THE MODULATING EFFECT OF *MYO*-INOSITOL AND PROTOTYPICAL ANTIDEPRESSANTS ON MARKERS OF CELLULAR RESILIENCE IN CULTURED HUMAN NEUROBLASTOMA CELLS

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"Life is no brief candle for me. It is a sort of splendid torch which I have got hold of for the moment, and I want to make it burn as brightly as possible before handing it on to the future generation"
~ George Bernard Shaw ~

Abstract

THE MODULATING EFFECT OF *MYO*-INOSITOL AND PROTOTYPICAL ANTIDEPRESSANTS ON MARKERS OF CELLULAR RESILIENCE IN CULTURED HUMAN NEUROBLASTOMA CELLS

Although several antidepressants are available for the treatment of depression, certain limitations are common, including drug resistance and significant side effects. Recently, several studies indicated the possibility of neurodegeneration in the pathophysiology of depression. Oral *myo*-inositol (*m*Ins) has been found to be effective in the treatment of depression. It is thought that understanding the mechanism of action of *m*Ins may contribute to the understanding of the pathophysiological basis of depression.

The aim of the current study was to determine the possible neuroprotective effects of *m*Ins, in comparison with other prototype or experimental antidepressants, against glutamate-induced excitotoxicity in human neuroblastoma cells. For this purpose, the cells were pretreated for 24 hours with different concentrations of either *m*Ins, one of a series of antidepressants, or a combination of *m*Ins and an antidepressant, all with or without 15 mM glutamate. The MTT cell proliferation assay was employed to determine cell viability, while the comet assay was used to determine DNA fragmentation.

After pretreatment with the different regimes it was found that most drugs had no significant effect on cell viability, while 10 mM *m*Ins decreased cell viability. Interestingly, fluoxetine (10 µM) and lithium (2, 5 or 10 mM) protect against the *m*Ins-induced neurodegeneration. Imipramine alone (10 µM), imipramine in combination with *m*Ins and 2 mM or 10 mM lithium in combination with *m*Ins caused an increase in glutamate sensitivity, while 0.23 µM gabapentin protected against glutamate-induced excitotoxicity. Data from the comet assay largely supports the data from the MTT cell proliferation assay.

In conclusion, *m*Ins is not neuroprotective, but rather neurodegenerative in this *in vitro* model and results also do not suggest an additive neuroprotective effect of the combination of *m*Ins with other antidepressants. The study provides little support for the neuroprotective hypothesis of antidepressant action.

Uittreksel

DIE MODULERENDE EFFEK VAN *MIO*-INOSITOL EN PROTOTIPE ANTIDEPRESSANTE OP MERKERS VAN SELLULÊRE LEWENSVATBAARHEID IN GEKWEEKTE MENSLIKE NEUROBLASTOOMSELLE

Alhoewel verskeie antidepressante vir die behandeling van depressie beskikbaar is, is sekere beperkings algemeen, insluitend geneesmiddelweerstandbiedendheid en beduidende newe-effekte. Onlangse studies het gedui op die moontlikheid dat *neurodegenerasie* 'n rol in die patofisiologie van depressie speel. Studies het gevind dat die orale toediening van *mio*-inositol (*m*Ins) effektief is vir die behandeling van depressie. Indien die werkingsmeganisme van *m*Ins verstaan word, kan dit daartoe bydra dat die patofisiologiese grondslag van depressie beter verstaan word.

Die doel van die huidige studie was om die moontlike neurobeskermende effekte van *m*Ins, in vergelyking met ander prototipe of eksperimentele antidepressante, teen glutamaat-geïnduseerde toksisiteit in menslike neuroblastoomselle te bepaal. Vir hierdie doel is die selle vir 24 uur voorafbehandel met verskillende konsentrasies van óf *m*Ins, óf een van 'n reeks antidepressante, óf 'n kombinasie van *m*Ins en 'n antidepressant, almal met of sonder 15 mM glutamaat. Die MTT-selproliferasietoets is gebruik om lewensvatbaarheid van die selle te bepaal, terwyl die DNA-komeetanalise gebruik is om DNA-fragmentering te bepaal.

Ná voorafbehandeling met die verskillende behandelingsregimes is gevind dat die meeste geneesmiddels geen statisties betekenisvolle effek op lewensvatbaarheid het nie, terwyl 10 mM *m*Ins lewensvatbaarheid verlaag. Fluoksetien (10 µM) en litium (2, 5 of 10 mM) beskerm teen die *m*Ins-geïnduseerde neurodegenerasie. Imipramien alleen (10 µM), imipramien in kombinasie met *m*Ins en 2 mM of 10 mM litium in kombinasie met *m*Ins veroorsaak 'n toename in glutamaatsensitiwiteit, terwyl 0.23 µM gabapentien die selle teen glutamaat-geïnduseerde toksisiteit beskerm. Data van die komeetanalise ondersteun grotendeels die data van die MTT-toets.

Ten slotte is *m*Ins in hierdie *in vitro*-model nie neurobeskermend nie, maar eerder neurodegeneratief en die resultate dui ook nie op 'n bykomende neurobeskermende effek van die kombinasie van *m*Ins met ander antidepressante nie. Hierdie studie verskaf weinig ondersteuning vir die neurobeskermingshipotese van die werkingsmeganisme van antidepressante.

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Table of Contents

Abstract i
Uittreksel ii
Acknowledgements iii
Table of Contents iv
List of Figures vii
List of Tables xii
Chapter 1: Introduction 1
1.1 Problem Statement 1
1.2 Research Objectives 2
1.3 Project Layout 3
Chapter 2: Literature Overview 4
2.1 *myo*-Inositol: Physiological Role and Therapeutic Use 4
2.1.1 The Phosphatidylinositol Cycle 5
2.1.2 *m*Ins and Psychiatric Disorders 8
2.1.2.1 Pathophysiology 8
2.1.2.2 Clinical Evidence for Therapeutic Efficacy of *m*Ins 9
2.1.2.3 Data from Animal Studies 12
2.1.2.4 Mechanistic *In Vitro* Investigations 13
2.2 Depression: Criteria & Pharmacotherapy 14
2.2.1 Symptoms of Depression 15
2.2.2 Pharmacotherapeutics 16
2.2.2.1 Monoamine Oxidase Inhibitors 16
2.2.2.2 Tricyclic Antidepressants 17
2.2.2.3 Selective Serotonin Reuptake Inhibitors 18
2.2.2.4 Lithium 19
2.2.2.5 Atypical Antidepressants 21
2.2.2.5.1 Memantine 21
2.2.2.5.2 Tianeptine 22
2.2.2.5.3 *myo*-Inositol 23
2.3 Depression and Neuroplasticity 23
2.3.1 Brain Regions Implicated 24
2.3.1.1 Neuroplasticity in the Hippocampus 24
2.3.1.2 Neuroplasticity in Other Brain Regions 28
2.3.1.2.1 Prefrontal Cortex 28
2.3.1.2.2 Amygdala 29

2.3.2 Glutamate ... 29
2.3.2.1 Glutamate as a Neurotransmitter ... 29
2.3.2.2 Involvement of Glutamate in Depression ... 31
2.3.2.2.1 Clinical Data ... 31
2.3.2.2.2 Data from Animal Studies ... 32
2.3.2.2.3 *In Vitro* Data ... 33
2.3.3 Stress and Cortisol ... 35
2.3.4 The cAMP/CREB/BDNF Signalling Pathway ... 37
2.3.4.1 CREB ... 39
2.3.4.2 BDNF ... 39
2.4 Synopsis ... 41
Chapter 3: Experimental Procedures ... 42
3.1 Introduction ... 42
3.2 Experimental Layout ... 43
3.3 Cell Lines Employed ... 44
3.4 Materials ... 45
3.4.1 Chemicals ... 45
3.4.1.1 Chemicals Used for Cell Cultures ... 45
3.4.1.2 Chemicals Used for Assays ... 45
3.4.2 Consumables ... 46
3.4.3 Instruments and Software ... 46
3.5 Experiments ... 47
3.5.1 Seeding of Cells in 24-well Plates ... 47
3.5.2 Drug Pretreatments ... 48
3.5.2.1 Introduction ... 48
3.5.2.2 Pretreatment Layout ... 48
3.5.2.3 Concentrations Used in Pretreatment ... 49
3.5.3 Assays ... 51
3.5.3.1 Cell Counting and Seeding Assay ... 51
3.5.3.2 MTT Cell Proliferation Assay ... 51
3.5.3.2.1 Introduction ... 51
3.5.3.2.2 Assay ... 52
3.5.3.3 Single Cell Gel Assay (Comet Assay) ... 53
3.5.3.3.1 Introduction ... 53
3.5.3.3.2 Assay ... 54
3.6 Statistical Data Analysis ... 56
Chapter 4: Results and Discussion ... 57
4.1 Control Experiment ... 57

4.1.1 Development of a Model for Glutamate-induced Excitotoxicity ... 57
4.2 Study Objective Experiments ... 58
4.2.1 Neuroprotective Properties of Drugs ... 59
4.2.1.1 *myo*-Inositol ... 59
4.2.1.2 Imipramine ... 63
4.2.1.3 Fluoxetine ... 67
4.2.1.4 Lithium ... 70
4.2.1.5 Memantine ... 74
4.2.1.6 Tianeptine ... 78
4.2.1.7 Gabapentin ... 81
4.2.2 Drugs Combined with *myo*-Inositol ... 83
4.2.2.1 Imipramine ... 83
4.2.2.2 Fluoxetine ... 86
4.2.2.3 Lithium ... 88
4.3 Summary ... 90
Chapter 5: Summary and Conclusions ... 92
5.1 Summary ... 92
5.2 Conclusions ... 93
5.3 Recommendations ... 95
References ... 96
Appendix A: Abbreviations ... 124

List of Figures

Figure 2-1: The chemical structure of *m*Ins ... 5
Figure 2-2: Representation of the PI cycle. ... 6
Figure 2-3: The chemical structure of imipramine, a prototypical tricyclic antidepressant. ... 17
Figure 2-4: The chemical structure of tianeptine. ... 22
Figure 2-5: Representation of the effects of stress and antidepressant treatment on hippocampal neurogenesis ... 37
Figure 2-6: Representation of the signalling cascades involved in neural plasticity ... 38
Figure 3-1: A schematic representation of the experimental design ... 43
Figure 4-1: The modulating effect of 24-hour pretreatment with different concentrations of glutamate on mitochondrial activity as measured by the MTT cell proliferation assay, expressed as percentage of the control (i.e. 0 M glutamate). ... 58
Figure 4-2: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of *m*Ins (without glutamate) on (A) mitochondrial activity, as measured by the MTT cell proliferation assay, and (B) DNA integrity, as measured by the DNA comet assay. (C) Representative photographs of the DNA integrity after 24 hour pretreatment with the indicated concentration of *m*Ins and corresponding to the bars in (B). ... 59
Figure 4-3: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of *m*Ins on glutamate sensitivity. (A) The difference in mitochondrial activity before and after 15 mM glutamate at the indicated co-treatment concentrations of *m*Ins, as measured by the MTT cell proliferation assay. (B) The difference in DNA integrity before and after 15 mM glutamate at the indicated co-treatment concentrations of *m*Ins, as measured by the DNA comet assay ... 61
Figure 4-4: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of *m*Ins, as measured by the MTT cell proliferation assay. ... 62
Figure 4-5: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of imipramine (without glutamate) on (A) mitochondrial activity, as measured by the MTT cell proliferation assay, and (B) DNA integrity, as measured by the DNA comet assay. (C) Representative

photographs of the DNA integrity after 24 hour pretreatment with the indicated concentration of *m*Ins and corresponding to the bars in (B). 63

Figure 4-6: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of imipramine on glutamate sensitivity. (A) The difference in mitochondrial activity before and after 15 mM glutamate at the indicated co-treatment concentrations of imipramine, as measured by the MTT cell proliferation assay. (B) The difference in DNA integrity before and after 15 mM glutamate at the indicated co-treatment concentrations of imipramine, as measured by the DNA comet assay 65

Figure 4-7: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of imipramine (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of imipramine, as measured by the MTT cell proliferation assay. 66

Figure 4-8: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of fluoxetine (without glutamate) on (A) mitochondrial activity as measured by the MTT cell proliferation assay and (B) DNA integrity, as measured by the DNA comet assay. (C) Representative photographs of the DNA integrity after 24 hour pretreatment with the indicated concentration of *m*Ins and corresponding to the bars in (B). 67

Figure 4-9: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of fluoxetine on glutamate sensitivity. (A) The difference in mitochondrial activity before and after 15 mM glutamate at the indicated co-treatment concentrations of fluoxetine, as measured by the MTT cell proliferation assay. (B) The difference in DNA integrity before and after 15 mM glutamate at the indicated co-treatment concentrations of fluoxetine, as measured by the DNA comet assay 69

Figure 4-10: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of fluoxetine (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of fluoxetine, as measured by the MTT cell proliferation assay. 70

Figure 4-11: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of lithium (without glutamate) on (A) mitochondrial activity as measured by the MTT cell proliferation assay and (B) DNA integrity, as measured by the DNA comet assay. (C) Representative photographs of the DNA integrity after 24 hour pretreatment with the indicated concentration of *m*Ins and corresponding to the bars in (B). 71

Figure 4-12: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of lithium on glutamate sensitivity. (A) The difference in mitochondrial activity before and after 15 mM glutamate at the indicated co-treatment concentrations of lithium, as measured by the MTT cell proliferation assay. (B) The difference in DNA integrity before and after 15 mM glutamate at the indicated co-treatment concentrations of lithium, as measured by the DNA comet assay 72

Figure 4-13: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of lithium (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of lithium, as measured by the MTT cell proliferation assay 73

Figure 4-14: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of memantine (without glutamate) on (A) mitochondrial activity as measured by the MTT cell proliferation assay and (B) DNA integrity, as measured by the DNA comet assay. (C) Representative photographs of the DNA integrity after 24-hour pretreatment with the indicated concentration of memantine and corresponding to the bars in (B) 75

Figure 4-15: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of memantine on glutamate sensitivity. (A) The difference in mitochondrial activity before and after 15 mM glutamate at the indicated co-treatment concentrations of memantine, as measured by the MTT cell proliferation assay. (B) The difference in DNA integrity before and after 15 mM glutamate at the indicated co-treatment concentrations of memantine, as measured by the DNA comet assay 76

Figure 4-16: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of memantine (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of memantine, as measured by the MTT cell proliferation assay 77

Figure 4-17: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of tianeptine (without glutamate) on (A) mitochondrial activity as measured by the MTT cell proliferation assay and B) DNA integrity, as measured by the DNA comet assay. (C) Representative photographs of the DNA integrity after 24-hour pretreatment with the indicated concentration tianeptine and corresponding to the bars in (B) 79

Figure 4-18: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of tianeptine on glutamate sensitivity. (A) The

difference in mitochondrial activity *before* and after 15 mM glutamate at the indicated co-treatment concentrations of tianeptine, as measured by the MTT cell proliferation assay. (B) The difference in DNA integrity before and after 15 mM glutamate at the indicated co-treatment concentrations of tianeptine, as measured by the DNA comet assay 80

Figure 4-19: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of tianeptine (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of tianeptine, as measured by the MTT cell proliferation assay 81

Figure 4-20: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with *different concentrations* of gabapentin (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of gabapentin, as measured by the MTT cell proliferation assay. 82

Figure 4-21: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of gabapentin (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of gabapentin, as measured by the MTT cell proliferation assay 83

Figure 4-22: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of imipramine in combination with 10 mM *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of imipramine in combination with 10 mM *m*Ins, as measured by the MTT cell proliferation assay 84

Figure 4-23: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of imipramine in combination with 10 mM *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of imipramine in combination with 10 mM *m*Ins, as measured by the MTT cell proliferation assay 85

Figure 4-24: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of fluoxetine in combination with 10 mM *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of fluoxetine in combination with 10 mM *m*Ins, as measured by the MTT cell proliferation assay 86

Figure 4-25: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of fluoxetine in combination with 10 mM *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of fluoxetine in combination with 10 mM *m*Ins, as measured by the MTT cell proliferation assay 87

Figure 4-26: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of lithium in combination with 10 mM *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of lithium in combination with 10 mM *m*Ins, as measured by the MTT cell proliferation assay 88

Figure 4-27: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of lithium in combination with 10 mM *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of lithium in combination with 10 mM *m*Ins, as measured by the MTT cell proliferation assay 89

List of Tables

Table 3-1: Drugs and the concentrations used in pretreatment 49
Table 4-1: Summary of the results obtained after 24 hour pretreatment of human neuroblastoma cells with the indicated drugs 90

Chapter 1: Introduction

1.1 Problem Statement

The World Health Organization has estimated that depression will be the second most important cause of disability by 2020, with ischaemic heart disease being the most important. Presently, depression is estimated to be the fourth most disabling of all medical disorders (Peveler, *et al.*, 2002). It is often referred to as the "common cold" of mental illness, since so many people are affected by depression (Price, 2004). Depression is a potentially fatal disorder (Disalver *et al.*, 1994; Strakowsky *et al.*, 1996) and also poses a burden on the economy of any country (Price, 2004). Furthermore, depression is often underdiagnosed and undertreated (Baldessarini, 2001). Major challenges in combating the burden of depression include the need for a better understanding of the pathophysiological basis of depression, as well as the need for new drugs with reduced bothersome side effect profiles, faster onset of action after initiation of therapy and therapeutic efficacy in cases that are currently resistant to treatment.

Of the hypotheses related to the biological basis of depression, the monoaminergic hypotheses are the best studied and described, based primarily on the modulating effects of classical antidepressants, such as the monoamine oxidase inhibitors, tricyclic antidepressants and the selective serotonin reuptake inhibitors, on the monoaminergic systems. Recently, a novel hypothesis regarding the pathophysiology of depression has emerged, which involves the plasticity of neural networks. According to this hypothesis, depression results from the inability of the brain to make the appropriate adaptive responses to environmental stimuli, due to alteration in neuroplasticity. It is believed that antidepressant drugs act by normalising this impairment (Duman *et al.*, 1999; Manji & Duman, 2001; Manji *et al.*, 2000, 2001). However, the link between alterations in neuroplasticity and depressive symptoms remains to be established (Fossati *et al.*, 2004). Data suggest that modulation of the glutamatergic system plays a pivotal role in the regulation of synaptic plasticity and that antidepressants may act, in part, by normalising the alterations in glutamate function (McEwen & Chattarji, 2004).

myo-Inositol (*m*Ins) is a simple isomer of glucose and is an essential constituent in many human cells (Holub, 1986). *m*Ins is a key metabolic precursor in the phosphatidylinositol (PI) cycle (Berridge & Irvine, 1989; Berridge *et al.*, 1989), being an important component of G protein-coupled receptor (GPCR) signalling systems, regulated by several neurotransmitters (Baraban *et al.*, 1989). Several subtypes of adrenergic, serotonergic, cholinergic and metabotropic glutamatergic receptors in the brain are coupled to the hydrolysis of phosphoinositides (PIs).

*m*Ins is vital to the resynthesis of PIs and, consequently, the maintenance and effectiveness of signalling (Fisher *et al.*, 2002). Activation of phospholipase Cβ (PLCβ) facilitates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol trisphosphate (IP_3) and diacylglycerol (DAG) as second messengers (Baraban *et al.*, 1989). IP_3 mediates the release of intracellular calcium (Ca^{2+}) via interaction with three IP_3 receptor subtypes, while DAG activates proteinkinase C (PKC; Harvey, 1997) to eventually affect cAMP-dependent nuclear pathways.

In recent years, it has been demonstrated that high-dose oral supplementation of *m*Ins (several-fold higher than normal dietary intake) may be effective in the treatment of several psychiatric disorders (Levine, 1997). Several clinical studies have demonstrated that *m*Ins may have therapeutic value in the treatment of depression (Levine, 1997; Levine *et al.*, 1993a, 1995a), depression associated with post-traumatic stress disorder (Kaplan *et al.*, 1996), panic disorder (Benjamin *et al.*, 1995) and obsessive-compulsive disorder (OCD) (Levine *et al.*, 1993b, 1994). However, it proved to be ineffective in schizophrenia (Levine *et al.*, 1993b, 1994), Alzheimer's disease (Barak *et al.*, 1996) and autism (Levine *et al.*, 1997). It seems as though *m*Ins may worsen attention deficit hyperactivity disorder (Levine *et al.*, 1995b). Although clinical evidence exists for the efficacy of oral *m*Ins in the treatment of several psychiatric disorders, the subcellular mechanism of action remains elusive. Being involved in the cellular signalling mechanisms of GPCRs associated with antidepressant action, a better understanding of the mechanism of action of *m*Ins holds great promise for understanding the pathophysiology of depression and other *m*Ins-responsive disorders (Harvey *et al.*, 2002a).

1.2 Research Objectives

The primary objectives of this study were to investigate whether *myo*-inositol (*m*Ins), in comparison with various prototype experimental and clinical antidepressants, display any protective effects against glutamate-induced excitotoxicity in an *in vitro* human neuroblastoma cell line, as well as in a non-neuronal mammalian cell line.

In order to achieve these primary objectives, the following specific outcomes were set:

- Establishing appropriate conditions for exposure to glutamate to induce neurodegeneration.
- Determining the effect of the pretreatment with the experimental drugs on the mitochondrial activity of cells.
- Determining the effect of the pretreatment with the experimental drugs on the DNA integrity of cells.

- Determining the effect of the pretreatment with the experimental drugs on glutamate-induced excitotoxicity, as defined by measuring:
  - ◊ mitochondrial activity
  - ◊ DNA integrity.

The experimental drugs used in the current study include *m*Ins, imipramine, fluoxetine, lithium, memantine, tianeptine and gabapentin – all prototypes of different classes of antidepressants. It was also investigated whether *m*Ins augments any putative neuroprotective properties of prototype antidepressants. Finally, it was determined whether the effects observed were specific to neuronal cells.

1.3 Project Layout

All experiments were performed in the Laboratory for Applied Molecular Biology at the North-West University (Potchefstroom Campus), Potchefstroom, South Africa. In order to address the abovementioned objectives a neuronal, human neuroblastoma cell line [SK-N-BE(2)], as well as a non-neuronal Chinese Hamster Ovary cell line (CHO-K1) were selected. The cells were pretreated for 24 hours with different concentrations of either *m*Ins, one of a series of prototype antidepressants, or a combination of *m*Ins and a prototype antidepressant, all with or without 15 mM glutamate added simultaneously (inducing neurodegeneration). Thereafter neuroplasticity was defined by determining the remaining mitochondrial activity and DNA integrity. Mitochondrial activity was determined utilising the MTT cell proliferation assay, while DNA integrity was evaluated by means of electrophoresis and a visual DNA fragmentation quantification (comet) assay.

Chapter 2: Literature Overview

Major depression is a common and debilitating psychiatric mood disorder, while the neuropathology is not yet fully understood. Drug treatments are considered effective, yet insufficient in several regards, where bothersome (or even sometimes intolerable) side effects, drug resistance and delayed onset of action represent the major challenges for ongoing research in drug treatment. Since oral *myo*-inositol (*m*Ins; a precursor in receptor signalling pathways associated with depression and its drug treatments) has been proven to be clinically effective in the treatment of depression when dosed, understanding the mechanism of action holds great promise in understanding the pathophysiological basis of depression and other *m*Ins-responsive disorders (Harvey *et al.*, 2002a).

This chapter will review the physiological role of *m*Ins and its therapeutic application in depression and other anxiety-related disorders. It will also briefly review current understanding of depression, as well as current antidepressants employed in its treatment. Lastly and important for the current study, this chapter will also extensively review current hypotheses and associated supportive data on the proposed role of neuroplasticity in depression.

2.1 *myo*-Inositol: Physiological Role and Therapeutic Use

The inositols are ubiquitous, cyclic carbohydrates with a basic 6-carbon ring structure. Inositol exists as nine isomers of which *m*Ins (Figure 2-1) is the most abundant, biologically active isomer in the central nervous system and other tissue of mammals (Ross, 1991; Frey *et al.*, 1998). *m*Ins contains a distinctive single axial hydroxyl group on the number 2 carbon atom (Vandal, 1997) and is a simple isomer of glucose (Holub, 1986). As essential constituent in many human cells, it is obtained either from the diet (Holub, 1986) or via *de novo* synthesis (Hauser & Finelli, 1963; Clements & Diethelm, 1979). The average dietary intake of *m*Ins is only about one gram per day and is considered a minor replenishing pathway (Petroff *et al.*, 1989; Colodny & Hoffman, 1998), with *de novo* synthesis from D-glucose-6-phosphate as the major source of *m*Ins. Only about 3% of plasma *m*Ins crosses the blood-brain barrier (Spector & Lorenzo, 1975; Spector, 1998). The concentration of *m*Ins is higher in brain tissue than in plasma or cerebrospinal fluid and concentrations of *m*Ins in human brain have been calculated to range between 2 and 15 mM and possibly even higher in certain neuronal cells (Fisher *et al.*, 2002). Neuronal *m*Ins concentrations are regulated by various physiological mechanisms,

including the Na^+/*m*Ins transporter (Berry *et al.*, 1995), the H^+/*m*Ins symporter (Uldry *et al.*, 2001) or efflux through a non-specific Cl^- channel (volume-sensitive organic osmolyte anion channel) following hypotonic stress (Jackson & Strange, 1993; Jackson & Madeson, 1997). *m*Ins serves as a physiologically important non-nitrogenous osmolyte in the brain (Fisher *et al.*, 2002).

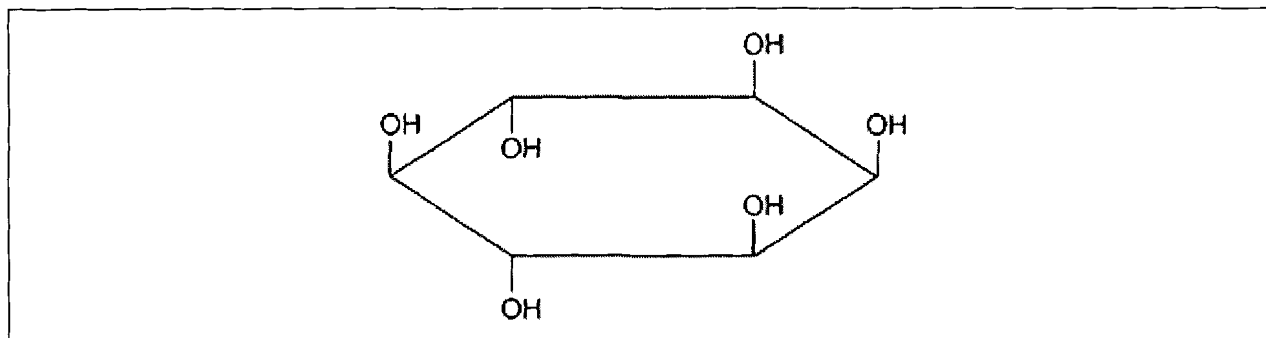


Figure 2-1: The chemical structure of *m*Ins.

The role of *m*Ins as a key metabolic precursor in the phosphatidylinositol (PI) cycle has been well established (Berridge & Irvine, 1989; Berridge *et al.*, 1989). The PI cycle, in particular with inositol trisphosphate (IP_3) and diacylglycerol (DAG) as second messengers, is an important component of G protein-coupled receptor (GPCR) signalling systems of several neurotransmitters (Baraban *et al.*, 1989). Several subtypes of adrenergic, serotonergic, cholinergic and metabotropic glutamatergic receptors in the brain are coupled to the hydrolysis of phosphoinositides (PIs). PIs are crucial for facilitating several cellular events, such as membrane trafficking, the maintenance of the actin cytoskeleton, the regulation of cell death and survival and the anchoring of plasma membrane proteins. *m*Ins is vital to the resynthesis of PIs and the maintenance and effectiveness of signalling (Toker & Cantley, 1997; Low, 2000; Vanhaesebroeck *et al.*, 2001; Fisher *et al.*, 2002; Harvey *et al.*, 2002a;).

2.1.1 The Phosphatidylinositol Cycle

The major components of the PI cycle, and in particular the significance of *m*Ins, is illustrated in Figure 2-2 and discussed below.

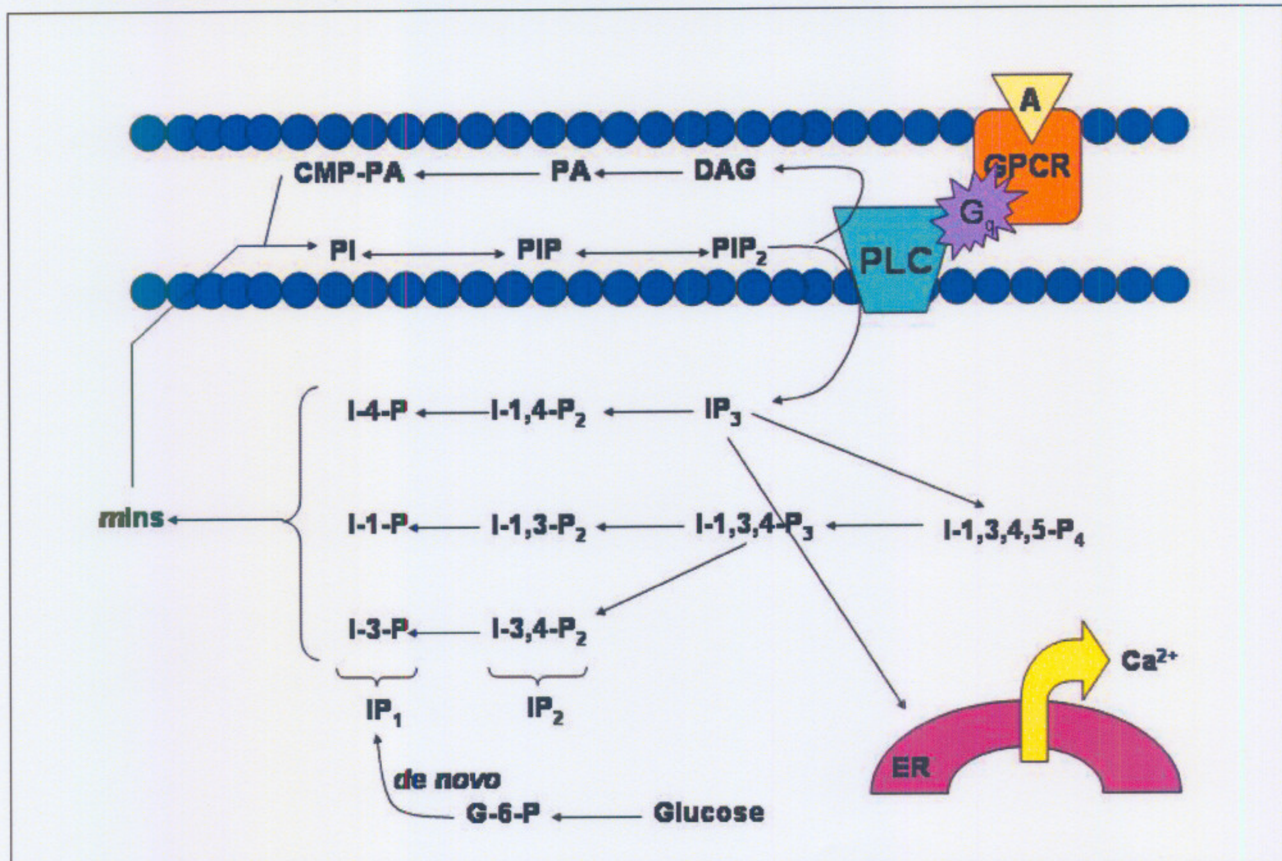


Figure 2-2: Representation of the PI cycle (adapted from Kim *et al.*, 2005). A = agonist; GPCR = G protein-coupled receptor; PLC = phospholipase C; CMP-PA = cytidine monophosphorylphosphatidate; G-6-P = D-glucose-6-phosphate; ER = endoplasmic reticulum.

*m*Ins is transported across the plasma membrane via specific carrier molecules, namely the Na^+/*myo*-inositol transporter (SMIT) and H^+/*myo*-inositol symporter (HMIT). SMIT is a saturable sodium (Na^+)-dependent uptake mechanism, which is pH-dependent and requires two Na^+ ions for each molecule of *m*Ins transported. The transporter is widely distributed throughout the central nervous system (CNS) and is found in both neural as well as non-neural cells. The highest levels of SMIT messenger RNA (mRNA) were observed in the choroid plexus, pineal, hippocampus, locus coeruleus and Purkinje cells. It has been reported that lithium reduces the activity of this transporter. *In vitro* studies have also demonstrated that the activation of protein kinase C (PKC) results in an inhibition of SMIT (Fisher *et al.*, 2002).

HMIT, on the other hand, is expressed almost exclusively within the CNS. Although it is expressed primarily within astrocytes, it is also present in other neural cells. While SMIT is inhibited by a reduction in pH, HMIT is maximally active at low pH. It is proposed that this transporter may be involved in the regulation of *m*Ins homeostasis (Fisher *et al.*, 2002).

Intracellular concentrations of *m*Ins are also increased by means of *de novo* synthesis. This process involves the conversion of D-glucose-6-phosphate to inositol-1-monophosphate (IP_1)

catalysed by IP_1 synthase. IP_1 is subsequently hydrolysed by inositol monophosphatase (IMPase) to produce *m*Ins. IMPase may reportedly be inhibited by lithium ion (Li^+; Kim *et al.*, 2005).

*m*Ins is incorporated into neuronal cell membranes as inositol phospholipids. PI is produced by PI synthase from *m*Ins and cytidine monophosphorylphosphatidate (CMP-PA; Batty & Downes, 1995), resulting in the formation of, amongst others, phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2 constitutes an important part of the inositol phospholipids incorporated into neuronal cell membranes and therefore also serves a structural role (Harvey, 1997). Binding of an agonist to a G_q protein-coupled receptor, including adrenergic, serotonergic, dopaminergic, glutamatergic and cholinergic receptor subtypes, activates phospholipase C (PLC; Kim *et al.*, 2005). PLC mediates the hydrolysis of PIP_2 into IP_3 and DAG (Atack, 2000). These two second messengers (IP_3 and DAG) in turn initiate separate cascades in cellular events (Berridge & Irvine, 1989; Berridge, 1997; Bootman *et al.*, 2002).

IP_3 mediates the release of intracellular calcium (Ca^{2+}) via interaction with three IP_3 receptor subtypes (Harvey, 1997). The type-1 receptor mediates Ca^{2+} mobilisation from the sarcoplasmic reticulum, while the type-3 receptor regulates Ca^{2+} influx across the plasma membrane. These are key functions of the PLC pathway (Wilcox *et al.*, 1998; Harvey *et al.*, 2002a). The action of IP_3 is short lived, since it is rapidly hydrolysed to inositol 1,4-biphosphate (IP_2), IP_1 and eventually to *m*Ins. Alternatively, IP_3 is converted to inositol 1,3,4,5-tetrakisphosphate (IP_4). IP_4 may in turn be converted to inositol 1,3,4,5,6-pentakisphosphate (IP_5) and inositol 1,2,3,4,5,6-hexakisphosphate (IP_6; Brailoiu *et al.*, 2003). These higher order phosphoinositides, namely IP_4, IP_5 and IP_6 can be converted once again to IP_3 by means of prolyl oligopeptidase (PO; Williams & Harwood, 2000).

