mentioned, melanocytes do not release glutamate but they express active NMDARs that regulate melanocyte survival and morphology (Genever, Maxfield et al. 1999, Hinoi, Takarada et al. 2004, Hoogduijn, Hitchcock et al. 2006). The deregulation of the glutamate signalling process in the skin may result in melanoma occurrence.

8.7. Proposed model for the role of NMDARs in melanoma progression

Based on the results and previous discussions of this thesis, we propose a model to describe the role of NMDARs in melanoma cell progression (Figure 8.4). The model considers that the glutamate levels in the extracellular environment can influence NMDAR activity through the impacts on subunits expression, post-translational modification, subunit composition, assembly and trafficking of subunits to the cell membrane, affinity for glutamate and the generation of a mesenchymal phenotype.

We hypothesise that under lower glutamate conditions akin to that of the CNS, NMDAR expression and activation is tightly regulated and promotes cell survival, invasion, migration and proliferation. Therefore, during early tumour development (when glutamate levels are lower), \textit{GRIN2A} mutations may have oncogenic effects that support cell survival, proliferation and invasion. In support, we observed that NMDAR and glutamate inhibitors, attenuated proliferation of melanoma cells \textit{in vitro} and cells carrying \textit{GRIN2A} mutations showed signs of a mesenchymal phenotype (Figure 8.4a).
As tumours progress and become more hypoxic, the role of NMDARs may change, specifically when glutamate levels rise. Surrounded by cytotoxic levels of glutamate, cells escape cell death by down-regulating NMDAR expression or accumulating mutations that impair receptor activation. In the presence of high glutamate levels, NMDARs would thus function as tumour suppressors and the presence of \textit{GRIN2A} mutations would enhance this effect (Figure 8.4a and b).

Previous work has shown that melanoma cells display glutamine dependence (Zacharias, Lima et al. 2003, Erickson and Cerione 2010, Wang, Erickson et al. 2010, Hernandez-Davies, Tran et al. 2015) and this is also seen in cells resistant to BRaf inhibitor, vemurafinib (Hernandez-Davies, Tran et al. 2015). Glutaminase converts glutamine to glutamate but impairing this activity inhibits oncogenic transformation (Zacharias, Lima et al. 2003, Erickson and Cerione 2010, Wang, Erickson et al. 2010, Hernandez-Davies, Tran et al. 2015). Furthermore, in comparison with the surrounding skin, melanoma were found to have higher glutaminase activity (Hernandez-Davies, Tran et al. 2015). Glutamate can be released by tumour cells themselves (Ye and Sontheimer 1999, Hinoi, Takarada et al. 2004, Li and Hanahan 2013) or other cells including tumour infiltrating immune cells (Curi, De Melo et al. 1997, Pithon-Curi, De Melo et al. 2004).

There is a lack of convincing evidence for the pathophysiological significance of elevated glutamate in the extracellular space surrounding tumour but intriguingly it has been suggested that in astrocytomas, glutamate may promote active cell death, thereby creating space for the tumour to expand (Ye, Rothstein et al. 1999, Ye and Sontheimer 1999, Hamadi, Giannone et al. 2014); this idea may be applicable to melanoma. This integrates well with another proposed model wherein cells on the periphery of the tumour are thought to express NMDARs through which they mediate invasion (Li and Hanahan 2013). Our model is in agreement with these two studies. We also advance these ideas by suggesting that progressive or aggressive melanomas release glutamate not only to incur a survival advantage but they also adapt and escape glutamate mediated cytotoxicity by down regulating NMDAR expression.
**A**

- **GRIN2A expression**
- **Glutaminase activity**
- **GRIN2A mutations**

**NMDAR activity**

- **Survival and proliferation**
  - MAPK
  - ERK

- **Cytotoxicity**
  - Calcium flux

- **Invasion, migration**
  - CREB

- **Glutamate concentration**

**CNS**  
**Peripheral tissue**

- **Tissue injury**
- **Tumour**
- **In vitro**

---

**B**  

**Glutamate level**

- **Low (normal)**
  - keratinocytes
  - Effects melanocyte:
    - differentiation
    - proliferation
    - morphology

- **Moderate**
  - melanoma cells
  - immune cells
  - ↑ GRIN2A mutation
  - supports proliferation and EMT

- **High**
  - melanoma cells
  - immune cells
  - Interstitial fluid
  - ↓ NMDAR expression
  - escape cytotoxicity

- **High**
  - melanoma cells
  - ↓ NMDAR expression
  - escape cytotoxicity
Figure 8.4: Model proposing the role of NMDARs and the relevance of \textit{GRIN2A} mutations in melanoma progression

(A) NMDAR activity on melanoma cells depends on glutamate concentrations in tissue. Under lower glutamate concentrations, \textit{GRIN2A} mutations are oncogenic, supporting cell survival, proliferation and migration. Under higher glutamate concentrations, further \textit{GRIN2A} mutations may help cells escape glutamate-mediated excitotoxicity. In addition, \textit{GRIN2A} expression is down regulated and glutaminase activity upregulated. (B) Panels highlight the role of tissue environment in melanoma progression. In normal skin, melanocytes do not secrete glutamate but glutamate is released by surrounding keratinocytes. In contrast, melanoma cells secrete glutamate into surrounding tissue to drive their own growth in an autocrine or paracrine manner. Molecular modifications (e.g. \textit{GRIN2A} mutations and decreased expression) help melanoma cells sustain high glutamate levels. Cell lines cultured \textit{in vitro} are subjected to even higher glutamate concentrations, which induces further adaptation for cell survival and proliferation.
8.8. Limitations and future directions

