CITALOPRAM AND MIRTAZAPINE EFFECTS IN CHANGES IN FURA2 AND FURAFF RATIOOMETRIC FLUORESCENCE LEVELS IN C6 AND SH-SY5Y CELL LINES USING MICROPLATE AND RATIOOMETRIC MICROSCOPY

A Thesis Submitted to the College of Graduate Studies and Research In Partial Fulfillment of the Requirements For the Degree of Doctor of Philosophy

In the Department of Pharmacology University of Saskatchewan Saskatoon

By

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Abstract

Research in the field of molecular neuroscience contributes a better perception of the events that trigger neurodegeneration. At the forefront of this work is the study of intracellular calcium as a consequence of mitochondrial dumping and NMDA receptor activation by glutamate. Increased intracellular calcium presages excitotoxicity with ultimate apoptosis of the cell. Among the many disorders involving this sequence is depression, a disorder that in and of itself is a risk factor for neurodegenerative disorders such as Alzheimer disease. The successful use of anti-depressants to alleviate the depressive state leads to the question about whether these pharmacological agents, as part of their effect to ameliorate depression, might have an effect on intracellular calcium. Until now, this has not been explored directly but such exploration was initiated with this thesis. As a model for astrocytes and neurons, two cell lines, C6 and SH-SY5Y were used. These were differentiated with all-trans retinoic acid into astrocyte-like and neuron-like cells.

Graphic imaging of intracellular calcium by ratiometrics is not new, but what is new is using this technique to evaluate the effect of the antidepressants mirtazapine and citalopram on intracellular calcium fluxes induced by glutamate. Furthermore, comparing the ratiometric intracellular calcium flux in the presence of mirtazapine and citalopram to that of known NMDA blockers was also done for the first time. Also studied were the acute and chronic effects of mirtazapine and citalopram on cell viability.
The antidepressant agents, mirtazapine and citalopram, were chosen for this study. Mirtazapine blocks the adrenergic and serotonergic inhibitory autoreceptors which results in the increased release of these neurotransmitters and increases their concentration in the synapse. And it also has been shown to have an anti-oxidant and a calcium modulatory effect. Citalopram has the highest degree of serotonin reuptake selectivity of all the selective serotonin reuptake inhibitors.

The ratiometric studies found that mirtazapine and citalopram reduce the effect of glutamate-induced increase in relative \([\text{Ca}^{2+}]\), by either a direct or indirect action on NMDA receptors. This effect is not similar to the NMDA blockers memantine and AP5. The supporting evidence is that CCCP, which normally releases calcium from mitochondria, has no effect in cells treated with acute mirtazapine or citalopram. This indicates that no calcium entered the cell – and subsequently none was taken up by mitochondria – in response to glutamate. However, these observations were with a limited number of cells and, therefore, these results will have to be verified by different techniques by different laboratories.

In microplate studies, all drugs studied reduced cell viability but the mechanism behind this reduced viability remains to be determined. This may be due to mutations in enzymatic expression, uptake of drug through the cell membrane, or other perturbations.

The reduction in cell viability induced by acute glutamate was attenuated by pretreatment with mirtazapine or citalopram. Moreover, chronic treatment of the cells with mirtazapine or citalopram for 10 weeks before acute treatment with glutamate either attenuated the effect on viability or reversed it.
Based on this present study, mirtazapine and citalopram may be useful as neuroprotective agents to alleviate not only depression but also to reduce cell death in neurodegenerative diseases, trauma and stroke.
UNIVERSITY OF SASKATCHEWAN

College of Graduate Studies and Research

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LIST OF ABBREVIATIONS

5HT 5-hydroxytryptophan
AD Alzheimer disease
ALS-FTD amyotrophic lateral sclerosis-frontotemporal dementia
AM acetoxymethyl ester
ANOVA analysis of variance
AP5 2-amino-5-phosphonopentanoic acid
ATRA all-trans retinoic acid
CCCP carbonyl cyanide m-chlorophenyl hydrazone
CCD charge-coupled device
CNS central nervous system
D differentiated
DMEM Dulbecco’s modified Eagle’s medium
DMSO dimethyl sulfoxide
DPBS Dulbecco’s phosphate buffered saline
EMCCD electron-multiplying charge-coupled device
ER endoplasmic reticulum
FBS fetal bovine serum
FF affinity (low affinity)
HBSS Hank’s balanced salt solution
HEPES N-[2-hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid]
MDD major depressive disorder
<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>MPT</td>
<td>mitochondrial permeability transition</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mt</td>
<td>mitochondrial</td>
</tr>
<tr>
<td>NaSA</td>
<td>noradrenergic serotonergic antidepressant</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>Pen-Strep</td>
<td>Penicillin-Streptomycin</td>
</tr>
<tr>
<td>PTI</td>
<td>Photon Technologies International</td>
</tr>
<tr>
<td>SSRI</td>
<td>selective serotonin reuptake inhibitors</td>
</tr>
<tr>
<td>U</td>
<td>undifferentiated</td>
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1. INTRODUCTION

1.1. Calcium

Calcium is a mineral that is essential for normal metabolism and function. Among its numerous physiological actions, it is a cofactor for many enzymatic reactions, and it is involved in muscular contraction, in the release of neurotransmitters and in providing compressional strength of bone and teeth.

1.1.1. Calcium in Neurons and Glia

Calcium, as the ion Ca$^{2+}$, is responsible for part of the current flow across excitable membranes in the nervous system (Friedman, 2006). Calcium concentrations are 2.1 to 2.6 mM in serum, 1 mM in extracellular fluid (Friedman, 2006) and 100 nM within the cell (Friedman, 2006).

Cytosol calcium requirements are met both by an influx of extracellular calcium and by the release of calcium from the endoplasmic reticulum (ER) stores (Chakroborty & Stutzmann, 2011). Extracellular calcium enters through channels in the neuronal and glial plasma membranes that open in response to a depletion of intracellular calcium (Putney, 2011). Calcium enters the cell predominantly through two types of channels: voltage-gated and ligand-gated channels (Chakroborty & Stutzmann, 2011). The types of voltage-gated channels are classified by the letters L, N, T, P/Q and R (Altier & Zamponi, 2008) and are activated by changes in the membrane potential of the cell. Ligand-gated channels can be grouped into indirect and direct acting. One type of indirect acting calcium channel activates G protein which induces a series of reactions that opens the channel. Another type of indirect-acting channel also involves a G protein, but relies on a diffusible second messenger to activate the calcium channel (Nicoll, 2007). The indirect-acting channels are referred to as metabotropic receptors. Direct-acting
channels open when a ligand combines with the receptor. These are further grouped into NMDA and non-NMDA receptors. Glutamate activates direct-acting and indirect-acting ligand receptors, including NMDA receptors, the principle receptor involved in fast excitatory synaptic transmission (Bloom, 2006).

In addition to the above channels in the cell membrane, intracellular calcium ([Ca$^{2+}$]$_i$) can be increased from intracellular calcium stores. Calcium is released from these stores when [Ca$^{2+}$]$_i$ becomes too low and are mediated by G-protein coupled receptors that enhance inositol triphosphate (IP$_3$) production (Carafoli, 2005; Salido, Sage, & Rosado, 2009). These are most prominent in the endoplasmic reticulum (ER) and cause the ER to discharge calcium. This is primarily regulated by two STIM sensor molecules, STIM1 and STIM2 (Putney, 2011), found on both the endoplasmic reticulum and plasma membrane. STIM1 and to a much lesser extent, STIM2, stimulate the activation of Orai channels in the plasma membrane. Store-operated Ca$^{2+}$ entry differs from the voltage-gated and ligand-gated pathways in that these channels directly provide Ca$^{2+}$ to specific intracellular targets that, in turn, initiate signaling pathways (Putney, 2011). This signaling then regulates calcium going through voltage-gated calcium channels in a process known as calcium-release-activated calcium (Bakowski, Nelson, & Parekh, 2012; Putney, 2012). ER release is mediated by two receptors, the ryanodine receptor (RyR) and IP$_3$ receptor (IP$_3$R) (Chakroborty & Stutzmann, 2011).
1.1.1.1. Calcium Signaling

Calcium signaling differs depending on the cell type. In non-excitable cells, the effect of external signals causes ligand binding to G-protein-coupled receptors and also to tyrosine kinase which then activates phospholipase. The phospholipase cleaves the phosphatidylinositol 4,5, bisphosphate (PIP$_2$) into 1,4,5,-inositol triphosphate (IP$_3$) and diacylglycerol (DAG). The IP$_3$ binds to its receptor on the endoplasmic reticulum (ER) which permits the release of calcium from the ER into the cytoplasm (Ehrlich, 1995). Once in the cytoplasm, it will interact with enzymes to enhance reactions.

1.1.1.2. Ryanodine Receptors

In excitable cells, calcium signaling is due to changes in the membrane potential and the introduction of calcium by way of different channels on the plasma membrane, mentioned above. In addition to IP$_3$ receptors, ryanodine receptors (RyRs) are also expressed on the ER and sarcoplasmic reticulum (SR) membranes of excitable cells (although they are also found in some non-excitable cells, such as epithelial cells, exocrine cells, and lymphocytes (Lanner, Georgiou, Joshi, & Hamilton, 2010). The ligand for these receptors is calcium itself (Van Petegem, 2012). In this context, in myocytes, upon depolarization of the membrane, calcium comes into the cell through L-type calcium channels and binds to the RyRs, opening them and permitting release of calcium from the sarcoplasmic reticulum. However, when calcium cytoplasmic calcium levels rise too high, calcium causes the closure of the RyRs, due to calcium binding at another site on the RyRs. When cytoplasmic levels become low in calcium, the RyRs open up permitting calcium to egress (Woods & Padmanabhan, 2012). There are three basic isoforms of RyRs. RyR1 was the first to be identified in skeletal muscle (Zorzato et al., 1990); RyR2 is found in the
heart (Otsu et al., 1990), and RyR3 was initially found in the brain (Hakamata, Nakai, Takeshima, & Imoto, 1992), although all three types were eventually found in other tissues (Lanner et al., 2010).

1.1.1.3. Nuclear Calcium

In addition to cytoplasmic interactions, calcium also freely enters the nucleus through the nuclear pore complex (NPCs) in neurons (Eder & Bading, 2007). However, NPCs are limited in number slowing the rise of intranuclear calcium in response to signals. Concomitantly, there is a slower decay of the nuclear calcium signal compared to that from the cytoplasm is due to the absence of plasma membrane exchangers and no reuptake by the endoplasmic reticulum (Bengtson & Bading, 2012). Increased synaptic activity in excitable cells will causes infoldings of the nuclear envelope, causing an increase in NPCs and enhancing the surface to volume ratio. In so doing, calcium is made available to deep nuclear regions causing increased kinetics and higher calcium signals. One result is faster histone-3-phosphorylation, a benchmark for transcription induction (Bengtson & Bading, 2012).

1.1.1.4. Cytoplasmic Regulation of Calcium

Cytoplasmic calcium is maintained at approximately 100nM by two calcium storage regulators, the ER/SR and mitochondria. In addition, there are several types of calcium pumps that continuously expel calcium to the external environment, much like emptying bilge water in a ship. Two types of ATPase pumps are high affinity but low capacity calcium exporters. The sarcoendoplasmic reticular Ca^{2+} ATPase (SERCA) pumps cytoplasmic calcium into the ER/SR, while the plasma membrane Ca^{2+} ATPase (PMCA) dumps calcium out of the cell (Strehler,
Sodium-calcium exchangers (NCX) are low-affinity but high capacity pumps in which either one Ca\textsuperscript{2+} is exchanged for three Na\textsuperscript{+} ions (NCX) or one Ca\textsuperscript{2+} and one K\textsuperscript{+} are exchanged for four Na\textsuperscript{+} ions (NCKX) (Schnetkamp, 2004).

1.1.1.5. VGCCs

The fastest calcium transport channels are the paddle-shaped voltage-gated calcium (VGCC) channels (Woods & Padmanabhan, 2012). Normally closed, these can undergo conformational changes and rapidly enable calcium to passively flow down the concentration gradient either from the cytoplasm to the exterior or from the exterior to the cytoplasm (Catterall, 1995). In the absence of extracellular calcium, these channels will also transport potassium or sodium. These channels, as listed above, are divided by pharmacological or physiological responses or characteristics into 6 types (L-, N-, P-, Q-, R-, and T-type channels). These channels are responsible for the release of neurotransmitters, neuropeptides, or neurohormones by presynaptic membranes and axon endings (boutons) on other neurons, glia, blood vessels or other structures.

1.1.2. Role of Calcium in Disease

Calcium plays a ubiquitous role in the mediation of neuronal death in a number of neurodegenerative conditions including Alzheimer disease (Zhou, Gopalakrishnan, & Richardson, 1996; Zatti et al., 2004; Boada et al., 2010), Parkinson disease (Furukawa et al., 2006; Danzer et al., 2007; Cali, Ottolini, Negro, & Brini, 2012), Huntington disease (Choo, Johnson, MacDonald, Detloff, & Lesort, 2004; Lim et al., 2008) and amyotrophic lateral sclerosis (Carri et al., 1997; Carriedo, Sensi, Yin, & Weiss, 2000; Martin et al., 2009), as well as...
in normal aging (Halliwell, 1992; Lees, 1993; Markesbery, 1997; Hansson Petersen et al., 2008). Of the many mechanisms which have been implicated, there are several pathophysiological events can result in elevated calcium levels. These include the build-up of beta amyloid, glutamate excitotoxicity (Coyle & Puttfarcken, 1993; Beal, 1995; Iversen, Mortishire-Smith, Pollack, & Shearman, 1995; Mattson, Goodman, Luo, Fu, & Furukawa, 1997), and activation of the NMDA receptor (Leslie et al., 1992; Werth & Thayer, 1994; Dugan et al., 1995; Baron, Wang, Padua, Campbell, & Thayer, 2003). Calcium promotes the formation of ROS, which, in turn, oxidize cellular lipids to form lipid peroxidation products (Mattson, Barger, Begley, & Mark, 1995; Reynolds & Hastings, 1995; Mark, Lovell, Markesbery, Uchida, & Mattson, 1997; Mohmmad et al., 2006). Among these products is oxidized LDL, which is often found in neurodegenerative disorders (Palinski et al., 1989; Coyle & Puttfarcken, 1993; Deibel, Ehmann, & Markesbery, 1996).

1.1.3. Intracellular Distribution of Calcium

Intracellular calcium \([\text{Ca}^{2+}]_i\) ranges from 50 nM to 100 nM in the basal state to 500 nM - 1 \(\mu\)M in an active state (Friedman, 2006). Most cytosolic calcium is stored in the endoplasmic reticulum which acts as a calcium reservoir, the lumen of which has of 0.2 to 1 mM of \([\text{Ca}^{2+}]_i\) (Salido et al., 2009), or up to approximately 10,000 times that of the surrounding cytosol \([\text{Ca}^{2+}]_i\) in its resting state (Putney, 1999; Wei, Wang, Zheng, & Cheng, 2012). The mitochondrial matrix normally has very low levels (about 100 nM) \([\text{Ca}^{2+}]_{\text{mt}}\) but this can increase up to 100 \(\mu\)M in response to a rise in \([\text{Ca}^{2+}]_i\) (Rizzuto, De, Raffaello, & Mammucari, 2012a). The nucleus also has calcium channels through the nuclear envelope and uses calcium in gene transcription, DNA metabolism and synthesis of new envelope.
In neurons and astrocytes (Lalo, Pankratov, Kirchhoff, North, & Verkhratsky, 2006), peripheral mitochondria are located near the cytosolic exit of the NMDA ligand-gated channel, whereas the granular mitochondria attach to the ER (Bakowski et al., 2012) by a trypsin-sensitive protein complex, one of which is mitofusin 2 (Rizzuto, De, Raffaello, & Mammucari, 2012b) and quickly take up and buffer any calcium emanating from the ER and then slowly releases it to the cytosol (Chakroborty & Stutzmann, 2011; Rizzuto et al., 2012b). Movement into the mitochondria is rapid and via a high-affinity calcium uniporter (Raffaello, De, & Rizzuto, 2012a), where it is stored in the matrix bound to phosphate (Rizzuto et al., 2012b). The \([Ca^{2+}]_{mt}\) is slowly exchanged for sodium by a mitochondrial \(2Na^+/Ca^{2+}\) or a \(H^+/Ca^{2+}\) exchanger (Raffaello, De, & Rizzuto, 2012b).

1.1.4. Mitochondrial Distribution in Response to \([Ca^{2+}]_i\)

In pancreatic acinar cells, there are three mitochondrial groups (Petersen, 2012). A peri-nuclear group which surrounds the nucleus, a peripheral group which lies just inside the cell membrane, and a peri-granular group which surrounds the apical acinar granules which produce pancreatic enzymes for exocrine excretion to the small intestine. In all three areas, the mitochondria serve as a buffer of calcium to prevent high levels of calcium from entering or leaving these compartments. It has been observed that the nuclear pores are not selective and that if mitochondria were not present, calcium would enter unabated. Moreover, since calcium is important in the production of a large number of calcium regulatory proteins, it also keeps the calcium environment of the nucleus at a constant level (Petersen, 2012).
1.1.5. Sudden Calcium Influx

In response to a sudden increase in intracellular calcium entering either via glutamate-activated NMDA receptors or released from the ER, mitochondria rapidly take up the excess calcium by both uniporter (actively transports only calcium) and RaM (rapid mode of calcium uptake) mechanisms (Gunter & Gunter, 2001). The sudden increase in intracellular calcium causes the release of calcium from other intracellular stores, most notably the endoplasmic reticulum in the calcium-induced calcium release (CICR) mechanism (Kotlikoff, 2003). The excess calcium is also taken up by mitochondria but saturation of the buffering capacity of the matrix will cause efflux of calcium by two different mechanisms (Pfeiffer, Gunter, Eliseev, Broekemeier, & Gunter, 2001). One of these is a response termed mitochondrial permeability transition (MPT). In MPT, pores are formed in the inner mitochondrial membrane which, if the MPT is maintained, will result in the depolarization of the inner membrane and the cessation of ATP synthesis (Dong, Saikumar, Weinberg, & Venkatachalam, 2006; Cali, Ottolini, & Brini, 2012). In the central nervous system, neuronal death results in excess glutamate – the major excitatory neurotransmitter in the CNS – being released. The glutamate storm combines with NMDA receptors on adjacent neurons to admit a flood of calcium into the cytosol. Mitochondria will soak this up, but if mutated, cannot do so effectively (Duchen, 2012). Oversaturation causes mitochondrial depolarization with release of cytochrome c, thereby initiating programmed cell death (apoptosis) (Cali et al., 2012).

One approach in pharmacologic therapy has been to search for neuroprotective drugs that reduce either the production or the effect of unstable free radicals (such as by inhibiting calcium influx into the mitochondria) and ultimately the lethal depolarization of the mitochondria. Another
approach would be to modulate the glutamate-induced entry of calcium into the cells.

Citalopram and mirtazapine are two antidepressants agents that may provide a neuroprotective effect by attenuating the glutamate-induced increase in intra-cellular calcium and preventing intracellular calcium from reaching excitotoxic levels.

1.1.6. Examining Calcium Levels by Ratiometric Determinations

The movement of calcium into the cytoplasm from the mitochondria can be studied using Fura2 AM and FuraFF AM (Kurebayashi, Harkins, & Baylor, 1993; Putney, 1999). Fura 2 is a high affinity fluorophore which means it will be saturated at low concentrations of calcium. Therefore it will not be sensitive to concentrations much above 1 μM. Because its K_d for calcium is 0.14 μM, rapid Ca^{2+} shifts are often missed because of the slow rate of Ca^{2+} dissociation from Fura 2. Fura FF, in contrast, has a low affinity for calcium so that it will not be saturated at low concentrations of calcium. Furthermore, its K_d for calcium is 5.5 μM, indicating that rapid shifts will not be missed.

Intracellular compartments examined for calcium levels were the cytoplasm and mitochondria. Extracellular calcium is unlimited and steady at about 1 mM (Salido et al., 2009; Rizzuto et al., 2012b). Intracellular distribution of calcium is unequal, ranging from 10 nM to 0.2 mM within the same cell (Bakowski et al., 2012). Most cytosolic calcium is stored in the endoplasmic reticulum (and in muscle the sarcoplasmic reticulum) which acts as a calcium reservoir and the lumen of which has a concentration of 0.2 to 1 mM (Salido et al., 2009; Rizzuto et al., 2012b), about 10,000 times that of the surrounding cytosol. The mitochondrial matrix normally has very
low levels (about 100 nM) of calcium when compared to the lumen of the endoplasmic reticulum.

Calcium fluxes can be followed using Fura-2 (Collins, Lipp, Berridge, & Bootman, 2001; Collins, 2007) or Fura FF. Using Fura-2 or Fura FF, the first absorption peak is at 380 nm which represents free fluorophore not bound to calcium. After binding to calcium, the dye absorbs UV light at 340 nm. Emission for both is 505-510 nm. The ratio of 340/380 is not dependent on the concentration of the fluorophore and so is a reliable indicator of calcium. Although there is some binding to calcium before glutamate administration, most of the dye will not be bound to calcium as the cytosolic concentration of free calcium will be low. After the administration of glutamate, there will be a sharp increase in intracellular calcium which binds to the dye saturating it. The calcium-saturated dye will then absorb at a lower wavelength, 340 nm, producing a second peak.

