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**The effect of memantine and the ladybird  
alkaloid, harmonine, on NMDA receptor  
function and Alzheimer's disease**

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## Abstract

Alzheimer's disease (AD) is the most common late onset form of dementia. *N*-methyl-D-aspartate (NMDA) receptors are excitatory glutamate receptors and are primary therapeutic targets due to their glutamate and glycine recognition sites. Excessive glutamate neurotransmission can ultimately result in neuronal cell death, potentially leading to AD pathological conditions. Current treatments of AD include *N*-methyl-D-aspartate (NMDA) receptor antagonists such as memantine and acetylcholine receptor inhibitors. In the first part of this study, clinical trials of memantine were examined to assess whether memantine had a positive or negative effect on the pathological symptoms of AD human patients. The results suggested that memantine has a positive effect on the following outcome measures: molecular biomarkers and imaging; cognition and behaviour; functional and linguistic communication. The most common dose used was 20 mg of memantine per day. The highest dose of 28 mg was found to have a more significant positive effect on AD patient symptoms compared to lower doses of 10-20 mg. From this, it was concluded that memantine has a therapeutic effect on AD patient symptoms.

In addition, a two-electrode voltage clamp was used to assess the effects of the memantine and *Harmonia axyridis* alkaloid extract (HAE) on NMDA receptors expressed in *Xenopus laevis* oocytes. Memantine and HAE were found to inhibit the NMDA receptor in a concentration-dependent manner. Furthermore, memantine was shown to inhibit NMDA receptor responses in GluN1-1a/GluN2A and GluN1-1a/GluN2B receptor subtypes in a voltage-dependent manner. The highest concentration of HAE only partially blocked the NMDA receptor whilst the memantine applied at the highest concentration demonstrated complete inhibition of NMDA receptor responses.  $IC_{50}$  values were shown to be lowest at -75 mV compared to the more negative holding potential of -100 mV. This was found for all subunits except the GluN1-1a/GluN2B subunit combination when HAE was applied. Overall, memantine and harmonine were found to show high levels of inhibition of NMDA receptor response at highest concentrations in both receptor subunits.

## List of Abbreviations

| Abbreviations      | Meaning                                                                                                                     |
|--------------------|-----------------------------------------------------------------------------------------------------------------------------|
| A $\beta$ peptide  | $\beta$ -amyloid peptide                                                                                                    |
| AD                 | Alzheimer's Disease                                                                                                         |
| ADAS-cog           | Alzheimer's Disease Assessment Scale                                                                                        |
| ADCS-ADL Inventory | Alzheimer's Disease Cooperative Study/Activities of Daily Living                                                            |
| ADCS-ADLsev-abv    | Alzheimer's Disease Cooperative Study Activities of Daily Living Inventory Modified for Severe Dementia Abbreviated Version |
| ArgTX              | Argiotoxin-636                                                                                                              |
| Asn                | Asparagine                                                                                                                  |
| ATD                | Amino terminal domain                                                                                                       |
| BBSI               | Brain Boundary Shift Integral                                                                                               |
| Ca <sup>2+</sup>   | Calcium                                                                                                                     |
| CGIC               | Clinical Global Impression of Change                                                                                        |
| CIBIC-plus         | Clinician's Interview-Based Impression of Change with Caregiver Input                                                       |
| Con G              | Conantokin G                                                                                                                |
| Cr                 | Creatine                                                                                                                    |
| CSF Biomarkers     | Cerebrospinal Fluid Biomarkers                                                                                              |
| CTD                | Carboxyl terminal domain                                                                                                    |
| FLCI               | Functional Linguistic Communication Inventory                                                                               |
| GABA               | $\gamma$ -amino butyric acid                                                                                                |
| <i>H. axyridis</i> | <i>Harmonia axyridis</i>                                                                                                    |
| HAE                | <i>Harmonia axyridis</i> alkaloid extract                                                                                   |
| HCV                | Total Hippocampal Volume                                                                                                    |
| HD                 | Huntington's Disease                                                                                                        |
| iGluRs             | ionotropic Glutamate Receptors                                                                                              |
| LTD                | Ligand-Binding Domain                                                                                                       |
| LTP                | Long-Term Potentiation                                                                                                      |
| Mg <sup>2+</sup>   | Magnesium                                                                                                                   |
| MK-801             | Dizocilpine                                                                                                                 |
| MMSE               | Mini Mental State Examination                                                                                               |
| MRS                | Magnetic Resonance Spectroscopy                                                                                             |
| NAA                | N-acetylaspartate                                                                                                           |
| nAChRs             | nicotinic acetylcholine receptors                                                                                           |
| NMDA               | N-methyl-D-aspartate                                                                                                        |
| NPI                | Neuropsychiatric Inventory                                                                                                  |
| PCP                | Phencyclidine                                                                                                               |
| PCR                | Polymerase Chain Reaction                                                                                                   |
| PD                 | Parkinson's Disease                                                                                                         |
| PhTX-343           | philanthotoxin-343                                                                                                          |
| SIB                | Severe Impairment Battery                                                                                                   |

|        |                                                 |
|--------|-------------------------------------------------|
| SRT-IR | Selective Reminding Test-Total Immediate Recall |
| TEVC   | Two-Electrode Voltage Clamp                     |
| TMD    | Transmembrane Domain                            |

# 1 Introduction

## 1.1 Neurodegenerative disorders and Alzheimer's Disease (AD)

Neurodegenerative diseases affect millions of people worldwide, particularly in elderly populations. Such diseases are rapidly becoming a global health issue (Draper, 2013). It is common that the incidence and severity of many neurodegenerative diseases increases with age (Erkkinen *et al.*, 2018). Alzheimer's disease (AD) is the most predominant late onset form of dementia (Draper, 2013). It impacts approximately 24 million people globally and it is predicted that by 2050, there will be an increase in the worldwide prevalence of AD to approximately 100 million (Brookmeyer *et al.*, 2007). AD pathology is characterised by various cellular and molecular changes in the brain such as the development of extracellular senile amyloid-beta ( $A\beta$ ) plaques; intracellular aggregates of phosphorylated tau protein; loss of synapses and neurons and elevated gliosis (Navarro *et al.*, 2018). Reactive gliosis results in the alterations in morphology and function of microglia and astrocytes. This is a key component in the pathogenesis of a number of neurological disorders (Heneka *et al.*, 2015). Senile plaques and neurofibrillary tangles are often prevalent in the cortex and limbic regions of the AD brain, as shown in Fig 1.1.1. Consequently, AD pathology spreads in the later stages of the disease (van Oostveen *et al.*, 2021). Plaques and tangles are closely associated with the destruction of neurons in the medial temporal lobe and hippocampus (as shown in Fig 1.1.1, displayed in blue) which control memory, and contextual aspects of language and reasoning (Draper, 2013). Symptoms of AD include cognitive dysfunction such as memory loss, language and learning difficulties. Non-cognitive dysfunction is also evident in AD and includes depression and behavioural disturbances (Barage and Sonawane, 2015).