DAG remains in the plasma membrane (Karp, 2002), where it activates PKC (Harvey, 1997), which is a multifunctional serine and threonine kinase that phosphorylates a wide variety of proteins. PKC is involved in a number of important processes in cellular growth and differentiation, cellular metabolism and transcriptional activation (Karp, 2002). DAG kinase catalyses the conversion of DAG to phosphatidic acid (PA), which is consequently converted to CMP-PA. The cycle continues as CMP-PA interacts with free *m*Ins to produce PI (Horrobin & Bennet, 1999).

Of the higher inositol phosphates, inositol hexakisphosphate (IP_6; phytate) is the most abundant in both neural and non-neural cells and is particularly prevalent in the CNS (Fisher *et al.*, 2002). IP_6 concentrations have been demonstrated to range between 10 and 15 µM in distinct brain regions (Yang *et al.*, 2001). A large number of high-affinity IP_6 binding sites have been detected

in the CNS. Consequently, it has been proposed that IP_6 is involved in various processes, such as receptor regulation, vesicle trafficking and neurotransmitter release. It is also reported to activate a repair mechanism for radiation- and chemically induced double-stranded breaks in DNA via activation of a DNA-dependent protein kinase. IP_6 is rapidly phosphorylated into the diphosphoinositol polyphosphates diphosphoinositol pentakisphosphate (PP-IP_5, also known as 'IP_7') and bisdiphosphoinositol tetrakisphosphate ($[PP]_2$-IP_4, also known as 'IP_8'). These products are in turn dephosphorylated back to IP_6 (Fisher *et al.*, 2002).

2.1.2 *m*Ins and Psychiatric Disorders

2.1.2.1 Pathophysiology

During the last few years, *m*Ins has emerged as a new possible treatment in psychiatry (Levine, 1997; Einat & Belmaker, 2001). In 1978, it was reported that patients with affective disorders had markedly reduced levels of *m*Ins in cerebrospinal fluid (CSF; Barkai *et al.*, 1978). This was the first suggestion that *m*Ins may be involved in psychiatric disorders. These findings were, however, not replicated in some subsequent studies. For example, in a study conducted by Levine and colleagues (1996), the CSF inositol levels of drug-free depressed patients did not differ significantly from those of normal control subjects (Levine *et al.*, 1996). However, reduced *m*Ins levels were reported in the frontal cortex of the brains of post-mortem patients with bipolar disorder and suicide victims (Shimon *et al.*, 1997). *m*Ins is not metabolised in the brain (Sherman, 1991), which eliminates post-mortem effects as the cause of reduced *m*Ins levels in the frontal cortex (Shimon *et al.*, 1997). However, *m*Ins levels could have been artificially lowered if the suicide victims or patients with bipolar disorder were more likely than control subjects to be hyponatremic for several days before death (Shimon *et al.*, 1997), because hyponatremia lowers brain levels of *m*Ins (Thurston *et al.*, 1989).

*m*Ins levels and the activity of IMPase (the enzyme for dephosphorylation of IP to *m*Ins and inhibited by Li^+), were measured in post-mortem brain samples of suicide victims, patients with bipolar disorder and normal control subjects. Free *m*Ins levels were measured by gas chromatography, while IMPase activity was measured by the release of inorganic phosphate. These parameters were determined in the frontal cortex, occipital cortex and cerebellum. *m*Ins levels in the frontal cortex of suicide victims and patients with bipolar disorder were significantly lower than those of the control subjects. No significant differences were observed in the occipital cortex or cerebellum. Concerning IMPase activity, no significant differences were observed in any of the three brain regions analysed. These results could suggest a deficiency of second messenger precursor in suicide victims and patients with bipolar disorder. The pathophysiological implications of low frontal cortex *m*Ins levels are not clear. It was reported

that *m*Ins levels might regulate PLC activity in a complex manner unrelated to levels of PI (Batty & Downes, 1995). Therefore, it is proposed that low *m*Ins levels could cause functionally deficient responses to receptors linked to the PI cycle (Shimon *et al.*, 1997).

2.1.2.2 Clinical Evidence for Therapeutic Efficacy of *m*Ins

Importantly, several clinical studies have demonstrated that *m*Ins may have therapeutic value in the treatment of depression (Levine *et al.*, 1993a, 1995a; Levine, 1997), depression associated with post-traumatic stress disorder (Kaplan *et al.*, 1996), panic disorder (Benjamin *et al.*, 1995) and obsessive-compulsive disorder (OCD; Levine *et al.*, 1993b, 1994). In a study of depressed patients who had been resistant to previous antidepressant treatment, *m*Ins treatment resulted in a decline in the mean scores of the Hamilton Depression Scale (Levine *et al.*, 1993a). The only side effects reported by subjects were nausea and flatulence (Levine *et al.*, 1995a). However, it proved to be ineffective in schizophrenia (Levine *et al.*, 1993b, 1994), Alzheimer's disease (Barak *et al.*, 1996) and autism (Levine *et al.*, 1997). It seems as though *m*Ins may worsen attention deficit hyperactivity disorder (Levine *et al.*, 1995b). Interestingly, the clinical spectrum of *m*Ins seemingly parallels that of the SSRIs (Levine, 1997; Einat & Belmaker, 2001).

Even though *m*Ins was shown to be clinically effective in several anxiety-related disorders, the subcellular mechanism of action remains elusive (Harvey *et al.*, 2002a). The disorders in which *m*Ins is effective are at best 60-70% responsive to current drug treatment (Mendels, 1987; Carpenter *et al.*, 1996). Therefore, understanding the mechanism of action of *m*Ins holds great promise for understanding the pathophysiology of these disorders (Harvey *et al.*, 2002a). The therapeutic response to *m*Ins demonstrates a time delay of 4 to 6 weeks (Levine, 1997). This is also observed with traditional antidepressants and is typical of the initiation and adaptation theory proposed for the mechanism of action of all psychotropic agents, including antidepressants. In patients responsive to treatment, considerable improvement in depressive symptoms occurs after long-term use (Hyman & Nestler, 1996).

The therapeutic efficacy of *m*Ins in depression was investigated under double-blind conditions. A dose of 12 g/day *m*Ins or placebo (glucose) was administered to depressed patients for four weeks. After two weeks of treatment, no significant difference in the Hamilton Depression Scale (HDS) was observed between the two groups. However, after four weeks it was apparent that *m*Ins reduced the HDS significantly more than placebo. In the *m*Ins group, one subject complained of nausea and one of flatulence, while no changes in haematology, kidney or liver function were noted (Levine *et al.*, 1995a). Relatively few side effects are expected, since *m*Ins is an isomer of glucose.

In a study conducted by Levine and colleagues (1993a), the effect of chronic administration of 6 g/day *m*Ins on depressive symptoms was evaluated. All of the subjects were clinically depressed and had not responded to previous antidepressant treatments for at least eight weeks before the trial. Current antidepressant treatment was continued (not changed) during the trial. All subjects were assessed with the HDS before treatment and after one, two, three and four weeks on *m*Ins treatment, as well as one week after cessation of *m*Ins treatment. Nine of the eleven subjects had at least a twelve point reduction of HDS, with the mean reduction being fifteen points. No changes in haematology, kidney or liver function were observed after two or four weeks of *m*Ins treatment. Since no controls were present in this study, a placebo response cannot be ruled out and the results cannot be viewed as conclusive. However, none of the subjects in the trial responded to at least eight weeks of standard antidepressant treatment before the trial (Levine *et al.*, 1993a), suggesting that responses could be ascribed to *m*Ins.

Proton (^{1}H) magnetic resonance spectroscopy imaging (MRSI) was used to determine whether oral *m*Ins supplements increase *m*Ins levels in the brain, since previous studies have demonstrated a decrease in *m*Ins levels in patients with bipolar disorder and suicide victims (Shimon *et al.*, 1997). *m*Ins levels were measured in occipital cortex grey matter and parietal white matter of healthy subjects taking an oral supplement of 12 g/day *m*Ins for eight days. These brain areas were selected to best represent cerebral grey matter and white matter, respectively. Compliance was excellent and no side effects of *m*Ins ingestion were reported. Measurements on day 4 revealed significantly higher *m*Ins levels in occipital grey matter, while the increase observed in parietal white matter did not reach statistical significance. At day 8, *m*Ins levels in occipital grey matter returned to baseline levels. The initial increase in *m*Ins levels, followed by a decrease is consistent with homeostatic changes and may reflect the role that *m*Ins plays in the maintenance of osmotic equilibrium in the brain. Homeostasis may also be restored as a consequence of changes in brain PI metabolism. If increased cellular *m*Ins levels led to increased synthesis of PI, *m*Ins ingestion might be associated with important changes in neuronal signal transduction, which could be responsible for the reported antidepressant effects of *m*Ins. The observed difference between *m*Ins uptake between the occipital cortex and parietal white matter suggests a regional specificity that has been noted in animal studies. Studies have also demonstrated that *m*Ins uptake into grey and white matter phospholipids differ. Important limitations exist in this study. Only two brain regions were examined and, if *m*Ins were to enter the brain in sufficient quantities to alleviate depression, the locus of action may not be in either of the selected brain regions. The subjects in the present study were not depressed and therefore probably had normal baseline *m*Ins levels. Depressed patients reportedly have reduced *m*Ins levels and this may alter the uptake of *m*Ins into the brain (Moore *et al.*, 1999).

Dwivedi and colleagues (1998) determined the density (B_{max}) and the affinity (K_D) of IP_3 receptors and the steady-state level of expressed IP_3 receptor proteins in the platelets of depressed patients and normal control subjects. The density and affinity of IP_3 receptors were determined by means of a $[^3H]IP_3$ binding assay. The density of IP_3 receptors was significantly increased in depressed patients as compared to control subjects. The observed increase was in the range of 81%. No significant differences in the affinity of IP_3 receptors were observed. The level of IP_3 receptor protein expression was also determined by means of immunolabelling. The expression level was significantly increased in depressed subjects compared to control subjects. The increased IP_3 receptor binding sites and protein levels in platelets of depressed patients suggest that IP_3 receptor-mediated functions in platelets are abnormal in depression and indicate the involvement of IP_3 receptors in depressive behaviour. This also raises the possibility of abnormal IP_3 receptor-mediated function in the CNS. Previous studies have revealed evidence that the PI cycle is overactive in depression. It has been reported that constant muscarinic receptor stimulation by agonists causes the down-regulation of IP_3 receptors in SH-SY5Y human neuroblastoma cells. The results of the present study provide evidence of increased IP_3 receptors in platelets of depressed subjects despite the fact that the PI cycle is over-stimulated in patients with depression (Dwivedi *et al.*, 1998).

A study was conducted to determine the effect of depression on 5-HT_{2A} binding parameters and the concentration of IP_3. The frontal cortex and hippocampus of the brains of post-mortem suicide victims and control subjects were analysed. None of the suicide victims had taken antidepressant medication for at least six months prior to death. No statistically significant differences in the maximum density and affinity of the 5-HT_{2A} binding sites were observed between frontal cortex of suicide and control subjects. A significantly decreased number of 5-HT_{2A} binding sites in the hippocampus of suicide victims were noted as compared to control subjects. Lower K_D values (better affinity) were also observed in the suicide victims. In contrast, IP_3 concentrations were significantly increased in the hippocampus of suicide victims. Although the present study revealed a decreased number of 5-HT_{2A} receptors in the hippocampus of suicide victims, the increased apparent affinity constant may suggest postsynaptic 5-HT_{2A} hypersensitivity and may therefore be a compensatory mechanism to the serotonin (5-HT) deficit widely described in depression. The activated 5-HT_{2A} receptors are capable of generating a significant increase in the production of their intracellular second messenger, IP_3, suggesting that the coupling efficacy of the receptor to its G protein may vary between regions (Rosel *et al.*, 2000).

2.1.2.3 Data from Animal Studies

In order to determine the effect of acute intraperitoneal administration of *m*Ins on activity levels of rats, Sprague Dawley rats were divided into five groups, which received either saline, glucose 1 g/kg, glucose 5 g/kg, *m*Ins 1 g/kg or *m*Ins 5 g/kg. Thereafter their behaviour was monitored for horizontal and vertical activity for 20 minutes. Significantly more rearings (vertical activity) was observed with the *m*Ins 1 g/kg group compared to the other groups. The same dose induced a similar, although not significant, trend in horizontal activity. These findings suggest antidepressant-like activity by *m*Ins (Kofman *et al.*, 1993).

In a closely related study, rats were divided into two groups to determine the effect of chronic oral *m*Ins administration. The rats received either *m*Ins-enriched food or a control diet for three weeks. Locomotion and rearing were significantly higher in the *m*Ins-treated group than in the control group, indicating antidepressant-like activity by *m*Ins. After the activity was monitored, the rats were decapitated to determine the levels of *m*Ins in cortex, hippocampus, caudate, hypothalamus and cerebellum following chronic *m*Ins treatment. The free *m*Ins levels were determined by gas-liquid chromatography. A significant overall increase of brain *m*Ins levels was observed in the *m*Ins group compared to the control group. A 36% increase in *m*Ins was found in the cerebral cortex and a 27% increase in the hippocampus of rats treated with *m*Ins. No statistically significant differences were observed in the caudate and cerebellum. The results suggest that chronic dietary *m*Ins is taken up into the brain in sufficient quantities to affect behaviour (Kofman *et al.*, 1998).

Einat and colleagues (1999b) investigated the effect of chronic *m*Ins administration on reserpine-induced immobility and the forced swim test. Intraperitoneal administered *m*Ins at a dose of 1.2 g/kg demonstrated significantly reduced immobility time and increased struggle time in the forced swim test (suggesting antidepressant-like activity). Although no significant differences were observed with lower doses, a similar trend was detected. Chronic *m*Ins treatment significantly reduced complete immobility time after three days treatment with reserpine. Therefore, chronic *m*Ins treatment is effective in two different animal models of depression. In the forced swim test, *m*Ins was observed to increase not only the total activity time, but also the struggle time, a measure that may reflect not just general hyperactivity but possibly a reduction in levels of despair. The PI cycle is involved in serotonergic neurotransmission and the serotonergic system is involved in motor activity, therefore the behavioural effects of *m*Ins may be related to the serotonergic system (Einat *et al.*, 1999b).

A study was conducted to determine the effect of acute and chronic *m*Ins treatment on levels of monoamines and their metabolites in rat brain. No significant changes in levels of monoamines or their metabolites were observed after acute or chronic *m*Ins administration. Turnover rates of

either dopamine or 5-HT did not differ between rats treated with *m*Ins and control rats. The therapeutic action of *m*Ins may not be directly related to the function of monoamines at the synapse. Although the acute effect of antidepressant drugs is localised at the synapse, this may not be the source of their therapeutic action. These drugs may, in fact, initiate a cascade of events that ultimately induces a therapeutic related change elsewhere downstream. *m*Ins possibly activates a similar cascade of events as do other antidepressants, but at a different point, perhaps by acting directly on the second messenger system. Alternatively, *m*Ins may activate a different cascade that eventually interacts or converges with the events related to other antidepressant drugs (Einat *et al.*, 1999a).

2.1.2.4 Mechanistic *In Vitro* Investigations

PIs play a mandatory role in sustaining the efficacy of signalling of receptors associated with PI hydrolysis and have therefore hinted at the potential value of *m*Ins in the neurobiology and treatment of various psychiatric disorders, including depression. Several PI-mobilising receptors, such as cholinergic muscarinic receptors (mAChRs) and 5-HT_{2A}-Rs have been shown to be involved in mediating depression (Harvey, 1997; Daws & Overstreet, 1999). A study examining the *in vitro* effects of *m*Ins on 5-HT_{2A}-Rs, has demonstrated that *m*Ins reduces 5-HT_{2A}-R function, mainly by reducing the signalling capacity through G_q proteins, while it does not alter 5-HT_{2A}-R binding. This suggests that *m*Ins reduces the signalling capacity of these receptors at the receptor-G-protein level (De Kock, 2003; Brink *et al.*, 2004). The prefrontal cortex exerts an inhibitory effect on the amygdala by modulating fear responsiveness, which may involve 5-HT_{2A}-Rs (Harvey *et al.*, 2003a). Activation of 5-HT_{2A}-Rs is also associated with anxiety and poor adaptation to environmental stressors (Harvey *et al.*, 2001, 2002a; Brink *et al.*, 2004).

It was also noted that fluoxetine has a similar modulating effect on 5-HT_{2A}-Rs, although it seems to have a smaller modulating capacity than *m*Ins, under the specific experimental conditions. This is an interesting observation, since the clinical spectrum of *m*Ins appears to parallel that of the SSRIs (Levine, 1997; Einat & Belmaker, 2001). Imipramine pretreatment caused a significant increase in 5-HT_{2A}-R function, while it does not alter receptor binding. It is known that imipramine, and other antidepressants with less serotonergic properties, are less effective in the treatment of certain anxiety disorders, such as social anxiety disorder and OCD (De Kock, 2003; Brink *et al.*, 2004).

Anticholinergic properties of antidepressants have often been associated with side effects, but evidence suggests that this may contribute significantly towards antidepressant activity (Daws & Overstreet, 1999). It has also been suggested that the M_1-mAChR in the nucleus accumbens

may mediate behavioural depression (Chau *et al.*, 2001), while clinical studies have described depression as a state of cholinergic hyperactivity (Rubin *et al.*, 1999, 2003).

Accordingly, *in vitro* studies indicated that *m*Ins, fluoxetine and imipramine reduce mAChR function, with *m*Ins having the most pronounced effect in this regard. Inclusion of a PLC inhibitor, a phosphatidylinositol 3-kinase (PI-3-kinase) and phosphatidylinositol 4-kinase (PI-4-kinase) inhibitor or a mAChR antagonist in the pretreatments, resulted in a diminished modulating effect by *m*Ins. This suggests that the observed effect is dependent, in part, on the PI metabolic pathway (Viljoen, 2002; Brink *et al.*, 2004).

Further mechanistic investigations regarding the effects of *m*Ins were performed, examining the *in vitro* effects of *m*Ins and other drugs on the mRNA and protein levels of PLCβ_1 and glycogen synthase kinase 3β (GSK-3β). Pretreatment with 10 mM *m*Ins caused a marked decrease in PLCβ_1 mRNA levels, while only a trend towards decreased protein expression was observed. However, *m*Ins in combination with fluoxetine or sildenafil caused a significant decrease in PLCβ_1 mRNA and protein levels. After pretreatment with *m*Ins alone or in combination with either fluoxetine or sildenafil mRNA levels of GSK-3β were significantly decreased, while only a trend towards decreased protein expression was found. The observed discrepancies between the mRNA levels and protein expression after pretreatment with *m*Ins may be due to the time delay between transcriptional and translational events (Van Rooyen, 2005).

2.2 Depression: Criteria & Pharmacotherapy

The major disorders of mood or affect include the syndromes of major depression and bipolar disorder. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV; APA, 1994) major depressive disorder follows a clinical course that is characterised by one or more episodes of major depression without a history of manic, hypomanic or mixed episodes. Episodes due to the direct effects of a drug of abuse, a medication or toxin exposure or of mood disorder due to a general medical condition do not count toward a diagnosis of major depressive disorder. In addition, the episodes must not be better accounted for by schizoaffective disorder and are not superimposed on schizophrenia, schizophreniform disorder, delusional disorder or psychotic disorders not otherwise specified. The primary clinical manifestations of major depression are clinical significant depression of mood and impairment of functioning. Clinical depression is distinguished from normal grief, sadness, disappointment and the dysphoria or demoralisation often associated with medical illness.

The lifetime risk for major depressive disorder varies between 10% and 25% for women and between 5% and 12% for men. The point prevalence varies from 5% to 9% for women and from 2% to 3% for men. Approximately 19 million Americans (9,5% of the population) are affected by depression in any one-year period. So many people are affected by depression that it is often referred to as the "common cold" of mental illness. Depression is also a burden on the economy of any community or country (Price, 2004).

Depression is strongly associated with physical disease. An estimated third of physically ill patients attending hospital have depressive symptoms. Depression is even more common in patients with:

- life threatening or chronic physical illness;
- unpleasant and demanding treatment;
- low social support and other adverse social circumstances;
- personal or family history of depression or other psychological vulnerability;
- alcoholism and substance misuse;
- drug treatments that cause depression as a bothersome side effect, such as antihypertensives, corticosteroids and chemotherapeutic agents (Peveler *et al.*, 2002).

According to an estimate by the World Health Organization, depression will become the second most important cause of disability by 2020, with ischaemic heart disease being the most important. At present, depression is estimated to be already the fourth most disabling of all medical disorders (Peveler *et al.*, 2002). Most patients suffering from depression think about suicide, while approximately 50% attempt suicide and up to 15% die from suicide. Therefore, depression is a potentially fatal disorder (Disalver *et al.*, 1994; Strakowsky *et al.*, 1996). Mood disorders are among the most prevalent causes of morbidity, disability and suicide throughout the world (Greden, 2001). Major depression is often underdiagnosed and undertreated. Only an estimated one-quarter to one-third of cases of depression are diagnosed and a similar proportion of these are adequately treated (Baldessarini, 2001).

2.2.1 Symptoms of Depression

Major depression is characterised by feelings of intense sadness and despair, mental slowing and loss of concentration, pessimistic worry, lack of pleasure, self-deprecation and agitation. Physical changes also occur, particularly in severe, vital or "melancholic" depression. These changes include insomnia or hypersomnia, altered eating patterns with changes in weight, decreased energy and libido, disruption of the normal circadian and ultradian rhythms, body temperature and many endocrine functions (Baldessarini, 2001).

According to the DSM-IV (APA, 1994), diagnosis depends on the presence of five or more of the following symptoms during the same two-week period:

- depressed mood;
- substantial weight loss or weight gain;
- insomnia or hypersomnia;
- feelings of worthlessness or inappropriate guilt;
- recurrent thoughts of death or suicide or actual suicide attempt;
- decreased interest or pleasure;
- psychomotor retardation or agitation;
- fatigue or loss of energy;
- diminished ability to think or concentrate.

Two cardinal symptoms of persistent and pervasive low mood and loss of interest or pleasure in usual activities must be present.

2.2.2 Pharmacotherapeutics

Not all grief, misery and disappointment are indications for medical treatment and even severe affective disorders have a high rate of spontaneous remission, provided that sufficient time passes. Antidepressant agents are generally reserved for the more severe and otherwise incapacitating depressive disorders. Most antidepressants exert important actions on the metabolism of monoamines, particularly *l*-norepinephrine (*l*-NE) and 5-HT, and their receptors. Despite considerable shortcomings (e.g. relatively high incidence of treatment resistance and many troublesome side effects), antidepressants are still considered clinically effective in treating and preventing depression and have been used for more than fifty years (Baldessarini, 2001).

Available antidepressants may be classified according to their primary mode of action into the monoamine oxidase (MAO) inhibitors, tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), lithium and an increasing host of atypical antidepressants. These groups will be discussed in more detail below.

2.2.2.1 Monoamine Oxidase Inhibitors

MAO inhibitors increase the concentration of monoamines by inhibiting their metabolism by the MAO enzyme. Older MAO inhibitors include phenelzine, isocarboxazid and tranylcypromine. Phenelzine and isocarboxazid inhibit MAO irreversibly, while tranylcypromine interacts reversibly with the enzyme but has a prolonged action. These drugs are non-selective inhibitors of both MAO-A and MAO-B (Potter & Hollister, 2004). Moclobemide, on the other hand, is a

reversible and selective inhibitor of MAO-A and causes only minimal pressor response to dietary tyramine. Therefore, the risk of developing potentially fatal hypertensive crisis is considerably reduced and the need for dietary precaution is consequently reduced. Moclobemide has been found to be superior in efficacy to tranylcypromine and equal to tricyclic antidepressants and SSRIs (Zerjav, 2004).

The ability of MAO inhibitors to induce mania was noted in the early 1950s and these drugs were subsequently studied intensively in the treatment of depression. However, early MAO inhibitors presented both toxic risks and potentially hazardous interactions with other drugs; therefore the tricyclic antidepressants were preferred to the MAO inhibitors (Baldessarini, 2001). Dietary limitations and abstinence from foods containing tyramine are mandatory for treatment with MAO inhibitors and are recommended for up to a month after cessation of therapy, since it may precipitate a potentially fatal hypertensive crisis (Eisendrath & Lichtmacher, 1999). Currently MAO inhibitors are reserved for patients who fail to respond to other treatment regimes. It may be combined with lithium or a low dose of triiodothyronine in an attempt to potentiate the antidepressant effect (Baldessarini, 2001).

2.2.2.2 Tricyclic Antidepressants

Imipramine (Figure 2-3), amitriptyline, their *N*-demethyl derivatives and other similar compounds were amongst the first successful antidepressants and, since the early 1960s, have been widely used for the treatment of major depression. These agents have been proven useful in a number of other psychiatric disorders. The tricyclic antidepressants are reasonably effective for the treatment of major depression, but their use is often associated with severe and intolerable side effects.

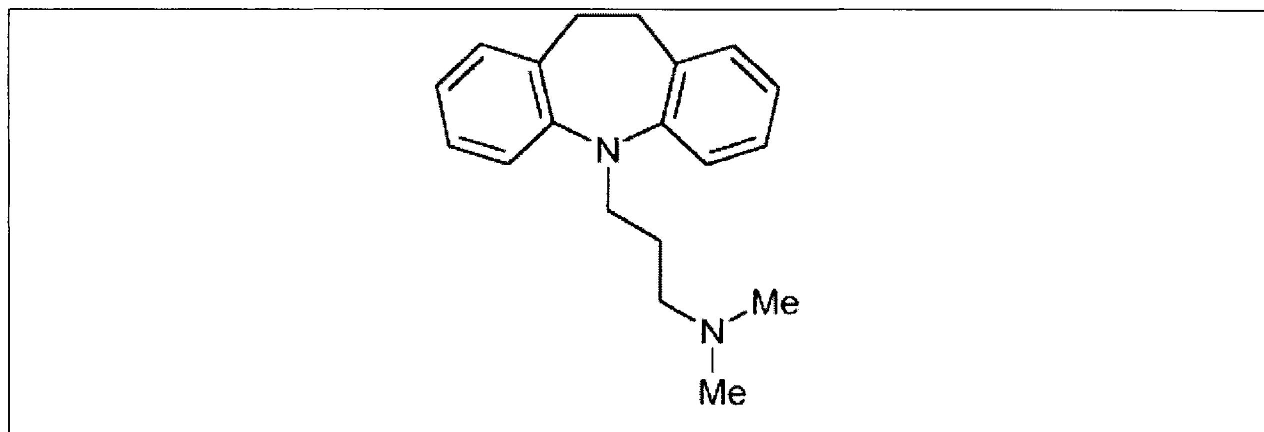


Figure 2-3: The chemical structure of imipramine, a prototypical tricyclic antidepressant.

Older tricyclic antidepressants with a tertiary amine side chain (including imipramine and amitriptyline) block neuronal reuptake of both 5-HT and *l*-NE, while clomipramine is relatively selective for the neuronal reuptake of 5-HT. Those with a secondary amine side chain or the *N*-demethylated ("*nor*") metabolites of the agents with tertiary moieties (including desipramine and nortryptiline) are relatively selective inhibitors or *l*-NE reuptake. The amine transport-inhibiting effects occur immediately and are sustained throughout therapy (Baldessarini, 2001).

Tricyclic antidepressants interact with various adrenergic receptors. Most of these drugs have at least moderate and selective affinity for α_1-adrenergic receptors (α_1-ARs), much less for α_2-ARs and almost no affinity for β-ARs. The α_2-ARs include presynaptic autoreceptors that limit the neurophysiological activity of noradrenergic neurons ascending from the locus coeruleus in the brain stem to supply mid- and forebrain projections, as well as descending projections to the spinal cord cholinergic preganglionic efferents to the peripheral autonomic ganglia. Activation of autoreceptors reduces the synthesis of noradrenalin through the rate-limiting step at tyrosine hydroxylase. Administration of antidepressants causes rapid activation of the α_2-AR-mediated negative-feedback mechanisms, but these responses are eventually diminished. Other adaptive changes have also been observed, including altered sensitivity of muscarinic acetylcholine receptors, decreases of $GABA_B$ receptors and possibly also *N*-methyl-D-aspartate (NMDA) receptors. The cyclic AMP-response-element binding protein (CREB) and brain-derived neurotrophic factor (BDNF) are also affected (Baldessarini, 2001). Imipramine and other tricyclic antidepressants have antagonistic effects on NMDA receptors (Hough *et al.*, 1996).

Tricyclic antidepressants are relatively lipophilic drugs and, once absorbed, are widely distributed. These drugs are strongly bound to plasma protein and constituents of tissue, which leads to large apparent volumes of distribution. Use of these drugs is associated with significant side effects and acute poisoning is potentially life threatening. Some of the most important and common side effects include antimuscarinic effects (including dry mouth, epigastric distress, tachycardia and palpitations) and cardiovascular effects (including orthostatic hypotension, sinus tachycardia and unpredictable prolongation of cardiac conduction times, with the potential of life-threatening arrhythmias (Baldessarini, 2001).

2.2.2.3 Selective Serotonin Reuptake Inhibitors

Although the tricyclic antidepressants were the most commonly used antidepressants from 1960 to 1990, the SSRIs and other atypical agents are now accepted as agents of first choice, particularly for medically ill or potentially suicidal patients and in the elderly. Even though these drugs are regarded as being safer, they are not without limitations, side effects and interactions

with other drugs (Baldessarini, 2001). The SSRIs are the first antidepressant drugs being rationally designed and are currently among the most frequently prescribed therapeutic agents in medicine. Prior to this, all psychotropic medications were the result of chance observation. Not only are the SSRIs effective in the treatment of major depressive disorder, but are also useful in anxiety disorders, pain disorders and premature ejaculation (Vaswani *et al.*, 2003).

SSRIs were developed for inhibition of the neuronal uptake pump for 5-HT, a property shared with the tricyclic agents, but without interacting with the various other receptors or fast sodium channels affected by the tricyclic antidepressants. Actions on these latter sites are responsible for many of the safety and tolerability problems of the tricyclic drugs (Vaswani *et al.*, 2003).

Fluoxetine is the prototype SSRI, while other SSRIs include paroxetine, fluvoxamine, citalopram and sertraline. These drugs act by inhibiting the active reuptake of 5-HT into the presynaptic nerve terminal, thereby increasing the concentration of 5-HT in the synaptic cleft (Baldessarini, 2001). However, the primary mechanism of action is not antagonism of 5-HT reuptake, since the drugs are not effective after acute administration. Delayed neurochemical adaptations may explain the therapeutic effects of SSRIs. A leading hypothesis for this action is desensitisation of somatodendritic 5-HT_{1A} autoreceptors in the midbrain raphae nucleus. Another hypothesis proposes that 5-HT modulates a homeostasis between dopamine, *l*-NE and gamma-amino butyric acid (GABA), which mediate thought process, anxiety and mood, respectively. Depression sets in when this homeostasis is disturbed. Therefore, serotonergic drugs may merely reinstate the homeostasis. A study on prior drug-free depressed patients showed that paroxetine down-regulates 5-HT_{2A} receptors in the cortex of young depressed subjects. It has also been demonstrated that the 5-HT reuptake inhibition-induced remission from depression is dependent upon the integrity of the 5-HT neuronal system (Vaswani *et al.*, 2003).

Comparison between tricyclic antidepressants and SSRIs indicate equal efficacy and onset of action but slightly different side effects, therefore the treatment decision needs to be based on considerations of patient acceptability, tolerability and cost (Vaswani *et al.*, 2003).

2.2.2.4 Lithium

Convincing and abundant evidence exists for the efficacy of lithium in the treatment of mania and the prevention of recurrent attacks of bipolar disorder. The primary indication for lithium treatment is for long-term prevention of recurrences of particularly both mania and depression in bipolar disorder, but it may also be used as an alternative or adjunct to antidepressants in severe recurrent depression or when response to an antidepressant alone is unsatisfactory.

Lithium, however, has a low therapeutic index and is associated with a wide range of side effects (Baldessarini & Tarazi, 2001).

Several studies have proved lithium to possess neuroprotective properties. Lithium has been found to increase activator protein-1 (AP-1) and CREB DNA-binding activities in cultured cerebellar granule cells, as well as in rat hippocampus, frontal cortex, amygdala and cerebellum (Ozaki & Chuang, 1997). Evidence suggests that this have profound effects in the regulation of cell viability (Chuang, 2004). It was reported that long-term lithium treatment of cultured rat cerebellar granule cells robustly reduces glutamate-induced excitotoxicity, which can be blocked by NMDA receptor antagonists, but not by non-NMDA receptor antagonists (Nonaka *et al.*, 1998). This neuroprotective effect seems to be long lasting and occurs at therapeutically relevant concentrations. However, six to seven days pretreatment is necessary for maximal protection, while short-term pretreatments are ineffective. Excessive *m*Ins supplementation fails to reverse the observed neuroprotection, suggesting this effect is unrelated to lithium's inhibition of IMPase. The neuroprotective effect of lithium involves the blockade of the apoptotic component of glutamate excitotoxicity and is due, in part, to a reduction in NMDA receptor-mediated Ca^{2+} influx. The necessity of long-term pretreatment to produce these neuroprotective effects suggests the involvement of gene expression. It was found that lithium induces a time- and concentration-dependent upregulation in Bcl-2 (an antiapoptotic protein) mRNA and protein levels and downregulation of p53 and Bax (both are pro-apoptotic proteins) mRNA and protein levels (Chen & Chuang, 1999). It has also been observed that lithium increases bcl-2 levels (Manji *et al.*, 2000b), while it reduces the levels of pro-apoptotic proteins p53 and Bax (Lu *et al.*, 1999) in human neuroblastoma SH-SY5Y cells *in vitro*.

Chronic treatment of rats with therapeutic concentrations of lithium produced an increase in bcl-2 levels in frontal cortex (Manji *et al.*, 1999; Manji *et al.*, 2000c). Chronic lithium treatment led to a significant increased number of bcl-2 immunoreactive cells in the dentate gyrus and striatum (Manji *et al.*, 1999).

Akt is a serine/threonine kinase down-stream of PI 3-kinase and functions as a cell survival factor. Lithium treatment of cultured cerebellar granule cells caused a rapid activation of PI 3-kinase followed by increased phosphorylation of Akt at Ser473 and, therefore, enhanced Akt activity (Chalecka-Franaszek & Chuang, 1999). Long-term lithium treatment facilitates the recovery of glutamate-induced Akt inactivation and prevents the complete loss of Akt activity, suggesting that Akt is a contributor in mediating protection against glutamate excitotoxicity (Chuang, 2004).