1.2. Mitochondria

Mitochondria are double membrane intracellular organelles of bacterial ancestry (Boussau, Karlberg, Frank, Legault, & Andersson, 2004) that are essential for normal cell function. On their inner membranes, mitochondrial produce 95% of cellular ATP (Cali et al., 2012), and in their matrix 100% of cellular heme (van Dooren, Kennedy, & McFadden, 2012). Both mitochondrial locations are also sites for other functions essential to cell survival. One such activity is the sequestration of excess intracellular calcium and the subsequent release of that calcium as cytosolic calcium lowers (Rizzuto et al., 2012b; Raffaello et al., 2012b).
In stroke, head trauma, aging, Alzheimer disease, amyotrophic lateral sclerosis, and other neurodegenerative disorders, there are consequential metabolic changes and mutations in mitochondria. These changes are the result of, and result in, the production of excess unstable free radicals as a result of excess calcium uptake by neurons and glial cells leading to cell dysfunction and death. (Duchen, 2012; Rizzuto et al., 2012b)

1.2.1. Mitochondrial Origins
Mitochondria originated from alpha-proteobacteria (Lang, Gray, & Burger, 1999) three billion years ago (de Castro, Martins, & Tufi, 2010) and acquired the ability to survive in the presence of a new environmental toxin – oxygen – and convert it to carbon dioxide, nitrate and water (Schon, 1997). Between two and three billion years ago these bacteria became an endosymbiont with eukaryotic cells as (Wallace, 2005). They retained much of the structure and biochemistry of their heritage, including a double membrane, with the inner one having multiple infoldings called cristae (Schon, 1997). Like bacteria, they have their own genes and the ability to divide over time as they increase from the 5 or 10 that are in the human ovum, to the $10^{15}$ that are present in the human body (Schon, 1997). As humans age, the mitochondria mutate, driven by normal genetic mutation and the presence of adverse environmental factors. In free-living bacteria, a driving force for mutation has been the presence of antibiotics in the environment that promote the survival of drug-resistant bacteria while killing off susceptible strains. In mitochondria, some mutations are the result of random DNA rearrangements which may have a detrimental impact on the health of the cell and of the individual. Or, the driving mutation force could be the presence of toxic factors such as oxidants or heavy metals. The presence of abnormal aggregates that are the hallmarks of many neurodegenerative diseases may impede
normal mitochondrial function. The outcome is the initiation of mitochondrial death and subsequently, apoptosis of the cell.

Mitochondria are the focal point for neurodegenerative disorders by virtue of their production of free radicals, regulation of intracellular calcium and iron, being the sole source of ATP for the cell and initiation of apoptosis. Within a single human, all of the constantly dividing and dying mitochondria have originated from just 5 to 10. The large number of mitochondria coupled with a high reproductive rate lends itself to mutations throughout the life span. Mutations that occurred early resulted in widespread mitochondrial defects in many different tissues. Those mutations occurred later further upstream from the initial 5 to 10 mitochondria, may have affected only a single organ or a specific location within that organ. These mutations may result in the formation of aggregates of substances within mitochondria, in mtDNA derangements that permit excess free radical production or diminished ATP formation, or in the opening or closing of pores through the inner membrane, permitting metabolic aberrations. Work must be continued in all of these areas to get a better idea of the disease process. Only then can specific targeted therapy be developed. Meanwhile, in the short term, drugs must be tested which will provide a palliative method of disease containment. In this thesis, two such drugs, citalopram and mirtazapine, will be screened for possible beneficial properties using glial-like and neuron-like cell lines.
1.3. Glutamate

1.3.1. Overview

The concentration of glutamate in the mammalian brain is 1,000 times as much as other neurotransmitters such as dopamine or serotonin (Kostandy, 2012). Glutamate was identified in the middle of the nineteenth century but its biological role was not studied until the beginning of the twentieth century (Vickery & Schmidt, 1931). It was found in the brain in the 1930s and twenty years later was shown to be a neuro-stimulant that causes seizures (Collingridge, Kehl, Loo, & McLennan, 1983; Sano, 2009). Since that time, it has been found to be the most abundant excitatory neurotransmitter in the mammalian brain and spinal cord, and that, in excess, it damages neurons and sets in motion a series of events which leads to the death of neurons (Budd & Nicholls, 1996; Cheng, Fass, & Reynolds, 1999; Deng, Yue, Rosenberg, Volpe, & Jensen, 2006; Caudle & Zhang, 2009). Among the most important of these events is the influx of large amounts of calcium and the subsequent uptake of this calcium load by mitochondria (Collins et al., 2001) resulting in mitochondrial dysfunction. Glutamate excitotoxicity is the process which causes cell death in many neurodegenerative diseases (Corona, Romo, & Tapia, 2007; Caudle & Zhang, 2009).

1.3.2. History of Glutamate and its Role as a Neurotransmitter

Glutamate is a non-essential amino acid that was discovered in 1866 by the German chemist Karl Heinrich Leopold Ritthausen (Vickery & Schmidt, 1931; Kurihara, 2009). Between 1899 and 1906, Emil Fischer put some glutamate into his mouth and found it first tasted sour and then became tasteless (Fischer, 1906; Kurihara, 2009). Following Fischer’s report, Kikunae Ikeda identified the tasteful substance in kelp as the sodium salt of glutamate which he called *umami,*
and commercialized the process of isolating sodium glutamate from kelp (Ikeda, 1912; Kurihara, 2009). At the end of the 20th century the taste of umami (sodium glutamate monophosphate) was added to the four classical tastes, sweet, sour, salty and bitter (Kurihara, 2009).

In the 1930s glutamate was found to be present in the brain and so led to dietary experiments with the amino acid in the 1940s to treat learning disorders and epilepsy (Watkins & Jane, 2006). In 1954, Hayashi found that injecting sodium glutamate into the carotid arteries or directly into the brain caused seizures and he speculated that glutamate might be a neurotransmitter in the brain (Watkins & Jane, 2006). In the 1960’s, Krnjevic and Phillis demonstrated that many brain cells were excited by the application of glutamate by micropipettes, and concluded that glutamate was an excitatory neurotransmitter in the brain (Krnjevic & Phillis, 1963a; Krnjevic & Phillis, 1963b). These authors correctly identified glutamate to be the principle excitatory neurotransmitter in the mammalian brain and spinal cord. Over the years, this single criterion for a neurotransmitter – namely, that neurons respond to the agent - has been refined and expanded to include four criteria. The first is that a neurotransmitter must be either synthesized by - or, if a natural substance such as an amino acid, sequestered in - a neuron. Second, it must be released in sufficient quantities from the presynaptic membrane to cross a synapse and combine with enough receptors on a post-synaptic membrane to exert the presumed effect. Third, when applied by micropipette or other method, it must mimic exactly the effect of the natural neurotransmitter. Finally, fourth, there must be a method by which the neurotransmitter is removed from the synapse, either by axonal reuptake or by enzymatic destruction (Parent, 1996). As will be discussed below, glutamate fits all of these criteria.
1.3.3. Synthesis of Glutamate

Glutamate does not cross the blood brain barrier but rather is synthesized in axon endings and in astrocytes (Deutch & Roth, 1999). Glutamate synthesis occurs in neuronal mitochondria from alpha ketoglutarate, one of the intermediates of the Krebs cycle (tricarboxylic acid cycle) (Schwartz, 2000). Glutamine is another source of glutamate. Glutamine is synthesized by astrocytes which then transfer it to neighbouring neurons. Within the neuron terminal mitochondrion, the amine group is removed by glutaminase to form the final product, glutamate. Once formed, glutamate is stored in synaptic vesicles and released in a calcium dependent process (Deutch & Roth, 1999).

1.3.4. Glutamate Receptors

The term ‘receptor’ was first used by Paul Ehrlich in 1900 to describe the hypothetical molecule on the cell with which toxins interacted and which was also responsible for antigen-antibody reactions. The first subclass of glutamate receptors be discovered was the NMDA receptor, followed by the AMPA and kainate receptors. The latter are often classified as non-NMDA receptors. By the mid-1990s three classes of ionotropic and eight classes of metabotropic glutamate receptors had been characterized (Dingledine & McBain, 1999). The three classes of ionotropic receptors are N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isozazole propionic acid (AMPA) and kainate (KA) receptors (Dingledine & McBain, 1999). NMDA receptors are further subdivided into subunits (NRI, NR2A-NR2D, and NR3A). More recently, the nomenclature has been changed to GluN1, GluN2A to GluN2D, GluN3A and GluN3B (Collingridge, Olsen, Peters, & Spedding, 2009; Pachernegg, Strutz-Seebohm, &
Hollmann, 2012). Most NMDA receptors are a combination of GluN1 and GluN2 subunits (Dingledine & McBain, 1999).

1.3.4.1. The NMDA Receptor
The NMDA receptor is part of a large transmembrane-cytosolic complex, the post-synaptic proteome consisting of over 1100 proteins (Collins et al., 2006) of which the NMDA component, called NMDA-PSD-95 was originally thought to contain 77 proteins (Grant & Husi, 2001) and now revised upward to 186 proteins (Collins et al., 2006). It is present on many cell types within and outside of the central nervous system (CNS) including neuroblastoma and glial cells (Waxman, Baconguis, Lynch, & Robinson, 2007; Singh & Kaur, 2007; Singh & Kaur, 2009). When stimulated, the NMDA receptor opens a Ca\(^{2+}\) channel and extracellular Ca\(^{2+}\) enters the neuron. In addition, the channel also permits Na\(^+\) and K\(^+\) to pass through (Zhou, Chen, & Pan, 2011).

1.3.4.2. Distribution and Function of NMDA Receptors
GluN1 subunits are found in all neurons from embryonic to adult stages of development (Monyer, Burnashev, Laurie, Sakmann, & Seeburg, 1994). During development GluN2B subunits are numerous and, based on electrophysiological studies, are found extrasynaptically in the CNS or perisynaptically (Stocca & Vicini, 1998). With development, GluN2B become more restricted to the forebrain where they are found in the cortex, hippocampus, striatum, thalamus and olfactory bulb (Monyer et al., 1994). More recently, synaptic GluN2A NMDA receptor subunits have been found to be important to neuronal survival while the extrasynaptic CNS GluN2B NMDA receptor subunits are coupled to apoptotic pathways (Kostandy, 2012). In
summary, synaptic NMDA receptors are neuroprotective whereas extrasynaptic NMDA receptors initiate apoptosis (Benarroch, 2011). GluN2A combined with GluN1 become the dominant subunits as synapses mature (Sheng, Cummings, Roldan, Jan, & Jan, 1994). GluN3 subunits can combine with either GluN1 or GluN2 subunits (Zhou et al., 2011). GluN1 combined with GluN3 is activated by glycine alone whereas GluN1 combined with GluN2 requires glycine and glutamate (Zhou et al., 2011).

1.3.5. Glutamate Excitotoxicity

Although glutamate and aspartate are the principle excitatory neurotransmitters in the brain and spinal cord (Ikonomidou et al., 1989), too much glutamate or aspartate causes neuronal dysfunction and death (Waxman et al., 2007). In cases of CNS trauma, seizure disorder, cerebrovascular accident and, possibly, some neurodegenerative diseases, excess extracellular glutamate and aspartate have been found (Ikonomidou et al., 1989; Greenwood & Connolly, 2007). This increased extracellular glutamate over stimulates glutamate receptors, causing large increases in \([\text{Ca}^{2+}]\), which results in neurone death (Lucas & Newhouse, 1957; Mattson, Culmsee, & Yu, 2000). The cell death is not only due to mitochondrial overloading with calcium but also the activation of \(\text{Ca}^{2+}\) dependent enzymes (Kostandy, 2012). The high concentration of extracellular glutamate may be due to either the release of glutamate from dead or dying neurons, or the reversal of glutamate transporter systems from the synaptic cleft across the membrane to the synaptic nerve terminal (Greenwood & Connolly, 2007).
Excitotoxicity is the process in which excess glutamate-activation of its receptors leads to cell death by apoptosis or necrosis. Apoptosis results from excess \([\text{Ca}^{2+}]\), while necrosis is due to the rapid influx of sodium followed by calcium into the cell (Choi, 1994; Choi, 1987).

Since most synapses occur between the nerve terminals of one neuron and the dendrite of another, most glutamate receptors are on dendrites. Consequently, dendrites are the most susceptible to injury by glutamate excitotoxicity. Glutamate-induced dendrotoxicity was first described in 1979 (Olney, Fuller, & de Gubareff, 1979). In dendrotoxicity, focal swellings which look like beads, form along the length of dendrites (Olney et al., 1979; Ikonomidou et al., 1989). Dendritic beads are, therefore, an early sign of neuronal damage and have been observed in ischemia, idiopathic seizure disorders, mechanical pressure, brain tumour and aging. The mechanism behind the beading is currently unknown (Greenwood & Connolly, 2007).

1.3.5.1. NMDA Antagonists in Clinical Practice
The use of NMDA antagonists to ameliorate the effects of glutamate excitotoxicity has not been successful to date (Hoyte, Barber, Buchan, & Hill, 2004). Memantine and amantadine are the only NMDA receptor antagonist in clinical use and that use is palliative not curative (Santangelo et al., 2012) in Alzheimer disease. All other NMDA receptor antagonists have been tried in neurodegenerative disorders, ischemic stroke and traumatic brain injury and have failed or have had too many side effects (Hoyte et al., 2004; Hallett & Standaert, 2004; Schmitt, Ryan, & Cooper, 2007; Santangelo et al., 2012).
1.3.5.2. Calcium and Mitochondria in Glutamate Excitotoxicity

In response to glutamate released from a nerve terminal, calcium enters neurons and glial cells through NMDA receptors (White & Reynolds, 1997). Most of this calcium is buffered by mitochondrial uptake (White & Reynolds, 1997) by a very fast reversible Ca\(^{2+}\) uniporter (Gunter & Gunter, 1994). Although there appears to be electrical coordination of the buffering function among the individual mitochondria, each mitochondrion takes up calcium independently (Gerencser & dam-Vizi, 2005). The energetic gradient is downhill, making the uniporter a facilitated passive transport process. It binds calcium with a high affinity (Gunter & Pfeiffer, 1990; Kirichok, Krapivinsky, & Clapham, 2004), and is activated by calcium, aminoglycosides and other antibiotics, protamine and triethylenetetramine. (Kröner, 1990; Saris & Kroner, 1990)

1.3.5.3. Accumulation of Calcium in Mitochondria

Isolated mitochondria will accumulate calcium when the concentration of calcium rises above a set point of approximately 0.5 μM. (Nicholls, 2009). When approximately 10 nM of calcium/mg of protein has been taken up, calcium phosphate complexes begin to form in the mitochondrial matrix (Nicholls, 2009). The calcium phosphate buffers the free matrix calcium between the values of 0.2 to 2 μM (Chalmers & Schapira, 2002; Nicholls, 2009). This buffering continues to lower the cytosolic (extra mitochondrial) calcium until more than 500 nmol of calcium/mg of protein has accumulated (Nicholls, 2009). Mitochondria act as temporary reversible stores of calcium which rise above the set point during a run of action potentials. They release the calcium back into the extra-mitochondrial cytosol when the calcium ATP pump in the cell membrane expels the excess calcium below the set point (Nicholls, 2009).
Mitochondrial calcium efflux is accomplished in three ways. First there is a Na\textsuperscript{+}-independent Ca\textsuperscript{2+} efflux antiporter that transports divalent cations (Ca\textsuperscript{2+}, Ba\textsuperscript{2+}, Sr\textsuperscript{2+} and Mn\textsuperscript{2+}) (Lukacs & Fonyo, 1985; Gunter, Wingrove, Banerjee, & Gunter, 1988), and is inhibited by cyanide (Gunter & Pfeiffer, 1990). The Na\textsuperscript{+} - independent transport predominates in the liver and kidney (Wingrove & Gunter, 1986). Second is the Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} efflux mechanism that exchanges calcium for two sodium or two lithium ions. Strontium can substitute for calcium, but manganese cannot (Crompton, Kunzi, & Carafoli, 1977; Carafoli & Crompton, 1978; Brand, 1985; Gavin, Gunter, & Gunter, 1990). Na\textsuperscript{+} - dependent mechanisms dominate in the heart, skeletal muscle, and brain and other tissues (Wingrove & Gunter, 1986). The third mechanism of efflux of calcium from the mitochondria is the opening of a proteinaceous “megachannel” (Jornot, Maechler, Wollheim, & Junod, 1999) called the permeability transition pore. This process is characterized by the sudden permeability of the mitochondrial inner membrane to ions and small molecules. This results in the loss of the ion gradient, and the movement of fluid into the matrix causing the mitochondria to swell. Swollen mitochondria are characteristics pathologists have known about for almost a half-century as a sign that the tissue is undergoing necrosis or apoptosis (Gunter & Pfeiffer, 1990; Gunter, Gunter, Sheu, & Gavin, 1994). Of further note is that when mitochondria fuse, the permeability transition merges with the outer membrane porins to form a channel linking the matrix of both mitochondria (Skulachev, 1990; Tanveer et al., 1996). This may be how mitochondria exchange genetic material and other macromolecules (Pletjushkina et al., 2006). Fused mitochondria have the same membrane potential but function independently with respect to calcium influx and efflux (Gerencser & dam-Vizi, 2005). Mitochondria also obtain material directly from the endoplasmic reticulum via microtubules,
using calcium as a signal (Mironov, Ivannikov, & Johansson, 2005). Most likely this is a direct means of obtaining mitochondrial proteins encoded in the nucleus.

1.3.5.4. Calcium-induced Changes in Dendrite Mitochondrial Morphology
Normally, mitochondria within dendrites are elongated and motile (Rintoul, Filiano, Brocard, Kress, & Reynolds, 2003). After glutamate exposure, with calcium entering the cell via NMDA receptors, the mitochondria stop moving and become rounded and/or swollen in appearance (Rintoul et al., 2003; Li, Okamoto, Hayashi, & Sheng, 2004).

1.3.5.5. The Effect of Mitochondrial Calcium Overload: Apoptosis
Apoptosis is a type of cell death in which the cell itself initiates a series of biochemical events that results in its demise. This is usually part of the normal process of tissue growth and renewal of all organisms during development and across the lifespan, but it becomes an abnormal pathological process when it is initiated prematurely or in cells that are essential for normal life and function. Apoptosis is found in all neurodegenerative diseases, including Alzheimer disease (AD) and amyotrophic lateral sclerosis-frontotemporal dementia (ALS-FTD) disease spectrum.

1.3.5.6. Comparison of Apoptosis with Necrosis
In any organism there are two types of cell death: necrosis and apoptosis. Necrosis is a sudden event caused by trauma or severe toxic insult which causes extensive physical damage resulting in the death of a large number of cells. One example is the damage caused by the sudden cessation of the blood supply to a structure in a living organism. Necrosis results in an inflammatory response. Necrosis is not found in Alzheimer disease although insults such as head
trauma which cause necrosis may be a factor in the onset of Alzheimer disease. Nor is necrosis found in ALS-FTD, in those cases where head trauma is not considered to be a risk factor.

Apoptosis comes from the Greek word meaning to shed, as the shedding of leaves in the Fall which is, ironically, the result of apoptosis of cells in the leaf stem (Nelson & Cox, 2008). Apoptosis does not initiate an inflammatory response (Taylor, Cullen, & Martin, 2008). Regardless of the initiating cause, apoptosis requires ATP from a still-respiring subset of mitochondria (Krysko, Roels, Leybaert, & D'Herde, 2001). Also, during apoptosis, the dying cell provides a means for it to be engulfed by macrophages without releasing pro-inflammatory cytokines (Brouckaert et al., 2004; Krysko, D'Herde, & Vandenabeele, 2006).

Apoptosis generally involves individual cells within a field of normal cells. In contrast, necrosis involves many cells or entire areas of a tissue or organ (Rubin & Strayer, 2005). Apoptosis can be a beneficial or a pathological process. As a beneficial process, it is a way of eliminating (1) individual cells that have mutated or have been infected by viruses and have become dysfunctional (Scorrano & Korsmeyer, 2003) (2) cells that are no longer needed (as postpartum uterine cells); (3) cells that have become senescent (e.g., leukocytes); and, (4) antigen presenting cells that erroneously produce antigens against “self.”

Apoptosis becomes pathological or part of a pathological process when (1) too many cells have become infected with a virus and eliminating these cells impairs the function of the organ (Scorrano & Korsmeyer, 2003; Rubin & Strayer, 2005); (2) cells have been damaged beyond repair as the result of ischemia/reperfusion toxicity in which too much ROS and NOS have
formed (e.g., in cardiac ischemia); and, (3) when healthy cells are destroyed even through their function is needed and necessary (such as occurs in dementias). In apoptosis, the destruction of the cell is slow enough to allow other cells to take up the degradation products such as individual amino acids or nucleotides (Nelson & Cox, 2008). Also possible is the uptake of larger aggregates by normal neighboring cells because the activation of macrophages and neutrophils, the scavenger cells in the inflammatory response, does not occur in apoptosis (Taylor et al., 2008).

1.4. Depression

Major depressive disorder (MDD) is the leading cause of long term disability in the industrialized world (Chen, Twyman, & Manji, 2010). It is estimated that 15% of the population is affected by major depression at some time in their life (Bremner et al., 2002). In addition to MDD, the depression phase of bipolar disorder accounts for most of the disabilities in people with bipolar disorder (Judd & Akiskal, 2003; Sidor & MacQueen, 2011). In the brain, the areas most affected in depression are the hippocampus, prefrontal cortex, amygdala, cingulate gyrus and nucleus accumbens (Duman, 2009). In the hippocampus, which is essential for declarative memory, there is a reduction in volume of 10% to 20% (Mervaala et al., 2000; Campbell & Macqueen, 2004; Campbell, Marriott, Nahmias, & MacQueen, 2004). There is also a reduction in the volume of the prefrontal cortex (Drevets et al., 1997; Bremner et al., 2002; Townsend et al., 2010; Murray, Wise, & Drevets, 2011; Turner, Furey, Drevets, Zarate, Jr., & Nugent, 2012) which functions in cognition, working memory, and also to inhibit those regions of the brain that control fear and emotion (Duman, 2009). The amygdala forms and recalls emotional memories and processes anxiety and fear and therefore is involved in mood disorders in which there are
dysfunctional responses to external events (Bezchlibnyk et al., 2007). There are conflicting reports even by the same author as to whether the amygdala increases or decreases in size in MDD (Sheline, Gado, & Price, 1998; Tebartz van, Woermann, Lemieux, & Trimble, 2000; MacMillan et al., 2003; Kronenberg et al., 2009).