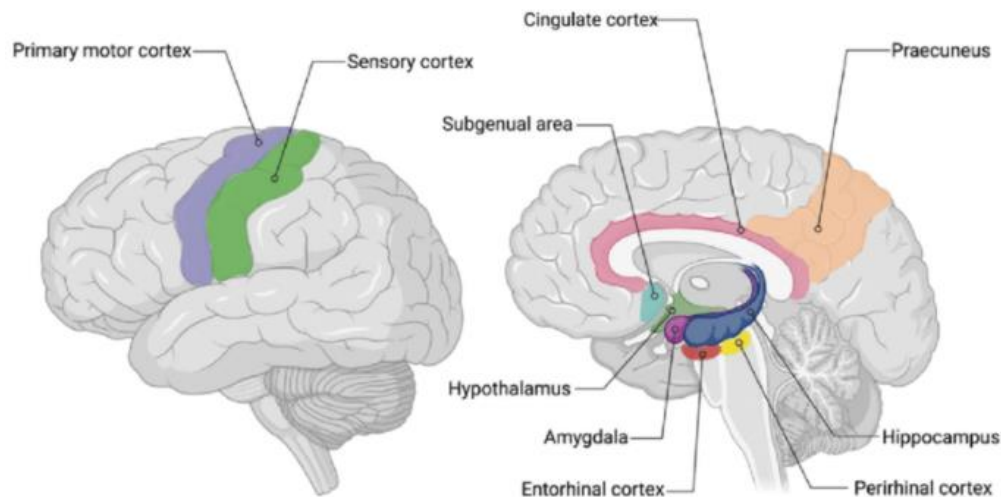


Figure 1.1.1 Regions in the brain which are primarily affected in Alzheimer's disease patients (van Oostveen *et al.*, 2021).

There are currently two approved drug types to treat the symptoms of patients with AD. The first type are *N*-methyl-D-aspartate (NMDA) receptor antagonists which normalises dysfunctional glutamate transmission by partially blocking the receptor. Glutamate is a key neurotransmitter involved in the pathology of AD (Parsons *et al.*, 2013). NMDA receptors are in the ionotropic glutamate receptors (iGluRs) family which are involved in learning, memory, synaptic plasticity and the normal development and function of the Central Nervous System (CNS). iGluRs are glutamate ion channels that regulate fast excitatory neurotransmission in the CNS (Popescu, 2016). Excessive glutamate neurotransmission can result in neuronal degeneration. Therefore, NMDA receptor antagonists can be beneficial in modulating and reducing glutamate neurotransmission. One key NMDA receptor antagonist is memantine, an uncompetitive, voltage-dependent NMDA receptor antagonist. Memantine has been found to be most effective under high glutamate conditions, higher membrane voltages and abnormal NMDA receptor activation caused by AD and other neurological disorders such as Parkinson's Disease. Memantine is voltage-dependent treatment, as memantine acts more effectively at higher voltages (Peskind *et al.*, 2006; Parsons *et al.*, 2013).

Cholinesterase inhibitors are the second class of drugs used to treat AD symptoms by inhibiting the action of acetylcholinesterase. They work by elevating the levels of acetylcholine to improve cognitive deficits. Examples of cholinesterase inhibitors include tacrine, donepezil, rivastigmine and galantamine (Parsons *et al.*, 2013). Treatments which target  $\beta$ -amyloid peptides directly have not yet been successful due to the inadequate interaction between antibodies and their target (Sevigny *et al.*, 2016). Aducanumab (BIIB037), a human antibody which selectively reacts with  $\beta$ -amyloid peptides, has been found to cross the blood-brain barrier, resulting in improved engagement with its target and clearing  $\beta$ -amyloid peptides in plaque-bearing transgenic mouse brains (Ferrero *et al.*, 2016). Although NMDA receptors are the primary receptor found to be involved in neuronal cell death, other glutamate receptors such as kainate and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptors are also involved in excitotoxic neuronal cell death. Excitotoxicity is the action of excitatory neurotransmitters, particularly glutamate, where prolonged activation of glutamate receptors initiate a cascade of neurotoxic events which ultimately results in the loss of neuronal function and cell death (Armada-Moreira *et al.*, 2020). Furthermore, other NMDA receptor antagonists such as MK-801 and ketamine, have also been found to be effective in *in vitro* studies, although they are not clinically tolerated (Olivares *et al.*, 2012; Song *et al.*, 2018).

## 1.2 The NMDA receptor

Ligand-gated ion channels (LGICs), such as nicotinic acetylcholine receptors (nAChRs),  $\gamma$ -amino butyric acid (GABA) and *N*-methyl-D-aspartate (NMDA) receptors, are activated via the chemical binding of an agonist to a specific site within the ion channel complex, resulting in a conformational change and the opening of the integral ion channel (Weir, 2019). NMDA receptors belong to a class of ionotropic glutamate receptors which mediate the normal function and development of the CNS and excitatory glutamate neurotransmission in the CNS (Popescu, 2016). Glutamate is released into the synaptic cleft and binds to NMDA

receptors at the post-synaptic membrane. The activity of NMDA receptors is physiologically inhibited by  $Mg^{2+}$  block at the regular membrane potential of approximately -70 mV, but the ion channel is readily unblocked via neurotransmission resulting in membrane depolarisation (-50 mV) which removes the  $Mg^{2+}$  from the pore and allows conduction to proceed. Additionally, microglia and astrocytes can non-vesicularly release glutamate into the extrasynaptic extracellular space where it can activate extrasynaptic NMDA receptors (Lewerenz and Maher, 2015). NMDA receptors are unique ligand-gated ion channels as their activation requires binding of glutamate, an NMDA receptor agonist, and glycine, a co-agonist. The structure of both glutamate and glycine binding sites is very similar. Glutamate and glycine binding sites often play equivalent roles in receptor activation. Although poorly studied, glycine has also been known to activate NMDA receptors in the absence of glutamate by a small percentage. Therefore, glycine may act as a partial agonist (Blancke and Van Dongen, 2009).

### 1.2.1 Structure and function of NMDA receptor

#### *Structure*

Most NMDA receptors are composed of at least two glycine-binding GluN1 subunits and two glutamate-binding GluN2 subunits (GluN2A-GluN2D). The magnesium block situated within the ion channel is removed by depolarisation, caused by the presence of glutamate and glycine. GluN1 subunits are synthesised and retained in the endoplasmic reticulum until they are bound to GluN2 subunits. Both GluN1 and GluN2 subunits are required to create a functional receptor. There are eight different GluN1 splice variants and four different isoforms of GluN2 subunit (GluN2A-GluN2D). Therefore, there are numerous combinations of the receptor subtype that can be generated in neurons. GluN3 subunits can also combine with both GluN1 and GluN2 subunits. The four GluN2 subunit subtypes are combined with four different genes showing both developmental and regional variability (Chatterton *et al.*, 2002).