Patients suffering from mood disorders and undergoing chronic treatment with lithium demonstrated significantly higher subgenual prefrontal cortex volume than in patients not receiving lithium treatment, while not differing significantly from the control (Drevets, 2000). It was also found that lithium treatment in patients suffering from bipolar disorder led to increased grey matter volume compared to untreated control subjects (Moore *et al.*, 2000a).

2.2.2.5 Atypical Antidepressants

2.2.2.5.1 Memantine

Memantine is a non-competitive inhibitor of NMDA receptors with moderate affinity for these receptors. Uncompetitive NMDA receptor antagonists block the NMDA receptor channel thereby preventing excessive flux of Ca^{2+} in the cell. Uncompetitive antagonism is a type of non-competitive antagonism; therefore it cannot be overcome by increasing the glutamate concentration (i.e. insurmountable). No interactions with food or drugs have been observed, although it may potentially interact with a number of drugs. Theoretically, any disorder of CNS characterised by glutamate excitotoxicity-induced neuronal death, should be treatable with NMDA receptor antagonists (Sonkusare *et al.*, 2005; refer to § 2.3.2.2). However, clinical trials with NMDA antagonists, before introduction of memantine, have failed, because of significant side effects due to high binding affinity towards NMDA receptors, differential actions on neurons from various brain regions and interactions with neurotransmitter receptors other than NMDA receptors (Kornhuber & Weller, 1997). These side effects include hallucinations, agitation, catatonia, centrally mediated increase in blood pressure and anaesthesia. Memantine, on the other hand, has shown acceptable safety and tolerability in clinical trials. Side effects associated with the use of memantine are of mild intensity and include hallucinations, confusion, dizziness, headache and tiredness. Memantine crosses the blood-brain barrier rapidly and has a high therapeutic index (Sonkusare *et al.*, 2005). Drugs with high affinity for NMDA receptors, such as phencyclidine (PCP) and MK-801, demonstrate strong psychotomimetic side effects. Memantine lacks such side effects possibly because of a lack of significant interference with physiological activity of NMDA receptors as opposed to PCP and MK-801 (Kornhuber & Weller, 1997).

Memantine blocks NMDA receptors when there is sustained release of low concentrations of glutamate, thereby preventing the influx of Ca^{2+}, resulting in neuroprotection. However, it does not interfere with glutamate's physiological actions required for learning and memory (Sonkusare *et al.*, 2005). Memantine causes an increase in BDNF mRNA and protein levels in the limbic cortex. This effect increases with an increase in dose. An induction of isoforms of the BDNF receptor trkB is also observed (Marvanova *et al.*, 2001).

2.2.2.5.2 Tianeptine

Tianeptine caused quite a commotion in the late 1980s and early 1990s, because even though it is an effective antidepressant, it enhances 5-HT reuptake (Kato & Weitsch, 1988; Fattaccini *et al.*, 1990; Brink *et al.*, 2006). This effect is contrary to the effects of SSRIs (Brink *et al.*, 2006). It challenged the traditional monoaminergic hypothesis of depression (Hindmarch, 2001). Because of its structure, tianeptine is viewed as a modified tricyclic antidepressant (Figure 2-4, compare with Figure 2-3; Pacher & Kecskemeti, 2004; Brink *et al.*, 2006). Two isomers exist, while the *l*-isomer seems to be therapeutically active (Oluyomi *et al.*, 1997).

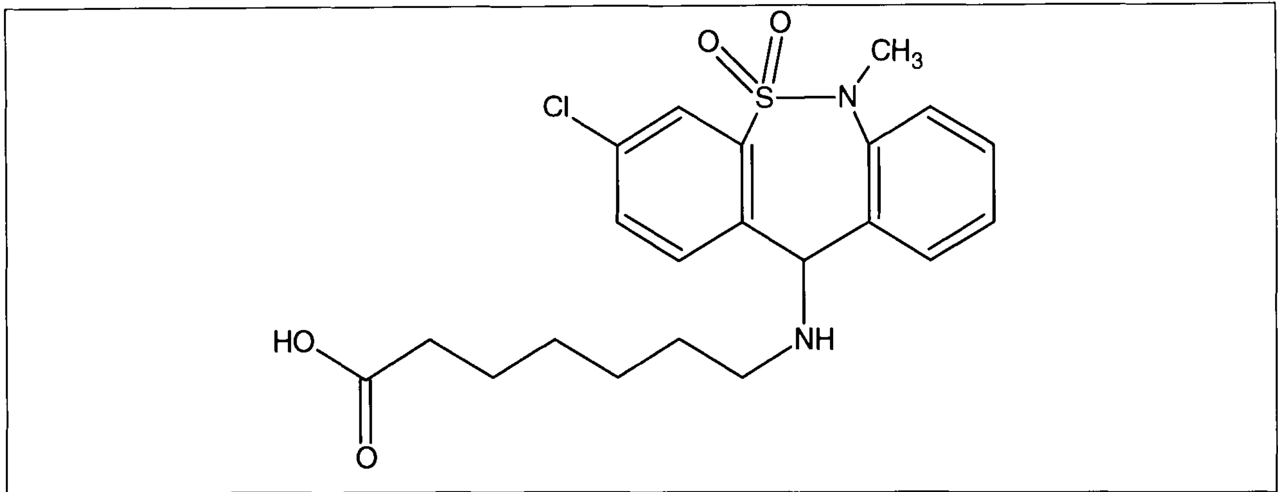


Figure 2-4: The chemical structure of tianeptine.

Clinical trials have reported therapeutic efficacy of tianeptine in more than 75% of treated depressed patients, including the elderly and anxiolytic properties were also demonstrated (Defrance *et al.*, 1988). It was also demonstrated that tianeptine is generally well tolerated, lacking significant side effects, such as sedation, anticholinergic effects, cardiovascular effects and disturbance of haematological or renal and hepatic function parameters (Delalleau *et al.*, 1988). In a study of tianeptine versus placebo in patients with major depression or bipolar disorder, tianeptine showed greater antidepressant efficacy than placebo and it was equally well tolerated, except for headache as a more prominent complaint in the tianeptine group (Costa e Silva *et al.*, 1997). Several clinical trials have reported tianeptine to be more effective, or at least as effective, as tricyclic antidepressants or SSRIs (Brink *et al.*, 2005). Tianeptine has been shown to improve not only depressive symptoms in the elderly, but also anxiety symptoms and cognitive function (Saiz-Ruiz *et al.*, 1998). Several reports regarding the misuse of tianeptine suggest less serious toxicity of overdosing (Brink *et al.*, 2005).

Extensive research led to the discovery of the neuroprotective properties of tianeptine (Czéh *et al.*, 2001; Fuchs *et al.*, 2004; McEwen & Chattarji, 2004). Studies have demonstrated the ability of tianeptine to prevent hippocampal neurodegeneration in rats, induced by chronic restraint

stress or corticosterone (Watanabe *et al.*, 1992). In particular, the reduction in the length and number of branch points of hippocampal CA3 neurons is prevented by tianeptine (Watanabe *et al.*, 1992). The reduced proliferation rate of the granule precursor cells in the dentate gyrus as well as the reduced hippocampal volume in a psychosocial stress paradigm in tree shrews were prevented by administration of tianeptine (Czéh *et al.*, 2001). It has also been shown to prevent stress-induced apoptosis in the temporal cortex and dentate gyrus of tree shrews (Lucassen *et al.*, 2004; refer to § 2.3).

Tianeptine is thought to alter the phosphorylation status of glutamate receptors, thereby normalising the stress-induced changes in the amplitude ratio of NMDA receptor to α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA)/kainate receptor-mediated currents in rat hippocampal CA3 neurons. This effect may contribute to its neuroprotective properties (Kole *et al.*, 2002). It has also been found that tianeptine inhibits nitric oxide synthesis (NOS) in the hippocampus (Wegener *et al.*, 2003). Nitric oxide (NO) is a neurotoxin that has been found to be elevated in patients with depression (Suzuki *et al.*, 2001). Chronic restraint stress induces an increase in glia glutamate transporter GLT-1 mRNA expression in rat hippocampus, while tianeptine administration inhibits this phenomenon (Reagan *et al.*, 2004).

2.2.2.5.3 *myo*-Inositol

Refer to § 2.1.2 for a review of the therapeutic use and effects of *m*Ins in depression.

2.3 Depression and Neuroplasticity

Recently a new hypothesis regarding the pathophysiology of depression has emerged which involves the plasticity of neural networks. According to this hypothesis, depression is thought to result from the inability of the brain to make the appropriate adaptive responses to environmental stimuli due to alteration in neuroplasticity and that antidepressant drugs act by normalising this impairment (Duman *et al.*, 1999; Manji & Duman, 2001; Manji *et al.*, 2001). However, the link between alterations in neuroplasticity and depressive symptoms remains to be established (Fossati *et al.*, 2004). Neuroplasticity is defined as the ability of the brain to undergo functionally relevant adaptations following external and/or internal stimuli. These dynamic processes are based on the capacity of neural systems, brain nuclei, single neurons, synapses and receptors to adapt and change their structural or functional repertoire in response to alterations in the internal and/or external environment (Zilles, 1992).

Major depression is a complex disorder that affects many different brain regions, often differentially. Changes associated with depression have been reported in the hippocampus,

amygdala, caudate nucleus, putamen and frontal cortex. All of these structures are extensively interconnected and comprise a neuroanatomical structure termed the limbic-cortical-striatal-pallidal-thalamic tract (Fuchs *et al.*, 2004). Various structural changes in the brains of post-mortem patients with depression have been observed, including a reduction in neuronal size in the orbitofrontal cortex, a reduction in glial number and size in the prefrontal and orbitofrontal cortex, and a decrease in cortical thickness and basal ganglia volume (Ongur *et al.*, 1998; Rajkowska, 2000a, b; Manji *et al.*, 2001). A loss of glia, being the most replicated finding, affects the orbital and medial prefrontal cortex, as well as several subdivisions of the anterior cingulated cortex (Rajkowska, 2000a). A decrease in glial density appears unique to mood disorders and is seen in both unipolar and bipolar depression (Rajkowska, 2000b).

2.3.1 Brain Regions Implicated

2.3.1.1 Neuroplasticity in the Hippocampus

Cellular and molecular alterations associated with depression have been studied using animal models of depression. Most of the research has been done on the hippocampus and elucidating the changes at hippocampal level may be crucial to understanding what happens in other brain areas implicated in the stress/depression circuitry (McEwen & Chattarji, 2004). The hippocampus is one of the main structures implicated in memory processes and the changes in hippocampal plasticity may possibly be responsible for the memory deficits often observed in depression (Fossati *et al.*, 2004). Inhibition of hippocampal neurogenesis is associated with memory impairments (Madsen *et al.*, 2003). Adult neurogenesis is a unique feature of the hippocampal dentate gyrus, which is accompanied by cell death and hence results in continuous cell turnover in this brain region (Biebl *et al.*, 2000; Kuhn *et al.*, 2001).

Several studies have investigated the effect of depression on hippocampal volumes by means of volumetric magnetic resonance imaging. Sheline and colleagues (1996, 1999, 2003) found a reduced hippocampal volume in depressed patients, a correlation with the duration of depression and recovery of volume loss after treatment with antidepressant drugs. It was also found that hippocampal atrophy worsens with repeated episodes. No relationship was detected between the number of days a depressive episode was being treated and the total hippocampal grey matter volume, or between the cumulative time treated with antidepressants and the total hippocampal volume. This study implies that antidepressants may protect against hippocampal volume loss associated with repeated depressive episodes. Since depression-related atrophy appears to be cumulative, immediate recognition and treatment is important in order to prevent damage occurring with repeated episodes (Sheline *et al.*, 2003). Any depressed or control subjects with medical problems were excluded from these studies, therefore it was speculated

that the subjects constitute "supernormals" (Sheline, 2000). Other groups have also found a reduction in hippocampal volume in currently depressed patients (Shah *et al.*, 1998; Bremner *et al.*, 2000; Steffens *et al.*, 2000; Mervaala *et al.*, 2000; Frodl *et al.*, 2002; MacQueen *et al.*, 2003; MacMaster & Kusumakar, 2004), while some found no abnormalities (Ashtari *et al.*, 1999; Vakili *et al.*, 2000). Some studies reported smaller left hippocampal volumes in depressed patients in remission (Sheline *et al.*, 1998, 2003; Bremner *et al.*, 2000). In studies that assessed depression severity and used high-resolution MRI techniques, depression was associated with bilateral hippocampal atrophy, ranging from 8% to 19%. The volume loss appears to have functional significance with an association between acute depression and abnormalities of declarative memory, as well as an association between severe depression in remission and verbal memory (Sheline, 2000).

Patients with significant depressive symptoms had smaller bilateral hippocampal grey matter volumes compared with patients with remitted depression (Caetano *et al.*, 2004). This is consistent with another study also comparing currently depressed patients to remitted patients that showed reduced grey matter density in the acutely depressed patients (Shah *et al.*, 1998). A significant inverse correlation between left hippocampus grey matter volume and duration of illness was observed (Caetano *et al.*, 2004).

The findings of Sheline *et al* (2003) together with the evidence that hippocampal volume is not reduced in the first depressive episode (MacQueen *et al.*, 2003), suggest that hippocampal atrophy is a result of the disorder rather than a cause (McEwen & Chattarji, 2004). Recent post-mortem studies have not found evidence for neuronal cell death in patients who suffered from depression (Muller *et al.*, 2001; Stockmeier *et al.*, 2003), however, evidence for hippocampal synaptic reorganisation (Muller *et al.*, 2001) and increased neuronal and glial cell packing density (Stockmeier *et al.*, 2003) have been found, suggesting a decrease in the hippocampal neuropil in major depression (Sheline *et al.*, 2003).

It seems as though morphological changes develop early in the course of depression (Frodl *et al.*, 2002; MacQueen *et al.*, 2003) and functional impairment, such as memory deficit, may precede the observable morphological changes in the hippocampus (MacQueen *et al.*, 2003). The reduction in hippocampal volume is apparently not caused by increased cell loss (Lucassen *et al.*, 2001), indicating that mood disorders may be characterised by atrophy instead of neuronal death (Castrén, 2004). Dendritic arbors and synaptic contacts, rather than cell bodies, take up most of the space in the mammalian cortex, therefore, neuronal atrophy without cell loss suggests that neuronal connectivity may be reduced in mood disorders (Castrén, 2004). The mechanisms responsible for the hippocampal volume loss have not been identified (Fuchs *et al.*, 2004). It is important, however, to view the hippocampal changes in a broader context,

since it is unlikely that disturbed neurogenesis and subsequent structural changes in the hippocampus alone will fully explain a disorder as complex as depression (D'Sa & Duman, 2002; Manji *et al.*, 2003; Fuchs *et al.*, 2004).

The hippocampus is one of the few brain regions where neural progenitor cells continue to divide and give rise to new neurons in adult animals (Duman, 2002). The proliferation and survival of the adult-generated granule neurons can be suppressed by exposure to both acute and chronic stress (Gould *et al.*, 1997; Czéh *et al.*, 2001, 2002; Pham *et al.*, 2003; Heine *et al.*, 2004). Stressful life events are among the most potent factors known to trigger or induce depression (Kendler *et al.*, 1999, Paykel, 2001).

Evidence suggests that successful antidepressant treatment may lead to the recovery of at least some of the morphological changes (Drevets, 2001; Drevets *et al.*, 2002). Chronic treatment with amitriptyline reversed the reduction in dendritic spine density in CA1, CA3 and dentate gyrus of rats, induced by olfactory bulbectomy, an experimental model of depression, while treatment with mianserin reversed this reduction only in the dentate gyrus (Norrholm & Ouimet, 2001). To determine the effects of chronic psychosocial stress and subsequent antidepressant treatment on hippocampal volume, adult male tree shrews were subjected to a psychosocial stress regime and received tianeptine treatment. It was demonstrated that chronic psychosocial stress significantly suppressed adult hippocampal cell proliferation by up to 33%. Chronic tianeptine treatment caused a significant increase in adult hippocampal cell proliferation. Interestingly, this effect was only observed in the stressed group and not in the control group receiving tianeptine treatment, suggesting that chronic tianeptine treatment affects cell proliferation only in stress-based conditions. Only a trend towards reduced hippocampal volume was observed as a result of chronic psychosocial stress. A significant increase in hippocampal volume was observed in stressed animals receiving antidepressant treatment as compared to stressed animals not treated with tianeptine. This suggests that hippocampal volume loss in depressed humans may possibly be prevented by antidepressant treatment (Czéh *et al.*, 2001).

In a similar study the effect of clomipramine treatment on adult hippocampal cell proliferation and hippocampal volume in adult male tree shrews was investigated. Chronic psychosocial stress caused a significant decrease in hippocampal cell proliferation as well as hippocampal volume. Chronic treatment with clomipramine demonstrated a significant increase in both cell proliferation and hippocampal volume (Van der Hart *et al.*, 2002).

Male Sprague Dawley rats were treated with fluoxetine, reboxetine, tranylcypromine, haloperidol, or electroconvulsive seizures to determine the effect of these treatments on

hippocampal neurogenesis. After chronic antidepressant treatment, significantly increased cell proliferation was observed in the hippocampus as compared to control rats receiving only vehicle. No increase in cell proliferation was noted after acute treatment. This is consistent with the time delay in clinical effect observed after antidepressant treatment. Interestingly, the non-antidepressant psychotropic drug, haloperidol, did not cause an increase in cell proliferation. This suggests that the upregulation of hippocampal cell proliferation may be selective to antidepressant treatment (Malberg *et al.*, 2000a).

Chronic treatment with fluoxetine failed to increase neurogenesis in 5-HT_{1A} receptor knockout mice, while imipramine, which also inhibits *l*-NE uptake, caused an increase in neurogenesis. These mice showed no behavioural response to fluoxetine administration in the novelty suppressed feeding test, but the response to imipramine was yet again normal. Hippocampal irradiation inhibits stem cell proliferation in the hippocampus and in this study it blocked the behavioural responses to several different types of antidepressants in the behavioural tests. This implies a correlation between the induction of neurogenesis and behavioural response to antidepressant drugs, suggesting that neuronal proliferation may be a prerequisite for the clinical mood-elevating effect of antidepressants (Santarelli *et al.*, 2003).

Post and colleagues (2000) conducted a study, investigating the effects of tricyclic antidepressants (amitriptyline and desipramine), SSRIs (paroxetine and fluoxetine) and MAO inhibitors (moclobemide and deprenyl) on the viability of the mouse hippocampal cell line HT-22. Treatment for 24 hours with tricyclic antidepressants and SSRIs resulted in a dose-dependent decrease in cell viability, while treatment with moclobemide and deprenyl had no significant effect on cell viability. Treatment with 100 μM tricyclic antidepressants or 50 μM SSRIs, respectively, caused massive cell death.

A significant increase in intracellular peroxide accumulation was observed after treatment with tricyclic antidepressants and SSRIs, suggesting a role for oxidative stress in the observed cytotoxicity. Consistent with the cell survival data, MAO inhibitors did not cause peroxide generation. Oxidative stress may initiate a cascade of cellular processes that ultimately lead to necrosis and/or apoptosis. At least part of the observed cell death is due to apoptosis, since treatment with tricyclic antidepressants and SSRIs caused a dose-dependent increase in the number of apoptotic cells as compared to untreated control cells. Once again, the MAO inhibitors did not induce significant amounts of apoptosis. Interestingly, exogenous administration of glutathione, which is responsible for the maintenance of the optimal redox state of the cell, protected the cell against the neurotoxic effects of the tricyclic antidepressants and SSRIs (Post *et al.*, 2000).

In another study, HT-22 cells and primary cortical cells were exposed to 5 mM glutamate and the tricyclic antidepressants, imipramine and clomipramine. Both drugs caused a significant increase in cell viability as compared to cells only exposed to glutamate, indicating a protective effect of tricyclic antidepressants against glutamate toxicity (Maher & Davis, 1996). These results are contrary to the results observed by Post and colleagues (2000), but it suggests that the particular cellular environment at any given time plays a role in determining neurotoxic versus neuroprotective outcomes (Post *et al.*, 2000).

Antidepressant-induced neurogenesis appears to be limited to the hippocampus, since no increased proliferation rate is observed in the cerebral cortex (Santarelli *et al.*, 2003). Antidepressants are known to exert neurotrophic or neuroprotective effects against various insults. They also modulate the expression of various factors involved in cell survival and growth, such as CREB, BDNF, bcl-2 and mitogen-activated protein kinases (MAP kinases; D'Sa & Duman, 2002; Manji *et al.*, 2003; Fuchs *et al.*, 2004).

New theories have arisen considering a fundamental role for hippocampal neurogenesis in the loss of neural plasticity. However, the exact functional significance of these newly generated neurons in the pathophysiology of mood disorders is still unclear (Fuchs *et al.*, 2004). According to Kempermann and Kronenberg (2003), "this does not mean that the secret of depression would lie in single new born or unborn neurons", while a recent study suggests that the behavioural effects of chronic antidepressants are mediated by the stimulation of neurogenesis in the hippocampus (Santarelli *et al.*, 2003).

2.3.1.2 Neuroplasticity in Other Brain Regions

2.3.1.2.1 Prefrontal Cortex

Strong reciprocal connections exist between the prefrontal cortex and hippocampus. Dysfunction of the prefrontal cortex is hypothesised to play an important role in the aetiology of depression (Fuchs *et al.*, 2004). Reduced neuronal densities, smaller neuronal somata and a significant decrease in cortical thickness have been found in post-mortem studies (Rajkowska *et al.*, 1999; Rajkowska, 2000b; Cotter *et al.*, 2002). The underlying processes for these observations have not yet been elucidated. Studies in rats showed that corticosterone and stress induced dendritic reorganisation in pyramidal neurons, particularly in the medial prefrontal cortex, thereby rendering a possible and indirect explanation (Wellmann. 2001; Verhovshek *et al.*, 2003). These findings suggest that similar neurochemical mechanisms could mediate the dendritic alterations observed in hippocampus and prefrontal cortex as a result of exposure to stress or high levels of glucocorticoids (Fuchs *et al.*, 2004).

2.3.1.2.2 Amygdala

The amygdala is a crucial structure in emotional regulation and formation of emotional memories and plays a fundamental role in the pathophysiology of mood disorders. Abnormalities of resting blood flow and glucose metabolism have been identified in the amygdala and the orbital and medial prefrontal cortex (brain areas that are extensively connected with the amygdala) of depressed patients. In patients that respond to treatment, the mean amygdala metabolism decreases during antidepressant treatment, while the persistence of elevated amygdala metabolism during remission is associated with a high risk of depressive relapse (Drevets, 2003). Magnetic resonance imaging studies revealed that women had a smaller amygdala core (Sheline *et al.*, 1998). Another study, in which men and women were involved, showed that depressed patients have a larger amygdala volume (Bremner *et al.*, 2000). Reduced number of glial cells, but not neuron numbers, in the amygdala of depressed subjects was observed in post-mortem histopathological studies (Bowley *et al.*, 2002).

The structural changes reported in the amygdala of depressed patients could explain the anxiety symptoms frequently associated with depressed mood as well as the aggressive behaviour observed, particularly in depressed adolescents (Frodl *et al.*, 2003; Fossati *et al.*, 2004). Varied observations have been reported regarding amygdala volumes. One study reported larger amygdala volumes in patients with first-episode depression, but no abnormalities in patients with recurrent depression (Frodl *et al.*, 2003), while another study did not find significant differences in amygdala volume (Bremner *et al.*, 2000). Yet another study found decreased amygdala core nuclei volumes in depressed women (Sheline *et al.*, 1998). Asymmetry in amygdala volumes (smaller right amygdala volume) was observed in depressed patients (Mervaala *et al.*, 2000). Other studies performed showed asymmetry as well, but smaller left amygdala volumes were observed (von Gunten *et al.*, 2000; Caetano *et al.*, 2004).

2.3.2 Glutamate

2.3.2.1 Glutamate as a Neurotransmitter

Glutamate has powerful excitatory effects on neurons in virtually every region of the central nervous system. The view that glutamate, and possibly aspartate, function as the principle fast excitatory transmitters throughout the CNS is generally accepted.

Glutamate receptors are classified functionally either as ligand-gated ion channels (ionotropic receptors) or as metabotropic (G protein-coupled) receptors. The ligand-gated ion channels are further classified as α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA), kainate and *N*-methyl-D-aspartate (NMDA) receptors. Fast depolarisation at most glutamatergic synapses in the brain

and spinal cord is mainly mediated by AMPA and kainate receptors. NMDA receptors are also involved in normal synaptic transmission, but are more closely associated with the induction of various forms of synaptic plasticity rather than with fast point-to-point signalling in the brain. The induction of long-term potentiation (LTP) involves NMDA receptors. LTP refers to a prolonged (hours to days) increase in the size of a postsynaptic response to a presynaptic stimulus of given strength. Activation of NMDA receptors is essential for the induction of one type of LTP that occurs in the hippocampus (Bloom, 2001).

NMDA receptors are normally blocked by Mg^{2+} at resting membrane potentials. Therefore, activation of NMDA receptors requires depolarisation of the postsynaptic membrane together with the binding of synaptically released glutamate. This is achieved by activation of AMPA or kainate receptors at nearby synapses from inputs from different neurons (Bloom, 2001).

The ability of high concentrations of glutamate to produce neuronal cell death has been known for quite some time although first insights into the mechanism have only recently been obtained. Initially it was thought that the cascade of events leading to neuronal cell death was triggered exclusively by excessive activation of NMDA or AMPA/kainate receptors, which allow significant influx of Ca^{2+} into the neurons. More recent studies implicate both local depletion of Na^{+} and K^{+}, as well as small but significant elevations of extracellular zinc ion (Zn^{2+}) as factors that may activate both necrotic and proapoptotic cascades leading to neuronal death. NMDA receptor antagonists can attenuate or block neuronal death induced by activation of these receptors, but they cannot prevent all degenerative effects (Bloom, 2001).

Another pathway for glutamate toxicity, apart from excitotoxicity as described above, has been identified namely oxidative glutamate toxicity. This pathway is mediated via a series of disturbances to the redox homeostasis of the cell. Interestingly, both excitotoxicity and oxidative glutamate toxicity result in the production of free radicals (Maher & Davis, 1996).

It has been demonstrated that the exposure of cells to glutamate results in an inhibition of cystine transport into the cell, which causes an inability to maintain intracellular glutathione levels. The low intracellular levels of glutathione result in a reduced ability to protect against oxidative reactions within the cell and, ultimately, cell death. Since the observed cell death can be prevented by the administration of antioxidants, it is proposed that the accumulation of excess free radicals seems to be responsible for the toxicity (Maher & Davis, 1996).

2.3.2.2 Involvement of Glutamate in Depression

As mentioned in § 2.3.3, stressful life events have been found to be among the most potent factors to trigger or induce depression (Kendler *et al.*, 1999, Paykel, 2001). Evidence suggests that the effects of chronic stress involve not only glucocorticoids, but also excitatory amino acids. Increased levels of extracellular glutamate have been reported in the hippocampus and other brain regions after stress (Lowy *et al.*, 1993, 1995; Maghaddam *et al.*, 1994). It has also been demonstrated that major depression is accompanied by alterations in the serine/glycine ratio as well as glutamate (Altamura *et al.*, 1995).

Administration of NMDA receptor antagonists enhances neurogenesis in the dentate gyrus (Cameron *et al.*, 1995) and prevents the stress- and corticosterone-induced dendritic remodelling (Magariños *et al.*, 1999; McEwen, 1999). These data suggest that modulation of the glutamatergic system plays a pivotal role in the regulation of synaptic plasticity and that antidepressants may act, in part, by normalising the alterations in glutamate function. This normalisation may be achieved by mechanisms directly targeting the glutamatergic synapse or indirectly via other neurotransmitters such as 5-HT, noradrenalin or GABA (McEwen & Chattarji, 2004).

2.3.2.2.1 Clinical Data

After treatment with antidepressants for five weeks, it has been noted that serum levels of aspartate and glutamate are significantly reduced in patients suffering from depression (Maes *et al.*, 1998). Post-mortem studies in depression document changes in the NMDA receptor complex in the frontal cortex of suicide victims (Nowak *et al.*, 1995), as well as a reduction in the principle subunit for the NMDA receptor, NMDAR1, in the hippocampus (Law & Deakin, 2001). Zn^{2+} prevents the permissive action of glycine on glutamate stimulation of the NMDA receptor, thereby functioning as a negative regulator of the NMDA ion channel. It has been found that Zn^{2+} is lower in depressed patients than in healthy control subjects (Maes *et al.*, 1994a). Glutamate receptor super-sensitivity has been observed in the platelets of depressed patients (Berk *et al.*, 2001). Elevated levels of NO metabolites have also been found in patients with depression (Suzuki *et al.*, 2001). A decreased activity in calcium-dependent constitutive NOS in prefrontal cortex of depressed patients has been reported (Xing *et al.*, 2002). NOS-containing neurons in the hypothalamus are also reduced in patients with depression (Bernstein *et al.*, 1998). Inhibitors of downstream events initiated by NMDA receptor activation, including NOS inhibitors (Harkin *et al.*, 1999) and guanylyl cyclase-cGMP inhibitors (Heiberg *et al.*, 2002), have all demonstrated distinct antidepressant-like effects (Harvey *et al.*, 2003b).

The abovementioned data suggest that major depressive disorder is accompanied by alterations in glutamate and subcellular NOS signalling and that antidepressants may act by evoking adaptations of glutamate-dependent calcium-calmodulin-NOS-guanylyl cyclase signalling pathways (Skolnick, 1999; Paul, 2001). This is supported by pharmacological studies where the action of all classes of antidepressants, including electroconvulsive therapy, has been found to involve suppression of NMDA receptor activity (Skolnick, 1999; Stewart & Reid, 2002).

Glial cell numbers are markedly reduced in the amygdala and orbital and medial prefrontal cortex, as well as the striatum and thalamus of depressed patients (Sheline *et al.*, 1998; Rajkowska, 2000b). Glial cells play an integral role in regulating synaptic glutamate concentrations, glucose uptake and releasing trophic factors that are involved in the development and maintenance of synaptic networks formed by neuronal and glial processes (Rajkowska, 2000b).

NMDA antagonists such as amantadine and ketamine have been found to be extremely effective in severe treatment-resistant depression (Skolnick, 1999; Stryjer *et al.*, 2003). Therefore, NMDA antagonists may be useful in enhancing antidepressant effects (Harvey *et al.*, 2003b).

2.3.2.2.2 Data from Animal Studies

The combination of NMDA antagonists with classical antidepressants exerts synergistic antidepressant effects in the forced swimming test (Rogoz *et al.*, 2002). The uncompetitive NMDA antagonist, MK801, can re-establish antistress efficacy after withdrawal of imipramine following chronic treatment (Harvey *et al.*, 2002b).

In an animal study, Harvey and colleagues (2002b) demonstrated that acute imipramine withdrawal after chronic treatment caused a loss in situational stress responsiveness. These behavioural changes were accompanied by an increase in hippocampal NMDA receptor density. The resulting behavioural and neurochemical effects are reversed by the administration of the NMDA antagonist, MK801 (Harvey *et al.*, 2002b). Antidepressant withdrawal leads to an increase in the number of NMDA receptors and subsequently an increased probability of receptor binding by available synaptic glutamate (Harvey *et al.*, 2003b).

Antidepressants reduce the proportion of high affinity glycine sites on the NMDA receptor, thereby reducing glutamatergic transmission (Skolnick *et al.*, 1996). It has also been observed that antidepressants reduce NMDA subunit mRNA in various limbic and subcortical structures

(Boyer *et al.*, 1998). NMDA antagonists, like classical antidepressants, induce β-AR down-regulation suggestive of a crosstalk mechanism between adrenergic and glutamatergic pathways. Interestingly, a functional adrenergic system is required for the aforementioned changes to occur at the NMDA receptor (Harkin *et al.*, 2000). D-cycloserine, a positive modulator of the NMDA receptor, can inhibit 5-HT function (Dall'Olio *et al.*, 2000); 5-HT depletion causes long-lasting increases in glutamatergic transmission (Di Cara *et al.*, 2001) as well as increases in downstream activation of NOS (Tagliaferro *et al.*, 2001). Both acute and chronic administration of an SSRI can reduce glutamate release in the prefrontal cortex (Golembiowska & Dziubina, 2000). NO and its second messenger, cGMP, are able to modify synaptic activity, such as 5-HT reuptake (Miller & Hoffman, 1994). While NMDA inhibition will modulate monoamine turnover, noradrenergic and serotonergic antidepressants will, in turn, induce effects at glutamatergic synapses. Both these mechanisms may contribute to antidepressant efficacy (Skolnick, 1999).

Glutamate and NO have important actions on cellular aspects of memory and neural plasticity (McLeod *et al.*, 2001). Emotional memory constitutes a fundamental part of symptoms of mood and anxiety disorders. Therefore, these transmitters may have an important impact on learning and memory, as well as other clinical symptoms, such as changes in appetite and sexual and aggressive behaviour (McLeod *et al.*, 2001).

In addition to the increased levels of circulating glutamate (Altamura *et al.*, 1995; Maes *et al.*, 1998), decreased expression of neuronal glutamate uptake transporters have been observed in major depression (McCullumsmith & Meador-Woodruff, 2002; Harvey *et al.*, 2003b).

It has been reported that glutamate induces a rapid upregulation of p53 and Bax and downregulation of bcl-2, while these changes are completely blocked by lithium. Glutamate also triggers the release of cytochrome c from mitochondria and the cleavage of nuclear lamin B_1, a substrate of caspase-3. Once again, these changes are suppressed by lithium (Chuang, 2004). Glutamate induces a rapid but reversible loss of Akt (Ser473) phosphorylation and activity through activation of protein phosphatases (Chalecka-Franaszek & Chuang, 1999). Phosphorylation of CREB, and therefore its activity, is decreased via an NMDA receptor-dependent mechanism by toxic concentrations of glutamate. Long-term lithium treatment suppresses the decrease in phosphorylation, while acute lithium has no effect (Chuang, 2004).

2.3.2.2.3 *In Vitro* Data

It has been found that the metabolism of catecholamines and indoleamines by MAO is a major source of hydrogen peroxide in nerve cells. Hydrogen peroxide, in turn, is a major source of

free radicals. In § 2.3.2.1 it was stated that glutamate induces oxidative toxicity apart from receptor-mediated excitotoxicity. Therefore, it is hypothesised that by blocking monoamine uptake into the cell, it may be possible to reduce the levels of intracellular free radicals, thereby reducing the effect of glutamate toxicity. A mouse hippocampal (HT-22) cell line as well as a primary rat cortical culture was employed to investigate this hypothesis.

The HT-22 cells and the primary cortical culture were exposed to either clomipramine (30 µM and 10 µM, respectively) or imipramine (75 µM and 25 µM, respectively), both in combination with 5 mM glutamate, for 24 hours. The MTT cell proliferation assay was performed directly after the pretreatment period and the results demonstrate that clomipramine and imipramine protects significantly against glutamate toxicity in both cases. Therefore, it can be seen that drugs that inhibit monoamine uptake may protect against glutamate toxicity (Maher & Davis, 1996).