1.4.1. Cause of Depression

The cause of MDD remains obscure, partly because it involves a number of brain structures rather than a single structural area or the whole organ. Genetics, environmental factors, early childhood trauma and stress are proposed as being involved (Teicher, Andersen, Polcari, Anderson, & Navalta, 2002; Iwata, Ota, & Duman, 2013). Research on antidepressant pharmacology has centered on enhancing serotonin, norepinephrine and dopamine as these were historically considered to be deficient and therefore considered to be a major factor in MDD. Currently, there is a growing agreement that depression is caused by altered synaptic plasticity affecting cognitive and behavioural functions (Chen et al., 2010) rather than a deficiency. This consensus arises from the fact that current antidepressants, such as citalopram, are associated with a remission rate of only 36.8 % and that 40% of patients on antidepressants relapsed within one year (Zisook, Ganadjian, Moutier, Prather, & Rao, 2008). The predominant causal hypothesis states that stress causes an increase in glucocorticoid synthesis due to a dysfunctional hypothalamic-pituitary-adrenal (HPA) regulation. The increased release of glucocorticoids causes a decrease in astrocytes, which, as part of their normal function, normally take up glutamate released from neurons. Astrocyte decline results in increased glutamate concentrations and concomitant glutamate excitotoxicity (Czeh, Simon, Schmelting, Hiemke, & Fuchs, 2006; Duman, 2009). The decline in hippocampal neuropil can be measured by the MRI imaging and is
seen as a reduction in the volume of the hippocampus, specifically atrophy of the dentate gyrus (Armanini, Hutchins, Stein, & Sapolsky, 1990; Gould & Tanapat, 1999; Campbell & Macqueen, 2004). That this structure and other areas of the hippocampus are targeted is not surprising since the entorhinal glutaminergic fiber endings terminate on the dentate gyrus as well as in the CA1 and CA3 regions (Campbell & Macqueen, 2004; Campbell et al., 2004). At the cellular level, there is a decrease in neurons and glial cells in all of the above hippocampal areas (Czeh et al., 2006). In the largest study to date (636 patients), Gerritsen et al., found reduced hippocampal volume in patients with early-age onset depression and who were still depressed. However, there was no decrease in the volume of the entorhinal cortex. For patients with later-age onset depression, there was a reduction in the entorhinal cortex volume without a reduction in the hippocampus. However, this effect and their symptoms could also have been the result of atherosclerosis, which was more prevalent in the latter group of patients (Gerritsen et al., 2011).

In support of the glucocorticoid theory is the finding that patients exposed to acute high dose corticosteroid therapy developed psychiatric symptoms (Brown, 2009), 40.5% of which were depression (Lewis & Smith, 1983). Also, there are glucocorticoid receptors in the hippocampus, suggesting that there is a relationship between glucocorticoid release and hippocampal function (McEwen, Weiss, & Schwartz, 1968; Brown, Rush, & McEwen, 1999). In addition, there are reports about cognitive deficits in patients or human subjects on 20 to 100 mg per day of prednisone (Varney, Alexander, & MacIndoe, 1984).

NMDA receptors are involved in depression (Skolnick, Popik, & Trullas, 2009). Ap-7 and MK-801 NMDA receptor antagonists produced an anti-depressant effect in forced-swim test in mice (Trullas & Skolnick, 1990). Escitalopram and memantine were effective in the treatment of 80
MDD patients comorbid with alcohol dependence (Muhonen, Lahti, Sinclair, Lonqvist, & Alho, 2008; Muhonen, Lonqvist, Juva, & Alho, 2008). Ketamine, a potent NMDA antagonist, was an effective antidepressant in a small study involving nine patients (Berman et al., 2000). Since that seminal paper, the first in which ketamine was used to treat human depression, other papers on ketamine as an antidepressant have been published, among which are two recent (2013) reviews by Murck and by Salvadore and Singh (Murck, 2013; Salvadore & Singh, 2013).

1.4.2. Long Term Effect of Depression

Long term, the consensus is that chronic depression causes an increase in oxidative stress (Stefanescu & Ciobica, 2012) and is a significant risk factor in Alzheimer disease (Hampel et al., 2004). This is based on a number of retrospective studies in which a high percentage of untreated and treated patients exhibit chronic depression.

1.4.3. Treatment of Depression

Regardless of the cause, the current treatment for depression is a pharmacological agent that enhances the concentration of serotonin, norepinephrine or a combination of these two at the synapse. Such drugs have proved beneficial in many patients. In one study (Insel & Wang, 2009) causing remission in 70% of the patients, 28% to 33% of whom were on citalopram. Moreover, chronic venlafaxine increases hippocampal cell proliferation in chronically stressed rats (Xu, Luo, Richardson, & Li, 2004). Mirtazapine and escitalopram, among other antidepressants, decreased oxidative stress as evidenced by an increase in SOD (Stefanescu & Ciobica, 2012).
1.5. The Cell Lines

1.5.1. C6 and SH-SY5Y Cell Lines

In depression, both neurons and astrocytes are affected. For this reason, the present study used cell lines that could be differentiated into neuron-like and astrocyte-like cells. The cell lines used were C6 and SH-SY5Y cells. C6 cells differentiated into astrocyte-like cells and SH-SY5Y cells differentiated into neuron-like cells. Differentiated SH-SY5Y cells are considered to be neuron-like but their membrane potential has not yet been reported in the literature.

Figure 1.1. Light micrographs of undifferentiated (left) and differentiated (right) C6 cells. Cells at left were at 200x (Pu, Kang, Li, Jiang, & Cheng, 2006) while the ones on the right were taken with a Nikon AIR Confocal using a 40x phase contrast objective nad Image Pro-Plus software 4.5.1. (Kataria, Wadhwa, Kaul, & Kaur, 2012). Permission for use had been obtained.
Figure 1.2. Light micrograph of differentiated SH-SY5Y neuroblastoma cells with 1 μM of 9-cis retinoic acid. Undifferentiated and differentiated look very similar except that differentiation was indicated by the presence of neurites greater than 50 μm long (arrows). Differential interference phase contrast (DIC) micrograph. Scale bar is 20 μm (Brown, Riddoch, Robson, Redfern, & Cheek, 2005). Permission for use has been obtained.

1.5.2. Calcium Channels in the Cell Lines

Both cell lines have calcium channels. In the cell types that were used in this thesis, it is not clear whether C6 cells do (Lee et al., 1999) or do not (Hinkle, Shanshala, & Nelson, 1992) have L-type calcium channels. SH-SY5Y cells do have L-type calcium channels (Li & Wu, 2007; Hettiarachchi et al., 2009), and both cell types have ligand-gated calcium channels (Sun & Murali, 1998; Singh & Kaur, 2004).
1.5.2.1. NMDA Receptors on C6 Cell Line

C6 is a rat cerebral glioma cell line established in 1968 from a nitrosourea-induced brain tumour in a Wistar-Furth strain rat (Benda, Lightbody, Sato, Levine, & Sweet, 1968). C6 cells also have NMDA receptors which responded to NMDA with significant increases in the activities of glutathione peroxidase and copper zinc-superoxide dismutase, all requiring calcium as a cofactor. These responses suggest that NMDA increased \([\text{Ca}^{2+}]\). These responses were blocked by MK-801 (Singh & Kaur, 2006; Singh & Kaur, 2009).

1.5.2.2. NMDA Receptors on SH-SY5Y Cells

SH-SY5Y is a human neuroblastoma three-time cloned subline of a neuroblastoma, SK-N-SH isolated in November, 1970 at Memorial Sloan-Kettering Cancer Center in New York from a bone marrow biopsy from a 4-year-old girl of a metastasis from a primary cerebral neuroblastoma (ECACC, 2007; ATCC, 2007). The patient subsequently died from cancer two months later (Biedler, Helson, & Spengler, 1973), and the cell line derived from her cancer, SH-SY5Y, has become one of the standard cell lines used for the study of neurons. The SH-SY5Y cells have (NMDA) receptors which are activated by glutamate (Sun & Murali, 1998; Singh & Kaur, 2005a).

1.5.3. Differentiation of Cell lines

In order to use the cell lines as models of the CNS neurons and glial cells, the C6 and SH-SY5Y cells lines were differentiated into astrocyte-like and neuron-like cells, respectively, with all trans-retinoic acid (ATRA) (Figures 1.1 and 1.2). ATRA was added to the media and was used in
the thawing out and seeding process described in Methods. Differentiation was accomplished within 48 hours in C6 cells and 96 hours in SH-SY5Y cells.

1.5.3.1. Differentiation of the C6 Cell Line

There is disagreement in the literature about the effect of all-trans retinoic acid has on C6 cells. One paper reports that C6 cells differentiate with retinoic acid into oligodendrocytes (Zhang, Tsuneishi, & Nakamura, 2001). However, other reports indicate that the differentiation is into astrocytes (Singh & Kaur, 2004; Singh & Kaur, 2006) as indicated by GFAP (Singh & Kaur, 2006) in differentiated C6 cells (Figure 1.1). Another finding indicates that it could be either cell type depending on the environmental conditions. One report used 10% fetal bovine serum (FBS) during normal growth and then reduced it to 0.1% FBS to demonstrate that the differentiation pathway was primarily oligodendrocytes by the presence of myelin proteolipid marker mRNA (Zhang et al., 2001), but did not rule out that astrocytes and neurons could also be in the mixture of cells (Bianchi, Gazzola, Tognazzi, & Bussolati, 2008).

1.5.3.2. Differentiation of SH-SY5Y Cell Line

The literature is in agreement that the SH-SY5Y cells will differentiate into neuron-like cells (Ammer & Schulz, 1994; Presgraves, Borwege, Millan, & Joyce, 2004; Jamsa, Hasslund, Cowburn, Backstrom, & Vasange, 2004; Brown et al., 2005) (Figure 1.2). SH-SY5Y differentiates into neurons or Schwann cells in the presence of appropriate exogenous stimuli, such as retinoic acid (Brown et al., 2005). Brown et al., used 9-cis retinoic acid differentiated SH-SY5Y cells and found they lack or have a reduced number of the capacitance Ca$^{2+}$ pathway, a voltage-independent pathway, while still retaining a voltage-dependent Ca$^{2+}$ pathways seen in
neurons, but do have the other voltage independent pathway and the voltage dependent pathway (Brown et al., 2005). Functionally, this may be significant since there is a decrease in the calcium influx (Brown et al., 2005). This may alter the predisposition towards apoptosis. If so, it may be that the undifferentiated cells, especially stem cells, may play a larger role in the excitotoxicity seen in the central nervous system. This attribute remains to be investigated. As well, the difference in the decrease in calcium channels when differentiated with 9-cis compared with all-trans retinoic acid (used in this thesis) remains to be elucidated. The SH-SY5Y cell line has N-methyl D-aspartate (NMDA) receptors which are activated by glutamate (Sun & Murali, 1998; Singh & Kaur, 2005b).

1.6. Mirtazapine and Citalopram

Mirtazapine and citalopram are antidepressant drugs that have different mechanisms of action. Mirtazapine increases the release of norepinephrine and serotonin while citalopram selectively blocks serotonin reuptake. Both drugs increase the concentration of serotonin at the synapse, with mirtazapine also increasing norepinephrine.

1.6.1. Historical Development of Antidepressants

In the late 1800’s Thiele and Holzinger synthesized iminodibenzyl. In the late 1940s, Hafliger and Schindler synthesized 40 derivatives of iminodibenzyl for potential clinical uses, including antihistamines. One of these derivatives was imipramine, a dibenzazepine. This drug differed from the phenothiazines only by the replacement of the sulfur with an ethylene group to produce a central ring. After screening in animals, the drugs were clinically tested. Kuhn found that imipramine was relative ineffective in treating psychotic disorders but was very beneficial in for
the treatment of depression (Kuhn, 1958). Within a short time, nine analogues of imipramine were developed including desipramine, amitriptyline nortriptyline, and doxepin. All of these had the three ring configuration and so were called tricyclic antidepressants (TCAs). The antidepressant effects were not immediately felt, but took place within a three week period.

At the same time, iproniazid was developed as an antitubercular drug in the early 1950s and soon became known as a monamine oxidase inhibitor (MAOI)(Kline, 1958) with antidepressant properties. The discovery of the mechanism of action of these drugs soon led to the catecholamine theory of depression in 1965 (Slattery, Hudson, & Nutt, 2004), which stated that some or all depression was the result of a decrease in one or more catecholamines, with an emphasis on a deficiency of noradrenaline (NA). This theory became rejuvenated when mirtazapine and related drugs entered the market as they also increased NA (Slattery et al., 2004). Venlafaxine, introduced in 1994 (Connolly & Thase, 2012), blocks the reuptake of NA and 5-HT and so it and similar drugs are referred to as serotonin-noradrenalin reuptake inhibitors. Mirtazapine is in an atypical class. By antagonizing inhibitory alpha2-adenoreceptors, mirtazapine blocks the negative feedback to noradrenaline-releasing neurons, especially in the locus coeruleus, thereby increasing the amount of noradrenalin in the synaptic cleft. In addition, mirtazapine blocks the 5-HT2 and 5-HT3 receptors. In summary, it increases both NA and 5-HT(Benjamin & Doraiswamy, 2011). It was introduced to the US market in 1997 (Connolly & Thase, 2012).

Developing a drug (fluoxetine) that would block the reuptake of 5-HT was begun by Eli Lilly in 1970 and trials were begun in the mid-1970. It did not become available in the US until 1987.
Citalopram is the most selective of the serotonin reuptake inhibitors (SSRI) and was introduced in the US in 1998, with its S-enantiomer introduced in 2003 (Connolly & Thase, 2012).

1.6.2. Mirtazapine

Mirtazapine, a 6-aza derivative of the tetracyclic antidepressant drug mianserin, (de Boer et al., 1988; Howland, 2008), is classed as a noradrenergic and serotonergic antidepressant (NaSA) and is widely used for treating major depression (Croom, Perry, & Plosker, 2009). It also blocks the presynaptic alpha-2 adrenergic inhibitory autoreceptors in the CNS, which results in the increased release of norepinephrine from adrenergic nerve terminals in the CNS. It also blocks the 5-HT2A serotonin inhibitory autoreceptor which results in the increase release of serotonin from serotonergic nerve terminals in the CNS (Pinder, 1997). In addition, mirtazapine stimulates 5-HT2C receptors causing agitation and restlessness, and the 5-HT3 receptors causing nausea, vomiting and decreased orgasm (Golden, Dawkins, Nicholas, & Bebchuk, 1998). Thus mirtazapine increases the concentration of both norepinephrine and serotonin (Tollefson & Rosenbaum, 1998; Baldessarini, 2001; Croom et al., 2009).

Mirtazapine has been shown to have antioxidant and calcium modulatory effects (Pan et al., 2006), but when given to patients with Huntington disease, mirtazapine alleviated the depression but had no effect on the symptoms or the progression of Huntington disease (Bonelli, Wenning, & Kapfhammer, 2004; Bonelli & Wenning, 2006).
1.6.3. Citalopram

Hyttel (1994) reported that citalopram has the highest degree of serotonin reuptake selectivity of all the selective serotonin reuptake inhibitors (SSRIs) (Hyttel, 1994). It is a racemate consisting of S (+) and R (-) enantiomers. The S (+) enantiomer sold as escitalopram is the active form, whereas the R (-) has much less affinity for the serotonin reuptake transporter (SERT) (Storustovu et al., 2004). In addition to blocking serotonin reuptake, citalopram has been shown to modify the synthesis of NMDA receptor components as determined by northern blot analysis (Khanzode, Dakhale, Khanzode, Saoji, & Palasodkar, 2003) and this could reduce the effects of glutamate excitotoxicity. In mouse primary cultures of astrocytes, citalopram up-regulates mRNA for 5-HT$_{2B}$ receptors (Zhang et al., 2010), and induces calcium transients in astrocytes in mouse prefrontal cortical slices (Schipke, Heuser, & Peters, 2011). Chronic citalopram administration reduces the expression of NMDA receptors in the mouse brain by altering the mRNA (Boyer, Skolnick, & Fossom, 1998). Citalopram has been reported to inhibit L-type calcium channels on isolated cardiac myocytes (Hamplova-Peichlova et al., 2002; Zahradnik, Minarovic, & Zahradnikova, 2008) and on Chinese hamster ovary cells in culture (Witchel, Pabbathi, Hofmann, Paul, & Hancox, 2002). Getz, Xu, Zaidi and Syed (2011) reported that citalopram, perfused at a concentration of 3 µg/ml over single brain neurons isolated from Lymnaea stagnalis snails, reduced presynaptic calcium influx but did not block calcium channels (Getz, Xu, Zaidi, & Syed, 2011). However, at concentrations of 100 µM citalopram induced apoptosis in Burkitt lymphoma cells (Serafeim et al., 2003). More recently, escitalopram, the active enantiomer of citalopram, increases the promotor of $p11$, a gene associated with reversing depressive-like behaviours in mice (Melas et al., 2012).
In addition to the above effects, mirtazapine and citalopram are antioxidants, acting within mitochondria to eliminate reactive oxygen species (Schmidt, Heiser, Hemmeter, Krieg, & Vedder, 2008; Bilici et al., 2009; Stefanescu & Ciobica, 2012).

1.7. NMDA Receptor Blocking Drugs

1.7.1. Memantine

Memantine is a derivative of the anti-viral drug amantadine derivative that is a noncompetitive, voltage-dependent, NMDA receptor blocker (Robinson & Keating, 2006). It is called ‘noncompetitive’ because increasing the concentration of glutamate does not overcome the blocking effects of memantine (Rogawski & Wenk, 2003). Although memantine is a non-competitive NMDA receptor antagonist, it has a low affinity for the NMDA receptor so that it dissociates from the receptor quite rapidly so that at any given time, 15-20% of the NMDA channels remain unblocked (Robinson & Keating, 2006). This enables some calcium to enter the cell for physiological signals such as normal learning, but it prevents excessive amounts of calcium from entering and possibly inducing excitotoxicity (Robinson & Keating, 2006). It does not act on any other calcium channel receptor (Robinson & Keating, 2006). It currently is one of only five drugs approved for use in treating Alzheimer disease and is the only one that acts on NMDA receptors (Schmitt et al., 2007). The other drugs are all cholinesterase inhibitors (Gauthier & Scheltens, 2009). Although it is being promoted for treating moderate to severe dementia, it is not very effective (Robinson & Keating, 2006; Thomas & Grossberg, 2009; Tabaton et al., 2010). The UK’s National Institute for Clinical Excellence concluded that the drug was not cost-effective for the National Health Service (Robinson & Keating, 2006).
1.7.2. AP5

AP5, a play on the acronym APV (the abbreviation for (2R)-amino-5-phosphovaleric acid), is also known as 2-amino-5-phosphonopentanoate (Olverman, Jones, & Watkins, 1988). It is a competitive selective NMDA receptor channel blocker (Morris, 1989; Bannerman, Rawlins, & Good, 2006; Jantas & Lason, 2009) that binds to the same site on the GluN2B subunit as glutamate. Gerard (2012) found that 10 μM AP5 blocked the effect of NMDA on NMDA receptors in primary cultures of rat astrocytes (Gerard & Hansson, 2012). Corcoran (2011) found that 10 μg/μl of AP5 infused into the CSF of mice impaired the retrieval of recent memory by its effect on the GluN2A subunit of the NMDA receptor (Corcoran et al., 2011). In the macaque monkey, injection of 30 nl of 50 mM AP5 into the primary visual cortex (V1) of live animals results in a slight reduction in the visual ability to discern an object from its background (Self, Kooijmans, Super, Lamme, & Roelfsema, 2012).

1.8 The Hypothesis and Rationale of the Study

The working hypothesis for the present study is that mirtazapine and citalopram reduce relative changes in [Ca^{2+}]_{i}, by their action on NMDA receptors. This effect of these drugs may be beneficial to patients who have neurodegenerative disorders, stroke, or other condition that could cause central nervous system disorder involving a sudden increase in [Ca^{2+}]_{i}.

This hypothesis was tested by using fluorescent ratiometric microscopy to look at fluorophore binding to calcium in two cell lines, C6 and SH-SY5Y, that have been differentiated into astrocyte-like and neuron-like cells, respectively, by all-trans retinoic acid. Cancer cell lines were used because they are more homogenous than primary cultures and they are immortal,
which permitted studies to be done over a 10-week interval. Furthermore, both cell lines adhered easily to glass without glass pretreatment, permitting growth on coverslips for microscopy studies. Both cell lines also can be detached by tituration, avoiding the introduction of chemicals to remove them from tissue culture flask surfaces. As controls, memantine and AP5 were used which are known NMDA receptor antagonists. A similar ratiometric system was used in primary rat astrocytes by Gerard (2012) who preferred using NMDA instead of glutamate and who used only AP5 to determine the effectiveness of the blockade (Gerard & Hansson, 2012).