GluN2A subunits are generally found in most brain regions, whilst GluN2B subunits are mostly limited to the forebrain. GluN2C subunits are often found in the cerebellum and GluN2D subunit levels are often low in an adult brain as they are mainly expressed during development. The GluN1/GluN2A/GluN2B receptor (Figure 1.2.1.1) combination is usually the most common combination found in many mature neurons of the forebrain (Waxman and Lynch, 2005; Javitt, 2004).

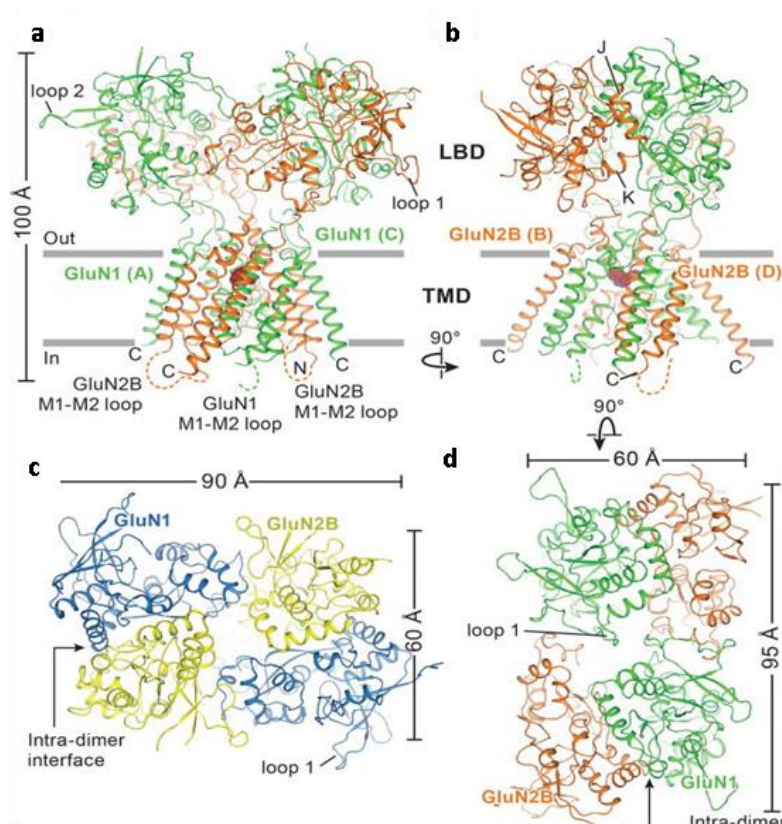


Figure 1.2.1.1 - Structure of the GluN1/GluN2B NMDA receptor- GluN1-1a subunit is represented by the green ribbon and GluN2B subunits are represented by the orange ribbon. A and B diagrams represent a side-view of the amino-terminal domain of the NMDA receptor. C and D represent top-down view of the amino-terminal domain(ATD) of the NMDA receptor. C represents the ATD receptor (blue and yellow), from the extracellular side of the membrane (D) (Song *et al.*, 2018).

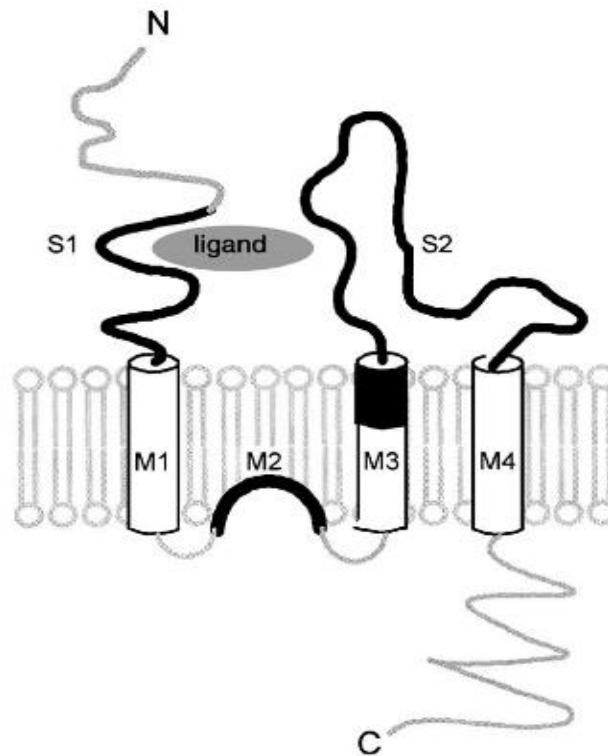


Figure 1.2.1.2 - Structure of a single NMDA receptor subunit. N indicates the extracellular N-terminal. C indicates the intracellular C-terminal. The three transmembrane regions are M1, M3 and M4 which are separated for clarity as the relative positions are currently unknown. M2 indicates the pore-loop. S1 and S2 are ligand-binding domains. The black portion of M3 may play a role in channel gating (taken from Qian and Johnson, 2002).

There are two key glutamate binding site domains present on the NMDA receptor, S1 and S2. The S1 and S2 regions are created by part of the sequence connecting the amino-terminal domain (ATD) to M1 and the extracellular loop connecting M3 to M4, retrospectively (Figure 1.2.1.2). The pore-loop, M2, forms a large part of the channel pore at the intracellular end as it includes the ion selectivity filter while M3 lines the outer parts of the pore and forms the gate (Figure 1.2.1.2). It has been suggested that the external end of the M3 region could be linked to channel gating as the mutations of amino acid of the M3 region affected the gating at the external channel entrance. It has also been hypothesised that the location of the channel gate is found near the selectivity filter. The selectivity filter is the region in which permeable ions interact most intimately with the channel. A slight change in the diameter of the selectivity filter could result in the gating of the channel (Qian and Johnson, 2002). Two

asparagine residues are thought to be involved in the voltage-dependent magnesium block and are located at the turn in the pore-loop (Lee *et al.*, 2014).

### *Function*

NMDA receptors are composed of various domains with specific functions to allow the NMDA receptor to be fully functional. The ion channel open probability and deactivation speeds are controlled by the amino terminal domain (ATD). Binding sites for allosteric modulator compounds are also present in the ATD, for instance, compounds such as zinc and polyamines. There is a vast spectrum of ligands that bind to the ATD of NMDA receptors and allosterically modulate the ion channel activity (Paoletti and Neyton, 2007). Ligand-binding domains (LTD) control ion channel opening through binding to agonists and competitive antagonists. The heteromeric ion channel is formed by the transmembrane domain (TMD). Furthermore, the carboxyl terminal domain (CTD) combines with postsynaptic proteins which facilitates intracellular signalling for synaptic plasticity. The GluN1 and GluN2B subunits are organised in a 1-2-1-2 orientation in all domains but with two-fold symmetry in the ATD and LBD and a four-fold symmetry in the TMD (Figure 1.2.1.3) (Karakas and Furukawa, 2014).