A study by Post and colleagues (2000) produced some unexpected results. The effect of a 24-hour exposure to amitriptyline, desipramine, fluoxetine and paroxetine on hippocampal cell viability was investigated. Mouse hippocampal (HT-22) cells were exposed to different concentrations of these drugs whereafter the MTT cell proliferation assay was performed to determine the cell viability. TUNEL staining was also performed to determine the presence of apoptosis.

Concentrations of 50 µM or higher amitriptyline caused a significant decrease in cell viability as compared to untreated control cells, while lower concentrations had no significant effect. The same trend was observed with desipramine, although 20 µM desipramine already caused a decrease in cell viability. Concentrations of 20 µM or higher of both SSRIs (fluoxetine and paroxetine) resulted in a statistically significant decrease in cell viability, while lower concentrations had no significant effect. It was also found that 50 µM amitriptyline, 20 µM and 50 µM desipramine, 20 µM and 50 µM fluoxetine, or 20 µM and 50 µM paroxetine resulted in a significant increase in the number of apoptotic cells as compared to untreated control cells. The increase in apoptotic cells was concentration-dependent. This suggests that antidepressants may induce neurodegeneration *in vitro* partly by inducing apoptosis (Post *et al.*, 2000).

These results contrast with the results obtained by Maher and Davis (1996), but the authors suggest that the particular cellular environment at any given time plays a role in determining neurotoxic vs. neuroprotective effects of antidepressants (Post *et al.*, 2000).

2.3.3 Stress and Cortisol

It is known that stress influences a wide array of neuronal systems and in the acute phase results in beneficial endocrine and behavioural responses, but repeated or severe stress or increased vulnerability due to genetic factors can lead to adverse effects on neuronal function. Exposure to stress or activation of the hypothalamo-pituitary-adrenal (HPA) axis and elevation of glucocorticoids are reported to damage hippocampal neurons (Duman *et al.*, 1999). Repeated stress paradigms have been shown to be reliable models of depression in rodents (Wilner, 1997) and tree shrews (Fuchs *et al.*, 1996).

Stressful life events are among the most potent factors known to trigger or induce depression (Kendler *et al.*, 1999, Paykel, 2001). Approximately 50% of all acutely depressed patients have elevated cortisol levels and once the depression is remitted, cortisol levels decrease to normal levels (Hoschl & Hajek, 2001).

When the hippocampus (or other brain regions) is exposed to stressful stimuli it can contribute to the emotional, cognitive and vegetative abnormalities found in depressed patients. The hippocampus is also involved in the feedback regulation of the HPA axis since it expresses a high level of adrenal steroid receptors (Jacobson & Sapolsky, 1991; McEwen, 1999), the dysfunction of which is associated with depression (Young *et al.*, 1991). Prolonged and severe stress may disrupt the negative feedback control of the hippocampus on the HPA axis (McEwen & Chattarji, 2004).

Studies suggest that stress-induced atrophy and loss of hippocampal neurons may contribute to the pathophysiology of depression (Yamada *et al.*, 2005). Animal studies demonstrated that stress and stress hormones caused significant dendritic remodelling in CA3 pyramidal neurons. Dendritic remodelling in these neurons is mediated by mechanisms involving high levels of glucocorticoid secretion and is characterised by a reversible shortening and debranching of apical dendrites (Watanabe *et al.*, 1992; Magariños & McEwen, 1995; McEwen, 1999). Dendritic remodelling was observed in the psychosocially stressed tree shrew (Magariños *et al.*, 1996) and a stress-induced reduction in total hippocampal volume has also been observed (Czéh *et al.*, 2001). It has also been reported in animal models that during acute stress the hippocampal CA3 dendrites atrophy, but they also recover as stress subsides (Hoschl & Hajek, 2001; McEwen, 2003; Brown *et al.*, 2004).

The hypothalamus secretes corticotropin-releasing factor (CRF) in response to stress. CRF stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary which, in turn, stimulates the release of glucocorticoids from the adrenal cortex (Liberzohn *et al.*, 1997). When the stress is chronic, a down-regulation of glucocorticoid receptors occurs. The

hippocampus, a glucocorticoid and mineralocorticoid feedback site, is extremely sensitive to endogenous glucocorticoid levels. According to the neuroendocrine theory of major depression sustained hypercortisolism becomes toxic to the hippocampus, thereby preventing its ongoing neurogenesis (Sheline *et al.*, 1996; Hoschl & Hajek, 2001; Mizoguchi *et al.*, 2003).

Adult hippocampal neurogenesis is regulated by a variety of pharmacological, internal and environmental signals (Fuchs & Gould, 2000; Eisch, 2002). Exposure to both acute and chronic stress can suppress the proliferation and survival of the adult-generated granule neurons (Gould *et al.*, 1997, 1998; Czéh *et al.*, 2001, 2002; Pham *et al.*, 2003; Heine *et al.*, 2004a). Chronic psychosocial stress has been found to decrease total hippocampal volume, while the volume reduction was prevented by antidepressant treatment with tianeptine (Czéh *et al.*, 2001) or clomipramine (Van der Hart *et al.*, 2002).

CA3 pyramidal neurons in the hippocampus are particularly susceptible to stress and adrenal glucocorticoids (Figure 2-5). The adverse effects of chronic exposure to stress can be divided into three areas, namely atrophy, death and neuroendangerment of CA3 neurons. Exposure to repeated restraint stress leads to atrophy of CA3 neurons in both rodents and nonhuman primates. Administration of glucocorticoids at a dose that approximates levels that are induced by stress, gives rise to the same effect. Atrophy is demonstrated by a decrease in the number and length of branch points of the apical dendrites of CA3 neurons. Severe and chronic stress or glucocorticoid administration has been reported to result in death of the CA3 neurons. Neuroendangerment of CA3 neurons by exposure to stress have been demonstrated. Damage of CA3 neurons caused by other neuronal insults, such as hypoglycaemia, hypoxia or excitotoxia, is reportedly exacerbated by acute exposure to stress or glucocorticoids (Duman *et al.*, 1999).

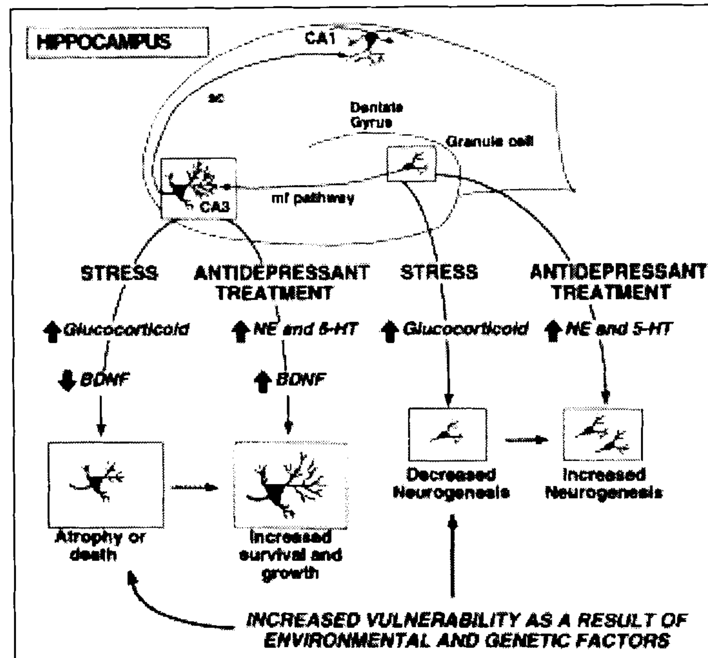


Figure 2-5: Representation of the effects of stress and antidepressant treatment on hippocampal neurogenesis (Duman *et al.*, 1999).

Studies concerning the effects of stress and glucocorticoids on neuronal atrophy and survival have identified three major effects, namely uptake and metabolism of glucose, increased glutamate and Ca^{2+} excitotoxicity and downregulation of neurotrophic factors (Duman *et al.*, 1999). Increased glutamate and Ca^{2+} excitotoxicity is thought to be involved in the actions of stress and glucocorticoids. Exposure to stress or glucocorticoid administration has been shown to increase levels of glutamate in extracellular dialysate in the hippocampus (Sapolsky, 1996a). Glutamate acts via NMDA and non-NMDA ionotropic receptors, thereby increasing the extracellular levels of Ca^{2+}. It has been established that sustained activation of glutamate-induced Ca^{2+} underlies the excitotoxic effects of repeated seizures and ischaemia. Taking these observations into account, enhanced glutamate release may contribute to glucocorticoid-induced neuroendangerment (Duman *et al.*, 1999).

Stressors may activate necrotic and apoptotic pathways as well as decreasing the expression of BDNF (Smith *et al.*, 1995), probably, in part, by affecting the function of the CREB signalling cascade (see § 2.3.4; Duman *et al.*, 1997). Withdrawal of neurotrophic support will trigger apoptosis, probably via the PI 3-kinase/Akt and the Ras/MAP kinase pathways (Yuan & Yankner, 2000). Subsequently, the CREB signalling pathway will be discussed in further detail.

2.3.4 The cAMP/CREB/BDNF Signalling Pathway

The involvement of the cAMP/CREB/BDNF cascade in neuroplasticity is depicted in Figure 2-6 and is discussed below:

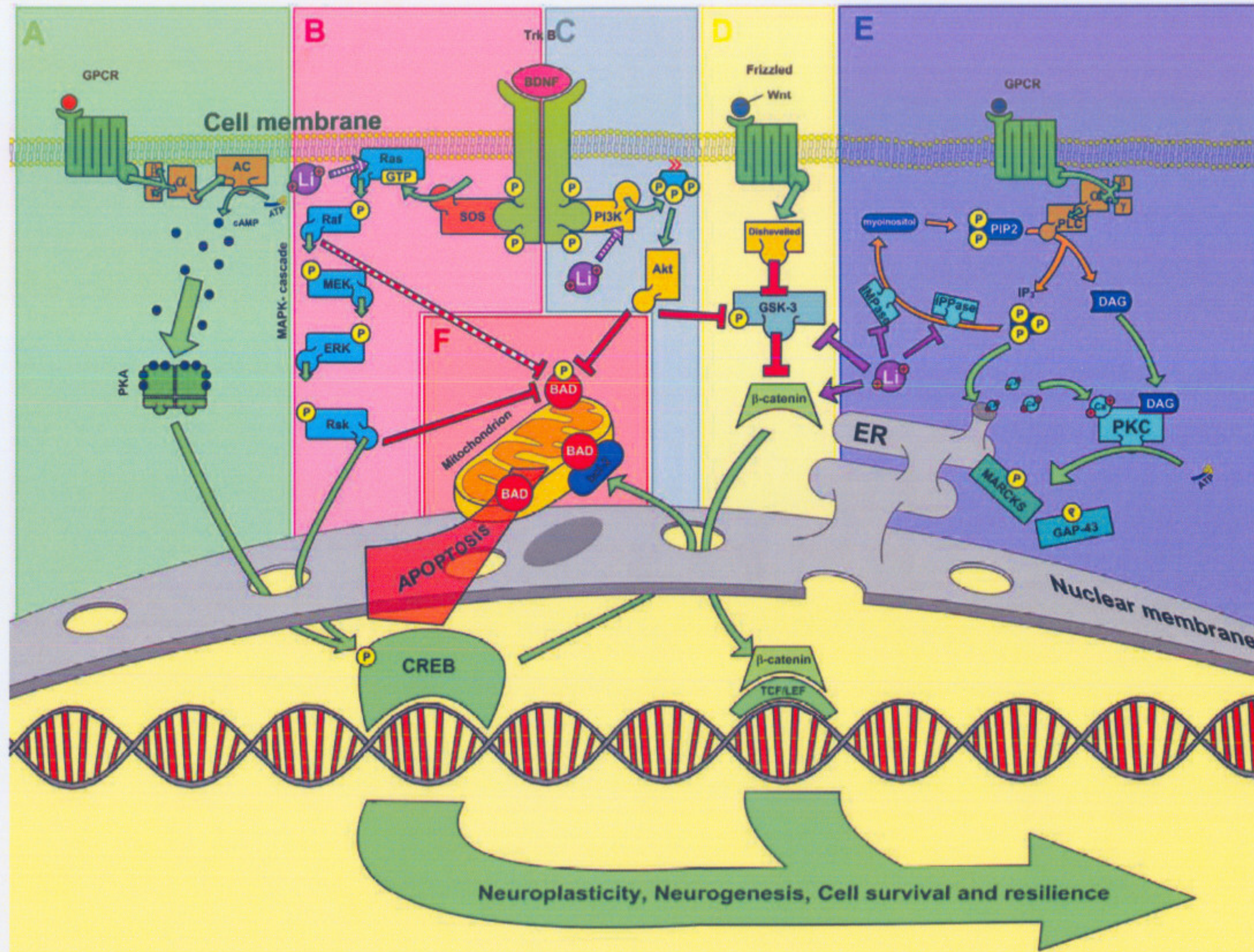


Figure 2-6: Representation of the signalling cascades involved in neural plasticity. (A) Adenylate cyclase (AC) signalling pathway. (B) MAP/ERK pathway. (C) Phosphatidylinositol 3-kinase (PI 3-kinase) pathway. (D) Glycogen synthase kinase 3 (GSK-3) and Wnt pathway. (E) Phosphoinositol and proteinkinase C (PKC) pathway. (F) Apoptosis (Yuan *et al.*, 2004).

Originally, neurotrophic factors, including BDNF, nerve growth factor and neurotrophin-3, were characterised for their actions during the development and maturation of neurons. These factors are also expressed in the adult brain and influence the survival and function of mature neurons. The expression of neurotrophic factors is regulated by various stimuli, such as stress and psychotropic drugs (Duman, 2002).

2.3.4.1 CREB

Several studies suggest that the cAMP signalling system is involved in the mechanism of action of antidepressants (Nestler *et al.*, 2002; D'Sa & Duman, 2002). Increased cAMP signalling phosphorylates and activates CREB, which, in turn, stimulates the transcription of cAMP responsive genes (Shaywitz & Greenberg, 1999; Figure 2-6 A). Antidepressants cause an induction of CREB mRNA, while expression of CREB in the hippocampus produces a similar behavioural response to that of antidepressant drugs (D'Sa & Duman, 2002). CREB activates the production of BDNF and is considered to play a pivotal role in mediating the antidepressant-induced increase in BDNF mRNA (D'Sa & Duman, 2002; Nestler *et al.*, 2002). No upregulation in BDNF mRNA levels are observed in CREB knockout mice (Conti *et al.*, 2002). CREB and BDNF form a positive feedback loop that may be essential in the trophic effects of antidepressants (Castrén, 2004). CREB activates BDNF production and BDNF, in turn, induces CREB phosphorylation and therefore, activation (Shaywitz & Greenberg, 1999; Saarelainen *et al.*, 2003; Figure 2-6 B).

Phosphorylated CREB may be implicated in antidepressant-induced neurogenesis and is localised to newborn neurons in the hippocampus (Nakagawa *et al.*, 2002a, 2002b). In a study examining the role of neurotrophins and trkB activation in antidepressant-induced neuronal proliferation in the hippocampus, it was observed that transgenic mice with reduced trkB signalling capacity showed a similar antidepressant-induced increase in stem cell proliferation than wild-type control mice. Three weeks later the survival of neurons was reduced in transgenic mice and no significant antidepressant-like effect could be observed. From this data it can be deduced that while neurotrophins seem to play no major role in antidepressant-induced stem cell proliferation, they are required for the long-term survival of newborn neurons (Sairanen & Castrén, 2003; Castrén, 2004).

2.3.4.2 BDNF

Synaptic remodelling and synaptogenesis are largely influenced by neurotrophic factors, including BDNF (Patapoutian & Reichardt, 2001). BDNF exerts its neurotrophic action and neuroprotective effect through a cascade composed of tyrosine kinase (Trk) receptors, the MAP

kinase signalling pathway (Figure 2-6 B) and activation of bcl-2 expression. Apoptosis plays an important role in the cell death observed in acute and chronic neurodegenerative diseases. It is controlled by the levels of proapoptotic (Bax and Bad; Figure 2-6 F) and antiapoptotic (bcl-2 and bcl-xL) proteins within the cell (Yuan & Yanker, 2000). The neuroprotective effect of BDNF may be mainly explained as an inhibition of cell death cascades (Fossati *et al.*, 2004).

Dysfunction of the cAMP/CREB signalling pathway may underlie the stress-induced downregulation of BDNF (Dowlatshahi *et al.*, 1998). Exposure to stress causes a significant downregulation in the expression of BDNF in the hippocampus of Sprague Dawley rats (Smith *et al.*, 1995). This is observed in the dentate gyrus, CA1 and CA3 pyramidal cell layers, after acute or chronic stress. The downregulation of BDNF may possibly contribute to the atrophy of CA3 neurons and reduced neurogenesis of granule cells in the hippocampus, but elevated levels of glucocorticoids could also account for these effects (Duman, 2002). In contrast, chronic administration of antidepressants has been shown to increase the expression of BDNF in the hippocampus and frontal cortex (Duman *et al.*, 1997; Duman *et al.*, 2000). Behavioural studies support the idea that upregulation of BDNF contributes to the therapeutic action of antidepressant drugs (Duman, 2002). An antidepressant effect in the forced swim test and learned helplessness models is observed after chronic infusion of BDNF into the midbrain (Siuciak *et al.*, 1997), while even a single infusion of BDNF into the hippocampus produces a potent and long-lasting antidepressant effect in these models (Shirayama *et al.*, 2000).

Several studies suggest that BDNF and its receptor, trk B, play a vital role in the mechanism of action of antidepressants (D'Sa & Duman, 2002; Nestler *et al.*, 2002; Popoli *et al.*, 2002). BDNF mRNA levels (Nibuya *et al.*, 1995; Russo-Neustadt *et al.*, 2000; Coppell *et al.*, 2003; Dias *et al.*, 2003; Ivy *et al.*, 2003) and protein (Altar *et al.*, 2003; Xu *et al.*, 2003) in rodent hippocampus and cortex are upregulated by means of chronic antidepressant administration and electroconvulsive therapy (ECT). The expression (Nibuya *et al.*, 1995) and activation of BDNF receptor trkB in rodent hippocampus and prefrontal cortex is induced by antidepressants (Saarelainen *et al.*, 2003). A normal behavioural reaction in response to antidepressants was observed in wild-type mice, while no behavioural changes were found in transgenic mice with reduced BDNF levels or reduced trkB signalling (Saarelainen *et al.*, 2003). These observations demonstrate that BDNF release and trkB signalling is required for antidepressant-like behavioural effects (Castrén, 2004).

The changes in BDNF signalling induced by antidepressants are relatively quick (Castrén, 2004). Within 30 minutes of a single antidepressant injection, increased trkB autophosphorylation in mouse prefrontal cortex is observed (Saarelainen *et al.*, 2003). ECT causes an almost immediate increase in BDNF mRNA levels in the hippocampus (Nibuya *et al.*,

1995), while daily antidepressant administration causes an increase within two days (Russo-Neustadt *et al.*, 2000). Antidepressant-like effects are observed within three days after a single intrahippocampal injection of BDNF (Shirayama *et al.*, 2002). ECT has been shown to induce sprouting in the mossy fibre pathway in normal rodent hippocampus (Vaidya *et al.*, 1999; Lamont *et al.*, 2001). This effect is dependent upon the expression of BDNF, while BDNF alone did not produce a similar effect (Vaidya *et al.*, 1999).

Neurotrophins are not only implicated in the mechanism of action of antidepressant drugs, but also in the pathophysiology of mood disorders (Castrén, 2004). Reduced BDNF mRNA levels are observed in rodent hippocampus following chronic stress or early maternal deprivation and this effect may be counteracted by antidepressants (Smith *et al.*, 1995; Roceri *et al.*, 2002). A Val66Met polymorphism in the BDNF coding region, which has been shown to influence activity-induced BDNF release (Egan *et al.*, 2003), has been linked to familial bipolar disorder (Neves-Pereira *et al.*, 2002; Sklar *et al.*, 2002), anxiety, obsessive-compulsive disorder and anorexia (Hall *et al.*, 2003; Ribases *et al.*, 2003; Sen *et al.*, 2003). The Met66 allele reduces BDNF release and has, paradoxically, been reported to be protective (Neves-Pereira *et al.*, 2002; Sklar *et al.*, 2002; Hall *et al.*, 2003; Sen *et al.*, 2003). This indicates that activity-dependent BDNF release may predispose to mood disorders and anxiety (Castrén, 2004). Increased levels of BDNF have also been observed in human brain after antidepressant treatment (Chen *et al.*, 2001).

Taking the abovementioned data into consideration, it seems that the cAMP/CREB/BDNF pathway may be implicated in the pathophysiology and treatment of depression.

2.4 Synopsis

Depression is a debilitating mood disorder and although several antidepressants are used for its treatment, certain problems accompany current antidepressant treatment. These involve, among others, side effects and resistance to treatment. Furthermore, the exact underlying pathophysiology of depression is still unknown. Several recent studies implicate a role for neurodegeneration in the pathophysiology of depression. High doses of oral *m*Ins have been found to be effective in the treatment of depression. However, the mechanism whereby *m*Ins exerts its therapeutic effect has not yet been elucidated. It has not been established whether *m*Ins possesses any neuroprotective properties, thereby potentially reversing neurodegeneration thought to be associated with depression. It is believed that a better understanding of the mechanism of action of *m*Ins may contribute to the understanding of the pathophysiological basis of depression. Understanding the underlying basis for depression may, in turn, result in the development of more effective antidepressants with fewer side effects.

Chapter 3: Experimental Procedures

3.1 Introduction

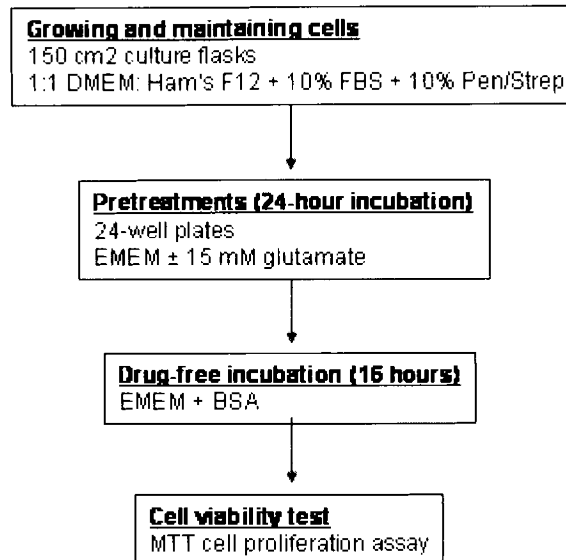
The primary objective of this study was to investigate whether *myo*-inositol (*m*Ins), in comparison with various prototype experimental and clinical antidepressants, display any *in vitro* protective effects against glutamate-induced excitotoxicity. For this purpose a neuronal, human neuroblastoma cell line [SK-N-BE(2)], as well as a non-neuronal Chinese Hamster Ovary cell line (CHO-K1) were selected. The cells were pretreated for 24 hours with different concentrations of either *m*Ins, one of a series of prototype antidepressants, or a combination of *m*Ins and a prototype antidepressant, all with or without 15 mM glutamate. Thereafter mitochondrial activity was determined using the MTT cell proliferation assay or DNA integrity was evaluated by means of an electrophoresis and DNA fragmentation quantification (comet) assay.

In this chapter, the materials, the experimental design, the biological model (i.e. cell line used) and the various assays used in the experiments are explained and discussed.

3.2 Experimental Layout

A rough schematic representation of the basic experimental layout is provided below.

Phase I: Development of a model for glutamate-induced excitotoxicity



Phase II: Study objective experiments

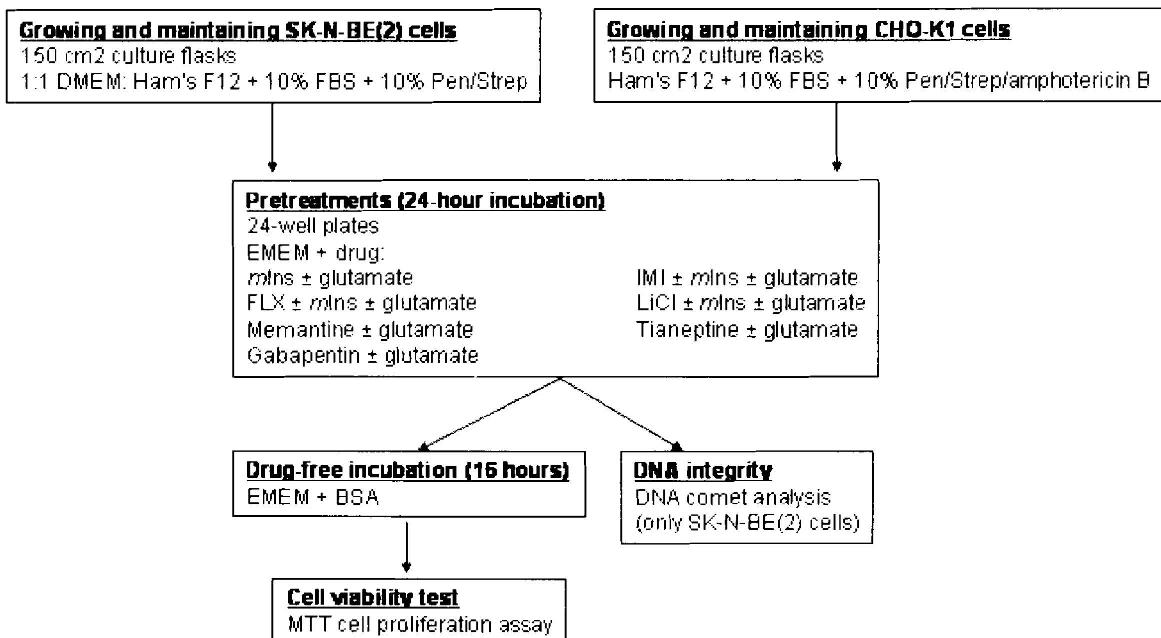


Figure 3-1: A schematic representation of the experimental design. DMEM = Dulbecco's Modified Eagles Medium; FBS = Foetal Bovine Serum; Pen = Penicillin; Strep = Streptomycin; EMEM = Minimum Essential Medium with Earle's Base; *m*Ins = *myo*-inositol; IMI = imipramine; FLX = fluoxetine; LiCl = lithium chloride.

3.3 Cell Lines Employed

For this study a human neuroblastoma cell line [the SK-N-BE(2) cell line from American Type Culture Collection (ATCC) – catalogue number CRL-2271] was used. This cell line was established in November 1972 from a bone marrow biopsy taken from a 2-year-old male with disseminated neuroblastoma after repeated courses of chemotherapy and radiotherapy. The well established and described SK-N-BE(2) cell line has been selected for this study because it is a neuronal cell line and therefore a putatively suitable biological model of cells of the central nervous system, where *m*Ins is expected to exert its psychotropic effects.

SK-N-BE(2) cells were maintained in 1:1 DMEM:Ham's F12, 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin in culture flasks at 37°C in 5% CO_2. Under these conditions the cells attached as a monolayer on culture flask surfaces and had a duplication time of approximately 30 hours. The medium was renewed every two to three days. At ± 95% confluency, cells were detached from the flask bottom by means of trypsination (10 minutes incubation with trypsin/versine) and seeded in new flasks at a density of no less than $^1/_6{}^{th}$ of confluent. One 95% confluent 150 cm^2 culture flask delivered sufficient cells for seeding ± 5 x 10^6 cells/well into three 24-well plates for cell treatment and assays (as described in § 3.5.2).

Chinese Hamster Ovary cells [the (CHO-K1) cell line from American Type Culture Collection (ATCC) – catalogue number CCL-61] were used to determine whether the effects observed in the human neuroblastoma cell line are also observed in a non-neuronal cell line, or whether it is specific to neuronal cells. This well established and described cell line was derived as a subclone from the parental CHO cell line obtained from a biopsy of an ovary of an adult Chinese hamster in 1957.

CHO-K1 cells were maintained in Ham's F12 medium, 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B in culture flasks at 37°C in 5% CO_2. Under these conditions the cells attached as a monolayer on culture flask surfaces and had a duplication time of approximately 24 hours. The medium was renewed every four to five days. At ± 95% confluency, cells were detached from the flask bottom by means of trypsination (10 minutes incubation with trypsin/versine) and seeded in new flasks. One 95% confluent 150 cm^2 culture flask delivered sufficient cells for seeding ± 4 x 10^6 cells/well into three 24-well plates for cell treatment and assays (as described in § 3.5.2).

3.4 Materials

3.4.1 Chemicals

3.4.1.1 Chemicals Used for Cell Cultures

- From Bio-Whittaker (Walkersville, MD, U.S.A.):
  Dulbecco's Modified Eagles Medium (DMEM):Ham's F12, Ham's F12 medium, foetal bovine serum (FBS), penicillin/streptomycin mixture, penicillin/streptomycin/amphotericin B mixture.

- From Highveld Biologicals (Johannesburg, South Africa):
  Minimum Essential Medium with Earle's Base (EMEM), bovine serum albumin (BSA), ciprofloxacin.

- From Scientific Group (Midrand, South Africa) (Gibco):
  Trypsin/versine (0.25% 1:250 + 0.38% EDTA).

- From Afrox (Johannesburg, South Africa):
  Liquid nitrogen (N_2).

3.4.1.2 Chemicals Used for Assays

- From Sigma Aldrich (Missouri, U.S.A.):
  myo-inositol (*m*Ins), imipramine (IMI), lithium chloride (LiCl), L-glutamic acid sodium salt hydrate (glutamate), memantine, thiazolyl blue tetrazolium bromide (MTT) ($C_{18}H_{16}N_5SBr$) reagent, Isopropanol (99+%) (C_3H_8O), Triton X100, Tris HCl, Trizma base.

- From Saarchem, Unilab (Krugersdorp, Gauteng, South Africa):
  Formic acid.

- From Merck (Johannesburg, South Africa/Damstadt, West Germany):
  Dimethyl sulfoxide (DMSO), NaCl, KCl, NaOH, Na_2HPO_4, KH_2PO_4.

- From MP Biomedicals (Eschwege, Germany):
  Ethidium bromide.

- From Eli Lilly (Johannesburg, South Africa):
  Fluoxetine hydrochloride (FLX) was a kind gift from Eli Lilly.

- From Servier (Neuilly sur Seine, France):
  Tianeptine.

- From Seperations (Eschwege, Germany):
  High melting point agarose (HMPA).

- From Whitehead Scientific (Johannesburg, South Africa):
  Low melting point agarose (LMPA).

3.4.2 Consumables

- From Corning (New York, U.S.A.):
  Culture flasks (25 cm^2 and 150 cm^2), 24-well plates, 96-well plates, 15 ml and 50 ml sterile conical tubes.

3.4.3 Instruments and Software

The following instruments were employed in the experiments as indicated.

- Haemocytometer (0.1 mm depth, 0.0025 cm^2) and Nikon TMS inverted microscope (model number: 31771) were used for cell counting.
- Eppendorf quantitative micropipettes (0.1-2.5 µl, 2-20 µl, 10-100 µl and 100-1000 µl) were used for analytical measurements.
- A 96-well plate reader with 560 nm filter (Labsystems Multiscan RC) was used for spectroscopic measurement of the MTT formazan product.
- Bench top centrifuge 3K15 (Sigma Aldrich, Johannesburg, South Africa).
- PowerPac 200 (from BioRad) was used for electrophoresis during the DNA comet assay.
- Olympus IX70 inverted system microscope was used for the visualisation of DNA comets.
- The analySIS® software program was used to capture the images of DNA comets visualised with the microscope.
- QuantityOne® software (from BioRad) was used for quantification of DNA comets captured during visualisation.

3.5 Experiments

3.5.1 Seeding of Cells in 24-well Plates

The following protocol was used to seed SK-N-BE(2) cells grown in 150 cm^2 flasks into 24-well plates:

- For experiments, cells were grown and cultured in 150 cm^2 culture flasks with normal culture medium (DMEM:Ham's F12 + 10% FBS for SK-N-BE(2) cells or Ham's F12 + 10% FBS for CHO-K1 cells) until 95% confluency (see § 3.3).
- Cells were detached from the flask bottom with trysin/versine and seeded with normal culture medium in 24-well plates at a density of either 5 x 10^6 [SK-N-BE(2)] or 4 x 10^6 (CHO-K1) cells per well, following cell counting (see § 3.5.3.1).
- For each experimental condition, three separate wells were seeded, allowing triplicate observations per condition per single experiment.
- Cells were then incubated for either 27 hours [SK-N-BE(2)] or 6 hours (CHO-K1) at 37°C in 5% CO_2 to allow cells to attach to the well bottoms.
- After incubation, cells were exposed to different pretreatment regimes for 24 hours (see § 3.5.2.2).
- At all times, whatever the procedure, all wells underwent the same rinsing and exposure steps to ensure comparable results at the time of analysis.

3.5.2 Drug Pretreatments

3.5.2.1 Introduction

As indicated in the experimental layout (see Figure 3-1), the pretreatment involved a 24-hour incubation of the cells with different concentrations of either *m*Ins, one of a series of prototype antidepressants, or a combination of *m*Ins and a prototype antidepressant, all with or without 15 mM glutamate. The aim of this pretreatment was to mimic the effect of chronic treatment of humans with these drugs. Most cell lines used for cell culture are of a cancerous type, with the growth medium supplemented with FBS, so that the duplication time (30 hours) of these cells is much faster than *in vivo*. This suggests that a 24-hour incubation period might be sufficient to mimic chronic treatment *in vivo* (Viljoen, 2002).

Several clinical studies have demonstrated that *m*Ins may have therapeutic value in the treatment of depression (Levine, 1997; Levine *et al.*, 1993a, 1995a), depression associated with post-traumatic stress disorder (Kaplan *et al.*, 1996), panic disorder (Benjamin *et al.*, 1995) and obsessive-compulsive disorder (OCD) (Levine *et al.*, 1993b, 1994). However, the mechanism by which *m*Ins exerts its therapeutic effects remains elusive. While putative neuroprotective effects of antidepressants are currently being advocated as potentially important in their therapeutic efficacy (see § 2.3) it was decided to investigate the possible effects of *m*Ins on markers of cellular resilience (as a potential contributing mechanism of action) in the SK-N-BE(2) human neuroblastoma cells.

3.5.2.2 Pretreatment Layout

SK-N-BE(2) cells were pretreated for 24 hours at 37°C in 5% CO_2 with one of the following drug series in serum-free EMEM, enriched with 0.1 mM *m*Ins (normal concentration for growth medium – see § 3.5.2.3). However, no additional *m*Ins was added when the effect of *m*Ins deprivation was investigated.

Table 3-1: Drugs and the concentrations used in pretreatment. *m*Ins = *myo*-inositol; IMI = imipramine; FLX = fluoxetine; LiCl = lithium chloride.