1.9. Objectives of the Study

The objectives of this project are to investigate:

1. The effectiveness of mirtazapine and citalopram in modulating the effect of glutamate on $[\text{Ca}^{2+}]_i$.

2. To compare the effectiveness of mirtazapine and citalopram to known NMDA blockers memantine and AP5.

3. To examine the effect of glutamate on C6 and SH-SY5Y cells which have been treated for 10 weeks with mirtazapine and citalopram.

4. To determine the cell viability of mirtazapine and citalopram by microplate studies.
2. Materials and Methods

2.1. Chemicals and Instruments

All-\textit{trans} retinoic acid (ATRA), 2-amino-5-phosphonopentanoic acid (AP5), calcium chloride-\(2\text{H}_2\text{O}\), carbonyl cyanide \textit{m}-chlorophenyl hydrazone (CCCP), citalopram, glutamate, glycine, magnesium sulfate-\(7\text{H}_2\text{O}\), memantine and mirtazapine were purchased from Sigma-Aldrich Canada Co., Oakville, Ontario. Dulbecco’s modified Eagle’s medium (DMEM) 4.5 g/l high glucose, Dulbecco’s phosphate-buffered saline (DPBS), Hank’s balanced salt solution (HBSS) without magnesium or calcium, penicillin-streptomycin solution, glutamine, 4-bromo-calcium ionophore A23187 (4BrA23187), 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES), calcein AM, Fura2 AM, FuraFF AM, Fluo4 AM, dimethyl sulfoxide (DMSO) and fetal bovine serum (FBS) were purchased from Life Technologies, Burlington, Ontario.

A Leica DM IL tissue culture microscope with a top C mount with a Canon camera was used for tissue culture observation and for initial microscopy studies. For linear calcium imaging, a Leica DMI 6000 B with a Hamamatsu C9100-12 back-thinned EMCCD with a Yokogawa CSU-10 scanhead was used. The Z-controller was a Mad City Labs Nanodrive. Light source used was an argon laser at 491 nm. The objective used was a 63× 1.3NA DIC glycine HCX Pl-Apo. The excitation controller was the Spectral Applied Research Laser Launch Module LMM5 and the emission control was a Ludl Mac5000 Controller. The acquisition software was Volocity version 5. Processing software was Volocity Demo 5.1 and 6.1.1. A Thermo-Fisher ultralow freezer (-80°C) was used to store the cells. The biosafety cabinet (BSC) was a NuAire Class II, Type A2 with laminar air flow. All bench work with tissue cultures was performed in the BSC. Cells were incubated in a Thermo-Fisher warm-air-jacketed tissue culture incubator with a HEPA filter and
CO₂ regulator. The above equipment was used with the kind permission of Dr. David Andrews at McMaster University. All microplates were read on two Tecan Safire microplate readers with XFluo software. One Tecan microplate reader was in Dr. David Andrew’s lab and the other was in Dr. Jonathan Bramson’s lab and were used with their kind permission. An Olympus 1x81 fluorescent inverted microscope with a spinning disc and confocal capabilities was used for ratiometric fluorescent microscopy. A 40x oil immersion objective was used for the ratiometric imaging. The camera was a Photometrics Cool Snap HQ CCD camera. The UV illumination was done with Photon Technologies International (PTI) monochromatic light source and controller. The acquisition software was ImageMaster 5.0. This microscope and software were used with the kind permission of Dr. Warren G. Foster at McMaster University. A Coulter counter was used to count cells and was in the lab of Dr. Xu-Dong Zhu at McMaster University and was used with her kind permission.

2.2. Tissue Culture

C6 and SH-SY5Y cell lines were obtained from American Type Culture Collection (ATCC) (Rockville, Maryland), grown in T-25 tissue culture flasks with media change every two to three days. Experiments were done with undifferentiated cells and with cells that had been exposed to ATRA as described below.

2.2.1. Differentiation of the Cell Lines

Differentiation was accomplished by 10 µM all-trans retinoic acid (ATRA) (Sigma-Aldrich, Oakville, Ontario) added to the stock media and was continuously present in the culture media from the time the cells were thawed to the time they were seeded for an experiment. After 48
hours (C6 cells) or 96 hours (SH-SY5Y cells) the cells were differentiated and ready for experimentation.

ATRA differentiated C6 cells into astrocyte-like cells (Singh & Kaur, 2006) and oligodendrocyte-like cells (Zhang, Tsuneishi, & Nakamura, 2001; Sidell et al., 2003), with astrocyte-like cells predominating (Singh & Kaur, 2006). Differentiated C6 cells were not tested for either GFAP (for astrocytes) or myelin proteolipid marker mRNA (for oligodendrocytes).

ATRA differentiated the SH-SY5Y cells into cells that morphologically resemble neurons (Brown et al., 2005). However, these neuron-like cells, because they are cancer cells, continue to divide after being differentiated (Brown et al., 2005; Singh & Kaur, 2005a).

2.2.2. Cell Culture Protocols
2.2.2.1. Incubation and Media Change
The cells are incubated in 5% CO₂ and 95% air at 37°C. The media in the tissue culture flasks was changed on Monday, Wednesday and Friday. Or on Tuesday, Thursday and Monday if there was a long weekend. If there was too rapid a build-up of cells and resultant carbonic acid, as evidenced by a media pH change from red (neutral) to orange (acid), the cells are thinned by trituration.
2.2.2.2. Passaging of Cell Lines

Passage in tissue culture is the transfer of cells from one tissue culture flask into another flask. This was done when cells reached confluence or there were enough cells to increase the production of acid over the buffering capacity of the media. The cells were grown to confluence and then were harvested by trituration. In this process, the cells were removed from the tissue culture flask by tilting the flask to 30° – 40° and using about 10 ml of a gentle stream of media (and dislodged cells) to dislodge other cells from the bottom of the flask. Once enough cells were removed – usually when the half of the bottom of a flask was free of cells – they are then plated in a new tissue culture flask and allowed to grow to confluence. Trituration was used for harvesting the cells as it is a gentler method than using trypsin.

2.2.2.3. Number of Passages

There was concern about the number of passages of the cell lines used in this project. With each passage, there is the possibility that cells will mutate, and with cancer cell lines, these mutations may occur frequently and they may affect the response of a cell to a drug (Hughes, Marshall, Reid, Parkes, & Gelber, 2007). Furthermore, as these cells are cultured in incubators housing other cell lines, the possibility of contamination by other cell lines (most notably HeLa cells) can occur (Hughes et al., 2007).

As the passage numbers of the two cell lines initially used were unknown, C6 and SH-SY5Y cell lines of known passage number were ordered from the American Type Culture Collection, (Manassas, Virginia, USA. The Canadian importer is Cedarlane, Burlington, Ontario). The new cells were grown to 75% confluence and then passaged into flasks or plates. They were frozen at
the end of the third passage after arrival. When thawed for use in experiments, the cells were passed a fourth time in flasks, and were passaged once more to 6- or 96-well plates for microplate studies or to 100 mm tissue culture plates with four to five 25 mm cover slips for microscopy. Except for the cells that had been chronically exposed to drugs for 10 weeks, all experimentation reported in this thesis was done with cells in their fifth passage since purchase.

2.2.2.4. Freezing of Cells

For freezing, cells were grown to confluence and then completely removed from the culture flask by the use of 2x trypsin (1 ml in DPBS for 15 -20 minutes) (Life Technologies, Burlington, Ontario). Although trypsinizing is not as gentle as trituration, it ensures that all cells will be removed for maximum efficiency. The trypsin was quenched by 10 ml of DMEM with 10% FBS. The enzymes in the FBS are responsible for inactivating the trypsin. The cells were aspirated from the flasks with 10 ml pipettes (one per flask) and placed in 15 ml tubes and spun down for 5 minutes at 1,000 rpm. The supernatant was discarded and the pellet and the first 0.5 ml above the pellet were re-suspended in 1 ml of freezing solution (32 ml DMEM, 4 ml FBS, 4 ml DMSO and 0.5 ml penicillin/streptomycin [pen/strep] for a total of 40.5 ml). The freezing solution was made up beforehand in a 50 ml tube. The re-suspended pellet in freezing solution was placed in a labeled 1.8 ml cryovial (Sarstedt, Montreal, Quebec) which in turn was placed in a Mr. Frosty jar in an ice bucket to begin the freezing process until placed in a -80°C freezer. Mr. Frosty (Thermo Fisher Scientific, Nepean, Ontario) is a double container, the bottom of which was filled with 250 ml isopropyl alcohol. When placed in a -80°C freezer with freezing vials, the container provides the critical, repeatable -1°C/minute cooling rate required for successful cell cryopreservation. The process prevents crystal formation within the cells. When the vials are
thawed out there was less cell destruction from expanding crystals. Mr. Frosty holds up to 18 vials. The top prevents vials from contacting the alcohol so that there was no contamination by wicking or removal of labels or printing on vials. Vials were kept in Mr. Frosty for 24 hours and then were removed and stored at -80°C until needed.

2.2.2.5. Thawing of Cell Lines

To thaw the cells, they were removed from storage and thawed by rolling for a few minutes between the palms of the hands. Once thawed, the compact cells from a freezing vial were transferred to a 15 ml tube and diluted with 10 ml of DMEM media to reduce the effect of the DMSO. If the cells were to be differentiated, then the cells were diluted in DMEM with 10 μM of ATRA. The tube was centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded down to 0.5 ml above the pellet and the pellet was re-suspended by using a pipette in 10 ml DMEM media (or DMEM with ATRA). The 10.5 to 11 ml of cells and media (the pellet plus the 0.5 ml above the pellet and the 10 ml of DMEM) were then plated into a T-25 tissue culture flask. The culture flask was incubated as described above and left undisturbed for 2-3 days. This gives the cells time to settle and attach to the bottom of the culture flask. Dead cells remain floating and are discarded with the first media change.

2.2.2.6. Mycoplasma Testing

Species of *Mycoplasma* contaminate between 5% and 35% of cell line cultures. (Hay, Macy, & Chen, 1989) Colonies of these small (less than 1 mm) obligate intracellular bacteria can easily reach $10^6$ to $10^7$ cells without causing turbidity (Hay et al., 1989). Although the ATCC cell lines are guaranteed not to be contaminated when purchased, *Mycoplasma* may be picked up during
incubation and can alter the metabolism of a cell thus affecting the results of experimental pharmacological studies if the culture is contaminated. The most common species that contaminate cell lines are *M. hyorhinis*, *M. arginini* and *M. orale* (Hay et al., 1989).

To rule out *Mycoplasma* contamination in the cultures in this thesis, routine testing for *Mycoplasma* was done by the McMaster Biophotonics facility staff using polymerase chain reaction (PCR) technique. All samples tested were negative for Mycoplasma.

2.2.2.7. Growing Cells for Microscopy

All preparatory work for microscopy and microplates was done in a biosafety cabinet (BSC). For microscopy, cells were grown on four or five 25 mm diameter glass cover slips that had at least 3 days earlier been placed in a 100 mm tissue culture dish and sterilized by flooding with 70% ethanol, which was then allowed to evaporate from the closed dish in a BSC. The C6 and SH-SY5Y cell lines grow well on uncoated glass.

After the cells had grown for a minimum of 4 days, each cover glass to be examined under the microscope was removed and placed in an Attofluor Cell Chamber (Life Technologies, Burlington, Ontario) (Figure 2.1), the bottom portion of which had been previously placed in an 60 x 15 mm tissue culture dish and closed because the chambers are not sterile. The top portion of the holder with its rubber O ring seal, had been soaking in 4 ml of HBSS+HEPES+MgSO₄+CaCl₂ in a 100 mm tissue culture dish to keep the O ring wet to ensure a good seal.
The top portion of the cell chamber was then removed from the 100 mm tissue culture dish where it had been soaking and screwed into the bottom holder. The entire cell chamber was then briefly removed and the bottom of the cover slip wiped with a Kimwipe (Thermo-Fisher Scientific, Burlington, Ontario) to remove any fluid. The inside bottom of the 60 mm tissue culture dish was also wiped to remove any fluid. 1000 µl of HBSS + HEPES + 0.9 mM MgSO₄-7H₂O +1.4 mM CaCl₂-2H₂O was added to the well of the cell chamber so the cells do not become dehydrated. A working solution of 40 ml of HBSS+20 mM HEPES, 4 µl MgSO₄7H₂O, 4 ml CaCl₂-2H₂O is made up before each run. Stock solutions of MgSO₄-7H₂O and CaCl₂-2H₂O
are made up before hand and kept refrigerated. Glutamate must be made up fresh from the powder each day it is to be used. Glycine stock solution is made up and refrigerated.

2.2.2.8. Staining Cells for Microscopy

Either fluorophore Fura 2-AM or FuraFF-AM at a concentration of 3 μM to 10 μM was mixed with 1 ml HBSS + HEPES+MgSO₄+CaCl₂ before adding it to the well of the cell chamber (the 1000 μl of HBSS previously added was removed beforehand). Fura 2 is a high affinity dye and will become quickly saturated with calcium so that large changes would not be detected. For this reason, a switch to FuraFF, a low affinity dye, was made to make certain that large changes in calcium would be detected. As Fura2 and FuraFF are light sensitive, the 60 mm tissue culture dish which contains the Attofluor cell chamber was wrapped in aluminum foil, and was either left at room temperature in the biosafety cabinet for 45 minutes, or incubated at 37°C. The fluorophore was then washed out with HBSS + HEPES+MgSO₄+CaCl₂. 1,000 μl of HBSS + HEPES+MgSO₄+CaCl₂ was added to the holder and the holder was left in the BSC or tissue culture incubator for another 45 minutes to enhance the enzymatic removal of the acetomethoxy ester which, once removed, activated the fluorescent fluorophore. However, it also enhanced the expulsion of the fluorophore from the cell, so incubation sometimes gave a mixed result.

The Attofluor cell chamber with the cells on the glass cover slip was placed on the microscope stage and taped in place with autoclave tape so that it could not move. To prevent drying out, 1 ml of HBSS+ HEPES+MgSO₄+CaCl₂ imaging solution was maintained in the well of the cell chamber.
Under the microscope, cells were selected for study that were not too close together or confluent, as confluent cells are reported to have less NMDA receptors. An average number was 8 to 10 cells in a single image frame as visualized on the computer screen. These cells must remain stationary throughout the run. If the cell chamber was touched by the tip of the transfer pipette (see below), the run would be negated. Or, if the flow of fluid from the transfer pipette was too strong, the cells would become detached and negate the run. As drugs would be added to these cells in the frame during the run, the remaining cells on the coverslip could not be used for further experimentation. The cells were further selected out on the basis of fluorophore uptake as imaged through the 380 nm filter and acquired in 24-bit colour on the computer screen. The screen also showed, simultaneously and in colour the 340 image and the 340/380 ratiometric image (but without the background subtraction). The 340 image was often so faint that it could not be detected. Direct visualization through the microscope eyepieces is not done as the illumination source is ultraviolet which damages the retina.

All drugs were delivered to the Attofluor cell chamber as a 1 ml solution in HBSS+HEPES+MgSO$_4$+CaCl$_2$ by a 2.5 ml transfer pipette (Fisher Scientific, Nepean, Ontario). To remove the fluid and wash out the extracellular calcium, the computer recording was stopped and a 2.5 ml transfer pipette aspirated HBSS+HEPES+MgSO$_4$+CaCl$_2$ and any drugs that were in solution. HBSS +HEPES without calcium or magnesium was used to gently wash the cells twice, taking care not to touch the glass coverslip or the cell chamber or the image would move making the run useless. Even when taped to the stage, there was enough movement if the glass coverslip or the cell chamber was inadvertently touched. Likewise, the stage could also not be touched as it was designed to move very easily.
After washing twice, 1 ml of HBSS+ HEPES without magnesium or calcium is placed in the well of the cell chamber to prevent cell dehydration. CCCP or 4BrA23187 in a 1 ml HBSS+HEPES calcium-and-magnesium -free solution was added by transfer pipette to 1 ml already in the well of the cell chamber. As the fluorophores Fura2 or FuraFF are light sensitive, the washing and microscopy had to be carried out with a minimum of light.

2.2.2.9. Seeding the 96-Well Plates

Cells to be used for experiments were grown in T-25 tissue culture flasks and harvested by trituration. After gentle mixing, a sample of cells was taken and the cells were counted in a hemocytometer (Malassez, 1873) or in a Coulter counter. The stock solution of cells was diluted with DMEM to give 2 x 10^3 cells per 100 µl. Once diluted (if necessary) the contents of the flask were placed in 55 ml tissue culture reservoirs with a V-shaped bottom (Fisher Scientific, Nepean, Ontario). The outer 36 wells of a 96-well plate are not used because they are thermally unstable which affects the growth of cells. These outer wells were filled with fluid or left empty but are not used for testing.

A 100 µl aliquot of cells and media was added to each well and the cells were allowed to settle and grow for 24 hours in an incubator (37°C, 5% CO₂/95% air). Twenty-four hours later, 100 µl of media with drugs were added to bring the total volume in each well to 200 µl. The plate was again incubated for 24 hours at which time the media and drugs were removed and replaced with 200 µl of 3 µM calceinAM in DPBS. The plate was wrapped in aluminum foil (the calceinAM is light sensitive). After incubation for 30 minutes at 37°C and 5% CO₂, the plates were transported
in a biosafety container to be read in a Tecan Safire plate reader (Tecan US Inc., Morrisville, North Carolina) with XFluo software. The total time that elapsed from adding calcein to finish reading the plate was approximately 45 minutes for the first plate, with an additional 5 minutes for a 96 well plate and 10 minutes for a 6-well plate to be added on to that figure for each plate to be read. The last plate of a group of four 96-well plates to be read would finish reading at 60 minutes after adding calcein.

2.3. Viability Studies

2.3.1. CalceinAM Assay

The viability assay began after the cells had been incubated with the drugs being studied for 24 hours. The media was then removed and to each well with cells was added 200 \( \mu \)l of 3 \( \mu \)M calcein AM in warm (37\(^{\circ}\)C) 1x DPBS. The plates were incubated for 30 minutes at 37\(^{\circ}\)C and read in a Tecan Safire plate reader. For calcein, absorption is at 485 nm and it is absorption that was read. The Tecan acquisition program (FluoX) readout is in Excel 2003. This is further processed by the addition of charts to visualize the readout. To determine the background signal 200 \( \mu \)l of warm DPBS was put in empty wells and read.

2.3.2. Pharmacological Agents

2.3.2.1. Glutamate

Initial studies used glutamate concentrations of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 milimole (mM) as previous studies done in Dr. Richardson’s lab had shown that 35 mM glutamate was not toxic to SH-SY5Y cells while 45 mM was toxic (Richardson, 2007). However, these preliminary microscopic studies showed that these doses were too toxic for
chronic studies over 24 hours. In the literature, the range of a toxic dose of glutamic acid is 50 μM to 100 μM (Ha et al., 2010; Szydlowska, Gozdz, Dabrowski, Zawadzka, & Kaminska, 2010). However, higher levels (500 μM and 1 mM with respect to C6 cells have also been reported (Singh & Kaur, 2006). The glutamate dosage range used in all subsequent microplate and microscopy experiments in this thesis were 0, 5, 25, 50, 125 and 250 μM. All calculations were checked on-line with Quick Calcs (Graph Pad, San Diego, California).

2.3.2.2. Microplate Acute Glutamate Studies
Undifferentiated or differentiated C6 or SH-SY5Y cells were plated in the inner 60 wells of a 96-well plate and incubated for 24 hours as described above. Then glutamate was added to the wells to produce final concentrations of 0, 5, 25, 50, 125 and 250 μM. Each of the 6 concentrations of glutamate was used on each of four cell types to give a total of 24 groups. Separate plates were used for each cell type. 24 hours after the addition of glutamate, the calcein AM viability test was done as described above.

2.3.3. Mirtazapine Studies
2.3.3.1. Microplate Acute Mirtazapine Studies
Mirtazapine was initially dissolved in DMSO and serially diluted in distilled water and added to media to give the target final concentrations in each well of a 96-well plate. Undifferentiated or differentiated C6 or SHSY5Y cells were plated in the inner 60 wells of a 96-well plate and incubated for 24 hours as described above. Then mirtazapine was added to the wells to produce final concentrations of 0, 5, 25, 50, 125 and 250 μM. Each of the 6 concentrations of mirtazapine was used on each of four cell types to give a total of 24 groups. Separate plates were used for
each cell type. Twenty-four hours after the addition of mirtazapine, calcein AM viability test was done and the plates were read as described above.

2.3.3.2. Microplate Chronic Mirtazapine Studies

Mirtazapine was dissolved in DMSO and serially diluted as described above. Over a 10-week period, cells in T-25 flasks were fed every 3 or 4 days with 10 ml of media containing 50 µM mirtazapine. The media given to the cells being differentiated also contained 10 µM ATRA. During the ten weeks, the cells were thinned every 4 days to avoid overcrowding. At the end of ten weeks, the DMEM and 50 µM mirtazapine was replaced with new DMEM and mirtazapine and the remaining cells were harvested by trituration and seeded in 96-well plates with DMEM and as described above. After 24 hours glutamate and mirtazapine were added to give final concentrations of 0, 5, 25, or 250 µM glutamate and 50 µM mirtazapine.