The extracellular portions of the four M3 segments converge to form a pyramid-like shape, resulting in the physical constriction of the ion channel permeation pathway. It has been speculated that the gate of the blocked state of the NMDA receptor is at the bundle crossing of M3 regions. Ion channel gating near the extracellular boundary of M3 helices is defined by Thr 646 of GluN1 and Ala 645 of GluN2B. In all iGluRs, regions are located within the highly conserved SYTANLAAF amino acid motif within amino acid sequences (Lee *et al.*, 2014).

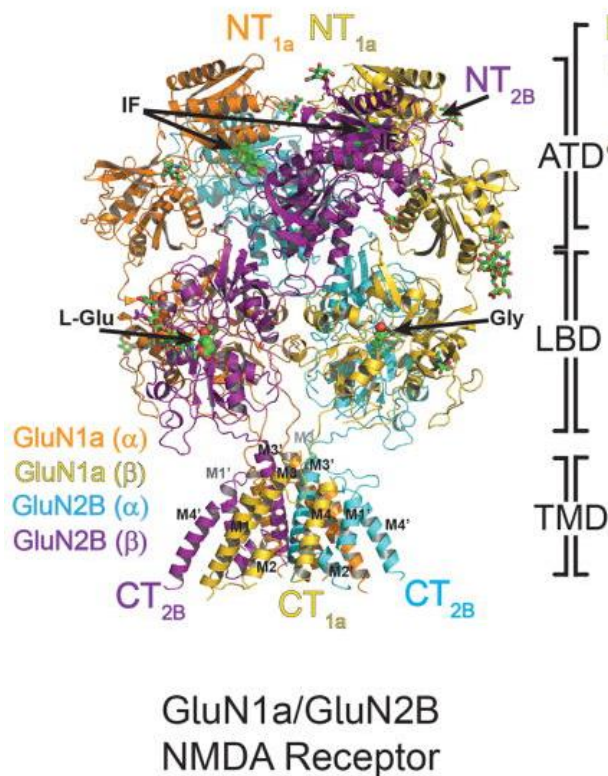


Figure 1.2.1.3 - Crystal structure of a NMDA receptor containing GluN1-1a and GluN2B subunits. Domains are indicated on the right of the image (Karakas and Furukawa, 2014).

After substituting the amino acids of M2, channel properties were investigated using the Two-Electrode Voltage Clamp (TEVC) following expression in *Xenopus laevis* oocytes.  $\text{Ca}^{2+}$  ion permeation was significantly reduced or abolished after the substitution of the Asn-616 residue found in the GluN1 subunit, suggesting that it plays a highly significant role in  $\text{Ca}^{2+}$  selectivity. Furthermore, the Asn-616 residue substitution was found to have no effect on the affinity for agonists. It was further demonstrated that the binding of  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$  and MK-801 also takes place at the Asn-616 residue (Sakurada *et al.*, 1993).

The GluN1 subunit is necessary for calcium conductivity of the channel while the GluN2 and GluN3 subunits determine electrophysiological and pharmacological properties of the receptor (Waxman and Lynch, 2005; Javitt, 2004). Furthermore, it has been found that GluN1-1a/GluN2B complexes *in vitro* have longer excitatory post-synaptic potentials and a higher level of permeability than GluN1/GluN2A complexes, resulting in the facilitation of

Long-Term Potentiation (LTP) and synaptic plasticity (Newcomer *et al.*, 2000; Javitt, 2004). The amino terminal domain attached to S1 is known to be a regulatory domain which potentially contains binding sites for modulators and antagonists (Kashiwagi *et al.*, 2002). The selectivity filter is a narrow constriction which acts as an ionic sieve. It has a key role in ion selectivity. The cross-sectional diameter of the GluN1-GluN2A receptor pore is narrower than other cation-selective ligand-gated channels including nicotinic acetylcholine receptors (Villarroel *et al.*, 1995; Wollmuth *et al.*, 1996). The S1 and S2 ligand-binding domains and their importance has also been investigated using site-directed mutagenesis and molecular modelling to assess the molecular dynamics of agonist selectivity between glutamate receptor subtypes. Mutations were introduced into the GluN2 subunit sequence using Polymerase Chain Reaction (PCR)-based strategies and the modified DNAs/RNAs were then injected into *Xenopus laevis* oocytes. Agonists were then applied, and responses were recorded using the TEVC technique. Mutations resulted in severe functional consequences of the receptor, highlighting the importance of these mutated regions found in GluN1 subunits in glycine coactivation of the NMDA receptor (Kuryatov *et al.*, 1994).

### 1.2.2 Neurotransmitter signalling and the role of NMDA receptors in AD

Pathological changes in neurotransmission, such as the impairments in glutamatergic and cholinergic signalling, are associated with the clinical symptoms displayed by AD patients (Danysz *et al.*, 2000; Larik *et al.*, 2018). NMDA receptors, excitatory glutamate receptors, are primary therapeutic targets due to their glutamate and glycine recognition sites.

Glutamate is a key neurotransmitter in the Central Nervous System (CNS) and is found in more than 80% of all neurons. It is known to have a significant control over a vast number of physiological functions in the CNS. However, changes in glutamatergic activity can result in psychological and neurodegenerative disorders such as AD, Parkinson's Disease (PD), Huntington's Disease (HD), Multiple Sclerosis and Stroke (Ezza and Khadrawy, 2014; Chen and Lipton, 2006). The reduced uptake of glutamate and/or the elevated levels of

glutamate release from presynaptic nerve terminals and astrocytes into extracellular space can trigger excitotoxicity, especially combined with the overstimulation of NMDA receptors (Ezza and Khadrawy, 2014). Astrocytes are a group of cells that have a role in regulating brain functions involved in neurogenesis and synaptogenesis. They have also been known to cause the induction and progression of the inflammatory state, resulting in  $\text{Ca}^{2+}$  signals, commonly observed in severe brain diseases and neurological disorders. The loss of astrocyte function has also been linked to having implications for neurodegenerative disorders, particularly AD and HD (Siracusa *et al.*, 2019).