Drug	Lower Concentration	Highest Concentration	Additional Interventions
*m*Ins deprivation	0.01 mM		± 15 mM glutamate
Control (no drug)	0.1 mM		± 15 mM glutamate
*m*Ins	1 mM	10 mM	± 15 mM glutamate
IMI	71 nM	10 μM	± 15 mM glutamate
FLX	97 nM	10 μM	± 15 mM glutamate
LiCl	2 mM or 5 mM	10 mM	± 15 mM glutamate
Memantine	0.46 μM	10 μM	± 15 mM glutamate
Tianeptine	1 μM	10 μM	± 15 mM glutamate
Gabapentin	0.23 μM	10 μM	± 15 mM glutamate
IMI + *m*Ins	71 nM + 10 mM	10 μM + 10 mM	± 15 mM glutamate
FLX + *m*Ins	97 nM + 10 mM	10 μM + 10 mM	± 15 mM glutamate
LiCl + *m*Ins	2 mM or 5 mM + 10 mM	10 μM + 10 mM	± 15 mM glutamate

3.5.2.3 Concentrations Used in Pretreatment

***myo*-Inositol concentrations**

DMEM:Ham's F12 1:1 mixture contains 12.51 mg/l *m*Ins (0.07 mM), while Ham's F12 medium contains 18 mg/l *m*Ins (0.1 mM). This concentration range is considered a "normal" concentration range for *m*Ins in culture medium. Therefore all pretreatments (regardless of the drug tested) were supplemented with 0.1 mM *m*Ins, except where *m*Ins deprivation was simulated.

Imipramine and fluoxetine concentrations

Both imipramine and fluoxetine have previously been used at a concentration of 10 μM for *in vitro* studies (Lightowler *et al.*, 1996; Willets *et al.*, 1996; Viljoen, 2002; De Kock, 2003; Brink *et al.*, 2004). Their respective maximal effective plasma concentrations in humans were obtained from appropriate literature and the free plasma concentration was subsequently calculated from the reported fraction bound to plasma protein.

Imipramine has a maximal effective plasma concentration of 200 ng/ml and is 90.1% bound to plasma protein (Thummel & Shen, 2001). The maximal effective free plasma concentration was calculated as 9.9% of 200 ng/ml ≈ 20 ng/ml (i.e. 71 nM). Fluoxetine, on the other hand, has a

maximal effective plasma concentration of 500 ng/ml and is 94% bound to plasma protein (Thummel & Shen, 2001). Similarly, the free plasma concentration was calculated as 6% of 500 ng/ml = 30 ng/ml (i.e. 97 nM). Therefore, concentrations of 71 nM and 10 µM imipramine and 97 nM and 10 µM fluoxetine were used.

Lithium chloride concentrations

Lithium has a maximal effective concentration of 2 mM and is not bound to plasma protein (Thummel & Shen, 2001). Previous *in vitro* studies have demonstrated that 5 mM LiCl is appropriate to pretreat cell cultures (Wachira *et al.*, 1998). Taking the small therapeutic index of lithium into consideration, and following from studies on cell cultures suggesting toxicity of concentrations slightly higher that the optimal, a concentration of 10 mM LiCl was selected as the highest concentration.

Memantine concentrations

Doses of 20 mg memantine per day, rendered steady-state concentrations ranging from 70 – 150 ng/ml in humans, with large variations between individuals. Memantine has been found to be 45% bound to plasma protein (RxList Inc., 2004). The free plasma concentration was subsequently calculated as 55% of 150 ng/ml = 82.5 ng/ml (i.e. 0.46 µM). While appropriate *in vitro* concentration ranges are commonly different from *in vivo*, a higher concentration of 10 µM memantine was selected randomly.

Tianeptine concentrations

Plaisant and colleagues (2003) have treated cortical cell cultures with 1, 10 and 100 µM tianeptine. No significant difference in mitochondrial activity was found after SK-N-BE(2) cells were pretreated with either 10 µM or 100 µM (results not shown), therefore it was decided to use 1 µM and 10 µM tianeptine respectively.

Gabapentin concentrations

Gabapentin is less than 3% bound to plasma protein with a maximal effective concentration of 4 µg/ml (Thummel & Shen, 2001). Since the 3% gabapentin bound to plasma protein was considered negligibly small, the concentration employed in the current project was taken as 100% of the maximal effective concentration, i.e. 0.23 µM. The higher concentration of 10 µM gabapentin was selected randomly.

3.5.3 Assays

3.5.3.1 Cell Counting and Seeding Assay

The following protocol was used for counting cells before seeding them into 24-well plates:

- DMEM:Ham's F12 medium [SK-N-BE(2)] or Ham's F12 medium (CHO-K1) was aspirated from the 150 cm^2 culture flasks.
- Trypsin/versine was added to each flask and incubated for 10 minutes until the cells were detached from the flask bottoms.
- The cell suspension was pipetted up and down to ensure a homogenous cell suspension and to inhibit the formation of cell clusters.
- A sample (20 µl) of the cell suspension was diluted in a 1:10 ratio, whereafter 20 µl of the diluted suspension was transferred to the haemocytometer.
- The number of cells per ml suspension was determined.
- The dilution factor required for the experimental suspension was calculated.
- Cells were seeded at a density of either 5×10^6 [SK-N-BE(2)] or 4×10^6 (CHO-K1) cells/well by transferring exactly 1 ml (using a micropipette to ensure uniform cell density in all wells per experiment) to each well in a 24-well plate.
- Seeded cells were incubated for either 27 hours [SK-N-BE(2)] or 6 hours (CHO-K1) at 37°C in 5% CO_2 to allow cells to attach to the bottoms of the wells.

3.5.3.2 MTT Cell Proliferation Assay

3.5.3.2.1 Introduction

The aim of the MTT cell proliferation assay was to determine the effect of various pretreatment regimes (see § 3.5.2.2) on cell viability.

The MTT cell proliferation assay, first described by Mosmann in 1983, is based on the ability of mitochondria from metabolically active cells to cleave the tetrazolium rings of the pale yellow, water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in order to produce dark purple, water-insoluble formazan crystals. Since the formazan crystals are largely impermeable to cell membranes, the crystals that are formed accumulate within healthy cells. An organic solvent is added to produce a homogenous solution suitable for measurement (Mosmann, 1983). The colour intensity can then be quantified using a multi-well scanning spectrophotometer (ELISA reader).

The conversion of MTT to its coloured by-product is attained by means of a group of non-specific mitochondrial dehydrogenase enzymes, including NADH dehydrogenase, malate dehydrogenase and succinic dehydrogenase (Liu *et al.*, 1997). NADH or NADPH function as coenzymes to convert the MTT salt to formazan crystals. The aforementioned enzymes belong to the respiratory chain present in the mitochondria and are thus only active in metabolically active cells.

3.5.3.2.2 Assay

The following protocol describes the MTT cell proliferation assay employed after the cells were pretreated with the different treatment regimes:

- MTT[1] was dissolved in phosphate buffered saline (PBS) for a stock solution of 5 mg/ml and filtered to sterilise and remove a small amount of insoluble residue present in some batches of MTT.
- A volume of the MTT stock solution was diluted with sterile PBS in a 1:20 ratio to produce a working solution with a concentration of 0.25 mg/ml.
- After the cells were incubated for 16 hours with serum-free medium, the medium was aspirated and 200 μl MTT reagent was added to every well.
- The 24-well plates were then incubated for two hours at 37°C at 5% CO_2 to allow the reduction of the MTT reagent.
- After two hours of incubation, the MTT reagent was aspirated.
- The purple formazan crystals formed were then dissolved by adding 250 μl isopropanol to every well and incubating the 24-well plates at room temperature for 5 minutes.
- From every well's solution, 100 μl was transferred to a 96-well plate.
- The absorbance was determined at 560 nm by means of a multi-well scanning spectrophotometer (ELISA reader).
- These results were used to determine the percentage mitochondrial function after pretreatment, as compared to control cells.

[1] The MTT reagent is sensitive to light and should be prepared in a laminar flow chamber in the dark.

- Percentage mitochondrial function was calculated with the following equation:

$$\% \ mitochondrial \ function = 100 \times \frac{\chi - avg(lysis)}{avg(control) - avg(lysis)}$$

where: $\chi \rightarrow$ measured absorbance of the experimental well

avg(lysis) $\rightarrow$ average of the absorbance values of cells destroyed with formic acid

avg(control) $\rightarrow$ average of the absorbance values of the cells pretreated with 0.1 mM *m*Ins

3.5.3.3 Single Cell Gel Assay (Comet Assay)

3.5.3.3.1 Introduction

The aim of the comet assay was to determine whether glutamate can induce DNA damage (single- and double-stranded breaks) in SK-N-BE(2) cells, as well as whether *m*Ins and prototype antidepressants can protect against any glutamate-induced DNA damage.

The comet assay, or single cell gel assay, was first introduced by Östling and Johanson in 1984 as a microelectrophoretic technique for the direct visualisation of DNA damage in individual cells. The assay involves the suspension of a small number of cells in a thin layer of agarose gel on a microscope slide, followed by lysis of the cells, electrophoresis and staining with a fluorescent DNA binding dye. During electrophoresis, the electric current pulls the charged DNA from the nucleus, causing the relaxed and broken DNA fragments to migrate further than the core (head) of intact DNA. The resulting images, which were named for their appearance as "comets", are measured to determine the extent of DNA damage (Fairbairn *et al.*, 1995).

The most commonly used parameters are tail length, relative fluorescence intensity of head and tail (normally expressed as a percentage of DNA in the tail) and the tail moment. The comets are usually divided into five classes, ranging from class 0 (no tail) to class 4 (almost all DNA in tail; Collins, 2004).

3.5.3.3.2 Assay

Preparation of microscope slides

- The following solutions were prepared freshly for every experiment:
  - ◊ 0.1 M EDTA (37.2 g dissolved in 1 litre ddH$_2$O).
  - ◊ High melting point agarose (1% HMPA)[2] – 0.5 g dissolved in 50 ml EDTA.
  - ◊ Low melting point agarose (0.5% LMPA)[2] – 0.25 g dissolved in 50 ml EDTA.
- HMPA was heated in the microwave oven until the solution reached a temperature just below boiling point and the agarose was dissolved.
- Of the HMPA, 350 μl was pipetted onto the rough side of the microscope slide and distributed evenly across the surface, using the steel template (1.3 mm) and a warmed steel scraper.
- HMPA was allowed to air dry (± 15 minutes). Slides were left in a container with a moist atmosphere until use.
- LMPA was heated in the microwave oven until the solution reached a temperature just below boiling point and the agarose was dissolved. The agarose solution was cooled down and kept in a water bath at 37°C, until the loading of the cells as described below.

Detaching and preparing cells for assay

- The cells were detached from the 25 cm^2 culture flasks with trypsin/versine.
- The cell suspension from every culture flask was transferred to microcentrifuge tubes.
- The cells were centrifuged at 5,500 x g for 5 minutes, whereafter the trypsin/versine (supernatant) was aspirated and the cells were resuspended in 1 ml PBS.

Loading of monolayer cells in LMPA onto microscope slides

- Of the cell suspension, 20 μl was suspended in 150 μl LMPA at 37°C in a microcentrifuge tube.
- From this suspension, 150 μl was transferred onto the centre of a pre-coated slide (see preparation of microscope slides above) and spread evenly across the surface, using the steel template and warmed steel scraper.
- The slide with cell-loaded gel was placed on moist paper towels to cool down and set.

[2] Both HMPA and LMPA should be prepared fresh once a week.

Cell lysis

- The following solutions were prepared on a weekly basis:
  - ◊ 1 M NaOH (40 g dissolved in 1 litre ddH_2O).
  - ◊ Lysis buffer:
    - ♦ 2.5 M NaCl
    - ♦ 10 mM Trizma base
    - ♦ 0.1 M EDTA
- The mixture was stirred while adding 8 g NaOH and allowing the NaOH to dissolve (30 minutes). The pH was adjusted to 10, if necessary, and the volume was adjusted to 890 ml with ddH_2O.
- Subsequently, the following chemicals were added:
  - ◊ 1% Triton x100 (0.1 ml / 10 ml)
  - ◊ 10% DMSO (10 ml / 100 ml)
- The cells on the microscope slides were lysed overnight in the lysis buffer at 4°C, while protecting them from light (light is believed to cause DNA damage).

Electrophoresis

- For the electrophoresis buffer, the following reagents, freshly prepared, were mixed:
  - ◊ 0.3 M NaOH (pH > 13)
  - ◊ 1 mM EDTA
- The pH of the buffer was measured to ensure pH > 13.
- The slides were rinsed in ddH_2O and then incubated in the electrophoresis buffer for 30 minutes at 4°C.
- Afterwards the slides were subjected to electrophoresis for 20 minutes at 37 V and 400 mA, at 4°C.
- After electrophoresis, the slides were rinsed in ddH_2O.

Neutralisation and staining

- The following solutions were prepared every week:
  - ◊ Tris buffer: 0.4 M Tris HCl (pH = 7.5)
  - ◊ 10 μM Ethidium bromide (light-sensitive)
- Slides were incubated in the Tris buffer for 15 minutes at 4°C.
- After neutralisation, the slides were rinsed with ddH_2O and then incubated in the ethidium bromide solution[3] for 15 minutes at 4°C.
- After staining with ethidium bromide, the slides were once again rinsed with ddH_2O.

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[3] After staining with ethidium bromide, slides may be stored for 3 – 4 hours at 4°C in a damp atmosphere.

- The images of 50 cells were captured on the same day. DNA from cells was enlarged 60 times.
- Comets of the captured cells were analysed using QuantityOne® (BioRad).

3.6 Statistical Data Analysis

Data presented in Chapter 4 are averages of triplicate observations from at least three separate and independent experiments, expressed as a percentage of control unless otherwise specified. The computer software, Microsoft® Excel 2002 and GraphPad Prism® (version 4.01 for Windows®, GraphPad Software, San Diego, CA, U.S.A., www.graphpad.com) were used to process and analyse all experimental data. Graphs were constructed using GraphPad Prism®.

GraphPad Prism® was also used for the statistical analysis of data. All measurements in every experiment were considered replicates as inter-day variations were overcame by converting every control group to 100%. For multiple comparisons, the one-way ANOVA comparison was performed followed by the Dunnett post-test (for comparing groups to control). Statistical probability values of $p < 0.05$ were regarded as statistically significant.

Chapter 4: Results and Discussion

As was mentioned in Chapter 1, the primary purpose of the current study was to investigate the potential neuroprotective properties of *myo*-inositol (*m*Ins) and other clinical and experimental antidepressants in cultured human neuroblastoma cells. This investigation was based on the novel hypothesis that depression may be associated with neurodegeneration and that antidepressants may restore neuroplasticity. In particular, glutamate is believed to play a central role in the loss of neuroplasticity during stress, so that the study also investigated whether or not the different drug treatment regimes protect against glutamate-induced excitotoxicity. In the current chapter, the results of the experiments conducted, as described in Chapter 3, will be presented and discussed.

4.1 Control Experiment

A control experiment was performed in Phase I of the current project to establish appropriate *in vitro* treatment conditions for inducing sufficient excitotoxicity with glutamate in cultured human neuroblastoma cells, as measured by the standard MTT cell proliferation assay. This was necessary to demonstrate any neuroprotective effects of the test drugs against glutamate-induced excitotoxicity.

4.1.1 Development of a Model for Glutamate-induced Excitotoxicity

In order to develop a model for glutamate-induced excitotoxicity in human neuroblastoma cells, it was necessary to determine the concentration of glutamate and duration of pretreatment that causes a statistically significant decrease in cell viability without destroying all the cells. Reports of studies in literature suggest that incubation of cells with glutamate at concentrations of 1 – 10 mM for up to 24 hours induces significant cytotoxicity (Schelman, *et al.*, 2004). In addition, human neuroblastoma cells were typically pretreated for 24 hours with antidepressants in serum-free medium to induce pharmacologically relevant changes (Brink *et al.*, 2004). 24-hour pretreatments with glutamate (eventually to be implemented simultaneously with antidepressants), was therefore a reasonable starting point to determine appropriate treatment concentrations for glutamate under our experimental conditions. Figure 4-1 depicts the results obtained after 24-hour pretreatment with different concentrations of glutamate.

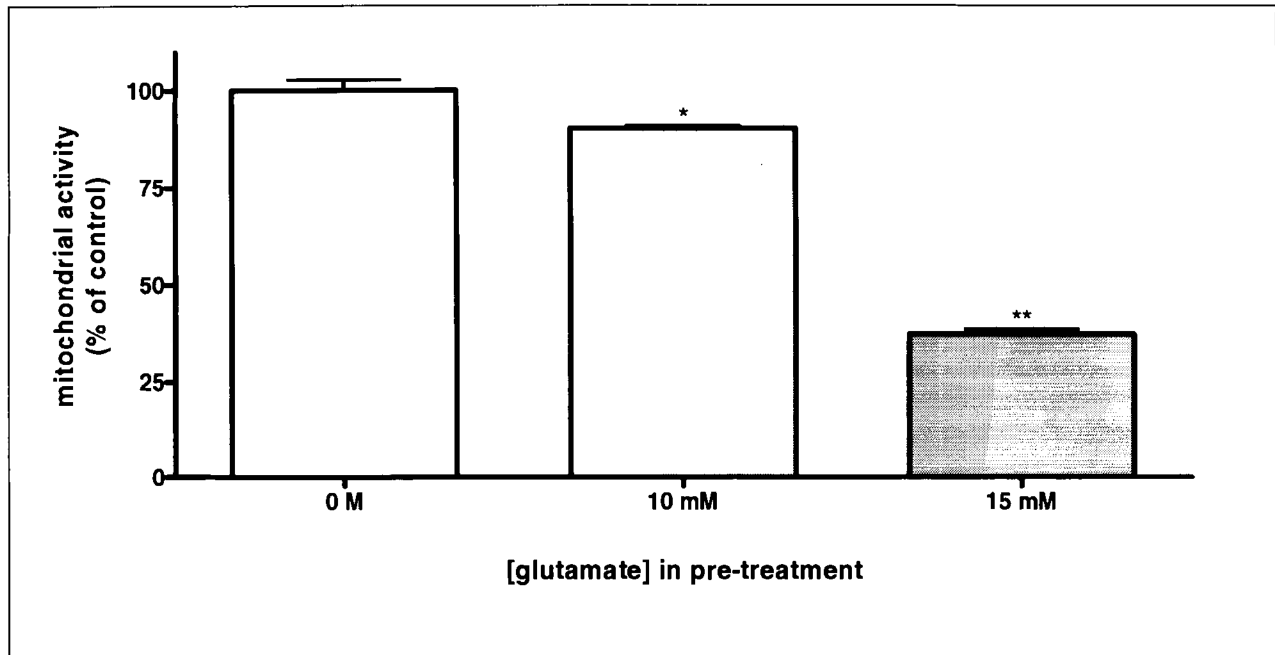

Figure 4-1: The modulating effect of 24-hour pretreatment with different concentrations of glutamate on mitochondrial activity as measured by the MTT cell proliferation assay, expressed as percentage of the control (i.e. 0 M glutamate). Data points represent averages ± standard error of the mean (S.E.M.) from three independent experiments, each with triplicate observations. Statistically significant differences between data were analysed by means of a one-way ANOVA, performing the Dunnett post-test, with * indicating $p < 0.05$ and ** indicating $p < 0.01$.

Although the mitochondrial activity reduced in statistically significant terms after pretreatment with 10 mM glutamate for 24 hours, the decrease was still relatively small (9.1 ± 0.7%; $p < 0.05$). It can be seen that incubation with 15 mM glutamate for 24 hours is sufficient to reduce the mitochondrial activity in statistically significant terms (62.8 ± 1.4%; $p < 0.01$), while some mitochondrial activity is retained. Therefore, 15 mM glutamate for 24 hours is a suitable treatment regime to induce excitotoxicity in human neuroblastoma SK-N-BE(2) cells and to evaluate any protective properties of antidepressants.

4.2 Study Objective Experiments

Study objective experiments were performed in Phase II of the current project, which includes all investigations into the effects of the test drugs on neuroplasticity.

Due to certain constraints (e.g. numerous infections of cell lines, limited time and the extent of the comet assay) during the study, the comet assay was not performed on the non-neuronal, Chinese hamster ovary (CHO-K1) cells, nor on all the drug pretreatments and concentrations used in the MTT cell proliferation assay. In general, the lower concentration of every drug pretreatment was used in the comet assay, except in the case of *m*Ins and lithium, where preliminary results from the MTT cell proliferation assay was used to identify the concentrations

most likely to cause statistically significant changes. Therefore, 10 mM *m*Ins and 5mM lithium, respectively, were selected.

4.2.1 Neuroprotective Properties of Drugs

4.2.1.1 *myo*-Inositol

*m*Ins is the main drug of investigation in the current study. Figure 4-2 depicts the effect of pretreatment with different concentrations of *m*Ins (without glutamate) on cell viability as measured by the MTT cell proliferation assay and DNA comet assay.

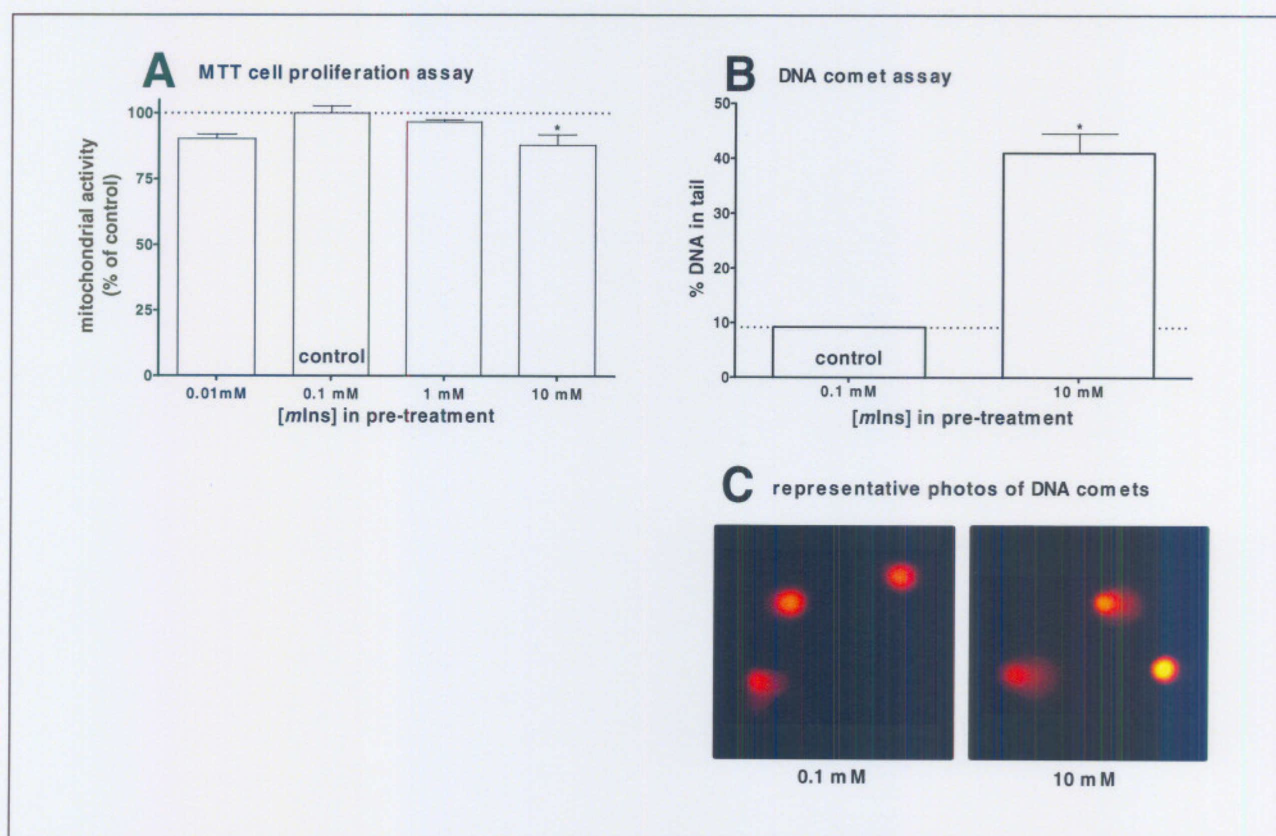

Figure 4-2: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of *m*Ins (without glutamate) on (A) mitochondrial activity, as measured by the MTT cell proliferation assay, and (B) DNA integrity, as measured by the DNA comet assay. (C) Representative photographs of the DNA integrity after 24 hour pretreatment with the indicated concentration of *m*Ins and corresponding to the bars in (B). Data from (A) are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as percentage of the control. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with * indicating $p < 0.05$. Data from (B) are averages ± S.E.M. from two independent and comparable experiments and are expressed as percentage DNA in the tail. Data were analysed statistically by performing a two-tailed Student's t-test, with * indicating $p < 0.05$.

In Figure 4-2 A, it can be seen that 1 mM *m*Ins and even *m*Ins deprivation (0.01 mM) have no significant effects on cell viability, while 10 mM *m*Ins causes a significant decrease in cell

viability (12.1 ± 3.9%; $p < 0.05$) as compared to the control (0.1 mM *m*Ins). There is a trend towards decreased mitochondrial activity after *m*Ins deprivation, although the difference is not statistically significant.

Since *m*Ins is effective in the treatment of depression and, when taking into account the indications that depression may be associated with neurodegeneration (see § 2.3), it was expected that *m*Ins may be neuroprotective; therefore the observed decrease in mitochondrial activity after pretreatment with 10 mM *m*Ins is unexpected. 10 mM *m*Ins is still well within the physiological range (Fisher *et al.*, 2002).

Consistent with the observations in Figure 4-2 A, a marked increase in the percentage of DNA in the tail (31.8 ± 3.6%; $p < 0.05$) is observed after pretreatment with 10 mM *m*Ins and performing the comet assay (Figure 4-2 B). This suggests substantial DNA fragmentation (i.e. decreased DNA integrity, suggesting neurodegeneration). Figure 4-2 C depicts representative photographs of the comet assay as is displayed in the bar graph (Figure 4-2 B).

Therefore, the evidence from investigations into both mitochondrial activity and DNA integrity suggests that 10 mM *m*Ins is neurodegenerative rather than neuroprotective in this *in vitro* model. In a study by Van Rooyen (2005) it was found that 24-hour pretreatment with 10 mM *m*Ins causes a trend towards decreased protein levels of GSK-3β, while it causes a statistically significant decrease in mRNA levels of GSK-3β in human neuroblastoma SH-SY5Y cells. This is contrary to the results obtained in the current study, since inhibition of GSK-3β is expected to result in neuroprotection.

Figure 4-3 depicts the calculated changes in two observed neuroplasticity parameters (i.e. mitochondrial activity and DNA integrity) before and after glutamate, each time in the presence of the indicated concentrations of *m*Ins.

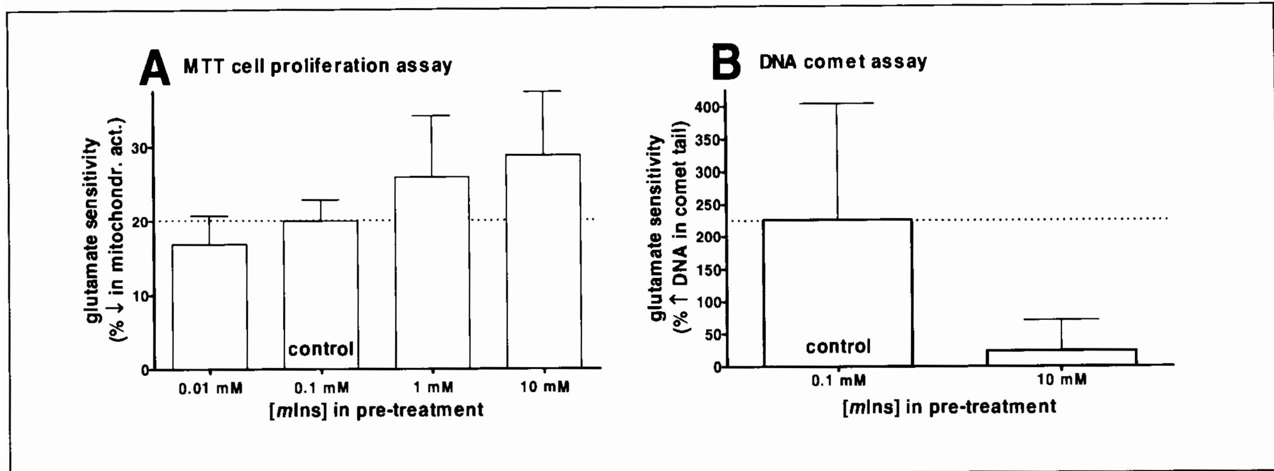

Figure 4-3: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of *m*Ins on glutamate sensitivity. (A) The difference in mitochondrial activity before and after 15 mM glutamate at the indicated co-treatment concentrations of *m*Ins, as measured by the MTT cell proliferation assay. (B) The difference in DNA integrity before and after 15 mM glutamate at the indicated co-treatment concentrations of *m*Ins, as measured by the DNA comet assay. Data from (A) are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test. No statistically significant differences were found. Data from (B) are averages ± S.E.M. from two independent and comparable experiments and are expressed as the percentage increase in DNA damage. Data were analysed statistically by performing a two-tailed Student's t-test. No statistically significant differences were found.

Although there is a trend towards increased glutamate sensitivity (Figure 4-3 A) after pretreatment with different concentrations of *m*Ins in combination with 15 mM glutamate, the differences are not statistically significant. In contrast, results from the comet assay (Figure 4-3 B) revealed a trend towards reduced glutamate sensitivity (although not statistically significant) after pretreatment with 10 mM *m*Ins in combination with glutamate.

This apparent divergence between the results obtained with the MTT cell proliferation assay and the comet assay may possibly be explained, in part, by the presence of a 16-hour drug-free incubation period before the MTT cell proliferation assay was performed. The comet assay, on the other hand, was performed directly after the 24-hour pretreatment period. Therefore, it is possible that it may take some time before *m*Ins causes the observed trend towards increased glutamate sensitivity. Another possible explanation for the observed divergence in the results obtained may be that two completely different parameters are measured by the assays employed. Whereas the results in Figure 4-2 suggest neurodegenerative effects of 10 mM *m*Ins, the results from Figure 4-3 regarding the effect of *m*Ins on glutamate sensitivity are inconclusive.

In order to determine whether these effects on mitochondrial activity are specific to neuronal cell lines, the same experiments were conducted on a non-neuronal cell line, namely Chinese

hamster ovary (CHO-K1) cells. The results obtained after pretreatment with different concentrations of *m*Ins, with or without 15 mM glutamate, are depicted in Figure 4-4.

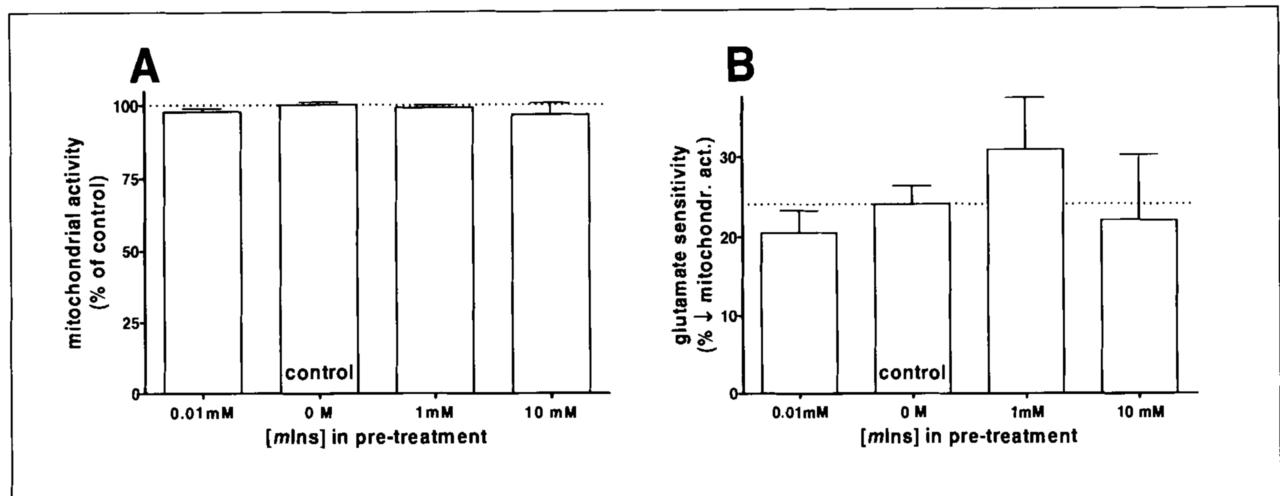

Figure 4-4: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of *m*Ins, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test. No statistically significant differences were found.

From Figure 4-4 A, it is evident that pretreatment with different concentrations of *m*Ins have no significant effect on mitochondrial activity. No decrease in mitochondrial activity is observed even after pretreatment with 10 mM *m*Ins, while this concentration of *m*Ins causes a statistically significant decrease in mitochondrial activity in human neuroblastoma cells (Figure 4-2 A).

In Figure 4-4 B, it can be seen that *m*Ins deprivation (0.01 mM) causes a trend towards decreased glutamate sensitivity, while pretreatment with 1 mM *m*Ins causes a trend towards increased glutamate sensitivity, although the differences did not reach statistical significance. The same trends were noted with neuronal cells in Figure 4-3 A. However, pretreatment with 10 mM *m*Ins causes a trend towards decreased glutamate sensitivity in CHO-K1 cells, contrary to what was observed in human neuroblastoma cells.

These results suggest that the observed neurodegenerative effects after pretreatment with 10 mM *m*Ins may be specific to neuronal cells, or at least that the effects may be cell-type specific and/or species specific.

4.2.1.2 Imipramine

Imipramine is a tricyclic antidepressant and is commonly used for the treatment of depression. The effects of pretreatment with different concentrations of imipramine (without glutamate) on human neuroblastoma cells are depicted in Figure 4-5.