2.3.4. Citalopram Studies

2.3.4.1. Microplate Acute Citalopram Studies

Citalopram was initially dissolved in DMSO and serially diluted in distilled water and added to media to give the target final concentrations in each well of a 96-well plate. Undifferentiated or differentiated C6 or SH-SY5Y cells were plated in the inner 60 wells of a 96-well plate and incubated for 24 hours as described above. Then citalopram was added to the wells to produce final concentrations of 0, 5, 25, 50, 125 and 250 µM. Each of the 6 concentrations of citalopram was used on each of four cell types to give a total of 24 groups. Separate plates were used for each cell type. Twenty-four hours after the addition of citalopram, calcein AM viability test was done and the plates were read as described above.
2.3.4.2. Microplate Chronic Citalopram Studies

Citalopram was dissolved in DMSO and serially diluted as described above. Over a 10-week period, cells in T-25 flasks were fed every 3 or 4 days with 10 ml of media containing 50 mM citalopram. During the ten weeks, the cells were thinned every 4 days to avoid overcrowding. At the end of ten weeks, the DMEM and 50 μM citalopram was replaced with new DMEM and citalopram, and the remaining cells were harvested by trituration and seeded in 96-well plates as described above. After 24 hours glutamate and citalopram were added to give final concentrations of 0, 5, 25, or 250 μM glutamate and 50 μM citalopram.

2.3.5. NMDA Receptor Blocking Agents

2.3.5.1. Microplate Acute Memantine Studies

Memantine was serially diluted in distilled water and added to media to give the target final concentrations in each well of a 96-well plate. Undifferentiated or differentiated C6 or SHSY5Y cells were plated in the inner 60 wells of a 96-well plate and incubated for 24 hours as described above. Then memantine was added to the wells to produce final concentrations of 0, 5, 25, 50, 125 and 250 μM. Each of the 6 concentrations of memantine were used on each of four cell types to give a total of 24 groups. Separate plates were used for each cell type. Twenty-four hours after the addition of memantine, calcein AM was added and the plates were read as described above.

2.3.5.2. Microplate Acute AP5 Studies

AP5 was serially diluted in distilled water and added to media to give the target final concentrations in each well of a 96-well plate. Undifferentiated or differentiated C6 or SHSY5Y cells were plated in the inner 60 wells of a 96-well plate and incubated for 24 hours as described
above. Then AP5 was added to the wells to produce final concentrations of 0, 5, 25, 50, 125 and 250 μM. Each of the 6 concentrations of AP5 were used on each of four cell types to give a total of 24 groups. Separate plates were used for each cell type. Twenty-four hours after the addition of AP5, calcein AM was added and the plates were read as described above.

2.4. Microscopy Results Processing

For microscopy, cells are prepared as described above. Once captured by ImageMaster, the resulting 340 and 380 TIF files were processed in ImageJ and each cell processed as an area of interest. A snapshot of all cells in the field is captured by Print Screen, placed in PowerPoint and labeled so each cell that was processed in 340 can be processed in 380 nm. Each cell image run was then converted to Excel 2010, the backgrounds were subtracted from the 340 and 380 cell runs respectively, and the resulting 340 data is divided by the 380 data and graphed. The final graph in Excel is then converted by Photoshop to a final TIF image.

2.5. Statistical Analysis

All statistical analyses for the microplate and microscopy were done with GraphPad Prism versions 5 or 6. Analyses performed included 2-way ANOVA and Bonferroni multiple comparisons tests.
3. Results

3.1. Cell Characteristics

Figures 3.1 to 3.9 are representative of the characteristics of the cells used in this project. The cells in Figures 3.1 to 3.4 were prepared as described in Methods and seeded at $1 \times 10^4$ cells per well of 24-well plates and incubated at 37°C, in 5% CO$_2$ and 95% air. No pharmacological agents were added to these cultures. They were photographed unstained in a Leica inverted tissue culture microscope 24 hours later with a top C-mounted Canon camera with a magnification factor of 4x in addition to the 40x phase contrast objective magnification. Figure 3.1 is of undifferentiated C6 cells with different types of cells growing in no organized pattern. Figure 3.2 is of differentiated C6 cells in which all of the cells are of one morphology and form a lace-like pattern. Figure 3.3 is of undifferentiated SH-SY5Y cells which contain a large number of neurites and generic types of cells. Figure 3.4 is of differentiated SH-SY5Y cells in which only neuron-like cells can be seen.
Figure 3.1. Representative C6 undifferentiated cells. Cells were seeded at a density of $1 \times 10^4$ cells/well in each well of a 24-well tissue culture plate and photographed 24 hours later using a 40x phase contrast dry objective on a Leica inverted tissue culture microscope with a top-C-mounted Canon 4x camera. Total magnification is 160x.
Figure 3.2. Representative C6 cells differentiated into astrocyte-like cells. Cells were continuously treated with ATRA since thawing seven days earlier, and were seeded at a density of $1 \times 10^4$ cells/well in each well of a 24-well tissue culture plate and photographed 24 hours later using a 40x phase contrast dry objective on a Leica inverted tissue culture microscope with a top-C-mounted Canon 4x camera. Total magnification is 160x.
Figure 3.3. Representative SH-SY5Y undifferentiated cells. Cells were seeded at a density of $1 \times 10^4$ cells/well in each well of a 24-well tissue culture plate and photographed 24 hours later using a 40x phase contrast dry objective on a Leica inverted tissue culture microscope with a top-C-mounted Canon 4x camera. Total magnification is 160x.
Figure 3.4. Representative SH-SY5Y cells differentiated into neuron-like cells. Cells were continuously treated with ATRA since thawing seven days earlier, and were seeded at a density of $1 \times 10^4$ cells/well in each well of a 24-well tissue culture plate and photographed 24 hours later using a 40x phase contrast dry objective on a Leica inverted tissue culture microscope with a top-C-mounted Canon 4x camera. Total magnification is 160x. The solid white arrow denotes a bipolar neuron-like cell, and the dashed white arrow points to a multi-polar motor neuron-like cell.
Figures 3.5 to 3.9 show [Ca\textsuperscript{2+}]\textsubscript{i} in the four cell types. Cells were seeded on 25 mm coverslips as described in Methods and then placed in an Attofluor cell chamber for imaging. The cell were loaded with 1.0 μM Fluo4, placed on the stage of a Leica DMI 6000 B inverted microscope with a 63x glycerol immersion objective, and excited at 485 nm by an Argon 488 nm laser. The cells were photographed with a Hamamatsu C9100-12 back-thinned EMCCD using a Yokogawa CSU-10 spinning disc scan head and Volocity 5.0 image software. Figure 3.5 is of undifferentiated C6 cells, Figure 3.7 is of differentiated C6 cells, Figure 3.8 is of undifferentiated SH-SY5Y cells. Figure 3.9 is of differentiated SH-SY5Y cells. Figure 3.6 is of confluent undifferentiated C6 cells.
Figure 3.5. Representative C6 nonconfluent undifferentiated cells loaded with Fluo4. Ten milliliters of DMEM containing cells were seeded at a density of 2 x 10^4 cells/ml and grown on coverslips in a 100 mm tissue culture dish for 4 days. The coverslips were placed in an Attofluor cell chamber, loaded with 1 μM Fluo4, incubated for 30 minutes, washed, and observed with a 63x (glycerol immersion) objective on a Leica DMI 6000 B spinning disc confocal microscope and photographed with a Hamamatsu C9100-12 back-thinned EMCCD camera. The scanhead was a Yokogawa CSU-10. Excitation was with an Argon laser emitting at 491 nm controlled by Spectral Applied Research Laser Launch Module LMM5 with emission control by Ludl Mac5000 controller. Acquisition software was Volocity 5.0.
Figure 3.6. Representative C6 confluent undifferentiated cells loaded with Fluo4. Ten milliliters of DMEM containing cells were seeded at a density of $2 \times 10^4$ cells/ml and grown on coverslips in a 100 mm tissue culture dish for 4 days. The coverslips were placed in an Attofluor cell chamber, loaded with 1 μM Fluo4, incubated for 30 minutes, washed, and observed with a 63x (glycerol immersion) objective on a Leica DMI 6000 B spinning disc confocal microscope and photographed with a Hamamatsu C9100-12 back-thinned EMCCD camera. The scanhead was a Yokogawa CSU-10. Excitation was with an Argon laser emitting at 491 nm controlled by Spectral Applied Research Laser Launch Module LMM5 with emission control by Ludl Mac5000 controller. Acquisition software was Volocity 5.0.
Figure 3.7. Representative C6 cells differentiated into astrocyte-like cells and loaded with Fluo4. Ten milliliters of DMEM containing cells were seeded at a density of 2 x10^4 cells/ml and grown on coverslips in a 100 mm tissue culture dish for 4 days. The coverslips were placed in an Attofluor cell chamber, loaded with 1 μM Fluo4, incubated for 30 minutes, washed and observed with a 63x (glycerol immersion) objective on a Leica DMI 6000 B spinning disc confocal microscope and photographed with a Hamamatsu C9100-12 back-thinned EMCCD camera. The scanhead was a Yokogawa CSU-10. Excitation was with an Argon laser emitting at 491 nm controlled by Spectral Applied Research Laser Launch Module LMM5 with emission control by Ludl Mac5000 controller. Acquisition software was Volocity 5.0.
Figure 3.8. Representative SH-SY5Y undifferentiated cells loaded with Fluo4. Ten milliliters of DMEM containing cells were seeded at a density of \(2 \times 10^4\) cells/ml and grown on coverslips in a 100 mm tissue culture dish for 4 days. The coverslips were placed in an Attofluor cell chamber, loaded with 1 \(\mu\)M Fluo4, incubated for 30 minutes, washed and observed with a 63x (glycerol immersion) objective on a Leica DMI 6000 B spinning disc confocal microscope and photographed with a Hamamatsu C9100-12 back-thinned EMCCD camera. The scanhead was a Yokogawa CSU-10. Excitation was with an Argon laser emitting at 491 nm controlled by Spectral Applied Research Laser Launch Module LMM5 with emission control by Ludl Mac5000 controller. Acquisition software was Volocity 5.0.
Figure 3.9. Representative SH-SY5Y differentiated into neuron-like cells with ATRA and loaded with Fluo4. These cells display large nuclei (white arrows) relative to the cytoplasm which is characteristic of all tumour cells. These are larger than would be found in the normal bipolar neuron. Ten milliliters of DMEM containing cells were seeded at a density of $2 \times 10^4$ cells/ml and grown on coverslips in a 100 mm tissue culture dish for 4 days. The coverslips were placed in an Attofluor cell chamber, loaded with 1 μM Fluo4, incubated for 30 minutes, washed and observed with a 63x (glycerol immersion) objective on a Leica DMI 6000 B spinning disc confocal microscope and photographed with a Hamamatsu C9100-12 back-thinned EMCCD camera. The scanhead was a Yokogawa CSU-10. Excitation was with an Argon laser emitting at 491 nm controlled by Spectral Applied Research Laser Launch Module LMM5 with emission control by Ludl Mac5000 controller. Acquisition software was Volocity 5.0.
Figures 3.10 to 3.12 show the same undifferentiated C6 cells before and after the addition of 112 μM glutamate/45 μM glycine. Ten milliliters of DMEM containing cells were seeded at a density of 2 x 10^4 cells/ml and grown on coverslips in a 100 mm tissue culture dish for 4 days. The coverslips were placed in an Attofluor cell chamber, loaded with 5 μM FuraFF, incubated for 30 minutes, washed and observed with a 40x (oil immersion) objective on a Olympus 1x81 fluorescent microscope and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1. This image was taken one minute before 112 μM glutamate/45 μM glycine glutamate was added, at 4.5 minutes into the run. Using the slider during processing, the image in Figures 3.11 (just after glutamate/glycine had been added at 5.5 minutes into the run) and Figure 3.12 (four minutes after CCCP had been added at 16.1 minutes into the run) show a decrease in white and an increase in red (arrow). This indicates that there had been a relative increase in \([Ca^{2+}]_i\), that[Ca^{2+}]_i had bound to the fluorophore and the fluorophore was now being excited at 340 instead of 380 nm.
Figure 3.10. A 380 nm 24-bit colour image of undifferentiated C6 cells loaded with FuraFF. Ten milliliters of DMEM containing cells were seeded at a density of $2 \times 10^4$ cells/ml and grown on coverslips in a 100 mm tissue culture dish for 4 days. The coverslips were placed in an Attofluor cell chamber, loaded with 5 μM FuraFF, incubated for 30 minutes, washed and observed with a 40x (oil immersion) objective on a Olympus 1x81 fluorescent microscope and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1. This image was taken one minute before 112 μM glutamate/45 μM glycine was added, at 4.5 minutes into the run. Using the slider during processing, the image in Figures 3.11 (just after glutamate had been added at 5.5 minutes into the run) and Figure 3.12 (four minutes after CCCP had been added at 16.1 minutes into the run) show a decrease in white and an increase in red (arrow). This indicates that there had been an increase in $[\text{Ca}^{2+}]$, $[\text{Ca}^{2+}]$, had bound to the fluorophore and the fluorophore was now being excited at 340 instead of 380 nm.
Figure 3.11. This 380 nm image is of the same undifferentiated C6 cells as in Figure 3.10. It was taken 2 minutes after the image in Figure 3.10 and 1 minute after the addition of glutamate. This frame was taken 6.5 minutes after the beginning of the run. In this frame, the white areas continue to steadily shrink to be replaced by red and green areas as $[\text{Ca}^{2+}]_{i}$ increases and becomes bound to the fluorophore (arrow). Ten milliliters of DMEM containing cells were seeded at a density of $2 \times 10^4$ cells/ml and grown on coverslips in a 100 mm tissue culture dish for 4 days. The coverslips were placed in an Attofluor cell chamber, loaded with 5 $\mu$M FuraFF, incubated for 30 minutes, washed and observed with a 40x (oil immersion) objective on a Olympus 1x81 fluorescent microscope and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Figure 3.12. A 380 image of the undifferentiated C6 cells seen in Figures 3.10 and 3.11, four minutes after the administration of the ionophore, CCCP, which reduced the unbound dye even further. This image (Figure 3.12) was taken 15 minutes after glutamate had been added and four minutes after CCCP had been added causing the release of calcium from the mitochondria, thus further increasing $[\text{Ca}^{2+}]$. Note that the red areas indicated by the arrow in this and in figures 3.10 and 3.11 are now increasing and some parts are changing to orange. Ten milliliters of DMEM containing cells were seeded at a density of $2 \times 10^4$ cells/ml and grown on coverslips in a 100 mm tissue culture dish for 4 days. The coverslips were placed in an Attofluor cell chamber, loaded with 5 $\mu$M FuraFF, incubated for 30 minutes, washed and observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
3.2. Glutamate Experiments

3.2.1. Phase Contrast Microscopy
Cells were seeded in each well of 24-well plates at a density of 1 x 10^4 per well. 24 hours later, the media was removed and replaced by media containing glutamate at a concentration of 5 mM, 30 mM, 50 mM or 1 M. The cells were examined 3, 6, and 27 hours later in a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount. Figures 3.13 to 3.24 provide representative pictures of cells exposed to 5 mM, 30 mM, 50 mM or 1M for 3, 6, or 27 hours. Total magnification was 160x. Figures 3.13 to Figure 3.24 show decreasing cell viability over time and with glutamate concentrations of 5 mM to 1 M. In addition, the concentration cytotoxicity response curves between undifferentiated and the differentiated cell lines showed that while there was little difference between undifferentiated and differentiated C6 (rat glioma) cells (Figure 3.25) there was a very marked difference between differentiated and undifferentiated SH-SY5Y cells (Figure 3.26).

This preliminary study suggested that these glutamate concentrations were too high for studies lasting 24 hours. Glutamate concentrations of 0, 5, 25, 50, 125, or 250 μM were used in further cell viability studies. This provided results that did not end in complete apoptosis within 24 hours. (Figures 3.25 to 3.26).
Figure 3.13. Phase contrast (40x) of C6 undifferentiated cells exposed to (clockwise from top left) 5, 30, 50 mM, and 1 M glutamate and photographed unstained at 3 hours. An apoptotic-like bleb is observed in the 30 mM picture (arrow). While chromatin condensation is another indication of apoptosis, the condensation was not clearly visible in all cells in this and succeeding illustrations in which there were blebs and so could not reliably be used as a second indicator of apoptosis. Cells are dead in the 1 M image. Cells were prepared as described in Methods, observed on a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount.
Figure 3.14. Phase contrast (40x) of C6 undifferentiated cells exposed to (clockwise from top left) 5, 30, 50 mM, and 1 M glutamate and photographed unstained at 6 hours. Cells were prepared as described in Methods, observed on a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount. Apoptotic changes are observed in the 5mM, 30 mM and 1 M concentrations (arrows).
Figure 3.15. Phase contrast (40x) of C6 undifferentiated cells exposed to (clockwise from top left) 5, 30, 50 mM, and 1 M glutamate and photographed unstained at 27 hours. Apoptotic blebs (white arrows) are evident in the 5 mM, 30 mM and 50 mM pictures. Some 1 M glutamate-treated cells (grey arrow) have become detached from the bottom a sign of cell death. Fragments of cells are also visible. Cells were prepared as described in Methods, observed on a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount.
Figure 3.16. Phase contrast (40x) of C6 cells differentiated into astrocyte-like cells exposed to (clockwise from top left) 5, 30, 50 mM, and 1 M glutamate and photographed unstained at 3 hours. Apoptotic cells, evident from their blebs are found in the 50 mM cells and numerous blebs are present in the 1 M glutamate cells after 3 hours (arrows). Apoptotic-like changes are also apparent in the 5mM and 30 mM cells (arrows). Cells were prepared as described in Methods, observed on a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount.
Figure 3.17. Phase contrast (40x) of C6 cells differentiated into astrocyte-like cells exposed to (clockwise from top left) 5, 30, 50 mM, and 1 M glutamate and photographed unstained at 6 hours. Apoptotic cells are present in the 5 mM, 30 mM and 1 M glutamate as evidenced by the appearance of blebs and bright spots in the cells (arrows). In the latter there is a lot of cellular debris in the image. Cells were prepared as described in Methods, observed on a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount.
Figure 3.18. Phase contrast (40x) of C6 cells differentiated into astrocyte-like cells exposed to (clockwise from top left) 5, 30, 50 mM, and 1 M glutamate and photographed unstained at 27 hours. Apoptotic cells are present in the 5 and 30 mM images as indicated by the numerous blebs and luminous spots on many cells (arrows). In the 1 M image there was only cellular debris. Cells were prepared as described in Methods, observed on a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount.
**Figure 3.19.** Phase contrast (40x) of SH-SY5Y undifferentiated cells exposed to (clockwise from top left) 5, 30, 50 mM, and 1 M glutamate and photographed unstained at 3 hours. Apoptotic blebs are evident in the 5 mM, 30 mM, 50 mM and 1 M images (arrows). Most of the 1 M glutamate-treated cells have become apoptotic. Cells were prepared as described in Methods, observed on a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount.
Figure 3.20. Phase contrast (40x) of SH-SY5Y undifferentiated cells exposed to (clockwise from top left) 5, 30, 50 mM, and 1 M glutamate and photographed unstained at 6 hours. Apoptotic blebs are evident in the 5, 30, 50 mM and 1 M images (arrows). Most of the 1 M glutamate-treated cells have become apoptotic. Cells were prepared as described in Methods, observed on a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount.
Figure 3.21. Phase contrast (40x) of SH-SY5Y undifferentiated cells exposed to (clockwise from top left) 5, 30, 50 mM, and 1 M glutamate and photographed unstained at 27 hours. Apoptotic blebs are evident in the 5, 30 and 50 mM images (arrows). All of the 1 M glutamate-treated cells have died and only cell fragments are visible (arrows). Cells were prepared as described in Methods, observed on a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount.
Figure 3.22. Phase contrast (40x) of SH-SY5Y differentiated cells exposed to (clockwise from top left) 5, 30, 50 mM, and 1 M glutamate and photographed unstained at 3 hours. Apoptotic blebs are evident in the 5 mM, 30 mM, 50 mM and 1M images (arrows). Cells were prepared as described in Methods, observed on a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount.
Figure 3.23. Phase contrast (40x) of SH-SY5Y differentiated cells exposed to (clockwise from top left) 5, 30, 50 mM, and 1 M glutamate and photographed unstained at 6 hours. Apoptotic blebs are evident in the 5 mM, 30 mM, 50 mM and 1 M images (arrows). Clumping is one of the characteristic growth patterns of SH-SY5Y cells (50 mM figure). Cells were prepared as described in Methods, observed on a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount.
Figure 3.24. Phase contrast (40x) of SH-SY5Y differentiated cells exposed to (clockwise from top left) 5, 30, 50 mM, and 1 M glutamate and photographed unstained at 27 hours. Apoptotic blebs are evident in the 5 mM, 30 mM and 50 mM images (arrows). In the 1 M glutamate image the apoptotic cells have lifted from the bottom of the well and are floating, indicating that the majority of them are dead (arrows). The image appears to be out of focus because the floating cells have balled up and so are not in a single plane of focus. Cells were prepared as described in Methods, observed on a Leica inverted tissue culture microscope with a 40x phase contrast dry objective and photographed with a 4x Canon camera attached to a top C mount.
3.2.2. Microplate Glutamate Studies

The phase contrast microscopy was followed by microplate experiments to determine the effects of glutamate on cell viability. Concentration cytotoxicity curves for glutamate are shown in Figures 3.25 for C6 cells. The C6 cells were prepared as described in Methods, and seeded in the inner 60 wells of 96-well plates at a density of 2 x 10^3 cells/100 µl. Twenty-four hours later, 0, 5, 25, 50, 125 or 250 µM glutamate in DMEM were added. After 24 hours, the media with the glutamate was removed, and replaced with 3 µM calcein AM in DPBS. The 96-well plates were then incubated at 37°C, 5%CO₂ and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

When looking at the effect of glutamate on C6 cells (Figure 3.25), the two-way ANOVA F value was 399.70 (DFn = 5, DFd = 70, p < .0001). Bonferroni post hoc tests showed that each dose of glutamate significantly reduced viability compared to the controls, but the doses were not different from each other. Additionally, there were no differences in the effects of glutamate on undifferentiated C6 cells verses differentiated C6 cells, except for the effect of 250 µM glutamate.