NMDA receptors are heterotetrametric ligand-gated receptors, consisting of assemblies of subunits which forms a central ion channel pore and allows ligands to bind (Karakas and Furukawa, 2014). They belong to the ionotropic glutamate receptor (iGluRs) family and are involved in vital brain functions, such as learning, memory and synaptic plasticity. They are also key receptors involved in the development of the CNS. The overstimulation of NMDA receptors has been found to trigger a cellular process in neurons which can result in apoptotic-like cell death, leading to early disease stages, such as synaptic or dendritic damage (Chen and Lipton, 2006). The continued search for NMDA receptor antagonists could potentially alleviate symptoms of these disorders through inhibiting the receptor, thus reducing glutamate neurotransmission (Javitt, 2004). An increasingly popular opinion is that many of the functional properties of NMDA receptors are mediated by their localisation. Extrasynaptic NMDA receptors can trigger apoptotic signalling pathways whilst synaptic NMDA receptors contribute to processes such as cell plasticity and neurotropic processes (Leveille *et al.*, 2008).

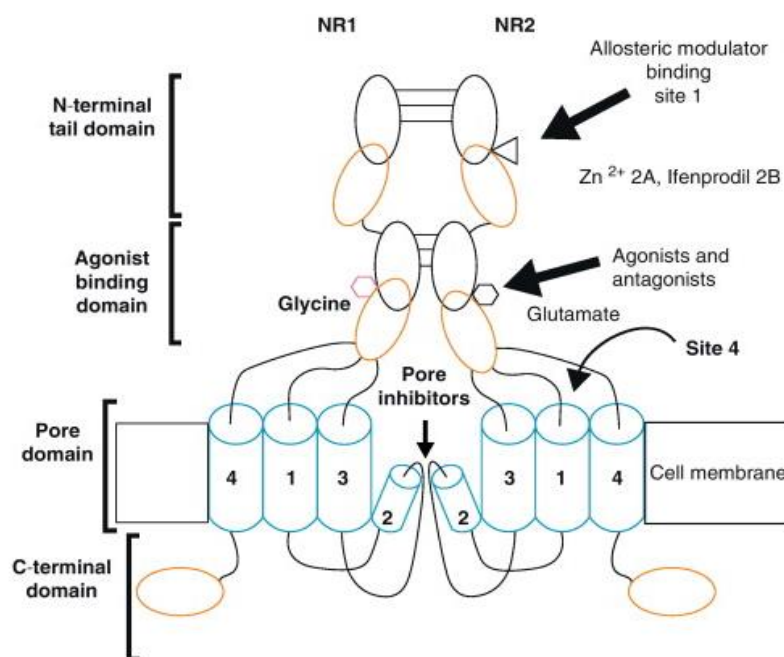


Figure 1.1.1.1- Schematic representation of the structure of the NMDA receptor (Flores-Soto *et al.*, 2012).

Calcium ( $\text{Ca}^{2+}$ ) ion influx plays a vital role in glutamate excitotoxicity (Wang and Qin, 2010). The notion that ( $\text{A}\beta$ ) peptides affect calcium ion homeostasis in neurons was first observed by Hardy and Higgins (1992). Excessive  $\text{Ca}^{2+}$  ion influx via NMDA receptors in a plasma membrane, is vital in the release of neurotransmitters and induction of post synaptic responses (Hartmann and Konnerth, 2005). Post-synaptic neurons can become overloaded by the excessive influx of  $\text{Ca}^{2+}$  which can cause further release of  $\text{Ca}^{2+}$  ions from intracellular stores, resulting in necrosis. It can also result in the dysfunction of mitochondria which can have a negative impact by causing excitotoxic neuronal death (Ezza and Khadrawy, 2014). Additionally, failure to balance  $\text{Ca}^{2+}$  homeostasis contributes to a number of pathological processes such as neuroinflammation, protein misfolding and protein aggregation, all causing neurotoxicity mediated by excitotoxic damage (Marotta *et al.*, 2020).

The progression of late-stage AD has been strongly linked to increased levels of glutamate neurotransmission. AD is believed to involve the deposition of  $\beta$ -amyloid ( $\text{A}\beta$ ) protein and the misfolding of hyperphosphorylated tau proteins. It has been suggested that  $\text{A}\beta$  peptides can

cause oxidative stress and intracellular calcium ion release, enhancing glutamate-mediated neurotoxicity *in vitro* (Danysz and Parsons, 2012). Furthermore, A $\beta$  peptides can indirectly and partially block NMDA receptors, contributing to the increase of glutamate by inhibiting its uptake and by causing the activation of NMDA receptor-dependent signalling cascades that are involved in synaptic loss. This can lead to an indirect increased glutamate levels at the synaptic cleft (Mucke and Selkoe, 2012). Nicotinic acetylcholine receptors also interact with A $\beta$  peptides, in a similar way to NMDA receptors, as the peptide antagonises the receptor, eliciting extracellular signal-regulated kinase MAPK activation, likely to be triggered by Ca<sup>2+</sup> ion influx (Dineley *et al.*, 2002). Additionally, a significant relationship has been established between glutamate levels and tau protein hyperphosphorylation in the hippocampus (Chen and Lipton, 2006; Ezza and Khadrawy, 2014). NMDA receptor activation has been observed to increase the process of tau phosphorylation in primary neuronal cells *in vitro* (Couratier *et al.*, 1994).

Several toxins, such as domoic acid (amino acid analog of the excitatory amino acid neurotransmitter, glutamate), can bind to AMPA-, kainate-, and NMDA-type iGluRs and have been shown to induce excitotoxicity in cell cultures. Therefore, has potential to induce the development neurological problems in both humans and animals (Qui *et al.*, 2006; Grant *et al.*, 2010). *In vitro*, aggregated A $\beta$  enhances NMDA receptor-mediated glutamate toxicity, most likely by interfering with the regulation of neuronal Ca<sup>2+</sup> ion neurotransmission. Soluble A $\beta$  oligomers were found to induce glutamate release from astrocytes (groups of cells which regulate neurogenesis and synaptogenesis), eventually leading to dendritic spine loss via over-activation of extrasynaptic NMDA receptors (Siracusa *et al.*, 2019). Extracellular glutamate concentrations were found to be increased in a transgenic mouse model of AD. A 3-month treatment with the NMDA receptor inhibitor NitroMemantine rescued dendritic spine loss (Talantova *et al.*, 2013; Lewerenz and Maher, 2015).

### 1.2.3 Current Drug Treatments for Alzheimer's disease

The current approved drugs used to treat Alzheimer's Disease are cholinesterase inhibitors (tacrine, donepezil, rivastigmine and galantamine) and NMDA receptor antagonists (memantine) (Marotta *et al.*, 2020). Tacrine was the first of the cholinesterase inhibitors to be used but showed hepatotoxic side effects. Donepezil is the most widely used followed by rivastigmine and galantamine (Briggs *et al.*, 2016). Memantine is also prescribed due to its concentration-dependent inhibitory effect on the NMDA receptor.