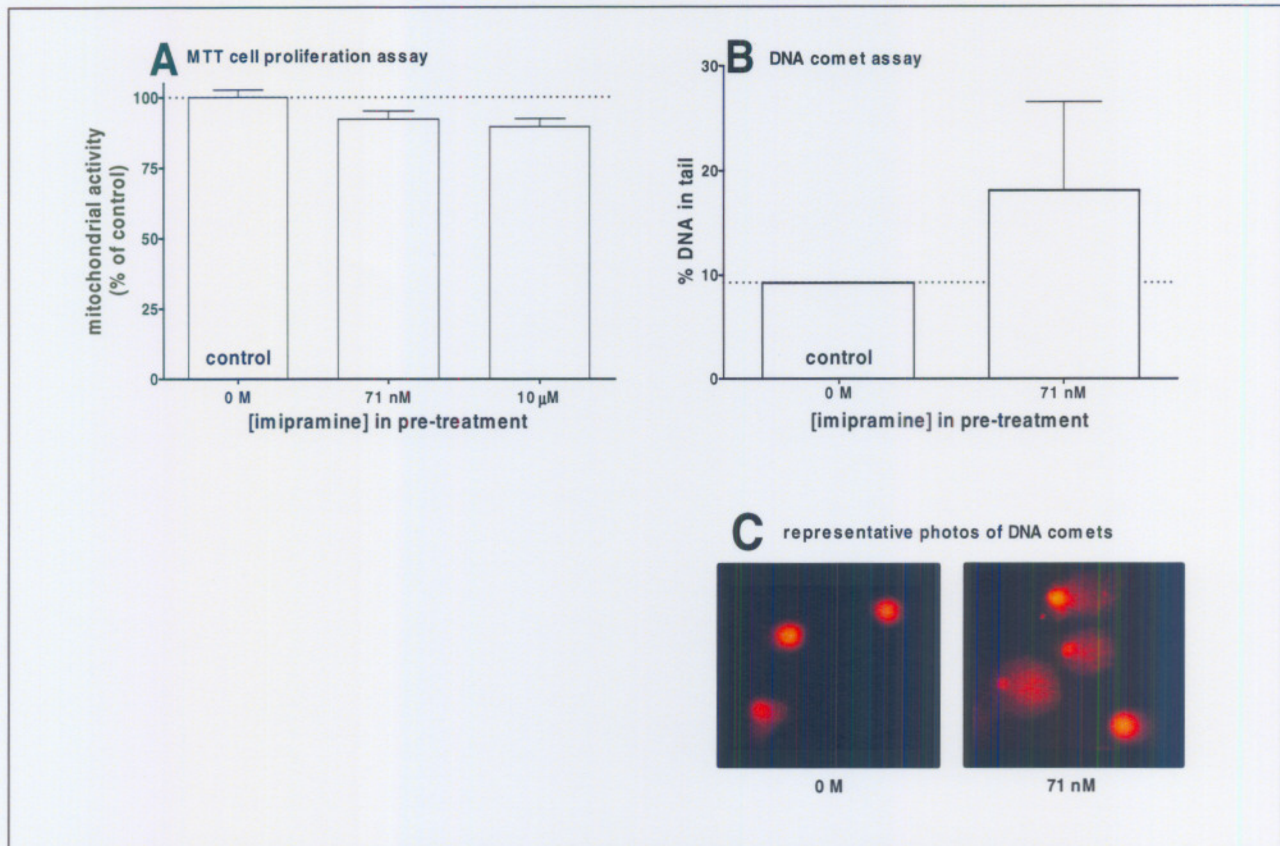

Figure 4-5: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of imipramine (without glutamate) on (A) mitochondrial activity, as measured by the MTT cell proliferation assay, and (B) DNA integrity, as measured by the DNA comet assay. (C) Representative photographs of the DNA integrity after 24 hour pretreatment with the indicated concentration of *m*Ins and corresponding to the bars in (B). Data from (A) are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as percentage of the control. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with * indicating $p < 0.05$. Data from (B) are averages ± S.E.M. from two independent and comparable experiments and are expressed as percentage of DNA in the tail. Data were analysed statistically by performing a two-tailed Student's t-test. No statistically significant differences were found.

In Figure 4-5 A, it can be seen that pretreatment with imipramine alone (even at 10 μM) has no statistically significant effect on cell viability, although a trend towards reduced mitochondrial activity is observed. The same tendency is observed in Figure 4-5 B, where a trend (without statistical significance) is seen towards increased DNA damage (neurodegenerative effect) after pretreatment with 71 nM imipramine. Figure 4-5 C depicts representative photographs of the comet assays corresponding to the bars in Figure 4-5 B.

Pretreatment with either amitriptyline (≥ 50 μM) or desipramine (≥ 20 μM) results in a statistically significant decrease in the viability of mouse hippocampal (HT-22) cells. The decrease is dose-dependent. These concentrations also result in a significant increase in the number of apoptotic cells compared to untreated control cells (Post *et al.*, 2000).

In another study, primary cultures of rat cerebellar granule cells were prepared from the cerebella of 7-day-old rat pups. Treatment was initiated either 1 day after plating (1 day *in vitro*, DIV) or 10 days after plating (10 DIV). It was found that a 48-hour *in vitro* pretreatment of the primary cerebellar cortical culture with 1 μM imipramine at 1 DIV causes a significant decrease in cell proliferation, while 48-hour pretreatment at 10 DIV causes a significant increase in cell proliferation. This suggests that imipramine has an inhibitory effect in younger cultures, while it has a stimulatory effect in older cultures (Manev *et al.*, 2001). It may be accepted that the human neuroblastoma [SK-N-BE(2)] cell line is a mature cell line, therefore the results obtained (i.e. a trend towards reduced cell viability after pretreatment with imipramine) is contradictory to the results of Manev and colleagues.

Figure 4-6 depicts the calculated changes in two observed neuroplasticity parameters (i.e. mitochondrial activity and DNA integrity) before and after glutamate, each time in the presence of the indicated concentrations of imipramine.

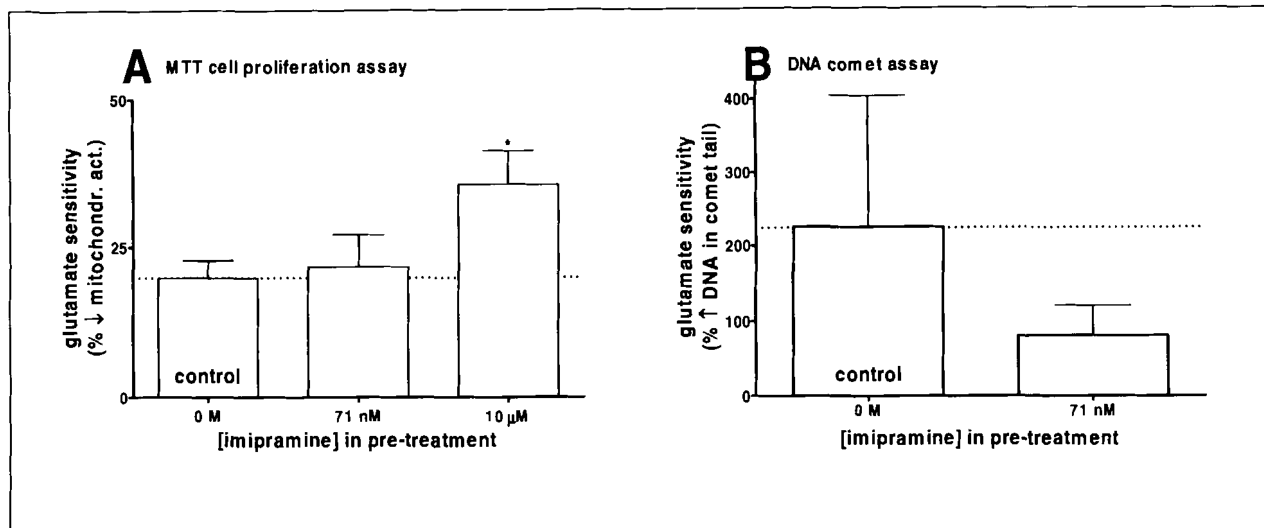

Figure 4-6: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of imipramine on glutamate sensitivity. (A) The difference in mitochondrial activity before and after 15 mM glutamate at the indicated co-treatment concentrations of imipramine, as measured by the MTT cell proliferation assay. (B) The difference in DNA integrity before and after 15 mM glutamate at the indicated co-treatment concentrations of imipramine, as measured by the DNA comet assay. Data from (A) are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with * indicating $p < 0.05$. Data from (B) are averages ± S.E.M. from two independent and comparable experiments and are expressed as the percentage increase in DNA damage. Data were analysed statistically by performing a two-tailed Student's t-test. No statistically significant differences were found.

In Figure 4-6 A, it can be seen that 24 hours pretreatment with 10 μM imipramine results in a significantly increased glutamate sensitivity (15.5 ± 5.7%; $p < 0.05$), while pretreatment with 71 nM imipramine causes only a trend towards increased glutamate sensitivity. Figure 4-6 B shows a trend towards decreased glutamate sensitivity after pretreatment with 71 nM imipramine, although the decrease is not statistically significant.

A mouse hippocampal cell line and a primary rat cortical culture were exposed to imipramine (75 μM and 25 μM, respectively) and 5 mM glutamate for 24 hours. The MTT cell proliferation assay was performed directly after the pretreatment period and the results demonstrate that imipramine protects significantly against glutamate toxicity in both cases (Maher & Davis, 1996).

These results are different to the results depicted in Figure 4-6 A, but are similar to the results obtained from the comet assay (Figure 4-6 B). While the effect of imipramine alone on neuroplasticity may differ from its effect on a glutamate challenge of the neuron (as suggested by the results), another explanation is also possible. As mentioned in § 4.2.1.1, the comet assay was performed directly after pretreatment, whereas the MTT cell proliferation assay was performed only after a 16-hour drug-free incubation period. It is possible that imipramine causes an initial reduction in glutamate sensitivity, while this effect is reversed as time passes.

In order to determine whether these effects on mitochondrial activity are specific to neuronal cell lines, the same experiments were conducted on a non-neuronal cell line, namely CHO-K1 cells. The results obtained after pretreatment with different concentrations of imipramine, with or without 15 mM glutamate, are depicted in Figure 4-7.

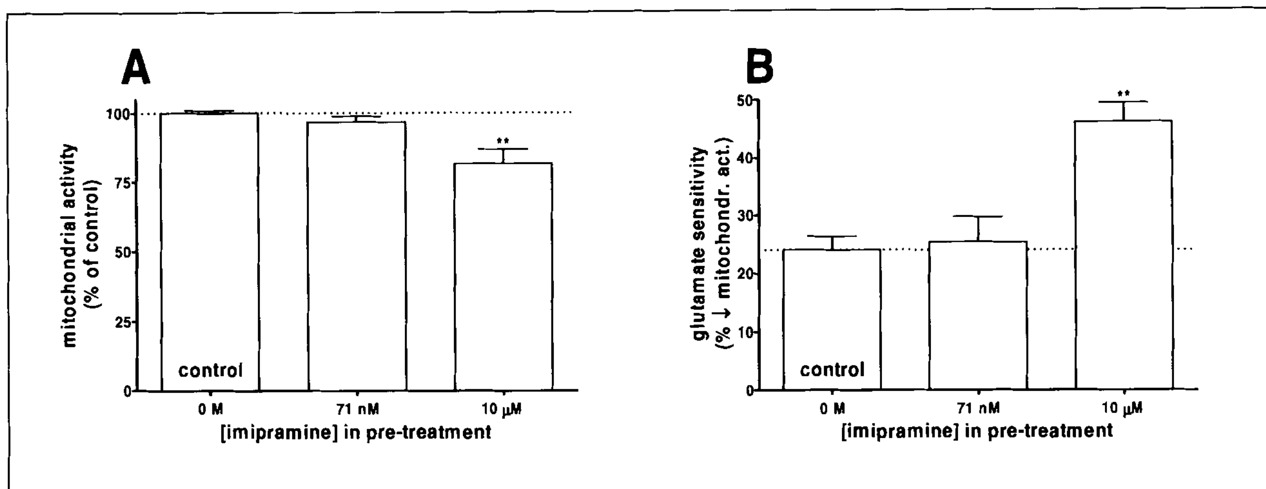

Figure 4-7: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of imipramine (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of imipramine, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with ** indicating $p < 0.01$.

The results portrayed in Figure 4-7 A suggest that pretreatment with 71 nM imipramine has no significant effect on mitochondrial activity of CHO-K1 cells, while pretreatment with 10 μM imipramine causes a marked decrease in mitochondrial activity (18.3 ± 5.3%; $p < 0.01$). The profile is similar to that observed in human neuroblastoma cells (Figure 4-5 A) although no significant differences were found in the neuronal cell line.

Glutamate sensitivity is significantly increased (22.2 ± 3.2%; $p < 0.01$) after pretreatment with 10 μM imipramine, while pretreatment with 71 nM imipramine has no statistically significant effect (Figure 4-7 B). The current profile is similar to that observed in human neuroblastoma cells (Figure 4-6 A). Therefore, these effects of imipramine seem to be neither neuroselective nor cell-type specific.

4.2.1.3 Fluoxetine

Since fluoxetine, a prototype SSRI, is frequently used to treat major depression, the effects of 24-hour pretreatment with different concentrations (without glutamate) on human neuroblastoma cells were investigated. The results are depicted in Figure 4-8.

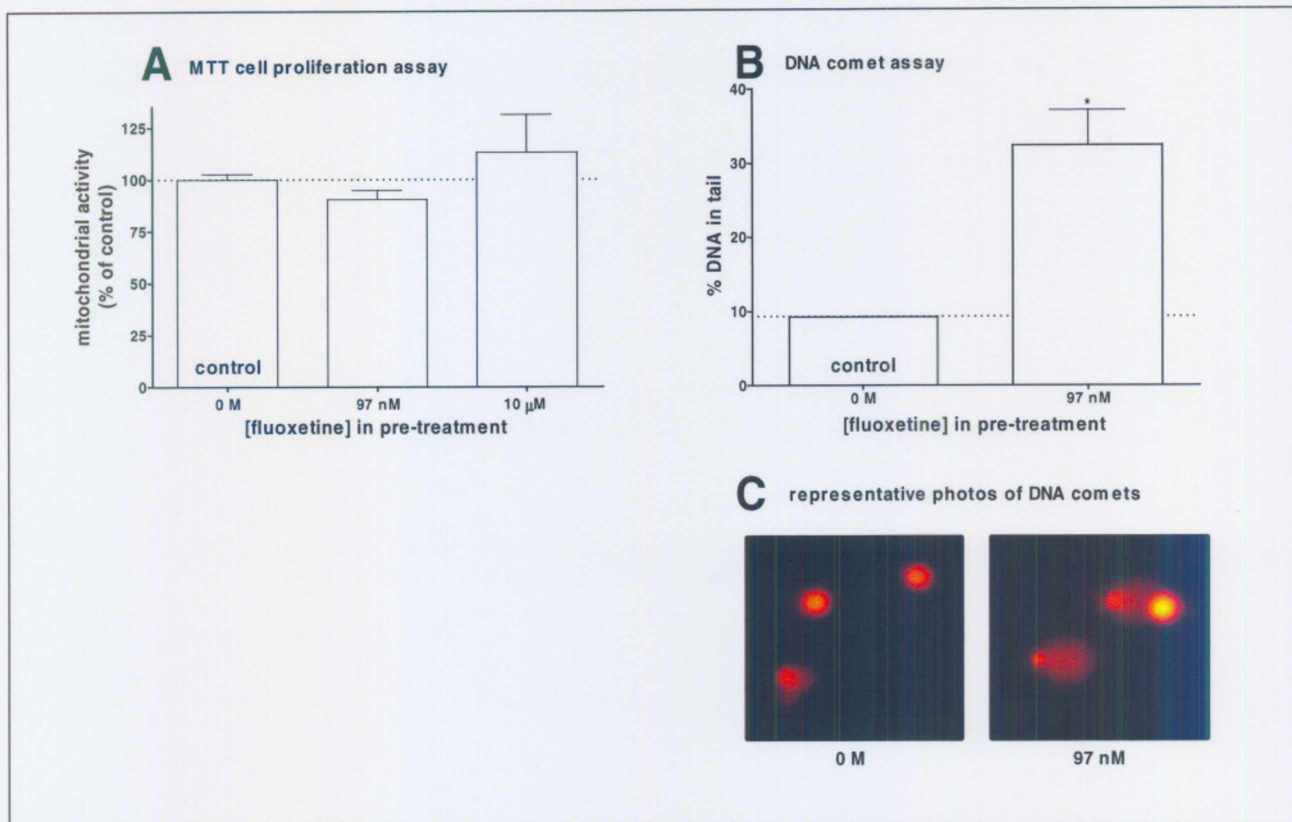

Figure 4-8: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of fluoxetine (without glutamate) on (A) mitochondrial activity as measured by the MTT cell proliferation assay and (B) DNA integrity, as measured by the DNA comet assay. (C) Representative photographs of the DNA integrity after 24 hour pretreatment with the indicated concentration of *m*Ins and corresponding to the bars in (B). Data from (A) are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as percentage of the control. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test. No statistically significant differences were found. Data from (B) are averages ± S.E.M. from two independent and comparable experiments and are expressed as percentage DNA in the tail. Data were analysed statistically by performing a two-tailed Student's t-test, with * indicating $p < 0.05$.

According to Figure 4-8 A, pretreatment with different concentrations of fluoxetine alone causes no statistically significant differences in mitochondrial activity, although there is a trend towards increased mitochondrial activity after pretreatment with 10 µM fluoxetine. Results from the comet assay (Figure 4-8 B) indicate a statistically significant increase in DNA damage (23.2 ± 4.7%; $p < 0.05$) after pretreatment with 97 nM fluoxetine. Figure 4-8 C depicts representative photographs of the comet assays corresponding to the bars in Figure 4-8 B.

In a study by Post and colleagues (2000), it was found that pretreatment with fluoxetine causes a dose-dependent reduction in HT-22 cell survival. A concentration of 20 μM results in a significant reduction in cell viability as compared to the untreated control cells. The use of higher concentrations resulted in even more cell death. The cell death was demonstrated to be, at least in part, due to apoptosis. Fluoxetine causes a dose-dependent increase in apoptotic cells.

In another study, it was found that a 48-hour pretreatment with fluoxetine at 1 DIV resulted in a concentration-dependent decrease in cell proliferation of a primary cerebellar cortical culture (concentrations of 0.1, 0.5 and 1 μM fluoxetine were used). However, after 48-hour pretreatment at 10 DIV, a significant increase in cell proliferation was noted, suggesting that fluoxetine has an inhibitory effect on cell proliferation of young cultures, whereas it has a stimulatory effect on older cultures (Manev *et al.*, 2001).

The results from the current study are comparable with the results obtained by Post and colleagues, although they used higher drug concentrations. However, the results differ from the results obtained by Manev and colleagues.

Figure 4-9 depicts the calculated changes in two observed neuroplasticity parameters (i.e. mitochondrial activity and DNA integrity) before and after glutamate, each time in the presence of the indicated concentrations of fluoxetine.

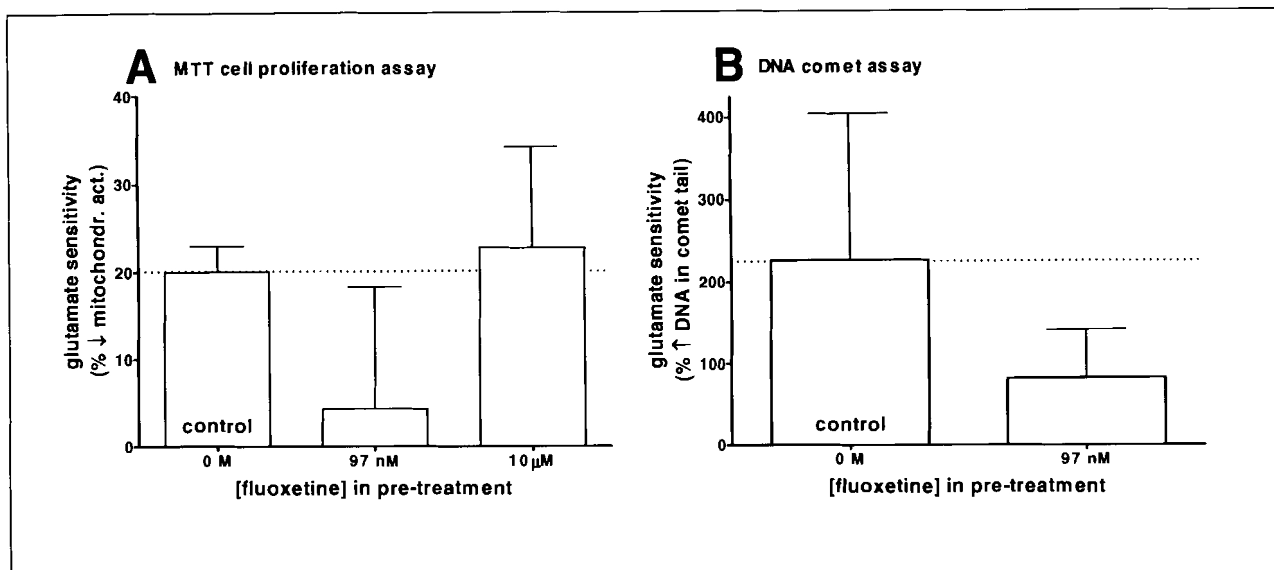

Figure 4-9: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of fluoxetine on glutamate sensitivity. (A) The difference in mitochondrial activity before and after 15 mM glutamate at the indicated co-treatment concentrations of fluoxetine, as measured by the MTT cell proliferation assay. (B) The difference in DNA integrity before and after 15 mM glutamate at the indicated co-treatment concentrations of fluoxetine, as measured by the DNA comet assay. Data from (A) are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test. No statistically significant differences were found. Data from (B) are averages ± S.E.M. from two independent and comparable experiments and are expressed as the percentage increase in DNA damage. Data were analysed statistically by performing a two-tailed Student's t-test. No statistically significant differences were found.

Pretreatment with 10 μM fluoxetine has no significant effect on glutamate sensitivity, while pretreatment with 97 nM reveals a trend towards reduced glutamate sensitivity, but the difference is not statistically significant (Figure 4-9 A). The same tendency is observed in the results of the comet assay (Figure 4-9 B), which demonstrates a trend towards decreased glutamate sensitivity after pretreatment with 97 nM fluoxetine. While the results do not reveal any statistically significant decrease in glutamate sensitivity, it is possible that repetition of the experiments may lead to reduced S.E.M. values and improved statistical significance of the results.

In order to determine whether these effects on mitochondrial activity are specific to neuronal cell lines, the same experiments were conducted on a non-neuronal cell line, namely CHO-K1 cells. The results obtained after pretreatment with different concentrations of fluoxetine, with or without 15 mM glutamate, are depicted in Figure 4-10.

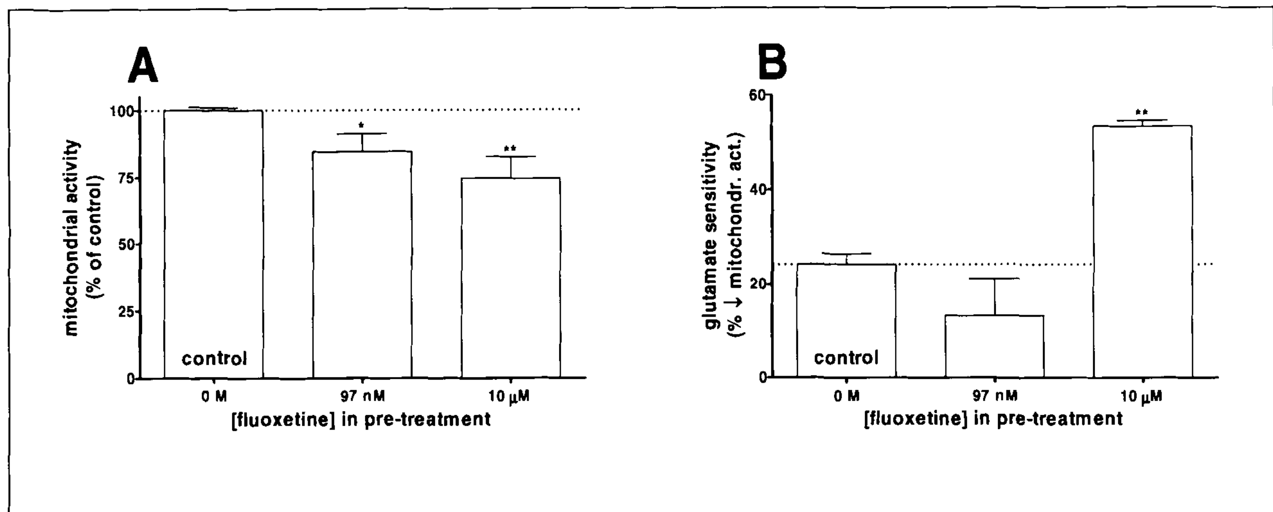

Figure 4-10: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of fluoxetine (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of fluoxetine, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with * indicating $p < 0.05$ and ** indicating $p < 0.01$.

According to Figure 4-10 A, it is obvious that pretreatment with either 97 nM (15.4 ± 6.5%; $p < 0.05$) or 10 µM (25.4 ± 8.0%; $p < 0.01$) fluoxetine causes a statistically significant decrease in mitochondrial activity. Therefore, it seems that fluoxetine is detrimental to the survival of CHO-K1 cells. In Figure 4-8 A, it was noted that pretreatment with either concentration of fluoxetine has no statistically significant effect on cell viability of human neuroblastoma cells, suggesting different effects of fluoxetine in neuroblastoma versus ovary cells.

Pretreatment with 97 nM fluoxetine causes a trend towards decreased glutamate sensitivity, although this is not statistically significant (Figure 4-10 B). The same trend was observed in human neuroblastoma cells (Figure 4-8 D). In Figure 4-10 B, it can also be seen that pretreatment with 10 µM fluoxetine causes a sharp increase (29.3 ± 1.2%; $p < 0.01$) in glutamate sensitivity. No significant effect on glutamate sensitivity of the human neuroblastoma cell line was observed (Figure 4-8 C). Therefore, non-neuronal cells may possibly be more sensitive to the detrimental effects of fluoxetine on cell survival.

4.2.1.4 Lithium

Lithium has been reported to be neuroprotective (see § 2.2.2.4) and, since the current study investigated the neuroprotective properties of certain antidepressant drugs, lithium is an obvious candidate for examination. The results obtained after pretreatment with lithium (without glutamate) are depicted in Figure 4-11.

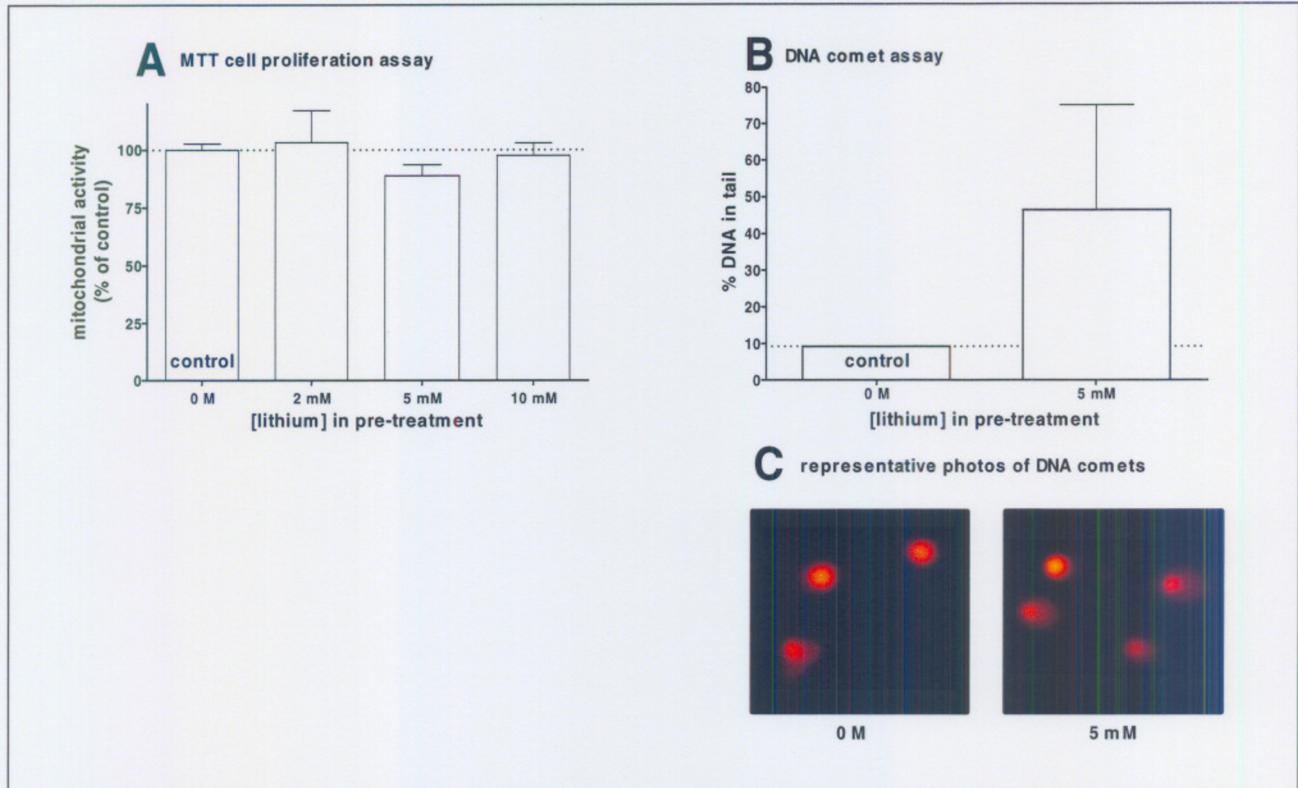

Figure 4-11: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of lithium (without glutamate) on (A) mitochondrial activity as measured by the MTT cell proliferation assay and (B) DNA integrity, as measured by the DNA comet assay. (C) Representative photographs of the DNA integrity after 24 hour pretreatment with the indicated concentration of *m*Ins and corresponding to the bars in (B). Data from (A) are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as percentage of the control. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test. No statistically significant differences were found. Data from (B) are averages ± S.E.M. from two independent and comparable experiments and are expressed as percentage of DNA in the tail. Data were analysed statistically by performing a two-tailed Student's t-test. No statistically significant differences were found.

In Figure 4-11 A, it can be seen that pretreatment with lithium alone causes no significant differences in mitochondrial activity. Even at higher concentrations, no decrease in cell viability is observed. Results from the comet assay (Figure 4-11 B) demonstrate a trend towards increased DNA damage after pretreatment with 5 mM lithium, although the difference is not statistically significant. Figure 4-11 C depicts representative photographs of the comet assays corresponding to the bars in Figure 4-11 B.

Figure 4-12 depicts the calculated changes in two observed neuroplasticity parameters (i.e. mitochondrial activity and DNA integrity) before and after glutamate, each time in the presence of the indicated concentrations of lithium.

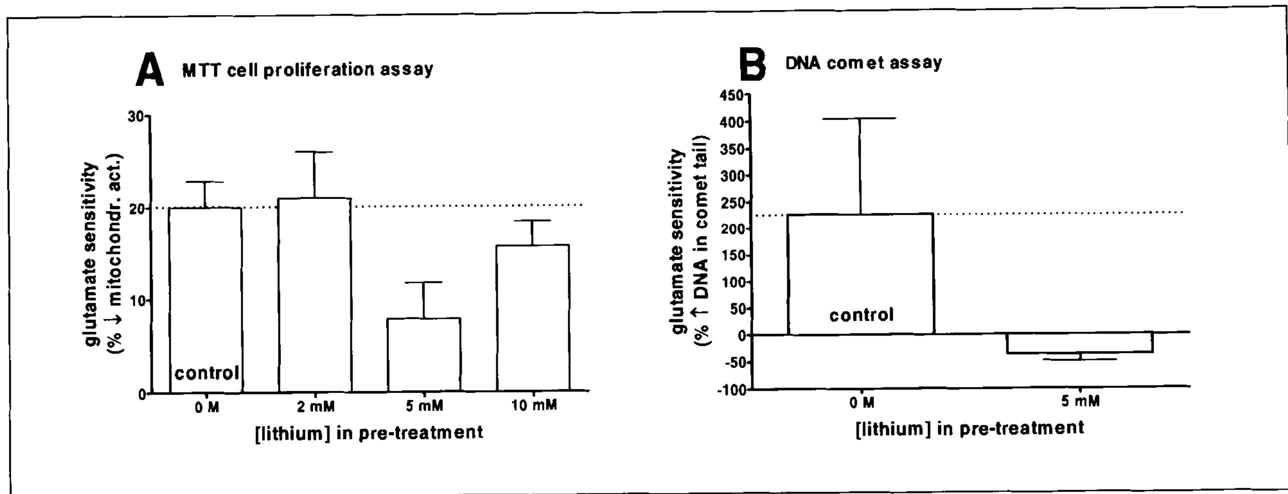

Figure 4-12: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of lithium on glutamate sensitivity. (A) The difference in mitochondrial activity before and after 15 mM glutamate at the indicated co-treatment concentrations of lithium, as measured by the MTT cell proliferation assay. (B) The difference in DNA integrity before and after 15 mM glutamate at the indicated co-treatment concentrations of lithium, as measured by the DNA comet assay. Data from (A) are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test. No statistically significant differences were found. Data from (B) are averages ± S.E.M. from two independent and comparable experiments and are expressed as the percentage increase in DNA damage. Data were analysed statistically by performing a two-tailed Student's t-test. No statistically significant differences were found.

Although pretreatment with different concentrations of lithium have no statistically significant effect on glutamate sensitivity, a trend towards reduced glutamate sensitivity is observed after pretreatment with 5 mM lithium. After pretreatment with 10 mM lithium, the observed trend towards reduced glutamate sensitivity is reduced (Figure 4-12 A). This may be explained by the well described small therapeutic index of lithium.

The same tendency observed in Figure 4-12 A is also present in Figure 4-12 B. Pretreatment with 5 mM lithium shows a trend towards protection against glutamate-induced excitotoxicity, although it is not statistically significant. Protection against glutamate-induced excitotoxicity was expected, since lithium has been reported to be neuroprotective.

A study by Nonaka and colleagues (1998) demonstrated that the protective effect of lithium is both time- and concentration-dependent. Primary cultures of cerebellar granule cells were pre-incubated with lithium whereafter they were exposed to glutamate. Maximal protection against glutamate toxicity occurred after pre-incubation for 6 – 7 days, while a 24-hour pre-incubation failed to produce any effect. Although lithium significantly protected the neurons at 0.5 mM, the maximal effect was observed at 3 mM with an EC_{50} of approximately 1.3 mM. Pre-incubation with lithium resulted in a dose-dependent inhibition of internucleosomal DNA cleavage caused by glutamate. It was also demonstrated that glutamate-induced neuronal death in cortical and

hippocampal cultures was significantly suppressed by pre-incubation for 7 days with 1 mM lithium (Nonaka *et al.*, 1998). Therefore, it is possible that the time of pretreatment with lithium may have been suboptimal to detect a significant decrease in glutamate sensitivity in the current model.

In order to determine whether these effects on mitochondrial activity are specific to neuronal cell lines, the same experiments were conducted on a non-neuronal cell line, namely CHO-K1 cells. The results obtained after pretreatment with different concentrations of lithium, with or without 15 mM glutamate, are depicted in Figure 4-13.