For the effect of glutamate on SH-SY5Y cells (Figure 3.26), the 2-way ANOVA F value was 262.25 (DFn = 5, DFd = 84, p < .0001). Post hoc Bonferroni tests showed that each dose of glutamate significantly reduced viability compared to controls, but as in C6 cells, the doses were not different from each other. Comparing the effects of glutamate on undifferentiated versus differentiated SH-SY5Y cells, the viability of the differentiated cells was significantly lower in the controls and at the 25, 50, 125, or 250 µM concentrations of glutamate.
Figure 3.25. Glutamate concentration cytotoxicity response curve comparison between undifferentiated (U) and differentiated (D) C6 cells. Cells in 96-well plates were incubated for 24 hours with 0, 5, 25, 50, 125 or 250 μM glutamate in DMEM at 37°C, 5%CO₂ and 95% air, and the viability of the cells was determined with the calcein AM indicator. Each data bar is the mean plus the standard deviation of 8 wells. In this and all subsequent figures, * means that the p value was at least less than 0.05 compared to the zero glutamate control group, and # means that the p value was at least less than 0.05 compared to the undifferentiated cells receiving the same treatment.
**Figure 3.26.** Glutamate concentration cytotoxicity response curve comparison between undifferentiated (U) and differentiated (D) SH-SY5Y cells. Cells in 96-well plates were incubated for 24 hours with 0, 5, 25, 50, 125 or 250 μM glutamate in DMEM at 37°C, 5% CO₂ and 95% air, and the viability of the cells was determined with the calcein AM indicator. Each data bar is the mean plus the standard deviation of 8 wells.
3.2.3. Ratiometric Glutamate Microscopy

All cells were seeded and grown on coverslips in a 100 mm tissue culture dish for four days. The cover slips were placed in an Attofluor cell chamber, loaded with 5 μM FuraFF in one milliliter of HBSS+HEPES, and observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.

One milliliter of glutamate/glycine was administered by a transfer pipette to the well of the Attofluor cell chamber. If the ionophores CCCP or Br4A23187 were to be added, the drugs and media were washed twice by calcium-and-magnesium-free HBSS+HEPES. The calcium response to acute glutamate administration in each cell type (differentiated and undifferentiated) was followed by ratiometric microscopy (Figures 3.27 to 3.32). These figures show a spike in response to glutamate/glycine, however the baseline did not return to where it had been before the glutamate administration. After stopping the run and washing the cells twice with one milliliter of calcium-and-magnesium-free HBSS+HEPES to remove the glutamate, glycine and calcium, the ionophore CCCP was administered to facilitate the release of stored calcium from the mitochondria. When released by CCCP, a second rise in the baseline occurred (Figures 3.27, 3.30 and 3.32). Figure 3.27 shows a typical spike followed by a slow decrease in the baseline until CCCP is added at which time there is an increase in the baseline. In Figure 3.28 there is a peak which returns to baseline but in Figure 3.29 the peak does not return to baseline. In neither Figure 3.28 nor Figure 3.29 was CCCP added. In Figure 3.30, an atypical 380 response shows a spike when glutamate and glycine are added and a second spike when CCCP is added. Normally, these spikes would be below the baseline, not above it. As a result, in Figure 3.31, the
ratiometrics for this same cell does not show much of a spike when the 340 nm values of the image are divided by the 380 nm values of the image. In Figure 3.32, there is a small increase in the baseline when glutamate and glycine are added, but then a negative spike in the baseline when Br4A23187, an ionophore, was added. In Figure 3.33 a large negative spike occurred when glutamate and glycine were added. CCCP was not used in that figure.
Figure 3.27. Undifferentiated C6 cell relative change in $[Ca^{2+}]_i$ ratiometric response of one cell following exposure to 112.5 µM glutamate/45 µM glycine (blue arrow). The cells were then washed twice with one milliliter of calcium-and-magnesium-free HBSS+HEPES (vertical purple line) and exposed to 1.5 µM of CCCP (green arrow).
Figure 3.28. Undifferentiated C6 cell relative change in $[\text{Ca}^{2+}]_r$, ratiometric response of one cell to acute administration of 75 μM glutamate/30 μM glycine (blue arrow). This graph is representative of 6 out of 8 cells analyzed from this run.
Figure 3.29. Undifferentiated C6 cell relative change in [Ca$^{2+}$], ratiometric response of a single cell to acute administration of 75 μM glutamate/30 μM glycine (blue arrow). This cell showed the typical rapid spike in [Ca2+]i to glutamate but an atypical slow return to baseline.
Figure 3.30. An atypical 380 nm recording of a single SH-SY5Y undifferentiated cell relative change in [Ca$^{2+}$], response to acute administration of 75 µM glutamate/30 µM glycine (blue arrow). These cells were then washed twice with calcium-free one milliliter of calcium-and-magnesium-free HBSS-HEPES imaging solution (vertical purple line) and then exposed to 1.5 µM of CCCP (green arrow). Typically, the 380 nm graph would show negative deflections, so this graph was atypical because of positive deflections in this recording.
**Figure 3.31.** The relative change in $[\text{Ca}^{2+}]_i$ response of a single SH-SY5Y undifferentiated cell to acute administration of 75 µM glutamate/30 µM glycine (blue arrow). These cells were then washed twice with one milliliter of HBSS+HEPES calcium-and-magnesium-free imaging solution (vertical purple line) and then exposed to 1.5 µM of CCCP (green arrow). This ratiometric graph is of the same cell illustrated in the 380 nm graph in Figure 3.30 and is the 340 nm graph divided by the 380 graph, as described in Methods. This graph is representative of the 8 undifferentiated SH-SY5Y cells that were analyzed from this run.
Figure 3.32. The relative change in \([\text{Ca}^{2+}]_i\), ratiometric response of a single differentiated SH-SY5Y cell following exposure to 75 μM glutamate/30 μM glycine (blue arrow). These cells were then washed twice with calcium-and-magnesium-free HBSS+HEPES (vertical purple line) and exposed to 1.5 μM of the ionophore Br4A23187 (green arrow). This cell is representative of 6 of the 8 cells analyzed from this run.
Figure 3.33. The relative change in $[\text{Ca}^{2+}]$, ratiometric response of a single differentiated SH-SY5Y cell following exposure to 75 µM glutamate/30 µM glycine (blue arrow). This cell showed a decrease in the Fura FF 340/380 ratio rather than the more typical increase seen in Figure 3.32. This atypical response was seen in 2 out of 8 cells analyzed for this run.
3.3. Studies with Mirtazapine

3.3.1. Microplate Mirtazapine Studies

Concentration cytotoxicity curves for mirtazapine are shown in Figures 3.34 for C6 cells. The C6 cells were prepared as described in Methods, and seeded in the inner 60 wells of 96-well plates at a density of $2 \times 10^3$ cells/100 μl. Twenty-four hours later, 0, 5, 25, 50, 125 or 250 μM mirtazapine in DMEM were added. After 24 hours, the media with the mirtazapine was removed, and replaced with 10 μM calcein AM in DPBS. The 96-well plates were then incubated at 37°C, 5%CO₂ and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

For the effect of mirtazapine on C6 cells, the two-way ANOVA F value was 443.50 (DFn = 5, DFd = 70, p <.0001). Post-hoc Bonferroni tests showed that each dose of mirtazapine was significantly different from the no drug controls, but no drug concentration was different from any other concentration. The cell type F value was 23.13 (DFn=1, DFd = 14, p = 0.0003). Bonferroni tests indicated that viability of differentiated cells was significantly different from undifferentiated cells in the control and the 25 μM mirtazapine groups.
**Figure 3.34.** Mirtazapine concentration cell viability response curve and comparison between undifferentiated (U) and differentiated (D) C6 cells. Cells were incubated for 24 hours with 0, 5, 25, 50, 125 or 250 μM mirtazapine in DMEM at 37°C, 5%CO₂ and 95% air and the viability of the cells was determined with the calcein AM indicator assay. Each data bar is the mean plus the standard deviation of calcein intensity in 8 wells. In this and all subsequent figures, * means that the p value is at least less than 0.05 compared to the zero mirtazapine control group, and # means that the p value is at least less than 0.05 compared to the undifferentiated cells receiving the same treatment. Post-hoc Bonferroni tests showed that each dose of mirtazapine significantly reduced viability compared to controls, and that the doses were not different from each other. Also the viability of differentiated C6 cells is significantly higher than the undifferentiated C6 cells in the control and in the 25 μM mirtazapine groups.
In Figure 3.35, the SH-SY5Y cells were prepared as described in Methods, and seeded in the inner 60 wells of 96-well plates at a density of $2 \times 10^3$ cells/100 µl. Twenty-four hours later, 0, 5, 25, 50, 125 or 250 µM mirtazapine in DMEM were added. After 24 hours, the media with the mirtazapine was removed, and replaced with 10 µM calcein AM in DPBS. The 96-well plates were then incubated at 37°C, 5%CO$_2$ and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

For the SH-SY5Y cells the F value was 303.07.77 (DFn = 5, DFd = 70, p < .0001), and Bonferroni tests indicated that each dose of mirtazapine was significantly different from the controls but the doses of mirtazapine were not different from each other. The cell type F value was 78.47 (DFn = 1, DFd = 14, p < .0001). Bonferroni tests showed that differentiated SH-SY5Y cells were significantly different from undifferentiated cells in the groups exposed to 5, 25, 50, or 125 µM mirtazapine.
Figure 3.35. Mirtazapine concentration cell viability response curve and comparison between undifferentiated (U) and differentiated (D) SH-SY5Y cells. Cells were incubated for 24 hours with 0, 5, 25, 50, 125 or 250 μM mirtazapine in DMEM at 37°C, 5%CO₂ and 95% air and the viability of the cells was determined with the calcein AM indicator assay. Each data bar is the mean plus the standard deviation of calcein intensity in 8 wells. In this and all subsequent figures, * means that the p value is at least less than 0.05 compared to the zero mirtazapine control group, and # means that the p value is at least less than 0.05 compared to the undifferentiated cells receiving the same treatment. Post-hoc Bonferroni tests showed that each dose of mirtazapine significantly reduced viability compared to controls, and that the doses were not different from each other. Also the viability of differentiated SH-SY5Y cells is significantly lower than the undifferentiated SH-SY5Y cells in the 5, 25, 50 and 125 μM mirtazapine groups.
The effects of glutamate on the viability of C6 cells exposed to mirtazapine for 10 weeks are shown in Figure 3.36. The cells were chronically exposed to 50 μM mirtazapine in t-25 flasks for 10 weeks, harvested by trituration as described in Methods, and seeded in DMEM from the flasks in the inner 60 wells of 96-well plates at a density of 2 x 10^3 cells/100 μl along with 50 μM mirtazapine. Twenty-four hours later glutamate in DMEM was added to give final 0, 5, 25, or 250 μM glutamate and 50 μM mirtazapine in DMEM. After 24 hours, the media with the glutamate and mirtazapine was removed, and replaced with 3 μM calcein AM in DPBS. The 96-well plates were then incubated at 37°C, 5%CO2 and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

For the glutamate effect in the presence of 50 μM mirtazapine on C6 cells chronically exposed to mirtazapine (Figure 3.36), the two-way ANOVA F value was 17.52 (DFn =3, DFd = 66, p <.0001). Post-hoc Bonferroni tests showed that each dose of glutamate was significantly different from the no drug controls in the undifferentiated C6 cells and in the 25 and 250 mM doses for differentiated cells, and the 25 and 250 μM glutamate concentrations were different from the control concentrations. The cell type F value for C6 was 190.45 (DFn=1, DFd = 22, p <0.0001). Post hoc Bonferroni tests showed that the 25 and 250 μM doses of glutamate significantly reduced viability of differentiated C6 cells compared to controls, and all doses of glutamate significantly increased viability of undifferentiated cells.
Figure 3.36. Effects of glutamate on C6 cells chronically treated with mirtazapine.

Undifferentiated (U) and differentiated (D) C6 cells were chronically exposed to 50 μM mirtazapine for 10 weeks in T-25 tissue culture flasks. Cells were harvested and seeded in the wells in 96-well plates at a density of 2 x 10^3 cells in 100 μl of DMEM with 50 μM mirtazapine. After 24 hours incubation at 37°C, 5%CO₂ and 95% air, 100 μl DMEM containing glutamate and mirtazapine to give final concentrations of 0, 5, 25, or 250 μM and 50 μM mirtazapine, were added to each well for a total of 200 μl. Twenty-four hours later, the DMEM with glutamate was removed and replaced with 200 μL DPBS containing 3 μM calcein indicator. The cells were read at 485 nm absorbance on a Tecan Safire plate reader with Excel output. Each data bar is the mean plus the standard deviation of 8 wells.
The effects of glutamate on the viability of SH-SY5Y cells exposed to mirtazapine for 10 weeks are shown in Figure 3.37. The cells were chronically exposed to 50 μM mirtazapine for 10 weeks, prepared as described in Methods, and seeded with DMEM with 50 μM mirtazapine in the inner 60 wells of 96-well plates at a density of 2 x 10³ cells/100 μl. Twenty-four hours later glutamate and mirtazapine in DMEM was added to give final 0, 5, 25, or 250 μM glutamate and 50 μM in DMEM. After 24 hours, the media with the glutamate was removed, and replaced with 3 μM calcein AM in DPBS. The 96-well plates were then incubated at 37°C, 5%CO₂ and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

For the SH-SY5Y cells chronically exposed to mirtazapine (Figure 3.37), the two-way ANOVA the F value was 42.71 (DFn = 3, DFd = 66, p < .0001). Post-hoc Bonferroni tests indicated that each dose of glutamate was significantly different from the controls except for the 5 μM glutamate. The cell type F value was 230.73 (DFn = 1, DFd = 22, p < .0001). The Bonferroni test showed that differentiated SH-SY5Y cells were significantly different from undifferentiated cells in the groups exposed to 5, 25 and 250 μM glutamate.
Figure 3.37. Effects of glutamate on SH-SY5Y cells chronically treated with mirtazapine.

Undifferentiated (U) and differentiated (D) SH-SY5Y cells were chronically exposed to 50 μM mirtazapine for 10 weeks in T-25 tissue culture flasks. Cells were harvested and seeded in the wells in 96-well plates at a density of 2 x 10⁳ cells in 100 μl of DMEM with 50 μM mirtazapine. After 24 hours incubation at 37°C, 5%CO₂ and 95% air, 100 μl DMEM containing glutamate and 50 μM mirtazapine to give final concentrations of 0, 5, 25, or 250 μM, were added to each well for a total of 200 μl. Each data bar is the mean plus the standard deviation of 8 wells. Post ANOVA Bonferroni tests indicated that pretreatment with 50 μM mirtazapine for 10 weeks significantly increased the viability of undifferentiated SH-SY5Y cells exposed to 5, 25, or 250 μM glutamate and decreased the viability of differentiated SH-SY5Y cells exposed to 25 or 250 μM glutamate.
In Figure 3.38, SH-SY5Y cells were treated with 50 μM mirtazapine for 10 weeks and seeded in 6 well-plates at a density of $1 \times 10^4$ cells/500 μl. A combination of glutamate and mirtazapine in DMEM were added to the cells for 24 hours before being removed and replaced with calcein-AM in DPBS. The 6-well plates were then incubated at $37^\circ$C, 5%CO$_2$ and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

For the SH-SY5Y cells in Figure 3.38, the two-way ANOVA the F value was 49.68 (DFn = 3, DFd = 18, p < .0001). Post-hoc Bonferroni tests indicated that each dose of glutamate was significantly different from the controls except for the 5 μM glutamate. The cell type F value was not significant ($F = 0.43$, DFn = 1, DFd = 6, p = 0.5351).

Similar studies on C6 cells are not available.
Figure 3.38. Effects of glutamate on SH-SY5Y cells chronically treated with mirtazapine.

Undifferentiated (U) and differentiated (D) SH-SY5Y cells were chronically exposed to 50 μM mirtazapine for 10 weeks. Cells were harvested and seeded in the wells in 6-well plates at a density of 1 x 10^4 cells in 500 μl of DMEM. After 24 hours incubation at 37°C, 5%CO₂ and 95% air, 500 μl DMEM containing 0 or 250 μM glutamate and or 0 or 5 μM of mirtazapine to give final concentrations of 250 μM, was added to appropriate wells for a total of 1000 μl. Each data bar is the mean plus the standard deviation of 4 wells. Post ANOVA Bonferroni tests indicated that, in cells exposed to 50 μM mirtazapine for 10 weeks, 250 μM glutamate in the absence of mirtazapine increased the viability of undifferentiated and differentiated SH-SY5Y cells, and in the presence of 5 μM mirtazapine increased the viability of differentiated SH-SY5Y cells and had no effect on undifferentiated SH-SY5Y cells.
3.3.2. Ratiometric Microscopy Mirtazapine Studies

Ratiometric studies of relative changes in $[\text{Ca}^{2+}]_i$ levels (Figure 3.39) with C6 cells showed a small peak when 50 μM mirtazapine was given followed by a drop in the baseline that was not changed when 75 μM of glutamate/30 μM glycine were added. Nor was it changed when, after washing twice with calcium-and-magnesium-free HBSS+HEPES imaging solution, 1.5 μM CCCP was added. Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 μM FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Figure 3.39. Representative C6 undifferentiated cell relative change in [Ca^{2+}], ratiometric response to acute administration of 50 μM mirtazapine (red arrow) followed by 75 μM glutamate/30 μM glycine (blue arrow). The cells were then washed twice with 1 ml of calcium-and-magnesium-free HBSS-HEPES imaging solution (vertical purple line) and 1.5 μM of CCCP (green arrow) was administered. The responses of this cell were seen in 8 out of 8 cells analyzed.
In Figure 3.40 there is a representative ratiometric response of C6 differentiated cell relative change in [Ca$^{2+}$], to acute administration of 50 μM mirtazapine (red arrow) which shows a sharp deflection followed by a return to baseline. There is no change in the baseline following 75 μM glutamate/30 μM glycine (blue arrow). The cells were then washed twice with calcium-and-magnesium-free HBSS+ HEPES imaging solution (vertical purple line) after which 1.5 μM of CCCP (green arrow) is administered resulting in a small negative spike before a return to a sloping baseline. The C6 cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 μM FuraFF and observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Figure 3.40. Representative C6 differentiated cell relative change in $[\text{Ca}^{2+}]_i$ ratiometric response to acute administration of 50 μM mirtazapine (red arrow) followed by 75 μM glutamate/30 μM glycine (blue arrow). The cells were then washed twice with 1 ml calcium-and-magnesium-free HBSS-HEPES imaging solution (vertical purple line) and 1.5 μM of CCCP (green arrow) was administered. The responses of this cell are representative of 8 out of 10 cells analyzed.
Figure 3.41 shows a representative 380 nm response of a single SH-SY5Y undifferentiated cell relative change in \([\text{Ca}^{2+}]_i\) to the acute administration two doses of mirtazapine in succession. At the left-most red arrow, the addition of 50 \(\mu\text{M}\) mirtazapine (red arrow) was followed by an immediate drop in the baseline. A similar drop was produced by the administration of 33 \(\mu\text{M}\) mirtazapine at the second red arrow from left. The administration of 56 \(\mu\text{M}\) glutamate and 22 \(\mu\text{M}\) glycine (blue arrow) did not produce the expected spike. The SH-SY5Y cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 \(\mu\text{M}\) FuraFF and observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Figure 3.41. Representative relative change in [Ca2+]i 380 nm response of a single SH-SY5Y undifferentiated cell. 50 μM mirtazapine was given at the first red arrow, 33 μM mirtazapine was given at the second red arrow from left, and 56 μM glutamate and 22 μM glycine was given at the blue arrow. The sharp changes in the baseline at the beginning of the run are due to focusing. This cell is representative of 8 of 8 cells analyzed.
Figure 3.42 is a representative ratiometric recording of a SH-SY5Y undifferentiated cell relative change in [Ca2+]. This pattern was seen in 8 out of 8 cells analyzed. Ratiometric response to acute administration of 50 μM mirtazapine (first red arrow) and 33 μM mirtazapine (second red arrow from left) followed by the administration of 56 μM glutamate and 22 μM glycine (blue arrow). In contrast to the 380 image, no baselines, peaks, or valleys can be discerned. Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for two days. They were placed in an Attofluor cell chamber and loaded with 5 μM FuraFF and observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
**Figure 3.42.** Representative SH-SY5Y undifferentiated cell relative change in $[\text{Ca}^{2+}]_i$ ratiometric response to acute administration of 50 $\mu$M mirtazapine (first red arrow) followed by 33 $\mu$M mirtazapine (second red arrow from left) then 56 $\mu$M glutamate and 22 $\mu$M glycine (blue arrow).
Figure 3.43 is representative SH-SY5Y differentiated cell relative change in \([\text{Ca}^{2+}]_i\) ratiometric response. This pattern was seen in 10 out of 10 cells analyzed. Acute administration of 50 \(\mu\text{M}\) mirtazapine (red arrow) results in a sharp drop in the baseline which is followed by a period of level baseline before returning to the original level. After the administration of 75 \(\mu\text{M}\) glutamate/30 \(\mu\text{M}\) glycine (blue arrow) there is no change in the baseline. The cells are then washed twice with calcium-and-magnesium-free HBSS+HEPES imaging solution (vertical purple line) followed by 1.5 \(\mu\text{M}\) of CCCP (green arrow) which does not change the baseline. Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 \(\mu\text{M}\) FuraFF and observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Figure 3.43 Representative SH-SY5Y differentiated cell relative change in $[\text{Ca}^{2+}]_i$ ratiometric response to acute administration of 50 μM mirtazapine (red arrow) followed by 75 μM glutamate/30 μM glycine (blue arrow). Then the cells are washed twice with calcium-and-magnesium-free HBSS+HEPES imaging solution (vertical purple line) and then 1.5 μM of CCCP (green arrow) is administered.
3.4. Studies with Citalopram