### 1.3 Memantine

Memantine is an uncompetitive, open-channel NMDA receptor antagonist with a moderate binding affinity. Although it is known to be ineffective when receptor activity is low, it is highly effective when the NMDA receptor is over-activated and glutamate concentrations are elevated (Chen and Lipton, 2006). Memantine is an 'open-channel blocker' as it can only block the current flow after the channel has opened, as shown in Figure 1.3.1. Its inhibitory action is also voltage-dependent. It has a profile that permits ion permeation during the strong membrane depolarisation associated with synaptic signalling, whilst inhibiting ion permeation under weak depolarisation caused by pathological leakage of glutamate (Figure 1.3.1). This is why it is therapeutically tolerated (Johnson and Kotermanski, 2006).

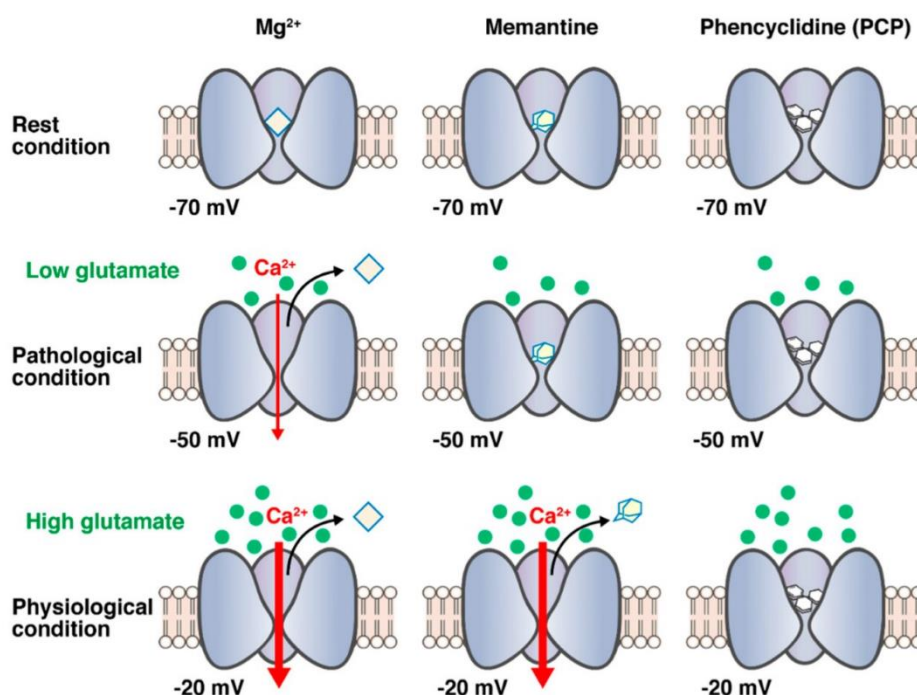


Figure 1.3.1 - Blocking kinetics of magnesium ion block, memantine and PCP on the NMDA receptor (Kikuchi, 2020).

Understanding the mechanisms by which memantine and other NMDA receptor antagonists perform and bind to receptors, can aid in the research of novel NMDA receptor antagonists. The binding of memantine to the NMDA receptor involves the interaction between the ammonium groups of memantine and asparagine (Asn) residues which are found in the GluN1 subunit (Chen and Lipton, 2006). The Asn residues are found at the tip of the pore loop (Figure 1.3.2), creating the narrowest constriction of the NMDA receptor pore (Limapichat *et al.*, 2013). Therefore, it has been suggested that the primary site memantine acts upon is the external  $Mg^{2+}$  blocking site, located at the selectivity filter region of the NMDA receptor-associated channels, as shown in Figure 1.3.1. The selectivity filter is formed by the asparagine residues found at the 'N-site' of GluN1 and N1 site of GluN2 subunits. It was found that the residues situated near the selectivity filter region in the M2 or M3 segment of either GluN1 or GluN2A, slightly affect the voltage dependence of memantine blockade and action (Chen and Lipton, 2005).

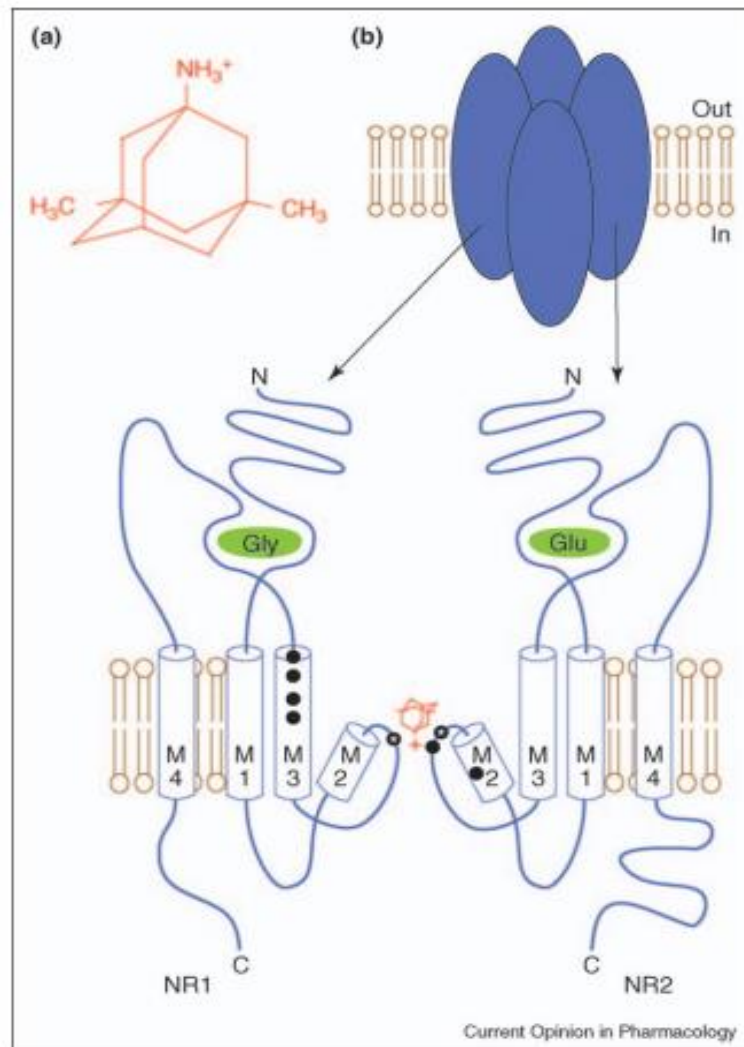


Figure 1.3.2 - Diagram of memantine and NMDA receptor interaction. (a) Structure of memantine, (b) Tetrameric structure of NMDA receptors . Glycine (shown in green) is bound to NR1 (GluN1) subunit whilst glutamate (shown in green also) is bound to the NR2 (GluN2) subunit. Memantine (shown in red) is blocking the channel pore (Johnson and Kotermanski, 2006).