As discussed above, the expression and function of NMDAR subunits in melanoma cell lines may be influenced by changes in extracellular glutamate levels and this in turn may dictate cell phenotype. For example, experiments conducted in Chapter Six to determine cell invasiveness revealed that only one cell line (NZM 40) was invasive even though all cell lines were derived from metastatic melanoma. In Chapter Seven, the associations between glutamate release and Slug expression were seen only when melanoma cells were grown in RPMI 1640 and not α-MEM. Furthermore, the invasiveness of NZM 40 cells was impaired when cells were cultured in RPMI 1640 and exposed to competitive modulators like NMDA and AP5. It may be that a particular concentration range of glutamate is needed to facilitate invasion and that invasiveness is impaired when this window gets shifted by the addition of an inhibitor or agonist. The relevance of glutamate and glutamate receptors such as NMDAR might be better evaluated under conditions where glutamate concentrations can be controlled or more reflective of physiological conditions. In the future, functional studies such as calcium flux analysis on melanoma cell lines cultured in RPMI 1640 instead of α-MEM may provide better insight into the NMDAR system in melanoma. Alternatively, the toxicity of this medium could be prevented by the NMDAR inhibitors MK-801 or AP5 (Ye and Sontheimer 1999).

NMDAR expression studies were difficult to achieve during this project as technical difficulties with immunoblotting were encountered that impaired protein quantification. In the future the method might be better optimised. Real-time PCR quantifying NMDAR subunit transcripts and mass spectrometry to detect proteins expressed at a lower level might be useful to validate the expression of subunits.

In order to further investigate the effects of NMDAR activity on proliferation, invasion and survival, NMDAR genes could be knocked-down by shRNA or the CRISPR-Cas9 system. Monitoring expression of the receptor under a glutamate concentration gradient would be useful in determining the effects on NMDAR gene expression. Determining the expression and cellular localisation of NMDAR subunits on whole tumour sections using histological techniques would also be useful in understanding the role of this receptor in tumour progression.

Animal models would be more relevant to study the role of glutamate in cancerous tissue so that tumour hypoxic or anoxic conditions can be reproduced and the effects of modulators can
be better represented. Establishing mouse xenograft models of melanoma cell lines, sectioning tumours that arise as a result of xenografts for further analysis, or treating mice with NMDAR modulators may assist in understanding the mechanism of the receptor in microenvironments better representing physiological conditions.

Electrophysiological analyses of cells following treatment with NMDAR modulators might also be considered, as in many cancer cell types a depolarised membrane favours cell proliferation. Ion channels are thought to control cell volume and migration, and emerging data suggest that the level of depolarisation has functional roles in cancer cell migration while hyperpolarisation is necessary for stem cell differentiation (Yang and Brackenbury 2013). Furthermore, invadopodia are essential for the crossing of the basal membrane by melanoma cells. Checking for the expression of ARF6, which is present in invadopodia would be a useful way in which to determine invasive phenotypes (Bonaventure, Domingues et al. 2013).

Human mGluR1 expressing melanoma cells release high amounts of extracellular glutamate (Yip, Le et al. 2009), and riluzole was shown to impair their migration, invasion and proliferation (Le, Chan et al. 2010). NZM cells used in our experiments showed greater sensitivity to riluzole compared with NMDAR inhibitors, memantine and MK-801 (Chapter Six). It may be useful to check the expression of mGluR1 in our panel of melanoma cell lines to determine whether they implement the use of metabotropic receptors in glutamate signalling system. Lastly, sequencing other GRIN and related genes could reveal a wider spectrum of mutations which will be useful in understanding the relevance of this system in melanoma development.

8.9. Final conclusions

This thesis provides evidence for the expression of functional NMDARs in melanoma, indicating that glutamate is an important component of melanoma biology, and that at least some effects of glutamate in melanoma cells are mediated by NMDARs. We conclude that glutamate levels in the tumour microenvironment contribute to melanoma progression by facilitating cell proliferation and encouraging a mesenchymal phenotype. These cellular effects are contributed by glutamate and NMDARs, and GRIN2A mutations may enhance these effects. We have proposed that GRIN2A can act as an oncogene or a tumour suppressor, depending on the context dictated by glutamate levels in the tumour. Lastly, our study of 20 melanoma patients revealed an association between GRIN2A mutations and poor survival.
This important clinical association justifies further investigations into the role of NMDAR in melanoma biology.
Supplementary figures

Supplementary Figure 9.1: Initial screen of GRIN2A transcripts in 15 melanoma cell lines

Supplementary Figure 9.2: Initial screen of GRIN2D transcripts in 16 melanoma cell lines
Supplementary Figure 9.3: Initial screen of \textit{GRIN1} transcripts in 16 melanoma cell lines using the GRIN1-1 primer set.

Supplementary Figure 9.4: Initial screen of \textit{GRIN1} transcripts in 16 melanoma cell lines using the GRIN1-2 primer set.
Supplementary Figure 9.5: Initial screen of GRIN1 transcripts in 16 melanoma cell lines using the GRIN1-3 primer set

Supplementary Figure 9.6: Initial screen of GRIN1 transcripts in 16 melanoma cell lines using the GRIN1-4 primer set
Supplementary Figure 9.7: Initial screen of $GRIN3A$ transcripts in 16 melanoma cell lines.

* Ladder may be running a little slower because of excess GelRed
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