3.4.1. Microplate Citalopram Studies

Concentration cytotoxicity curves for citalopram are shown in Figures 3.44 for C6 cells. The cells were prepared as described in Methods, and seeded in the inner 60 wells of 96-well plates at a density of $2 \times 10^3$ cells/100 μl. Twenty-four hours later, 0, 5, 25, 50, 125 or 250 μM citalopram in 100 μl DMEM were added. After 24 hours, the media with the citalopram was removed, and replaced with 3 μM calcein AM in DPBS. The 96-well plates were then incubated at 37°C, 5%CO and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

For the effect of citalopram on C6 cells (Figure 3.44), the two-way ANOVA F value was 372.83 (DFn = 5, DFd = 70, p <.0001). Post-hoc Bonferroni tests showed that each dose of citalopram was significantly different from the no drug controls, but no drug concentration was different from any other concentration. The cell type F value was 15.79 (DFn=1, DFd = 14, p = 0.0014). Bonferroni tests indicated that viability of differentiated cells was significantly higher from undifferentiated cells in the 5 μM citalopram group.
Figure 3.44. Citalopram concentration viability response curve and comparison between undifferentiated (U) and differentiated (D) C6 cells. Cells were incubated for 24 hours with 0, 5, 25, 50, 125 or 250 μM citalopram in 100 μl DMEM at 37°C, 5%CO2 and 95% air and the viability of the cells was determined with the calcein AM indicator assay. Each data bar is the mean plus the standard deviation of calcein intensity in 8 wells. In this figure, * means that the p value is at least less than 0.05 compared to the zero citalopram control group, and # means that the p value is at least less than 0.05 compared to the undifferentiated cells receiving the same treatment. Post-hoc Bonferroni tests showed that each dose of citalopram significantly reduced viability compared to controls, and that the doses were not different from each other. Also the viability of differentiated C6 cells is significantly higher than the undifferentiated C6 cells in the 5 μM citalopram group.
Concentration cytotoxicity curves for citalopram are shown for the SH-SY5Y cells in Figure 3.45. The cells were prepared as described in Methods, and seeded in the inner 60 wells of 96-well plates at a density of $2 \times 10^3$ cells/100 μl. Twenty-four hours later, 0, 5, 25, 50, 125 or 250 μM citalopram in 100 μl DMEM were added. After 24 hours, the media with the citalopram was removed, and replaced with 3μM calcein AM in DPBS. The 96-well plates were then incubated at 37°C, 5%CO and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

The effect of citalopram on SH-SY5Y cells was F value was 405.54 (DFn = 5, DFd = 70, p < .0001), and Bonferroni tests indicated that each dose of citalopram was significantly different from the controls but the doses of citalopram were not different from each other. The cell type F value was 24.06 (DFn = 1, DFd = 14, p = .0002). Bonferroni tests showed that differentiated SH-SY5Y cells were significantly different from undifferentiated cells in the groups exposed to 5, 25 or 50, μM citalopram.
**Figure 3.45.** Citalopram concentration viability response curve and comparison between undifferentiated (U) and differentiated (D) SH-SY5Y cells. Cells were incubated for 24 hours with 0, 5, 25, 50, 125 or 250 μM citalopram at 37°C, 5%CO₂ and 95% air and the viability of the cells was determined with the calcein AM indicator. Each data bar is the mean plus the standard deviation of 8 wells. The data bars are expressed in relation to calcein intensity. In this figure, * means that the p value is at least less than 0.05 compared to the zero citalopram control group, and # means that the p value is at least less than 0.05 compared to the undifferentiated cells receiving the same treatment.
The effects of glutamate on the viability of C6 cells exposed to citalopram for 10 weeks are shown in Figure 3.46. The cells were chronically exposed to 50 μM citalopram for 10 weeks, prepared as described in Methods, and seeded in media containing 50 μM citalopram in the inner 60 wells of 96-well plates at a density of 2 x 10^3 cells/100 μl in DMEM. Twenty-four hours later glutamate and citalopram in DMEM was added to give final concentrations of 0, 5, 25, or 250 μM glutamate and 50 μM citalopram. After 24 hours, the media with the glutamate was removed, and replaced with 3 μM calcein AM in DPBS. The 96-well plates were then incubated at 37°C, 5%CO₂ and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

For the glutamate effect in the presence of 50 mM citalopram on C6 cells chronically exposed to citalopram (Figure 3.46), the two-way ANOVA F value was 74.06 (DFn =3, DFd = 66, p <.0001). Post-hoc Bonferroni tests showed that each dose of glutamate was significantly different from the no drug controls in the undifferentiated and differentiated C6 cells. The cell type F value for C6 was 67.64 (DFn=1, DFd = 22, p <0.0001). Post hoc Bonferroni tests showed that the 5, 25 and 250 μM doses of glutamate significantly reduced viability of undifferentiated C6 cells compared to differentiated cells.
**Figure 3.46.** Effects of glutamate on C6 cells chronically treated with citalopram.

Undifferentiated (U) and differentiated (D) C6 cells were chronically exposed to 50 μM citalopram for 10 weeks in T-25 tissue culture flasks. Cells were harvested and seeded in the wells in 96-well plates at a density of 2 x 10³ cells in 100 μl of DMEM with 50 μM citalopram.

After 24 hours incubation at 37°C, 5%CO₂ and 95% air, 100 μl DMEM containing glutamate and citalopram to give final concentrations of 0, 5, 25, or 250 μM and 50 μM citalopram, were added to each well for a total of 200 μl. Twenty-four hours later, the DMEM with glutamate was removed and replaced with 200 μL DPBS containing 3 μM calcein indicator. The cells were read at 485 nm absorbance on a Tecan Safire plate reader with Excel output. Each data bar is the mean plus the standard deviation of 8 wells.
The effects of glutamate on SH-SY5Y cells chronically treated with citalopram are shown in Figure 3.47. Undifferentiated (U) and differentiated (D) SH-SY5Y cells were chronically exposed to 50 μM citalopram for 10 weeks in T-25 tissue culture flasks. Cells were harvested and seeded in the wells in 96-well plates at a density of 2 x 10³ cells in 100 μl of DMEM with 50 μM citalopram. After 24 hours incubation at 37°C, 5% CO₂ and 95% air, 100 μl DMEM containing glutamate and citalopram to give final concentrations of 0, 5, 25, or 250 μM and 50 μM citalopram, were added to each well for a total of 200 μl. Twenty-four hours later, the DMEM with glutamate was removed and replaced with 200 μL DPBS containing 3 μM calcein indicator. The cells were read at 485 nm absorbance on a Tecan Safire plate reader with Excel output. Each data bar is the mean plus the standard deviation of 8 wells.

For the effect of glutamate on SH-SY5Y cells chronically treated with citalopram (Figure 3.47), the two-way ANOVA F value was 14.83 (DFn =3, DFd = 66, p <.0001). Post-hoc Bonferroni tests showed that cell viability was not affected by any dose of glutamate except that the cell viability in differentiated SH-SY5Y cells receiving the 5 μM dose of glutamate was significantly higher than the no drug controls. The cell type F value for SH-SY5Y was not significant (F= 3.79, DFn=1, DFd = 22, p = 0.0644).
Figure 3.47. Effects of glutamate on SH-SY5Y cells chronically treated with citalopram.

Undifferentiated (U) and differentiated (D) SH-SY5Y cells that were chronically exposed to 50 μM citalopram for 10 weeks. Cells were incubated for 24 hours with 50 μM citalopram and 0, 5, 25 or 250 μM glutamate at 37°C, 5%CO₂ and 95% air and the viability of the cells was determined with the calcein AM indicator. Each data bar is the mean plus the standard deviation of 8 wells. In this figure, * means that the p values were at least less than 0.05 compared to the zero glutamate control group.
3.4.2. Ratiometric Microscopy  Citalopram Studies

Ratiometric studies of relative changes in [Ca$^{2+}$], levels (Figure 3.48) with a representative C6 undifferentiated cell showed a peak and an elevation in the baseline when 50 $\mu$M citalopram was given. The baseline remained unchanged when 75 $\mu$M of glutamate/30 $\mu$M glycine were added. Nor was it changed when, after washing twice with calcium-and-magnesium-free HBSS+HEPES imaging solution, 1.5 $\mu$M CCCP was added. Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 $\mu$M FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Figure 3.48. Representative C6 undifferentiated cell relative change in [Ca$^{2+}$], ratiometric response to acute administration of 50 μM citalopram (red arrow) followed by 75 μM glutamate/30 μM glycine (blue arrow). The cells were then washed twice with calcium-free imaging solution (vertical purple line) and 1.5 μM of CCCP (green arrow) is administered. Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 2 days. They were placed in an Attofluor cell chamber and loaded with 5 μM FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Ratiometric studies of relative changes in [Ca^{2+}], levels (Figure 3.49) with a representative C6 differentiated cell showed a drop in the baseline when 50 μM citalopram was given. The baseline remained unchanged when 75 μM of glutamate/30 μM glycine were added. Nor was it changed when, after washing twice with calcium-and-magnesium-free HBSS+HEPES imaging solution, 1.5 μM CCCP was added. Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 μM FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Figure 3.49. Representative C6 differentiated cell relative change in \([\text{Ca}^{2+}]_i\) ratiometric response to acute administration of 50 µM citalopram (red arrow) followed by 75 µM glutamate/30 µM glycine (blue arrow). The cells were then washed twice with calcium-free imaging solution (vertical purple line) and 1.5 µM of CCCP (green arrow) is administered. The hump in the baseline between the mirtazapine and glutamate/glycine administrations was due to focusing. Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 2 days. They were placed in an Attofluor cell chamber and loaded with 5 µM FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Ratiometric studies of relative changes in [Ca\(^{2+}\)]\(_i\) levels (Figure 3.50) with a representative SH-SY5Y differentiated cell showed a spike in the baseline when 50 \(\mu\)M citalopram was given. The baseline remained unchanged when 75 \(\mu\)M of glutamate/30 \(\mu\)M glycine were added. Nor was it changed when, after washing twice with calcium-and-magnesium-free HBSS+HEPES imaging solution, 1.5 \(\mu\)M CCCP was added. Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 \(\mu\)M FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Figure 3.50. Representative SH-SY5Y differentiated cell relative change in $[Ca^{2+}]_i$, ratiometric response to acute administration of 50 µM citalopram (red arrow) followed by 75 µM glutamate/30 µM glycine (blue arrow). The cells were then washed twice with calcium-free imaging solution (vertical purple line) and 1.5 µM of CCCP (green arrow) is administered. Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 2 days. They were placed in an Attofluor cell chamber and loaded with 5 µM FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
3.5. Studies with Memantine

3.5.1 Microplate Memantine Studies

Concentration cytotoxicity curves for memantine are shown in Figures 3.51 for C6 cells. The cells were prepared as described in Methods, and seeded in the inner 60 wells of 96-well plates at a density of $2 \times 10^3$ cells/100 µl. Twenty-four hours later, 0, 5, 25, 50, 125 or 250 µM memantine in DMEM were added. After 24 hours, the media with the memantine was removed, and replaced with 3 µM calcein AM in DPBS. The 96-well plates were then incubated at 37°C, 5% CO and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

For the effect of memantine on C6 cells (Figure 3.51), the two-way ANOVA F value was 323.03 (DFn = 5, DFd = 70, p < .0001). Post-hoc Bonferroni tests showed that each dose of citalopram was significantly different from the no drug controls, but no drug concentration was different from any other concentration. The cell type F value was not significant (F = 0.87, DFn=1, DFd = 14, p = 0.3673).
Figure 3.51. Memantine concentration cytotoxicity response curve and comparison between undifferentiated (U) and differentiated (D) C6 cells. Cells were incubated for 24 hours with 0, 5, 25, 50, 125 or 250 μM memantine in DMEM at 37°C, 5% CO₂ and 95% air and the viability of the cells was determined with the calcein AM indicator assay. Each data bar is the mean plus the standard deviation of calcein intensity in 8 wells. In this figure, * means that the p value is at least less than 0.05 compared to the zero memantine control group. Post-hoc Bonferroni tests showed that each dose of memantine significantly reduced viability compared to controls, and that the doses were not different from each other in their effect on differentiated and undifferentiated cells.
Concentration cytotoxicity curves for memantine are shown in Figures 3.51 for SH-SY5Y cells. The cells were prepared as described in Methods, and seeded in the inner 60 wells of 96-well plates at a density of $2 \times 10^3$ cells/100 µl. Twenty-four hours later, 0, 5, 25, 50, 125 or 250 µM memantine in DMEM were added. After 24 hours, the media with the memantine was removed, and replaced with 3 µM calcein AM in DPBS. The 96-well plates were then incubated at 37°C, 5%CO and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

For the effect of memantine on SH-SY5Y cells (Figure 3.51), the two-way ANOVA F value was 333.15 (DFn = 5, DFd = 70, p <.0001). Post-hoc Bonferroni tests showed that each dose of citalopram was significantly different from the no drug controls, but no drug concentration was different from any other concentration. The cell type F value was 61.67 (DFn=1, DFd = 14, p <.0001). Bonferroni tests indicated that viability of differentiated cells was significantly lower than that of undifferentiated cells in 5, 25, 50, 125 and 250 µM concentrations.
Figure 3.52. Memantine concentration viability response curve and comparison between undifferentiated (U) and differentiated (D) SH-SY5Y cells. Cells were incubated for 24 hours with 0, 5, 25, 50, 125 or 250 µM memantine in DMEM at 37°C, 5%CO₂ and 95% air and the viability of the cells was determined with the calcein AM indicator assay. Each data bar is the mean plus the standard deviation of calcein intensity in 8 wells. In this figure, * means that the p value is at least less than 0.05 compared to the zero memantine control group, and # means that the p value is at least less than 0.05 compared to the undifferentiated cells receiving the same treatment. Post-hoc Bonferroni tests showed that each dose of memantine significantly reduced viability compared to controls, and that the doses were not different from each other. Also the viability of differentiated SH-SY5Y cells is significantly lower than that of the undifferentiated SH-SY5Y cells in 5, 25, 50, 125, and 250 µM memantine groups.
3.5.2. Ratiometric Microscopy Memantine Studies

Ratiometric studies of relative changes in [Ca\textsuperscript{2+}]\textsubscript{i} levels (Figure 3.53) with a representative C6 undifferentiated cell showed a peak and an elevation in the baseline when 50 μM memantine was given. The baseline remained unchanged when 75 μM of glutamate/30 μM glycine were added. Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 μM FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Figure 3.53. Representative C6 undifferentiated cell relative change in [Ca\(^{2+}\)], ratiometric response to acute administration of 50 µM memantine (red arrow) followed by 75 µM glutamate/30 µM glycine (blue arrow). Disturbance in the baseline at the beginning of the run was due to focusing. Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 µM FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Ratiometric studies of the relative change in \([\text{Ca}^{2+}]_i\) levels (Figure 3.54) with a representative SH-SY5Y differentiated cell showed no change in the baseline when 50 \(\mu\text{M}\) memantine was given. The baseline remained unchanged when 75 \(\mu\text{M}\) of glutamate/30 \(\mu\text{M}\) glycine were added. Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 \(\mu\text{M}\) FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Figure 3.54. Representative SH-SY5Y differentiated cell relative change in \([\text{Ca}^{2+}]_i\), ratiometric response to acute administration of 50 \(\mu\text{M}\) memantine (red arrow) followed by 75 \(\mu\text{M}\) glutamate/30 \(\mu\text{M}\) glycine (blue arrow). Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 2 days. They were placed in an Attofluor cell chamber and loaded with 5 \(\mu\text{M}\) FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
3.6. Studies with AP5

3.6.1. Microplate AP5 Studies

Concentration cytotoxicity curves for AP5 are shown in Figure 3.55 for C6 cells. The cells were prepared as described in Methods, and seeded in the inner 60 wells of 96-well plates at a density of $2 \times 10^3$ cells/100 µl. Twenty-four hours later, 0, 5, 25, 50, 125 or 250 µM AP5 in DMEM were added. After 24 hours, the media with the AP5 was removed, and replaced with 3 µM calcein AM in DPBS. The 96-well plates were then incubated at 37°C, 5%CO2 and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

For the effect of AP5 on C6 cells (Figure 3.55), the two-way ANOVA F value was 214.05 (DFn = 5, DFd = 70, p <.0001). Post-hoc Bonferroni tests showed that each dose of AP5 was significantly different from the no drug controls, but no drug concentration was different from any other concentration. The cell type F value was not significant (F= 2.49, DFn=1, DFd = 14, p = 0.1366).
Figure 3.55. AP5 concentration cytotoxicity response curve and comparison between undifferentiated (U) and differentiated (D) C6 cells. Cells were incubated for 24 hours with 0, 5, 25, 50, 125 or 250 μM AP5 at 37°C, 5%CO₂ and 95% air and the viability of the cells was determined with the calcein AM indicator. Each data bar is the mean plus the standard deviation of 8 wells. In this figure, * means that the p values was at least less than 0.05 compared to the zero AP5 control group. Post hoc Bonferroni tests showed that each dose of AP5 significantly reduced viability compared to controls.
Concentration cytotoxicity curves for AP5 are shown in Figure 3.56 for SH-SY5Y cells. The cells were prepared as described in Methods, and seeded in the inner 60 wells of 96-well plates at a density of $2 \times 10^3$ cells/100 µl. Twenty-four hours later, 0, 5, 25, 50, 125 or 250 µM AP5 in DMEM were added. After 24 hours, the media with the AP5 was removed, and replaced with 3 µM calcein AM in DPBS. The 96-well plates were then incubated at 37°C, 5%CO2 and 95% air for 30 minutes and examined with a Tecan Safire microplate reader with Excel readout.

For the effect of AP5 on SH-SY5Y cells (Figure 3.56), the two-way ANOVA F value was 417.99 (DFn = 5, DFd = 70, p < 0.0001). Post-hoc Bonferroni tests showed that each dose of AP5 was significantly different from the no drug controls, but no drug concentration was different from any other concentration. The cell type F value was 76.75 (DFn = 1, DFd = 14, p < 0.0001). Bonferroni tests indicated that viability of differentiated cells was less than undifferentiated cells in the 0, 5, 25, 50, 125, and 250 µM concentrations.
Figure 3.56. AP5 concentration cytotoxicity response curve and comparison between undifferentiated (U) and differentiated (D) SH-SY5Y cells. Cells were incubated for 24 hours with 0, 5, 25, 50, 125 or 250 μM AP5 at 37°C, 5%CO₂ and 95% air and the viability of the cells was determined with the calcein AM indicator. Each data bar is the mean plus the standard deviation of 8 wells. In this figure, * means that the p values was at least less than 0.05 compared to the zero AP5 control group, and # means that the p value was at least less than 0.05 compared to the undifferentiated cells receiving the same treatment. Post hoc Bonferroni tests showed that each dose of AP5 significantly reduced viability compared to controls, and the responses between differentiated and undifferentiated cells showed reduced viability of the differentiated cells at the 0, 5, 25, 50, 125, and 250 μM concentrations of AP5.
3.6.2. Ratiometric Microscopy AP5 Studies

Ratiometric studies of the relative changes in $[\text{Ca}^{2+}]_i$ levels (Figure 3.57) with a representative SH-SY5Y differentiated cell showed no change when 50 $\mu$M memantine was given. The baseline remained unchanged when 75 $\mu$M of glutamate/30 $\mu$M glycine were added. Cells were seeded and grown on glass cover slips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 $\mu$M FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
Figure 3.57. Representative SH-SY5Y differentiated cell relative change in \([\text{Ca}^{2+}]_i\) ratiometric response to acute administration of 50 \(\mu\text{M}\) AP5 (red arrow) followed by 75 \(\mu\text{M}\) glutamate/30 \(\mu\text{M}\) glycine (blue arrow). Cells were seeded and grown on coverslips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 \(\mu\text{M}\) FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.
### 3.7. Ratiometric Results for Combinations of Drugs

A statistical analysis of the ratiometric relative change in $[\text{Ca}^{2+}]_{i}$ in response to 75 μM glutamate/30 μM glycine, 50 μM mirtazapine, 50 μM citalopram and 1.5 μM CCCP and their combinations was done by taking the baseline and measuring the ratiometric amplitude of the signals. Cells were differentiated as described in Methods. All cells were seeded and grown on glass coverslips in a 100 mm tissue culture dish for 4 days. They were placed in an Attofluor cell chamber and loaded with 5 μM FuraFF, observed with a 40x (oil immersion) objective on an Olympus 1x81 fluorescent microscope, and photographed with a Cool-Snap camera. Excitation was with a monochromatic tungsten filament emitting at 488 nm and controlled with a PTI laboratory controller. Acquisition software was ImageMaster 5.1.