Mutagenesis studies on NMDA receptors confirm the importance of the 'N-site' asparagine residues in the M2 segments of the NMDA receptors. Mutation of the selectivity filter and memantine binding site is found to severely effect the inhibition of NMDA receptors by memantine,  $\text{Mg}^{2+}$ , and other open-channel blockers, highlighting the importance of this site. The memantine  $\text{IC}_{50}$  of GluN1-1a/GluN2A mutant receptors increased to  $2.88 \mu\text{M}$  from the  $1.25 \mu\text{M}$  which was reported for the wild-type GluN1-1a/GluN2A receptors (Kotermanski *et al.*, 2009; Kashiwagi *et al.*, 2002). Oocyte patch clamp studies have also been conducted

resulting in the partial trapping of memantine to the GluN1/GluN2A NMDA receptor leading to an  $IC_{50}$  of 2.3  $\mu$ M. Memantine has been found to be highly potent due to fairly low  $IC_{50}$  values which is to be expected (Parsons *et al.*, 1995). In terms of different receptor subtypes, it has been found that memantine has a higher potency on GluN1/GluN2D NMDA receptor-mediated responses compared to responses mediated by GluN1/GluN2A NMDA receptor. This suggests that differences in the structure and combination of NMDA receptor subunits, can result in differences in the potency of memantine (Parsons *et al.*, 1999).

The neuroprotective agents which are available to block an NMDA receptor usually block virtually all NMDA receptor activity and thus have problematic and sometimes severe adverse effects. These effects include psychosis, nausea, vomiting and sometimes a state known as dissociative anaesthesia. Complete NMDA receptor blockade may also result in neuronal cell death. To be clinically accepted as an effective treatment, an agent must block the excessive activation of the NMDA receptor whilst leaving normal function relatively intact. This will avoid adverse side effects (Olivares *et al.*, 2012). Memantine and other NMDA receptor antagonists have been found to selectively antagonise current responses of rat hippocampal neurons to NMDA in a concentration- and voltage-dependent manner. Although alternative methods and cells were used, both studies demonstrate inhibitory effects on the NMDA receptor (Parsons *et al.*, 1996; Olivares *et al.*, 2012). Early studies have found memantine to selectively reduce whole cell current responses in hippocampal cells whilst other NMDA receptor antagonists have no effect, further supporting the idea that memantine is an appropriate therapeutic treatment for AD as NMDA response is inhibited at acceptable levels (Parsons *et al.*, 1999).

Phencyclidine (PCP), dizocilpine (MK-801) and ketamine have also been found to have antagonistic effects on the NMDA receptor (Figure 1.3.1). Molecular studies suggest the PCP binding sites are confined to the M2 segments, whereas blockade by MK-801 requires

both residues associated with the M2 and M3 regions of the NMDA receptor. Ketamine can bind to, or dissociate from, the  $Mg^{2+}$  ion blocking site located within the channel pore in the same way as memantine (Figure 1.3.1) (Orser *et al.*, 1997). MK-801 which is more potent than PCP, was not approved to be safe for human use due to its side effects on the cardiovascular system. It has been found to be a non-competitive antagonist of the NMDA receptor but appears to induce the same psychomimetic properties as found for PCP in different species including rhesus monkeys (Koek *et al.*, 1988; Krystal *et al.*, 1994). Subsequently, these drugs are not clinically safe and therefore, not approved to be a treatment for AD.

#### 1.4 Clinical Trials of Memantine

Clinical trials have been undertaken to assess the therapeutic effect of memantine on Alzheimer's disease (AD) patients. The outcomes and measures vary with each clinical trial, particularly when specific AD symptoms are being examined such as cognition and behaviour, language, and function. There is a need for an up-to-date systematic review to understand whether memantine has an overall positive or negative effect and which dose is the most useful.

The range of different outcomes of such clinical trials can be grouped according to the pathological symptoms of AD that were measured. The outcome groupings include molecular biomarkers and imaging, cognition and behaviour, and functional and linguistic communication. Molecular biomarkers and imaging measures include changes in N-acetylaspartate (NAA)/Creatine (Cr) ratios which are measured using Magnetic Resonance Spectroscopy (MRS), Brain Boundary Shift Integral (BBSI) and Hippocampal Volume Slope. Cognitive and behavioural changes and symptoms are commonly measured using the Mini-

Mental State Examination (MMSE); Alzheimer's Disease Assessment Scale - Cognitive (ADAS-cog); Neuropsychiatric Inventory (NPI); Severe Impairment Battery (SIB); Clinician's Interview-Based Impression of Change with Caregiver Input (CIBIC-plus); the Alzheimer's Disease Cooperative Study Activities of Daily Living Inventory Modified for Severe Dementia Abbreviated Version (ADCS-ADLsev-abv); Selective Reminding Test-Total Immediate Recall (SRT-IR); and Clinical Global Impression of Change (CGIC). Functional capabilities are assessed using Alzheimer's Disease Cooperative Study/Activities of Daily Living (ADCS/ADL) Inventory and ADCS-ADLsev-abv. Linguistic communication is measured using Functional Linguistic Communication Inventory (FLCI), SIB and CIBIC-plus. An overview of these trial outcomes is presented in Table 1.4.1 below.

| <b>Clinical Trial Study outcomes</b>                                                                                            | <b>Definitions/details</b>                                                                                                                                                                                                                                                                                                                            |
|---------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| N-acetylaspartate (NAA)/Creatine (Cr) (and other metabolite combinations) ratios measured using Magnetic Resonance Spectroscopy | Metabolite ratios were compared pre-and post-treatment to observe the effects of memantine on metabolite concentrations.                                                                                                                                                                                                                              |
| Brain Boundary Shift Integral (BBSI)                                                                                            | Measures direct changes in total brain volume per visit interval.                                                                                                                                                                                                                                                                                     |
| Hippocampal Volume slope                                                                                                        | Measures the change of Hippocampal Volume pre-and post-treatment.                                                                                                                                                                                                                                                                                     |
| Alzheimer's Disease Cooperative Study/Activities of Daily Living (ADCS/ADL) Inventory                                           | Assesses the functional abilities to perform activities of daily living in AD patients with a broad range of dementia severity.                                                                                                                                                                                                                       |
| Mini-Mental State Examination (MMSE)                                                                                            | Assess cognitive status in psychiatric patients with cognitive impairment. The MMSE questions are grouped into seven categories, each representing a different cognitive domain.                                                                                                                                                                      |
| Alzheimer's Disease Assessment Scale - Cognitive (ADAS-cog)                                                                     | Assess the severity of cognitive and non-cognitive behavioural impairments in patients with Alzheimer's disease.                                                                                                                                                                                                                                      |
| Neuropsychiatric Inventory (NPI)                                                                                                | Assesses psychological and behavioural problems in patients with dementia.                                                                                                                                                                                                                                                                            |
| Severe Impairment Battery (SIB)                                                                                                 | Evaluation of cognitive function in patients with more advanced dementia, and evaluates the areas of memory, language, praxis, orientation, and attention.                                                                                                                                                                                            |
| Clinician's Interview-Based Impression of Change with Caregiver Input (CIBIC-plus)                                              | Measured in units on a scale ranging from 1 to 7, where 1 is markedly improved, 4 is unchanged, and 7 is markedly worse.                                                                                                                                                                                                                              |
| Change in Selective Reminding Test-Total Immediate Recall (SRT-IR)                                                              | Measures word recall (maximum 12 words per trial, across 6 trials). Maximum total recall score across 6 trials are 72; minimum recall is 0 across 6 trials. The higher the raw score, the better the patient did at recalling the target words. The unit of measure is the raw score, or the sum of the number of words recalled across all 6 trials. |