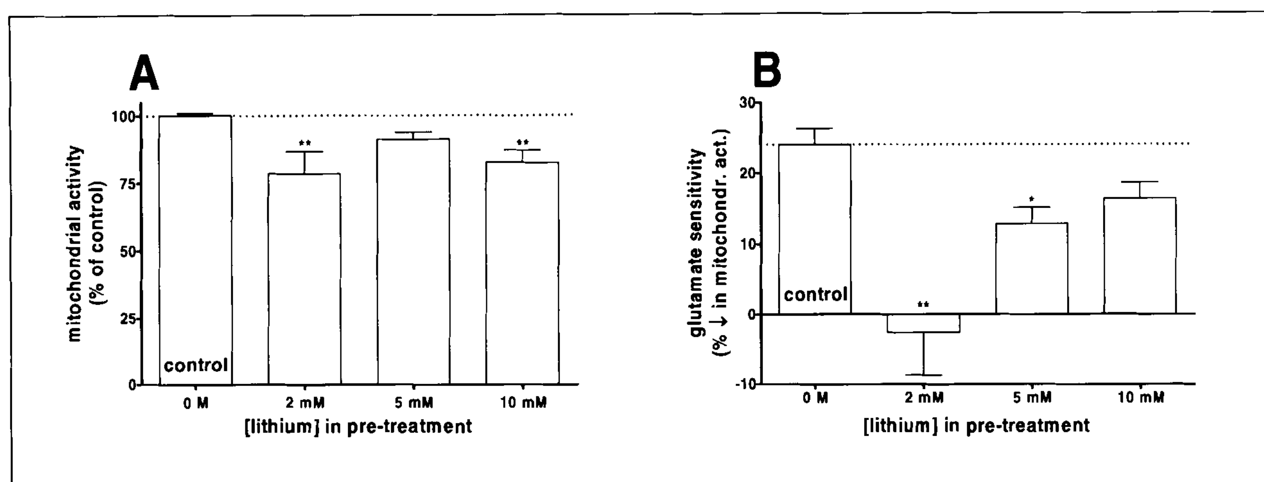

Figure 4-13: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of lithium (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of lithium, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with * indicating $p < 0.05$ and ** indicating $p < 0.01$.

In Figure 4-13 A, it can be seen that pretreatment with either 2 mM or 10 mM lithium causes a statistically significant reduction (21.5 ± 8.2%; $p < 0.01$ and 17.6 ± 4.7%; $p < 0.01$, respectively) in mitochondrial activity, while pretreatment with 5 mM lithium causes only a trend towards reduced mitochondrial activity. When human neuroblastoma cells were pretreated with different concentrations of lithium, no significant differences in cell viability were observed (Figure 4-11 A).

Figure 4-13 B indicates that pretreatment with 2 mM lithium protects significantly against glutamate-induced excitotoxicity (a reduction of 26.6 ± 6.1%; $p < 0.01$), while pretreatment with 5 mM lithium causes a statistically significant reduction (11.2 ± 2.3%; $p < 0.05$) in glutamate sensitivity. Although pretreatment with 10 mM lithium has no significant effect on glutamate

sensitivity, there is a trend towards reduced glutamate sensitivity. After human neuroblastoma cells were pretreated with lithium, no protection against glutamate-induced excitotoxicity is observed, but only a trend towards decreased glutamate sensitivity after pretreatment with either 5 mM or 10 mM lithium (Figure 4-12 A). From these observations, it may be postulated that CHO-K1 cells are more sensitive than human neuroblastoma cells to the effects of lithium (both cytotoxic when used alone and cytoprotective when used in combination with glutamate). The observed effects may be cell-type specific and/or species specific.

4.2.1.5 Memantine

The current study investigated the effects of various antidepressants on glutamate-induced excitotoxicity. Memantine is a non-competitive NMDA receptor antagonist and therefore it is expected to counteract the effect of glutamate. For the purpose of the current study, human neuroblastoma cells were pretreated with different concentrations of memantine with or without glutamate. The results obtained after pretreatment with different concentrations of memantine (without glutamate) are depicted in Figure 4-14.

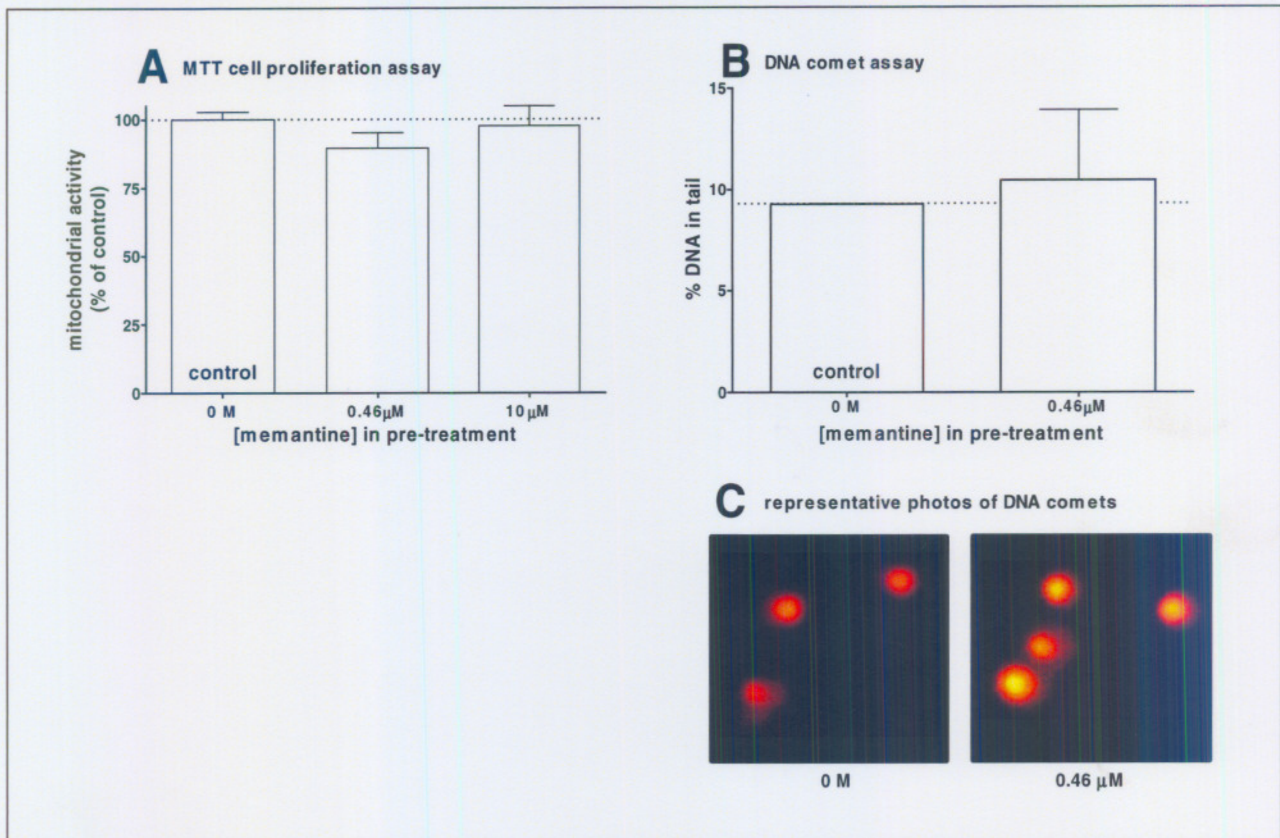

Figure 4-14: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of memantine (without glutamate) on (A) mitochondrial activity as measured by the MTT cell proliferation assay and (B) DNA integrity, as measured by the DNA comet assay. (C) Representative photographs of the DNA integrity after 24-hour pretreatment with the indicated concentration of memantine and corresponding to the bars in (B). Data from (A) are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as percentage of the control. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test. No statistically significant differences were found. Data from (B) are averages ± S.E.M. from two independent and comparable experiments and are expressed as percentage of DNA in the tail. Data were analysed statistically by performing a two-tailed Student's t-test. No statistically significant differences were found.

According to Figure 4-14 A, pretreatment with memantine has no significant effect on cell viability. This is comparable to the results shown in Figure 4-14 B, which demonstrates that pretreatment with memantine has no significant effect on DNA damage. Figure 4-14 C depicts representative photographs of the comet assays corresponding to the bars in Figure 4-14 B.

Figure 4-15 depicts the calculated changes in two observed neuroplasticity parameters (i.e. mitochondrial activity and DNA integrity) before and after glutamate, each time in the presence of the indicated concentrations of memantine.

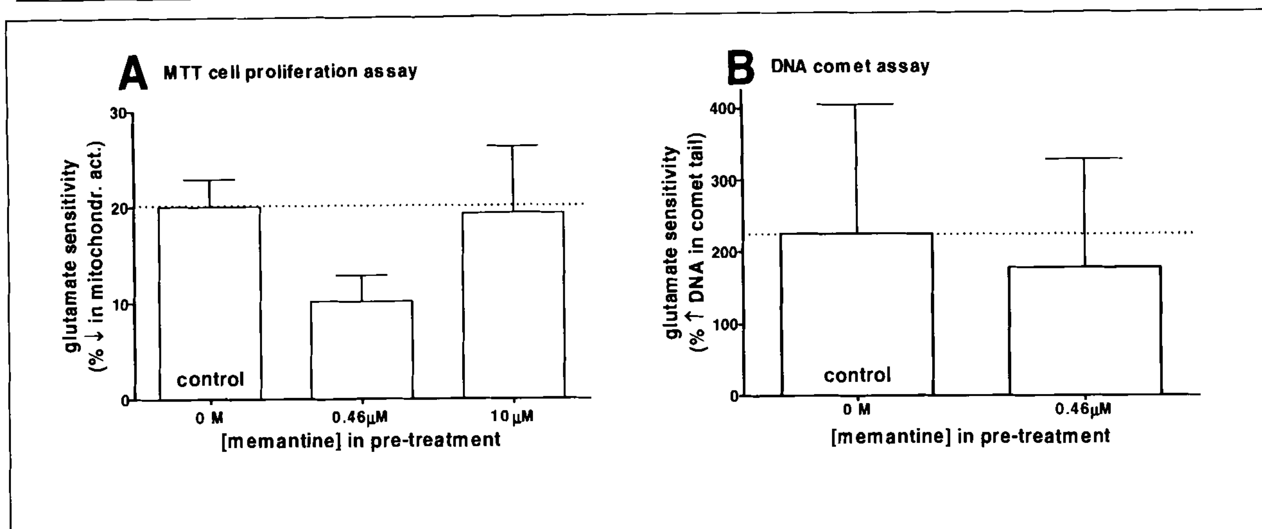

Figure 4-15: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of memantine on glutamate sensitivity. (A) The difference in mitochondrial activity before and after 15 mM glutamate at the indicated co-treatment concentrations of memantine, as measured by the MTT cell proliferation assay. (B) The difference in DNA integrity before and after 15 mM glutamate at the indicated co-treatment concentrations of memantine, as measured by the DNA comet assay. Data from (A) are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test. No statistically significant differences were found. Data from (B) are averages ± S.E.M. from two independent and comparable experiments and are expressed as the percentage increase in DNA damage. Data were analysed statistically by performing a two-tailed Student's t-test. No statistically significant differences were found.

In Figure 4-15 A, it can be seen that pretreatment with 0.46 μM memantine causes a trend (although not statistically significant) towards decreased glutamate sensitivity, while this trend is reversed after pretreatment with 10 μM memantine. Results from the comet assay (Figure 4-15 B) demonstrate a trend towards decreased glutamate sensitivity after pretreatment with a therapeutic concentration of memantine, although the difference is not statistically significant. It is possible that the observed trends may have shown statistical significance if more repetitions of the experiments had been performed. According to these results it may be postulated that memantine may have a beneficial effect on glutamate-induced excitotoxicity in this model, which is consistent with what is expected because of its mode of action (as mentioned earlier in this section).

A mouse hippocampal cell line (HT-22) without ionotropic glutamate receptors has been employed in investigations regarding the toxicity of glutamate. It was found that the NMDA receptor antagonists MK-801 and 2-amino-5-phosphonovalerate (APV) do not protect against glutamate toxicity, while cystine blocks glutamate toxicity. This suggests the involvement of an oxidative pathway (Maher & Davis, 1996).

In Figure 4-15 A, only a trend towards reduced glutamate sensitivity is observed after pretreatment with memantine. When taking the aforementioned information into account, the data may suggest that the observed toxicity as a result of glutamate may not only involve the excitotoxicity pathway, but also the oxidative pathway in the current model. Therefore, since memantine does not act as an antioxidant, it may not be able to sufficiently block the effects caused by glutamate.

In order to determine whether these effects on mitochondrial activity are specific to neuronal cell lines, the same experiments were conducted on a non-neuronal cell line, namely CHO-K1 cells. The results obtained after pretreatment with different concentrations of memantine, with or without 15 mM glutamate, are depicted in Figure 4-16.

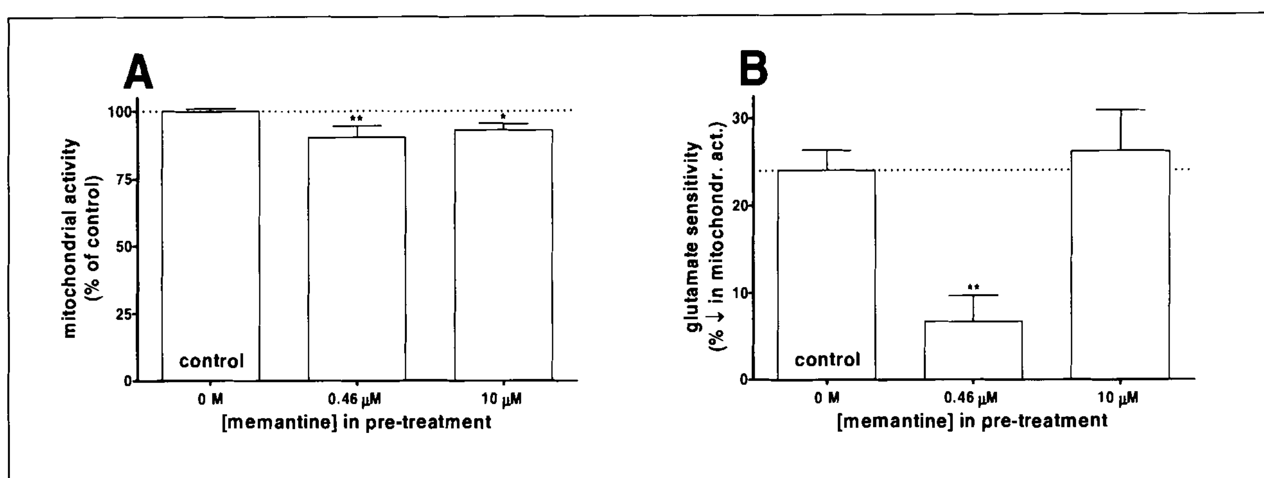

Figure 4-16: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of memantine (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of memantine, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with * indicating $p < 0.05$ and ** indicating $p < 0.01$.

Pretreatment with 0.46 μM or 10 μM memantine reduces the mitochondrial activity (9.5 ± 4.0%; $p < 0.01$ and 7.2 ± 2.5%; $p < 0.05$, respectively) of CHO-K1 cells in statistically significant terms (Figure 4-16 A), while it has no significant effect on the mitochondrial activity of human neuroblastoma cells (Figure 4-14 A).

The results depicted in Figure 4-16 B suggest that pretreatment with 0.46 μM memantine statistically significantly decreases glutamate sensitivity (17.4 ± 3.0%; $p < 0.01$), while pretreatment with 10 μM memantine has no statistically significant effect on glutamate sensitivity. Analysis of the results obtained after the pretreatment of human neuroblastoma cells

(Figure 4-15 A) with memantine reveal only a trend towards reduced glutamate sensitivity, while no statistically significant differences were found.

The cytotoxic effects of memantine alone, as well as the protective effect against glutamate-induced excitotoxicity, are more pronounced in CHO-K1 cells than in human neuroblastoma cells.

4.2.1.6 Tianeptine

Clinical studies have demonstrated that tianeptine is effective in the treatment of depression. It has also been found that tianeptine prevents the neurodegeneration in rat hippocampus induced by chronic stress. The results obtained after human neuroblastoma cells were pretreated with different concentrations of tianeptine (without glutamate) are depicted in Figure 4-17.

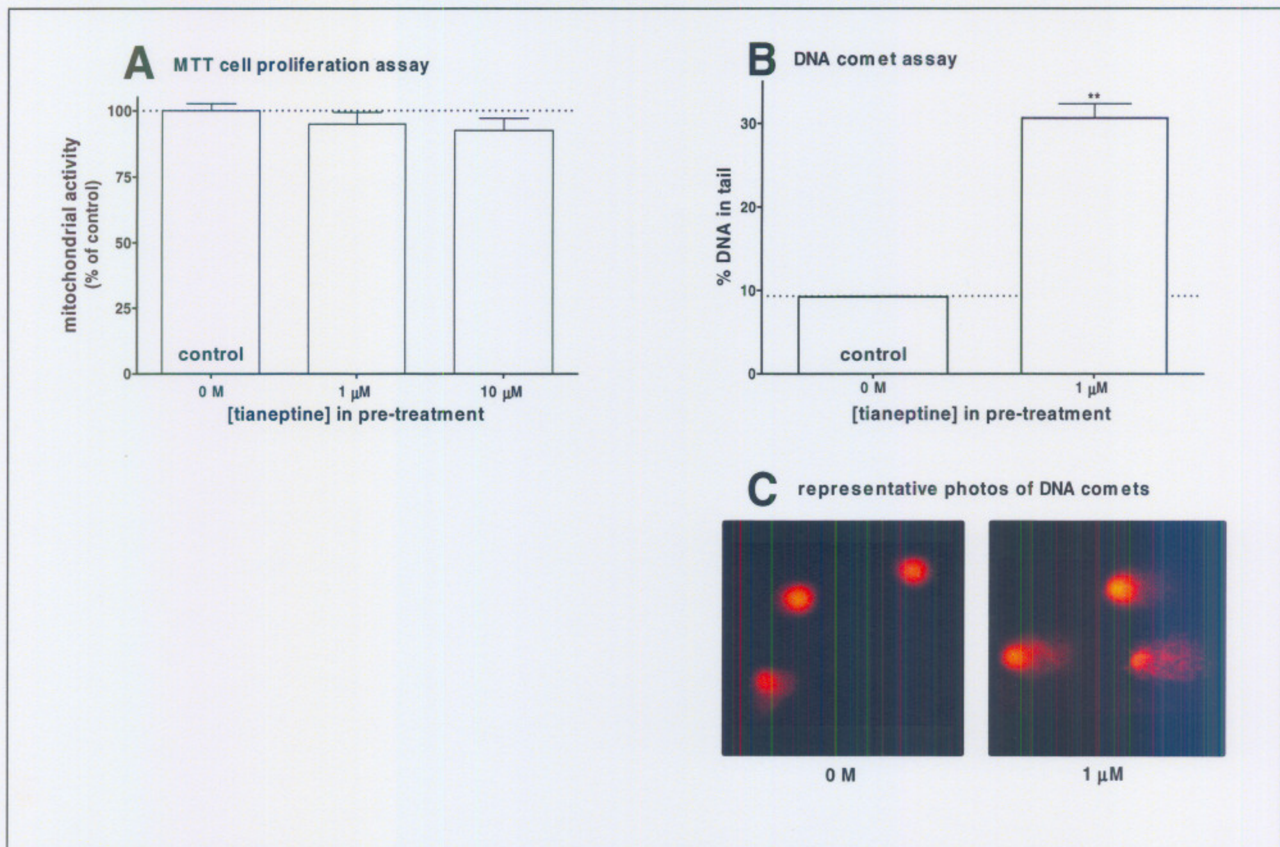

Figure 4-17: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of tianeptine (without glutamate) on (A) mitochondrial activity as measured by the MTT cell proliferation assay and B) DNA integrity, as measured by the DNA comet assay. (C) Representative photographs of the DNA integrity after 24-hour pretreatment with the indicated concentration tianeptine and corresponding to the bars in (B). Data from (A) are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as percentage of the control. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test. Data from (B) are averages ± S.E.M. from two independent and comparable experiments and are expressed as percentage DNA in the tail. Data were analysed statistically by performing a two-tailed Student's t-test, with ** indicating $p < 0.01$.

According to Figure 4-17 A, it can be seen that pretreatment with tianeptine has no significant effect on mitochondrial activity. In contrast, a significant increase (21.4 ± 1.7%; $p < 0.01$) in DNA damage is observed after pretreatment with 1 µM tianeptine (Figure 4-17 B). Figure 4-17 C depicts representative photographs of the comet assays corresponding to the bars in Figure 4-17 B.

As mentioned in § 4.2.1.1, the comet assay was performed directly after the 24-hour pretreatment, whereas the MTT cell proliferation assay was performed only after a 16-hour drug-free incubation period. Taking this into account, it is possible that pretreatment with tianeptine causes initial DNA damage that recovers over time and therefore the observed DNA damage does not result in decreased cell viability. On the other hand, it must be taken into account that two completely different parameters were measured.

Figure 4-18 depicts the calculated changes in two observed neuroplasticity parameters (i.e. mitochondrial activity and DNA integrity) before and after glutamate, each time in the presence of the indicated concentrations of tianeptine.

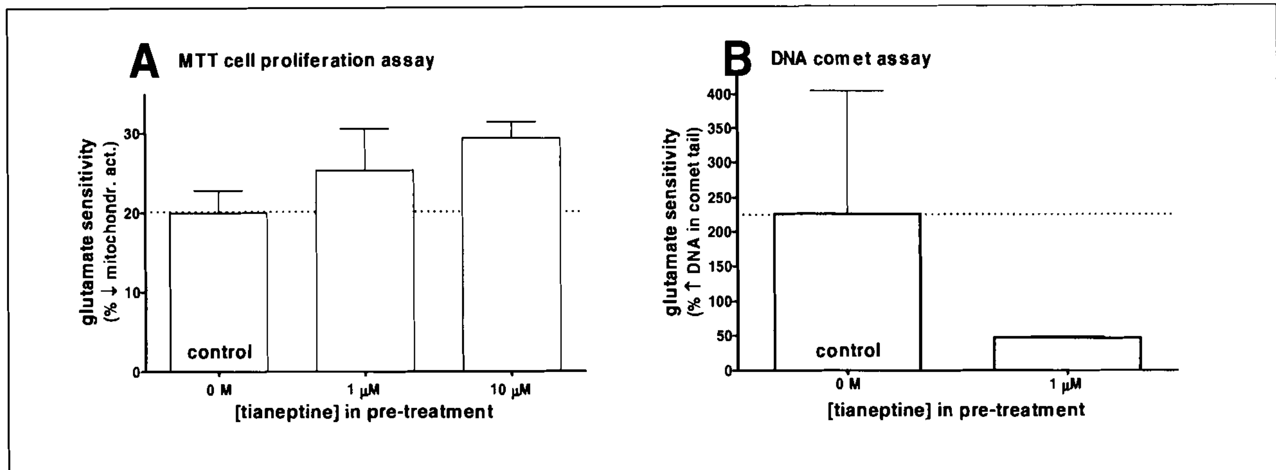

Figure 4-18: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of tianeptine on glutamate sensitivity. (A) The difference in mitochondrial activity before and after 15 mM glutamate at the indicated co-treatment concentrations of tianeptine, as measured by the MTT cell proliferation assay. (B) The difference in DNA integrity before and after 15 mM glutamate at the indicated co-treatment concentrations of tianeptine, as measured by the DNA comet assay. Data from (A) are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test. No statistically significant differences were found. Data from (B) are averages ± S.E.M. from two independent and comparable experiments and are expressed as the percentage increase in DNA damage. Data were analysed statistically by performing a two-tailed Student's t-test. No statistically significant differences were found.

Pretreatment with different concentrations of tianeptine causes a trend towards increased glutamate sensitivity (Figure 4-18 A), although this is not statistically significant. This is unexpected, since tianeptine's mode of action has been reported to involve the modulation of glutamate receptors. In Figure 4-18 B, it can be seen that pretreatment with 1 μM tianeptine causes a trend towards decreased glutamate sensitivity, although, once again, the difference is not statistically significant. The results regarding the effect of tianeptine on glutamate sensitivity are inconclusive.

In order to determine whether these effects on mitochondrial activity are specific to neuronal cell lines, the same experiments were conducted on a non-neuronal cell line, namely CHO-K1 cells. The results obtained after pretreatment with different concentrations of tianeptine, with or without 15 mM glutamate, are depicted in Figure 4-19.

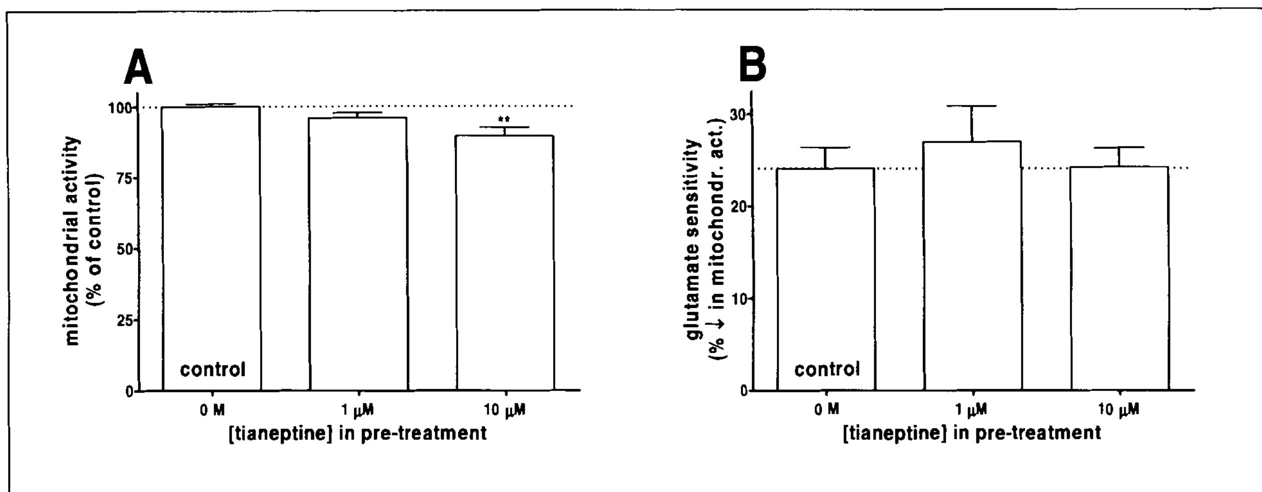

Figure 4-19: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of tianeptine (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of tianeptine, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with ** indicating $p < 0.01$.

In Figure 4-19 A, it can be seen that pretreatment with 1 µM tianeptine has no statistically significant effect on mitochondrial activity of CHO-K1 cells, while pretreatment with 10 µM tianeptine causes a statistically significant decrease in mitochondrial activity (10.6 ± 3.0%; $p < 0.01$). It seems that CHO-K1 cells are more sensitive to the effects caused by tianeptine than human neuroblastoma cells, since pretreatment with either 1 µM or 10 µM tianeptine had no significant effect on the mitochondrial activity of human neuroblastoma cells (Figure 4-17 A).

According to Figure 4-19 B, pretreatment with tianeptine has no significant effect on glutamate sensitivity. Pretreatment with 1 µM tianeptine causes a trend towards increased glutamate sensitivity, but the difference is not statistically significant. After human neuroblastoma cells were pretreated with different concentrations of tianeptine (Figure 4-18 A), a trend towards increased glutamate sensitivity was noted although the differences were not statistically significant.

4.2.1.7 Gabapentin

Studies have demonstrated that adjunctive gabapentin may be effective in the treatment of treatment-resistant depression. The results obtained after human neuroblastoma cells were pretreated for 24 hours with different concentrations of gabapentin, with or without 15 mM glutamate, are depicted in Figure 4-20.

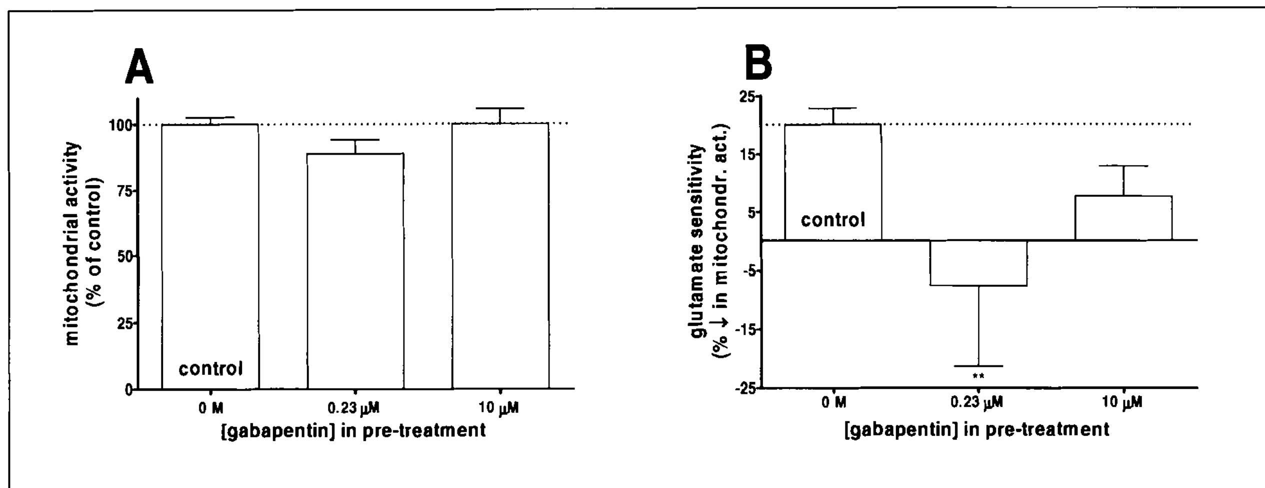

Figure 4-20: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of gabapentin (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of gabapentin, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with ** indicating $p < 0.01$.

In Figure 4-20 A, it can be seen that pretreatment with different concentrations of gabapentin has no statistically significant effect on mitochondrial activity, while Figure 4-20 B indicates that pretreatment with 0.23 µM gabapentin significantly protects against glutamate-induced excitotoxicity (a reduction of 27.6 ± 13.7%; $p < 0.01$). Pretreatment with 10 µM gabapentin merely causes a trend towards reduced glutamate sensitivity, which is not statistically significant.

It was found that oxygen/glucose deprivation (OGD) induces neuronal damage and that the neurons can be rescued by NMDA antagonists. Therefore, OGD also involves NMDA receptors. Rat hippocampal slice cultures were subjected to OGD and then exposed to several anticonvulsants for one hour. However, gabapentin was not neuroprotective in this study up to a concentration of 300 µM (Rekling, 2003).

In order to determine whether these effects on mitochondrial activity are specific to neuronal cell lines, the same experiments were conducted on a non-neuronal cell line, namely CHO-K1 cells. The results obtained after pretreatment with different concentrations of gabapentin, with or without 15 mM glutamate, are depicted in Figure 4-21.

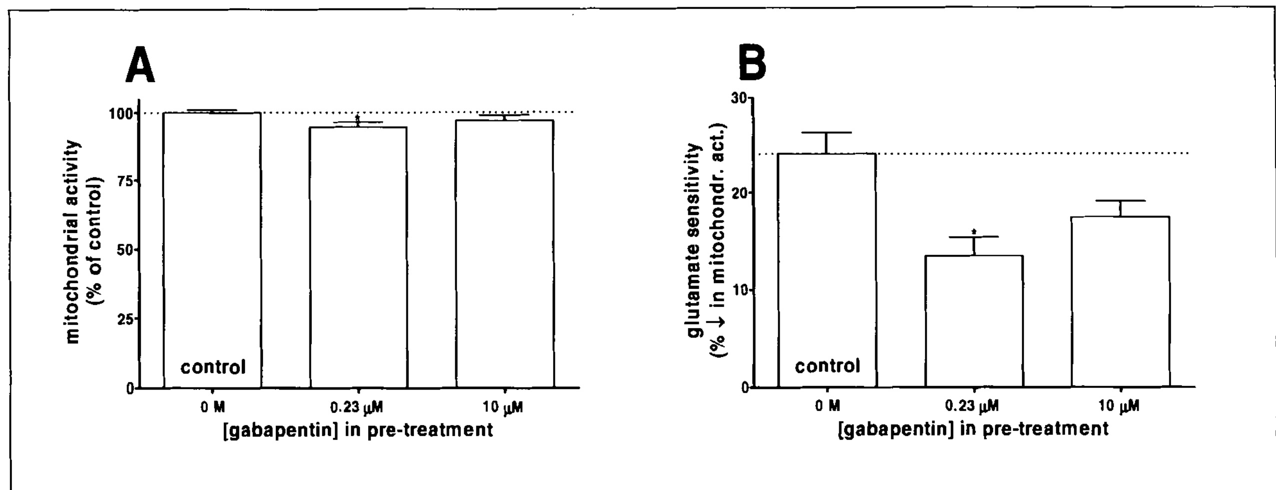

Figure 4-21: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of gabapentin (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of gabapentin, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with * indicating $p < 0.05$.

Pretreatment with 0.23 μM gabapentin causes a statistically significant decrease in mitochondrial activity (5.4 ± 1.8%; $p < 0.05$; Figure 4-21 A), while 10 μM gabapentin causes only a trend towards reduced mitochondrial activity. In Figure 4-20 A, it was noted that pretreatment with gabapentin has no significant effect on the cell viability of human neuroblastoma cells.

In Figure 4-21 B, it can be seen that pretreatment with 0.23 μM gabapentin causes a significant decrease in glutamate sensitivity (10.6 ± 1.9%; $p < 0.05$). Pretreatment with 10 μM gabapentin causes a trend towards decreased glutamate sensitivity, although the difference is not statistically significant. This is similar to the profile observed after human neuroblastoma cells were pretreated with gabapentin (Figure 4-20 B), although only reduced glutamate sensitivity is observed in CHO-K1 cells as opposed to protection against glutamate-induced excitotoxicity in human neuroblastoma cells.

4.2.2 Drugs Combined with *myo*-Inositol

4.2.2.1 Imipramine

The different concentrations of imipramine used in pretreatment were combined with 10 mM *m*Ins, with or without 15 mM glutamate. The MTT cell proliferation assay was performed in order to determine the effect of the various treatment regimes on the mitochondrial activity of cultured human neuroblastoma cells. The results are depicted in Figure 4-22.