For the drug treatment effect on C6 cell ratiometric amplitude (Figure 3.58), the two-way ANOVA F value was 31.77 ($DF_n = 7$, $DF_d = 70$, $p < .0001$). Post-hoc Bonferroni tests showed that the effect of mirtazapine or citalopram was significantly different from the glutamate only. The differentiated vs. undifferentiated F value was not significant ($F = 1.59$, $DF_n = 1$, $DF_d = 10$, $p = 0.2365$).
**Figure 3.58.** The effect of mirtazapine and citalopram on glutamate and CCCP response in differentiated and undifferentiated C6 cells based on the difference in baseline-to-peak ratio. The data bars are the mean plus the standard deviation of 6 cells. In this figure, * means that the p value was at least less than 0.05 compared to the glutamate-only control group.
4. Discussion

4.1. Characteristics of the Cells

Differentiated and undifferentiated C6 and SH-SY5Y cells (Figures 3.1 to 3.4) resemble those in the literature (Figures 1.1 and 1.2) and as described by Kataria et al., (2012) for C6 and Brown et al., (2005) for SH-SY5Y cells (Brown et al., 2005; Kataria et al., 2012).

When loaded with Fluo4, a linear calcium fluorescent fluorophore, the cells are quite visible (Figures 3.5, 3.7 to 3.9). Cells that are not confluent tend to spread out (Figure 3.5) but when they are almost confluent tend to form round balls (Figure 3.6). Also when they are confluent, they are not as bright (Figure 3.6) as when they are 30% confluent (Figures 3.5, 3.7 to 3.9) which may suggest that confluent cells have less [Ca^{2+}]. Undifferentiated cells tend to have shorter neurites (Figures 3.5 and 3.8) while differentiated cells have longer neurites (Figure 3.7 and 3.9) as described by Brown et al. (2005) (Brown et al., 2005). Moreover, differentiated SH-SY5Y cells often look motor neuron-like in morphology as identified in Figure 3.9.

Ratiometric fluorophores will become excited at different wavelengths depending on whether calcium is bound to them (excited at 340) or not (excited at 380). In Figures 3.10 to 3.12, which shows unbound Fura FF in the same cell in images taken a few minutes apart, as calcium binds to Fura FF, the concentrations of unbound FuraFF decreases and the unbound fluorophore is no longer as concentrated and the 380 fluorescent signal begins to fade as indicated in the change from white to red to orange to yellow to green. This change may be subtle but it is measurable.
4.2. Glutamate Studies

In order to explore the effects of mirtazapine and citalopram on glutamate-induced calcium influx, it was necessary to determine a range of glutamate concentrations that exerted some action on the cell types but did not result in extensive apoptosis. Literature reports of mM glutamate concentrations proved to be too toxic for 3-, 6- or 27-hour studies with C6 and SH-SY5Y cells (Figures 3.13 to 3.24). Glutamate concentrations in the μM range were found to be more appropriate for these cells over a 24-hour exposure (Figures 3.25 and 3.26). This study also showed that while there was no difference between C6 undifferentiated and differentiated C6 cells in their response to glutamate, differentiated SH-SY5Y cells were more susceptible to the 5, 25, and 50 μM concentrations of glutamate (p = < 0.01) than the undifferentiated SH-SY5Y cells.

In microplate studies, all drugs reduced cell viability but the mechanism behind this reduced viability is not clear. Mutations in enzymatic expression, uptake of drug through the cell membrane, or other perturbations could account for this perceived uniformity of effect. When looking at the effect of glutamate on C6 cells (Figure 3.25), the two-way ANOVA F value was 399.70 (DFn = 5, DFd = 70, p < .0001). For the effect of glutamate on SH-SY5Y cells (Figure 3.26), the 2-way ANOVA F value was 262.25 (DFn = 5, DFd = 84, p < .0001).

The ratiometric image in Figure 3.27 showed a spike in relative changes in [Ca²⁺], after 112.5 μM glutamate and 45 μM glycine were added indicating that there was a sudden influx of calcium. The subsequent addition of 1.5 μM of CCCP elicited another rise in relative change in [Ca²⁺], due to the release of calcium from the mitochondria (Figure 3.27). Glutamate (75 μM) and glycine (30 μM) also caused a relative increase in [Ca²⁺], but the characteristics of the
glutamate spike differed between individual cells (Figures 3.28 and 3.29). In Figure 3.30, the 380 nm graphic image of a single undifferentiated SH-SY5Y cell, 75 µM glutamate and 30 µM of glycine produced an increase in the signal which was followed by a positive curve when 1.5 µM CCCP was added to release calcium from the mitochondria. This change in the 380 nm signal cannot be explained because there should be a reduction in the unbound calcium instead of an increase. The ratiometric graph (Figure 3.31) from this same cell did not show an appreciable spike, nor was there a spike when 1.5 µM of CCCP was administered. With differentiated SH-SY5Y cells, there was a gradual rise instead of a sharp spike when the same dose of glutamate and glycine was administered, and CCCP produced a negative spike rather than a positive one (Figure 3.32). In another cell in another run, the administration of 75 µM glutamate produced a negative spike instead of a positive one in the ratiometric graph of differentiated SH-SY5Y cells. (Figure 3.33)

4.3. Mirtazapine Studies

Cell viability studies gave similar curves for mirtazapine as for glutamate (Figures 3.34 and 3.35). As with glutamate, there was no difference between undifferentiated and differentiated C6 cells in their cell viability response to µM concentrations of mirtazapine. However, there was significant difference in the viability between the differentiated and undifferentiated SH-SY5Y cells, with the differentiated cells showing a significant reduction in cell viability compared to the undifferentiated cells at all concentrations of mirtazapine (p < 0.0001) except the 250 µM concentration which reduced the viability of both cell types equally. This is in agreement with Pan, et al., (2006) who showed that at concentrations of 250 µM to 2 mM, mirtazapine was increasingly toxic to osteosarcoma tumour cells (Pan et al., 2006). For the effect of mirtazapine
on C6 cells, the two-way ANOVA F value was 443.50 (DFn = 5, DFd = 70, p <.0001). For the effect of mirtazapine on SH-SY5Y cells the two way ANOVA F value was 303.07.77 (DFn = 5, DFd = 70, p < .0001).

Chronic administration of 50 µM mirtazapine was done for ten weeks followed by 0, 5, 25, and 250 µM glutamate and 50 µM mirtazapine for 24 hours. Mirtazapine-treated cells showed a significant increase in viability over controls (p < 0.0001) with undifferentiated cells of both C6 and SH-SY5Y in all values of glutamate, but decreased viability with the differentiated cells of both cell lines (Figures 3.36 and 3.37). Results with a combination of 250 µM glutamate and 5 µM of mirtazapine on differentiated and undifferentiated cells showed increased viability with glutamate alone, but viability was depressed when mirtazapine was added. When both glutamate and mirtazapine were added, the viability was increased for the differentiated over the undifferentiated cells (Figure 3.38). At the same time, the cell lines are cancer cells with the ability to mutate over time. In triturating these cells, it is possible that those cells that do not adhere so well are removed, selecting for those that not only adhere more tightly but also may have other characteristics. These results also imply that mirtazapine-treated undifferentiated cells may be more resistant to glutamate, because they are less specialized whereas differentiated cells, because they are more specialized, may be more susceptible to toxic effects of glutamate.

For the glutamate effect in the presence of 50 µM mirtazapine on C6 cells chronically exposed to mirtazapine, the two-way ANOVA F value was 17.52 (DFn =3, DFd = 66, p <.0001). For SH-SY5Y cells similarly treated the two-way ANOVA the F value was 49.68 (DFn = 3, DFd = 18, p < .0001).
Ratiometric studies with mirtazapine (Figure 3.39) show that undifferentiated C6 cells given 50 \( \mu M \) mirtazapine showed a short spike followed by a sharp drop in the baseline below what it had been prior to the mirtazapine administration. Subsequent administration of 75 \( \mu M \) glutamate and 30 \( \mu M \) glycine failed to elicit any spike or any change in the baseline. The subsequent addition of 1.5 \( \mu M \) CCCP in calcium-and-magnesium-free HBSS + HEPES had no effect which was to be expected since no calcium had entered the cell. With differentiated C6 cells (Figure 3.40), there was a negative spike in the baseline after the administration of 50 \( \mu M \) mirtazapine followed by a return to the baseline. However, there was no response to either 75 \( \mu M \) glutamate with 30 \( \mu M \) glycine and, as anticipated, no response to 1.5 \( \mu M \) CCCP. In undifferentiated SH-SY5Y cells (Figure 3.41), when mirtazapine was given twice (50 \( \mu M \) and 33 \( \mu M \), respectively), the 380 nm graph image showed successive drops in the baseline which cannot be explained. The ratiometric graph (Figure 3.42) showed no obvious response to either doses of mirtazapine or to glutamate. In differentiated SH-SY5Y cells (Figure 3.43), 50 mM mirtazapine produced a drop in the baseline followed by a time interval of 14 seconds after which the baseline rose up to its pre-mirtazapine level. The subsequent administration of 75 \( \mu M \) glutamate and 30 \( \mu M \) glycine and had no effect on baseline. Because no calcium had entered the cell there was no response to 1.5 \( \mu M \) of CCCP. Mirtazapine is depressing the response to glutamate but the mechanism is not known.

4.4. Citalopram Studies

Dose response studies of \( \mu M \) concentrations of citalopram on the viability of C6 and SH-SY5Y cells are shown in Figures 3.44 and 3.45. All doses of citalopram equally reduced the viability of the cells compared to controls. There was no difference between undifferentiated and
differentiated C6 cells in their cell viability response to any of the concentrations of citalopram that were used. However, differentiated SH-SY5Y cells exposed to citalopram in the 5 μM to 50 μM range (Figure 3.45) had reduced viability compared to undifferentiated SH-SY5Y cells exposed to the same concentration of citalopram. For the effect of citalopram on C6 cells, the two-way ANOVA F value was 372.83 (DFn = 5, DFd = 70, p < .0001). For the effect of citalopram on SH-SY5Y cells the two-way ANOVA F value was 405.54 (DFn = 5, DFd = 70, p < .0001).

C6 cells chronically treated with citalopram for 10 weeks showed decreased viability in both cells but the decrease is greater in the undifferentiated C6 cells (Figure 3.46), but almost no change between differentiated and undifferentiated SH-SY5Y cells (Figure 3.47) except at the 5 μM level of glutamate. This implies that citalopram had a protective effect on SH-SY5Y cells exposed to glutamate but not in C6 cells. In those cases where viability increased over controls, it may signal a regulatory effect independent of the protective effect against glutamate that promotes cell survival. This correlates with Boyer, et al, who showed that in mouse brain chronic administration of citalopram reduced the expression of NMDA subunits by its effect on mRNA (Boyer et al., 1998). For the effect of glutamate on SH-SY5Y cells chronically treated with citalopram, the two-way ANOVA F value was 14.83 (DFn = 3, DFd = 66, p < .0001). For the effect of glutamate on SH-SY5Y cells chronically treated with citalopram, the two-way ANOVA F value was 14.83 (DFn = 3, DFd = 66, p < .0001).

Ratiometric graphic image with undifferentiated C6 cells showed a spike above baseline when 50 μM citalopram was administered. There was no relative change in [Ca^{2+}]_i when 75 μM glutamate and 30 μM glycine where subsequently added. As expected, when no calcium enters
the cell, there was no relative change in [Ca^{2+}]_{i} when 1.5 \mu M CCCP was added (Figure 3.48). The response to citalopram by differentiated C6 cells (Figure 3.49) was also changed in the baseline, but in these cells citalopram lowered the baseline instead of raising it, as it had done in undifferentiated cells. There was no response to the subsequent administration of 75 \mu M glutamate and 30 \mu M glycine nor to 1.5 \mu M CCCP which followed the glutamate/glycine administration. With differentiated SH-SY5Y cells (Figure 3.50), there was a spike when 75 \mu M citalopram was administered with a return to the same baseline. There was no response to the subsequent administrations of 75 \mu M glutamate/30 \mu M glycine followed by 1.5 \mu M of CCCP.

The interpretation of the above ratiometric data indicates that citalopram is blocking the effect of glutamate on the relative change in [Ca^{2+}]_{i} and since no calcium entered the cell, the mitochondria could not release calcium. Normally, mitochondria contribute calcium to the relative changes in [Ca^{2+}]_{i}, but were unable to do so because of the calcium-blocking effect of citalopram at the cell membrane. The mechanism is unclear but would be by a different mechanism than memantine or AP5, as neither of these drugs produce a change in the baseline (see below) yet both block NMDA receptors, thereby preventing a spike in relative change in [Ca^{2+}]_{i} that would normally be caused by glutamate. Clinical correlation can be found in Muhonen who found in a double blind study of 80 patients that escitalopram or memantine, 20 mg/day, was effective in the treatment of MDD comorbid with alcohol dependence (Muhonen et al., 2008)
4.5. Memantine Studies

Dose response studies with memantine (Figure 3.51 and 3.52), a known NMDA receptor blocker, decreased the viability in C6 cells and SH-SY5Y cells. There was a significant decrease in viability in differentiated C6 cells compared to undifferentiated ones, indicating that more specialized cells may be more susceptible to memantine. For the effect of memantine on C6 cells (Figure 3.51), the two-way ANOVA F value was 323.03 (DFn = 5, DFd = 70, p<.0001). For the effect of memantine on SH-SY5Y cells (Figure 3.51), the two-way ANOVA F value was 333.15 (DFn = 5, DFd = 70, p <.0001).

Ratiometric studies undifferentiated C6 and differentiated SH-SY5Y cells (Figures 3.53 and 3.54) showed no spike or change in baseline when 50 μM memantine was administered. This differs significantly from that observed with mirtazapine and citalopram in which there was, in most cases, a significant shift up or down in the baseline. Like mirtazapine or citalopram, memantine blocked the response to 75 μM glutamate and 30 μM of glycine (Figures 3.53 and 3.54). While the mechanism of mirtazapine and citalopram may be difference because of this shift in the baseline, the effect is the same in that it blocks calcium influx and, in some unknown way, also prevents the release of calcium from mitochondria. Clinical correlation was the study by Muhonen (cited above) as well as the study of Berman who found that ketamine, a powerful NMDA receptor antagonist, was effective in 9 patients with MDD (Berman et al., 2000). A number of papers on ketamine as an antidepressant have been published, among which are two recent (2013) reviews by Murck and by Salvadore and Singh (Murck, 2013; Salvadore & Singh, 2013).
4.6. AP5 Studies

Dose response studies with AP5, another known NMDA receptor blocker (Figure 3.55 and 3.56), showed a significant decrease in viability in C6 cells and SH-SY5Y cells. There was a decrease in viability in differentiated SH-SY5Y cells when compared to undifferentiated cells, indicating that more specialized neuron-like cells may be more susceptible to AP5. At the same time, there was less of a decrease in viability of differentiated C6 cells when compared to undifferentiated ones indicating that astrocyte-like cells may have some metabolic ability to survive AP5. For the effect of AP5 on C6 cells (Figure 3.55), the two-way ANOVA F value was 214.05 (DFn = 5, DFd = 70, p < .0001). For the effect of AP5 on SH-SY5Y cells (Figure 3.56), the two-way ANOVA F value was 417.99 (DFn = 5, DFd = 70, p < .0001).

Ratiometric studies with AP5 were done on differentiated SH-SY5Y cells only (Figure 3.57). These were similar to memantine in that there was no spike and no change in the baseline after the administration of 50 µM of AP5. Also, similar to memantine, AP5 blocked the response to 75 µM glutamate and 30 µM of glycine. Animal correlation can be found in Trullas and Skolnick who found that an analog of AP5, AP-7, was effective in relieving depression in forced-swim mice (Trullas & Skolnick, 1990).

4.7. Ratiometric Results for a Combination of Drugs

Measuring the C6 ratiometric differences from baseline to peak (Figure 3.58) for glutamate and CCCP following mirtazapine and citalopram administration and comparing these to the control, provided a good graphic summary of the blocking effect of mirtazapine and citalopram on glutamate and the normal CCCP increase in relative changes in [Ca$^{2+}$], if calcium had entered
the cell. For the drug treatment effect on C6 cell ratiometric amplitude (Figure 3.58), the two-way ANOVA F value was 31.77 (DFn = 7, DFd = 70, p <.0001). This approach is similar to, but developed independently from, that of Gerard and Hanson (2012) who, in rat astrocytes, also measured the amplitude of ratiometric imaging. Using NMDA as the agonist, they found that AP5 had the same effect on NMDA activated relative changes in [Ca^{2+}]_i as was found for AP5 on glutamate in this present study (Gerard & Hansson, 2012). Their use of primary cultures of rat astrocytes also supports the use of C6 rat glioma cells differentiated into astrocyte-like cells.

4.8. Discussion Summary

As discussed above, the results of this research project support the hypothesis that mirtazapine and citalopram appear to affect relative changes in [Ca^{2+}]_i. These drugs do this by appearing to block the effects of glutamate on NMDA receptors in a manner that appears to be similar to that of the known NMDA receptor antagonists memantine and AP5, and thereby preventing an influx of calcium into the cell, prevent mitochondrial uptake of calcium and subsequent release by CCCP. These two actions are most evident in the ratiometric microscopy studies where these drugs appear to prevent in the inflow of calcium induced by glutamate acting on NMDA receptors and, therefore, since calcium did not enter the cell, there was no mitochondrial uptake of calcium and hence no subsequent release of calcium from mitochondrial stores by CCCP. The ability of mirtazapine and citalopram to suppress the response to glutamate is summarized in Figure 3.58 which is based on the amplitudes of their changes in the ratiometric baselines reflective of relative changes in [Ca^{2+}]_i. Statistically, this suppression of the glutamate effect is significant (p<0.0001). Whether this protection is the result of a direct effects on NMDA receptors and on mitochondria, or whether it is indirect by way of an unknown metabolic pathway remains to be determined. Regardless, the effect of these drugs on modulating relative
changes in $[Ca^{2+}]_i$ suggest that they may be beneficial to patients who have neurodegenerative disorders, stroke, or other condition that could cause central nervous system dysfunction because of an excessive increase in the relative change in $[Ca^{2+}]_i$. 
5. Conclusions

The main findings of this thesis are:

1. Mirtazapine and citalopram block the glutamate effect on relative changes in $[\text{Ca}^{2+}]_i$ by either a direct or indirect action on NMDA receptors. This observation is supported by the lack of response of mitochondria to CCCP as there is no influx of calcium into the cell and from there into the mitochondria. If there had been uptake of calcium by mitochondria, CCCP would have released it.

2. The NMDA blocking effect of mirtazapine and citalopram is different from the known NMDA blockers memantine and AP5.

3. The effect of the acute glutamate on cells chronically treated with mirtazapine for 10 weeks either attenuated or reversed the reduction of cell viability. The effect of the acute glutamate on cells chronically treated with citalopram for 10 weeks attenuated the reduction in viability in C6 cells, but blocked the effect in SH-SY5Y cells.

4. While the effects of mirtazapine and citalopram attenuate the effects of glutamate on the cell lines used, more research is needed to demonstrate whether this is relevant to the action of the drugs in patients.
6. Future Work and Directions

Much of the research time was devoted to perfecting the ratiometric technique and making it applicable to the study of drugs, especially those that have an effect on calcium flux in the central nervous system. This work should be duplicated by other laboratories. The following experiments and changes to the protocol are suggested to gain more insight into the effect of drugs that impact calcium influx in CNS cells.

1. Fura 2 should be used instead of Fura FF to study the ratiometrics of comparatively low concentrations of \([\text{Ca}^{2+}]_i\), induced by glutamate, glycine, or any drug under study. Fura2 is a high affinity fluorophore and is better able to better detect small changes in \([\text{Ca}^{2+}]_i\). FuraFF is a low affinity dye and would be able to detect large changes in calcium and should be used only when large changes in \([\text{Ca}^{2+}]_i\) are expected.

2. A 63x objective should be used to get better and more accurate results. While important information was obtained from the 40x oil immersion lens, the 63x would be more sensitive.

3. Utilize higher concentrations of glutamate (e.g., 75, 150, 250 \(\mu\)M and 1 and 2 mM), and a range of concentrations (e.g., 1.5, 3, 5, and 10 \(\mu\)M) for CCCP.

4. The mechanisms behind the change in the ratiometric baseline induced by mirtazapine or citalopram should be investigated further.

5. Ratiometric microscopy studies with primary cultures of neurons would provide more direct information on the effects of mirtazapine and citalopram on \([\text{Ca}^{2+}]_i\) in neurons.
6. The reason why good 380 nm results are found but do not translate into a good ratiometric findings in SH-SY5Y cells should also be investigated.

7. The finding, in the 380 nm results, that repeated doses of mirtazapine sequentially lowered the baseline should be examined, as well as the effects of repeated glutamate doses.

8. CCCP should be used after memantine and AP5 to confirm that there is no calcium going into the cell and into the mitochondria, and thus CCCP would not be able to cause mitochondrial release of calcium.

9. The differences between differentiated and undifferentiated cells and their response to mirtazapine and citalopram should be examined further. Mirtazapine, chronically administered, seems to reduce the viability of differentiated cells when exposed to glutamate whereas citalopram seems to enhance the viability of differentiated cells.

10. For all work that was done and not reported using 6-well plates, it should be redone using 96 well plates. Although the idea was to minimize the number of naive cells that had never been exposed to a drug, the larger wells in the 6-well plate also permitted a greater number of mutant cells and their offspring that could affect the results of the drug administration tests.

12. A computer program should be developed, or obtained, that automatically subtracts baselines from peaks in ratiometric imaging for drug studies.

The above potentially interesting studies are for future research. The results of these studies would enhance the technique of ratiometric imaging of relative changes in [Ca^{2+}], for the study of the effects of drugs that impact on neuronal function. Such studies may prove beneficial in studies of neurodegeneration.
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Ref Type: Personal Communication


Ref Type: Internet Communication


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