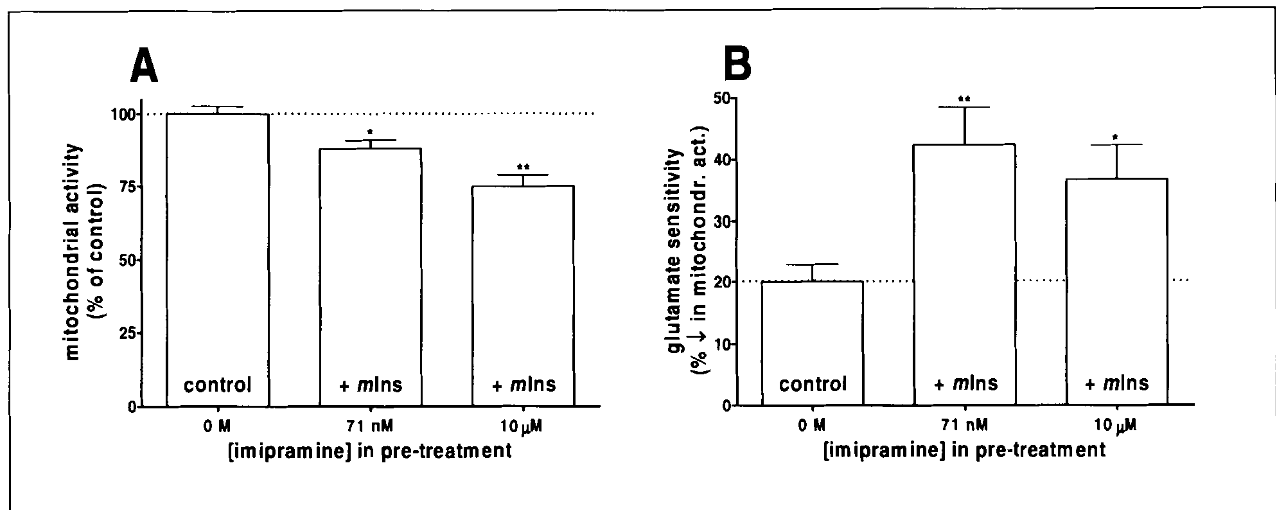

Figure 4-22: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of imipramine in combination with 10 mM *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of imipramine in combination with 10 mM *m*Ins, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with * indicating $p < 0.05$ and ** indicating $p < 0.01$.

In Figure 4-5 A, it was observed that pretreatment with different concentrations of imipramine have no significant effect on mitochondrial activity. However, when either 71 nM or 10 µM imipramine is combined with 10 mM *m*Ins (Figure 4-22 A), it results in a statistically significant decrease in mitochondrial activity (11.8 ± 2.8%; $p < 0.05$ and 24.9 ± 3.8%; $p < 0.01$, respectively). In Figure 4-2 A, it was noted that pretreatment with 10 mM *m*Ins alone results in a significant decrease in mitochondrial activity. Consequently, it can be seen that imipramine does not protect against the neurodegeneration induced by a higher concentration of *m*Ins.

As observed in Figure 4-6 A, pretreatment with 71 nM imipramine has virtually no effect on glutamate sensitivity, while 10 µM causes a significant increase in glutamate sensitivity. In Figure 4-22 B, it can be seen that when imipramine is combined with 10 mM *m*Ins, both 71 nM and 10 µM imipramine lead to an increase in glutamate sensitivity (22.3 ± 6.0%; $p < 0.01$ and 16.7 ± 5.6%; $p < 0.05$, respectively). Taken together, these results suggest that pretreatment with imipramine in combination with *m*Ins is detrimental to cell survival in this model.

In order to determine whether these effects on mitochondrial activity are specific to neuronal cell lines, the same experiments were conducted on a non-neuronal cell line, namely CHO-K1 cells. The results obtained after pretreatment with different concentrations of imipramine combined with 10 mM *m*Ins, with or without 15 mM glutamate, are depicted in Figure 4-23.

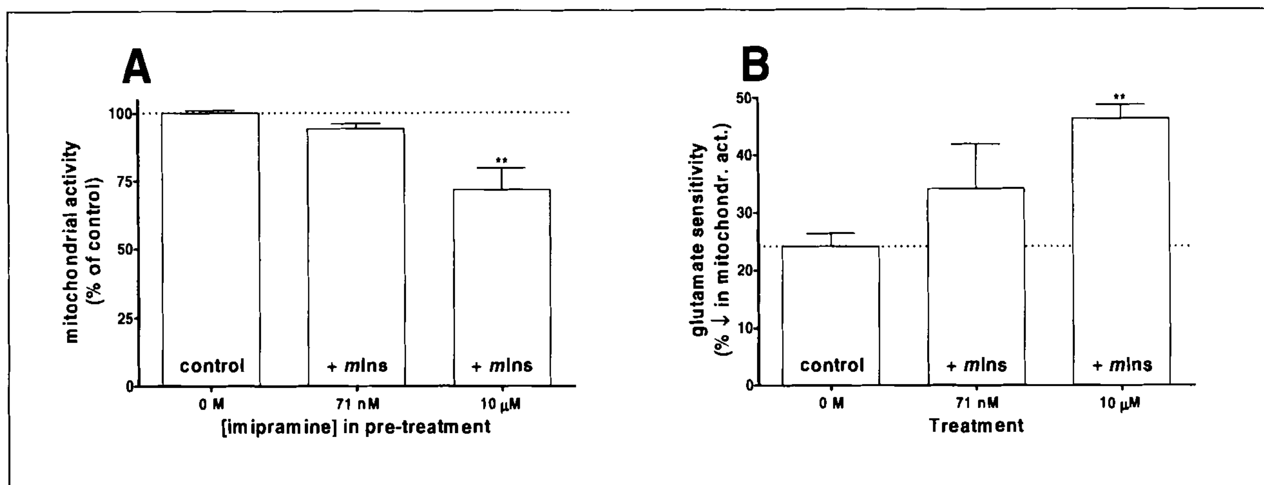

Figure 4-23: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of imipramine in combination with 10 mM *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of imipramine in combination with 10 mM *m*Ins, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with ** indicating $p < 0.01$.

It is evident from Figure 4-23 A that pretreatment with 10 μM imipramine in combination with *m*Ins causes a marked reduction in mitochondrial activity (28.4 ± 8.0%; $p < 0.01$), while 71 nM imipramine causes only a trend towards reduced mitochondrial activity. In Figure 4-22 A, it was noted that pretreatment with either 71 nM or 10 μM imipramine in combination with *m*Ins causes a decrease in the mitochondrial activity of human neuroblastoma cells.

In Figure 4-23 B, it can be seen that pretreatment with 71 nM imipramine in combination with *m*Ins causes a trend towards increased glutamate sensitivity, although the difference is not statistically significant. Pretreatment with 10 μM imipramine in combination with *m*Ins causes a significant increase in glutamate sensitivity (22.3 ± 2.4%; $p < 0.01$). According to Figure 4-22 A, pretreatment with imipramine (71 nM or 10 μM) in combination with *m*Ins leads to a considerable increase in glutamate sensitivity.

These results, as portrayed in Figure 4-23, are similar to the results in Figure 4-7, which depict the effect of pretreatment with imipramine alone on CHO-K1 cells. Therefore, this suggests that *m*Ins has no significant effect on the mitochondrial activity of CHO-K1 cells.

4.2.2.2 Fluoxetine

The different concentrations of fluoxetine used in pretreatment were combined with 10 mM *m*Ins, with or without 15 mM glutamate. The MTT cell proliferation assay was performed in order to determine the effect of the various treatment regimes on the mitochondrial activity of cultured human neuroblastoma cells. The results are depicted in Figure 4-24.

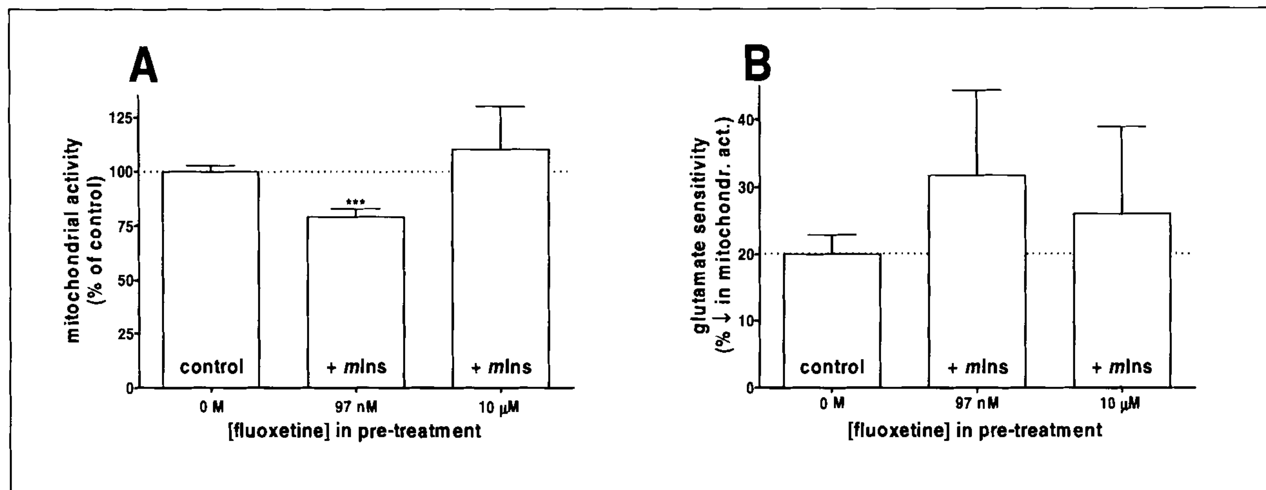

Figure 4-24: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of fluoxetine in combination with 10 mM *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of fluoxetine in combination with 10 mM *m*Ins, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a two-tailed Student's t-test, performing Bonferroni correction, with * indicating $p < 0.001$.**

Pretreatment with fluoxetine alone (Figure 4-8 A) has no significant effect on glutamate sensitivity. When fluoxetine is combined with 10 mM *m*Ins (Figure 4-24 A), 97nM fluoxetine leads to a statistically significant decrease in mitochondrial activity (21.0 ± 3.8%; $p < 0.001$), but this reduction is reversed after pretreatment with 10 μM fluoxetine in combination with *m*Ins. This suggests that 97 nM fluoxetine does not protect against *m*Ins-induced neurodegeneration (as observed in Figure 4-2 A), while 10 μM fluoxetine protects against this phenomenon.

In Figure 4-24 B, it can be seen that pretreatment with fluoxetine in combination with *m*Ins has no significant effect on glutamate sensitivity. Only a trend towards increased glutamate sensitivity is observed, since the differences did not reach statistical significance.

In order to determine whether these effects on mitochondrial activity are specific to neuronal cell lines, the same experiments were conducted on a non-neuronal cell line, namely CHO-K1 cells.

The results obtained after pretreatment with different concentrations of fluoxetine combined with 10 mM *m*Ins, with or without 15 mM glutamate, are depicted in Figure 4-25.

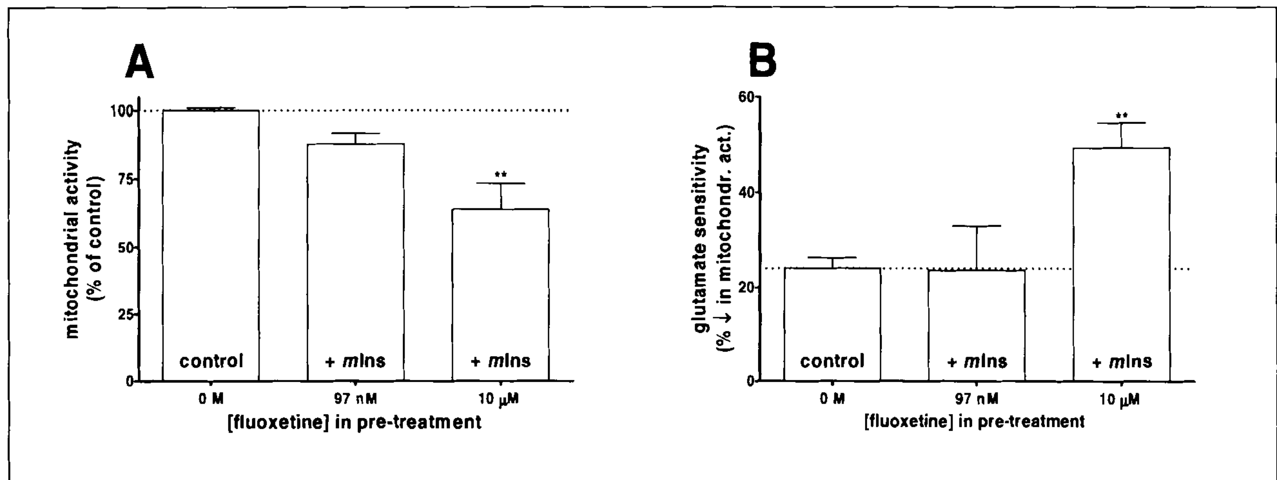

Figure 4-25: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of fluoxetine in combination with 10 mM *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of fluoxetine in combination with 10 mM *m*Ins, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with ** indicating $p < 0.01$.

Pretreatment with 97 nM fluoxetine in combination with *m*Ins has no significant effect on mitochondrial activity, although a trend towards decreased mitochondrial activity is observed (Figure 4-25 A). It is also noted that pretreatment with 10 μM fluoxetine in combination with *m*Ins causes a significant decrease in mitochondrial activity (36.2 ± 9.5; $p < 0.01$). Pretreatment with 97 nM fluoxetine in combination with *m*Ins causes a significant decrease in the mitochondrial activity of human neuroblastoma cells, while 10 μM fluoxetine in combination with *m*Ins has no significant effect on the mitochondrial activity (Figure 4-24 A).

In Figure 4-25 B, it can be seen that pretreatment with 97 nM fluoxetine in combination with *m*Ins has no statistically significant effect on glutamate sensitivity, while pretreatment with 10 μM fluoxetine in combination with *m*Ins leads to a marked increase in glutamate sensitivity (25.3 ± 5.5; $p < 0.01$). Only a trend towards increased glutamate sensitivity is noted in human neuroblastoma cells (Figure 4-24 B).

The effects of fluoxetine in combination with *m*Ins on both cell viability and glutamate sensitivity are possibly cell-type specific or species specific.

4.2.2.3 Lithium

The different concentrations of lithium used in pretreatment were combined with 10 mM *m*Ins, with or without 15 mM glutamate. The MTT cell proliferation assay was performed in order to determine the effect of the various treatment regimes on the mitochondrial activity of cultured human neuroblastoma cells. The results are depicted in Figure 4-26.

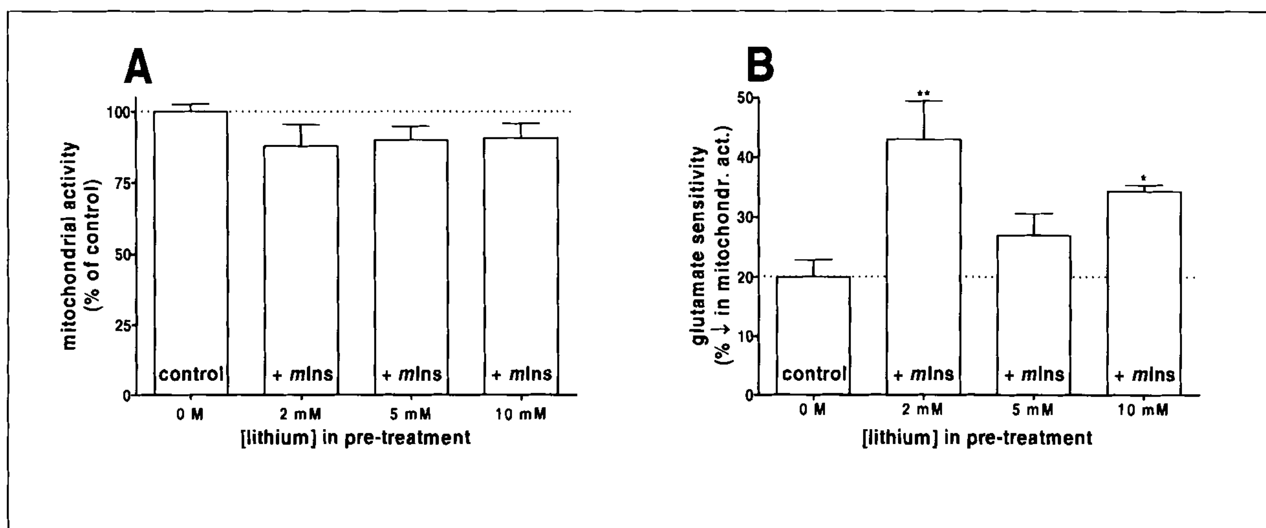

Figure 4-26: The effect of 24-hour pretreatment of human neuroblastoma [SK-N-BE(2)] cells with different concentrations of lithium in combination with 10 mM *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of lithium in combination with 10 mM *m*Ins, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with * indicating $p < 0.05$ and ** indicating $p < 0.01$.

Pretreatment with lithium in combination with *m*Ins has no statistically significant effect on mitochondrial activity (Figure 4-26 A). This indicates that lithium protects against the neurodegenerative effect observed after pretreatment with 10 mM *m*Ins alone. This was expected, as lithium has been reported to be neuroprotective.

Pretreatment with lithium alone (Figure 4-12 A) has no significant effect on glutamate sensitivity. However, an increase in glutamate sensitivity is observed after pretreatment with 2 mM (22.9 ± 6.4%; $p < 0.01$) and 10 mM (14.3 ± 1.0%; $p < 0.05$) lithium, both in combination with 10 mM *m*Ins (Figure 4-26 B). Pretreatment with 5 mM lithium in combination with *m*Ins has no significant effect on glutamate sensitivity. Therefore, in this *in vitro* model, the combination of lithium and *m*Ins seems to be detrimental to neuronal survival in the presence of glutamate-induced excitotoxicity.

In order to determine whether these effects on mitochondrial activity are specific to neuronal cell lines, the same experiments were conducted on a non-neuronal cell line, namely CHO-K1 cells. The results obtained after pretreatment with different concentrations of lithium combined with 10 mM *m*Ins, with or without 15 mM glutamate, are depicted in Figure 4-27.

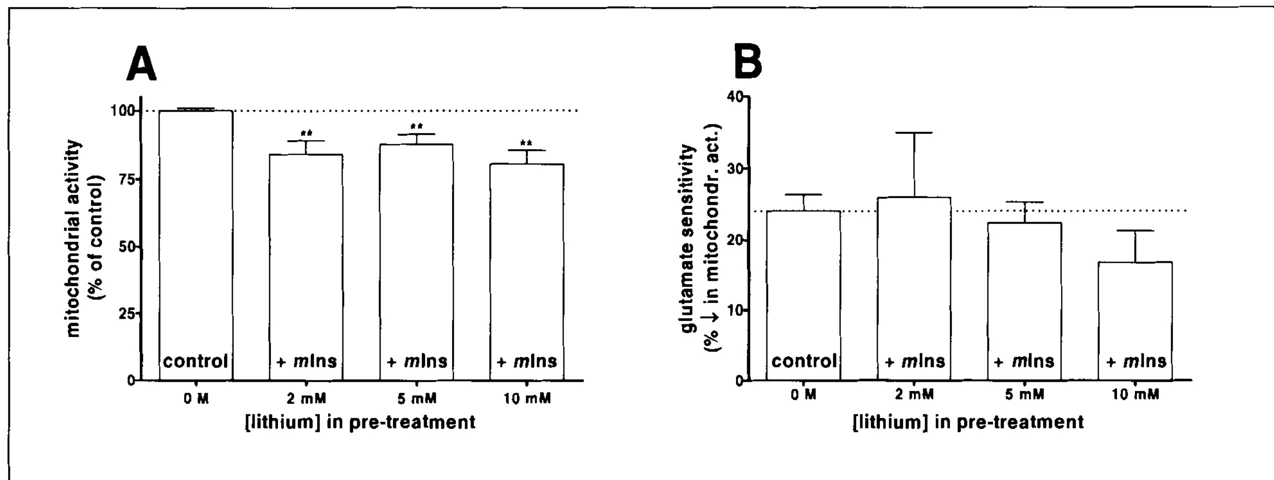

Figure 4-27: The effect of 24-hour pretreatment of Chinese hamster ovary (CHO-K1) cells with different concentrations of lithium in combination with 10 mM *m*Ins (A) without glutamate and (B) the difference in mitochondrial activity with and without 15 mM glutamate at the indicated co-treatment concentrations of lithium in combination with 10 mM *m*Ins, as measured by the MTT cell proliferation assay. Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments and are expressed as (A) percentage of the control or (B) the percentage decrease in mitochondrial activity. Data were analysed statistically by means of a one-way ANOVA, performing the Dunnett post-test, with ** indicating $p < 0.01$.

Interestingly, pretreatment with different concentrations of lithium (2, 5 or 10 mM) in combination with *m*Ins causes a significant decrease in mitochondrial activity (16.0 ± 5.1%; $p < 0.01$, 12.3 ± 3.8%; $p < 0.01$ and 19.4 ± 5.0%; $p < 0.01$, respectively; Figure 4-27 A), while it has no significant effect on glutamate sensitivity (Figure 4-27 B). The profile in Figure 4-26 A resembles that of Figure 4-27 A, although no statistically significant differences were found in the mitochondrial activity of human neuroblastoma cells (Figure 4-26 A).

The combination of lithium and *m*Ins causes either a trend towards increased glutamate sensitivity (5 mM lithium) or a significant increase in glutamate sensitivity (2 mM and 10 mM lithium) of human neuroblastoma cells (Figure 4-26 B). The treatment regimes have no significant effect on glutamate sensitivity in CHO-K1 cells, although a trend towards reduced glutamate sensitivity is observed after pretreatment with either 5 mM or 10 mM lithium in combination with *m*Ins (Figure 4-27 B).

4.3 Summary

The effects observed after human neuroblastoma cells were subjected to 24-hour pretreatment with various drugs, are summarised in Table 4-1.

Table 4-1: Summary of the results obtained after 24 hour pretreatment of human neuroblastoma cells with the indicated drugs, with N/A indicating not applicable, ↔ indicating no statistically significant difference, ↑ indicating a statistically significant increase and ↓ indicating a statistically significant decrease.

Drug Treatment	Cell Viability	DNA Damage	Glutamate Sensitivity (MTT)	Glutamate Sensitivity (Comet)
0.01 mM *m*Ins	↔	N/A	↔	N/A
1 mM *m*Ins	↔	N/A	↔	N/A
10 mM *m*Ins	↓	↑	↔	↔
71 nM imipramine	↔	↔	↔	↔
10 μM imipramine	↔	N/A	↑	N/A
97 nM fluoxetine	↔	↑	↔	↔
10 μM fluoxetine	↔	N/A	↔	N/A
2 mM lithium	↔	N/A	↔	N/A
5 mM lithium	↔	↔	↔	↔
10 mM lithium	↔	N/A	↔	N/A
0.46 μM memantine	↔	↔	↔	↔
10 μM memantine	↔	N/A	↔	N/A
1 μM tianeptine	↔	↑	↔	↔
10 μM tianeptine	↔	N/A	↔	N/A
0.23 μM gabapentin	↔	N/A	↓	N/A
10 μM gabapentin	↔	N/A	↔	N/A
71 nM imipramine + 10 mM *m*Ins	↓	N/A	↑	N/A
10 μM imipramine + 10 mM *m*Ins	↓	N/A	↑	N/A
97 nM fluoxetine + 10 mM *m*Ins	↓	N/A	↔	N/A
10 μM fluoxetine + 10 mM *m*Ins	↔	N/A	↔	N/A
2 mM lithium + 10 mM *m*Ins	↔	N/A	↑	N/A
5 mM lithium + 10 mM *m*Ins	↔	N/A	↔	N/A
10 mM lithium + 10 mM *m*Ins	↔	N/A	↑	N/A

In conclusion, the results obtained suggest that the current model, although useful, may not be optimal for the investigation of glutamate-induced toxicity in neural cells, and some unexpected results were obtained. From the current results, it can be seen that most drugs had no significant effect on cell viability in the absence of glutamate, except 10 mM *m*Ins, both 71 nM and 10 μM imipramine in combination with 10 mM *m*Ins and 97 nM fluoxetine in combination with 10 mM mIns, all of which led to a decline in cell viability. Furthermore, 10 μM fluoxetine and all the concentrations of lithium that were used, protected against the neurodegenerative

effect of a high concentration *m*Ins observed in this model, while imipramine did not protect against this neurodegeneration.

Finally, *m*Ins is not protective against glutamate-induced toxicity and it does not augment the neuroprotective effects of prototype antidepressants in this model.

Chapter 5: Summary and Conclusions

5.1 Summary

Several recent studies reported reduced volumes of certain brain areas in patients suffering from depression, suggesting neurodegeneration. A possible role has been suggested for glutamate (which, in excessive concentrations, is associated with neurodegeneration) in the pathophysiology of depression and other stress-related disorders. The current study aimed to investigate the possible protective effects of *m*Ins against glutamate-induced excitotoxicity in established *in vitro* cell lines (at a range of physiologically relevant concentrations) in comparison with various prototype experimental and clinical antidepressants. Cell viability before and after drug treatments was measured by changes in mitochondrial activity and DNA integrity.

Conditions for glutamate-induced excitotoxicity were successfully established (i.e. 15 mM for 24 hours) and, subsequently, human neuroblastoma cells were pretreated for 24 hours with different concentrations of either *m*Ins, one of a series of prototype antidepressants, or a combination of *m*Ins plus a prototype antidepressant, all with or without 15 mM glutamate. Thereafter, mitochondrial activity was determined using the MTT cell proliferation assay or DNA integrity was evaluated by means of an electrophoresis and visual DNA fragmentation quantification (comet) assay. The following results were obtained:

Mitochondrial activity

It was found that most treatment regimes in the absence of glutamate had no statistically significant effect on mitochondrial activity, with the exceptions of 10 mM *m*Ins, 71 nM imipramine + 10 mM *m*Ins, 10 µM imipramine + 10 mM *m*Ins and 97 nM fluoxetine + 10 mM *m*Ins, all of which decreased cell viability in statistically significant terms. Similarly, most drugs had no statistically significant effect on the glutamate sensitivity of cells, with the exceptions of 10 µM imipramine, 71 nM imipramine + 10 mM *m*Ins, 10 µM imipramine + 10 mM *m*Ins, 2 mM lithium + 10 mM *m*Ins and 10 mM lithium + 10 mM *m*Ins, all of which indicated an increase in glutamate sensitivity, while 0.23 µM gabapentin indicated a statistically significant decrease in glutamate sensitivity. In general, the combination of drugs with 10 mM *m*Ins augmented the neurodegenerative effects induced by 10 mM *m*Ins, except when 10 mM *m*Ins is combined with 10 µM fluoxetine or the different concentrations of lithium used in this study, which seem to protect against the mIns-induced neurodegeneration.

DNA integrity

Pretreatment with 10 mM *m*Ins, 97 nM fluoxetine or 1 µM tianeptine caused a small increase in DNA damage, while pretreatment with the other drugs had no statistically significant effect. None of the treatment regimes had any statistically significant effect on glutamate sensitivity. Although differences in effect were not statistically significant in most cases, observed trends generally supported data from measurements of mitochondrial activity.

5.2 Conclusions

In the current study, a glutamate treatment regime (15 mM for 24 hours) was established whereby the viability of human neuroblastoma cells was significantly reduced (but not abolished), enabling the study of any putative neuroprotective (or neurodegenerative) effects of drugs.

In general, results on cellular plasticity obtained after the various drug pretreatments were less pronounced and conclusive than anticipated. Contrary to what was expected from the neuroprotection hypothesis of antidepressant action, none of the antidepressants caused a dramatic neuroprotective effect in any of the assays employed. Certain trends were, however, observed for particular drugs. The lack of conclusive results may, in part, be due to numerous infections of cell lines (that had to be treated rigorously between experiments) that may have altered cellular behaviour. Certain antibiotic drugs (e.g. penicillin and ciprofloxacin, both of which were used to treat infections of the cell lines) are known to cause CNS toxicity. Studies suggest that ciprofloxacin induces DNA damage in a primary culture of rat astrocytes in a concentration-dependent manner. The ciprofloxacin-induced DNA damage may be related to oxidative stress (Gürbay *et al.*, 2006). In addition, the mean values of control data were sometimes associated with large standard errors of the mean, hampering conclusive statistical results. This may be overcome by more repetitions of experiments in future studies. It may also be advisable to optimise the *in vitro* model by experimenting with different established neuronal and non-neuronal cell lines, investigating primary culture cell lines, altering drug treatment regimes and diverse experimental conditions.

The results obtained from this study suggest that pretreatment with *m*Ins is not protective against glutamate-induced excitotoxicity in this model, while pretreatment with 10 µM *m*Ins decreases cell viability and leads to significant DNA fragmentation. It was also found that *m*Ins does not augment any putative neuroprotective properties of prototype antidepressant drugs. However, pretreatment with lithium (2 mM, 5 mM or 10 mM) or 10 µM fluoxetine, protects against the neurodegenerative effect observed after pretreatment with 10 mM *m*Ins. The combination of *m*Ins and imipramine is detrimental to cell survival. The results suggest that the

combination of *m*Ins with other antidepressants may not be additive / synergistic in terms of any neuroprotective effects, although its applicability to different experimental conditions should be determined.

Pretreatment with most of the other drugs had no significant effect on cell viability. Pretreatment with 10 μM imipramine caused a significant increase in glutamate sensitivity, while pretreatment with 0.23 μM gabapentin caused significant protection against glutamate-induced excitotoxicity. Trends towards decreased glutamate sensitivity were observed for several drugs treatments, including 97 nM fluoxetine, 5 mM lithium, 10 mM lithium and 0.46 μM memantine, although these trends were not statistically significant.

Inconsistent results have been found in this regard by other researchers (refer to § 2.3.2.2.3). A study by Maher and Davis (1996) found that pretreatment of neuronal cells with either clomipramine (30 μM and 10 μM, respectively) or imipramine (75 μM and 25 μM, respectively) for 24 hours, resulted in significant protection against glutamate-induced toxicity. However, another study found that antidepressants may reduce cell viability *in vitro.* Amitriptyline (≥ 50 μM) or desipramine (≥ 20 μM) resulted in significantly decreased cell viability after 24 hour pretreatment. 24-hour pretreatment with fluoxetine or paroxetine (both at concentrations higher than 20 μM) caused a significant reduction in cell viability. Furthermore, pretreatment with these antidepressants resulted in significantly more apoptotic cells as compared to untreated control cells (Post *et al.*, 2000). The results from the current study are therefore not in conflict with the results from similar reported studies. There is a need for *in vitro* models that more reliably correspond with neuromodulatory drug actions.

The results obtained from the MTT cell proliferation assays were largely replicated in the comet assays although, in some cases, more pronounced effects were seen on DNA fragmentation, specifically after pretreatment with 97 nM fluoxetine and 1 μM tianeptine. However, in some cases divergent results were found, namely after pretreatment with 10 mM *m*Ins, 71 nM imipramine and 1 μM tianeptine, whereafter the MTT cell proliferation assay indicated either a trend towards increased glutamate sensitivity or a significant increase in glutamate sensitivity, while the DNA comet assay indicated a trend towards reduced glutamate sensitivity as compared to the control cells.

In general, the neuroprotection hypothesis regarding the mechanism of action of antidepressants is not supported by the current study.

Differences were found between the results from the human neuroblastoma cells and those from the non-neuronal CHO-K1 cells, suggesting that the observed effects may be largely specific to neuronal cells or, at least, that the effects may be cell-type or species specific.

5.3 Recommendations

As mentioned before, several trends toward neuroprotective properties of various drugs have been observed, although the differences were not statistically significant. Due to constraints during the study, only a limited number of experiments were performed. Therefore the quality of the data might be improved if the experiments were repeated.

In light of the study by Nonaka and colleagues (1998; see § 4.2.1.4), it may be beneficial to experiment with different times for pretreatment, especially with lithium, since the researchers found that the neuroprotective effects of lithium are both time and concentration dependent. Different neuronal cell lines may also be implemented to find the optimal cell line and to investigate differences between cell lines.

In § 2.3.3, the possible involvement of stress and glucocorticoids in depression and the observed neurodegeneration was discussed. Therefore, it is possible to repeat the experiments that were conducted in the current project using glucocorticoids to induce neurodegeneration instead of glutamate.

Furthermore, the modulatory effects of the treatment regimes on enzymes and proteins concerned with neuroplasticity, including BDNF and CREB (upregulation of these proteins result in decreased cell death), the antiapoptotic protein bcl-2, the proapoptotic proteins Bax and Bad as well as the different caspase enzymes involved in apoptosis, may also be investigated both *in vitro* and *in vivo.* This can be done by means of quantitative protein measurements (e.g. Western blots) or quantitative polymerase chain reaction (PCR) to determine mRNA levels for the expression of these proteins, before and after drug treatments.

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Appendix A: Abbreviations

5-HT	serotonin
$5\text{-}HT_{2A}\text{-}R$	$serotonin_{2A}$ receptor

A	
ACTH	adrenocorticotrophic hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole
AP-1	activator protein-1
APA	American Psychiatric Association
AR	adrenergic receptor
ATCC	American Type Culture Collection

B	
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin

C	
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CMP-PA	cytidine monophosphorylphosphatidate
CNS	central nervous system
CO_2	carbon dioxide
CREB	cAMP response element binding protein
CRF	corticotropin-releasing factor
CSF	cerebrospinal fluid

D	
DAG	diacylglycerol
ddH_2O	double distilled water

DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition

E	
EDTA	ethylenediaminetetra acetic acid
EMEM	Minimum Essential Medium with Earle's Base

F	
FBS	foetal bovine serum
FLX	fluoxetine

G	
GABA	gama-amino butyric acid
GPCR	G protein-coupled receptor
GSK-3β	Glycogen synthase kinase 3β

H	
HCl	hydrochloric acid
HDS	Hamilton Depression Scale
HMIT	H^+/*myo*-inositol symporter
HMPA	high melting point agarose
HPA	hypothalamo-pituitary-adrenal

I	
IMI	imipramine
IMPase	inositol monophosphatase
IP_1	inositol-1-monophosphate
IP_2	inositol 4,5-bisphosphate
IP_3	inositol trisphosphate

IP_4	inositol 1,3,4,5-tetrakisphosphate
IP_5	inositol 1,3,4,5,6-pentakisphosphate
IP_6	inositol 1,2,3,4,5,6-hexakisphosphate

K	
K^+	potassium ion
KCl	potassium chloride

L	
Li^+	lithium ion
LiCl	lithium chloride
LMPA	low melting point agarose
l-NE	*l*-norepinephrine
LTP	long-term potentiation

M	
mAChR	cholinergic muscarinic receptors
MAO	monoamine oxidase
MAP	mitogen-activated protein
*m*Ins	*myo*-inositol
MK801	dizocilpine
mRNA	messenger ribonucleic acid
MTT	thiazolyl blue tetrazolium bromide

N	
Na^+	sodium ion
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NMDA	*N*-methyl-D-aspartate
NO	nitric oxide

NOS	nitric oxide synthase

O	
OCD	obsessive-compulsive disorder

P	
PA	phosphatidic acid
PBS	phosphate buffered saline
PCP	phencyclidine
Pen	penicillin
PI	phosphatidylinositol
PIs	phosphoinositides
PI 3-kinase	phosphatidylinositol 3-kinase
PI 4-kinase	phosphatidylinositol 4-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PO	prolyl oligopeptidase
$[PP]_2$-IP_4	bisdiphosphoinositol tetrakisphosphate
PP-IP_5	diphosphoinositol pentakisphosphate

S	
Ser	serine
SMIT	Na^+/*myo*-inositol transporter
SSRI	selective serotonin reuptake inhibitor
Strep	streptomycin

T	
trkB	tyrosine kinase B

Z	
Zn^{2+}	zinc ion