| <b>Clinical Trial Study outcomes</b>                                                                                                              | <b>Definitions/details</b>                                                                                                                                                                                                                                                                                                      |
|---------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Clinical Global Impression of Change (CGIC)                                                                                                       | The clinician compares the participant's current condition to the condition at admission to the project.                                                                                                                                                                                                                        |
| The Alzheimer's Disease Cooperative Study Activities of Daily Living Inventory Modified for Severe Dementia Abbreviated Version (ADCS-ADLsev-abv) | A structured questionnaire where each item consists of a series of hierarchical questions designed to determine a patient's ability to perform the activities of daily living as assessed by the caregiver. Possible scores range from 0 to 39, where a higher score is indicative of greater capacities. Baseline to 28 weeks. |
| Caregiver Activity Survey (CAS)                                                                                                                   | Measures the time caregivers spend aiding Alzheimer patients with their day-to-day activities.                                                                                                                                                                                                                                  |
| Functional Linguistic Communication Inventory (FLCI)                                                                                              | Standardised & validated instrument for evaluating functional communication in pts with moderate-to-severe Alzheimer's that can be used to obtain Baseline information and to track patients' capabilities thereafter.                                                                                                          |

Table 1.4.1 – Definitions and detail of each outcome measure used in eligible memantine clinical trial studies

## 1.5 Natural NMDA receptor antagonists

One effective way to explore the range of NMDA receptor antagonists available is to investigate natural compounds which demonstrate NMDA receptor antagonistic activity. It is also important to find compounds which do not completely block all activity of NMDA receptors, as this can also have detrimental effects on neuronal cell communication.

Various solitary wasp species including, *Dielis collaris* and *Podalonia tydei*, produce venoms which have demonstrated voltage-dependent inhibition and potentiation of NMDA receptors similar to the memantine action (Williams *et al.*, 1991). This observation suggests they bind using similar mechanisms, thus potentially have therapeutic effects. Using a whole-cell patch clamp technique, 1µg/mL of venom was co-applied with NMDA and inhibited the receptor almost completely at -100 and -125 mV holding potential. Therefore, the venoms, which possibly contain polyamines, have potential to be open channel blockers for NMDA receptors. This was also found to be the case for nicotinic acetylcholine receptors (Zalat *et al.*, 2005).

Furthermore, active toxins, such as argiotoxin-636 (ArgTX-636) (Figure 1.5.1), have been identified in spider venoms extracted from the spider, *Argiope lobata*. Whole-cell patch clamp recordings were performed to investigate the effect of ArgTX-636 on NMDA and non-NMDA receptors. ArgTX-636 was found to significantly block NMDA-induced currents in a voltage-dependent manner. These antagonistic actions of ArgTX-636 depended on the holding potential which ranged from -100 to +60mV. The antagonistic action only occurred at potentials of -20 to -40 mV, however, suggesting that ArgTX-636 would be a successful open-channel blocker for NMDA-activated ion channels present in mammalian neurons (Priestley *et al.*, 1989). ArgTX-636 is structurally related to the polyamine-containing toxin, philanthotoxin-343 (PhTX-343), isolated from the Egyptian digger wasp (*Philanthus triangulum*) (Figure 1.5.1). PhTX-343 has also exhibited a high selectivity for NMDA receptors. More in-depth research into ArgTX-636, led to the finding that ArgTX-636 was approximately 60 times more potent and effective in completely antagonising both native and cloned NMDA receptors than the wasp toxin, PhTX-343. Concentration-dependent inhibition was demonstrated for NMDA receptors by both PhTX-343 and ArgTX-636 (Brackley *et al.*, 1993).

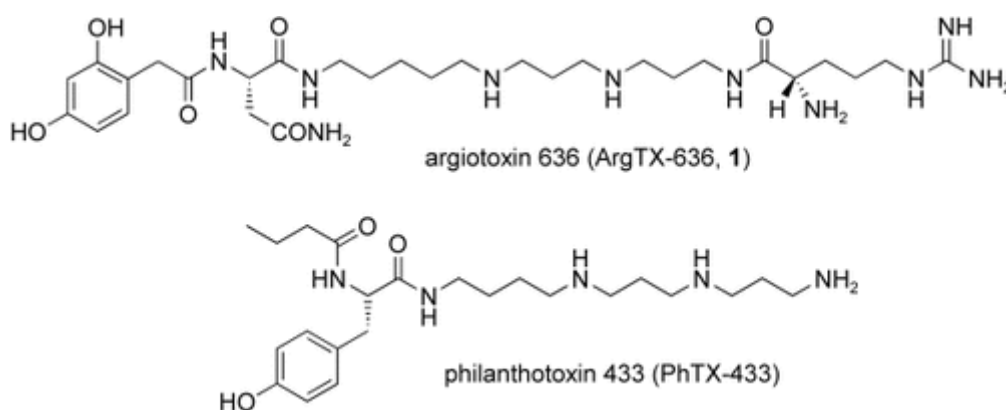


Figure 1.5.1 - Structure of two poly-amine toxins, argitoxin-636 (ArgTX-636) and philanthotoxin 433 (PhTX-433) (Nelson *et al.*, 2009).

Conantokin G (Con G), a peptide NMDA receptor antagonist, is isolated from the venom of the *Conus geographus*, a marine cone snail. Studies were carried out by investigating the antagonistic effect of Con G on the NMDA receptor expressed in the *Xenopus laevis* oocytes, using the two-electrode voltage clamp technique. Results indicated that Con G successfully acted as a competitive NMDA receptor antagonist, particularly selective to the GluN2B subunit, compared to alternative GluN2A subunits. This inhibitory effect was also displayed in whole-cell patch clamp recordings in cultured rat cortical cells, demonstrating that Con G is a potent competitive NMDA receptor antagonist. It was also found that it does not bind inside the channel pore and demonstrated a slow onset of blocking the receptor. Moreover, further tests concluded that polyamines such as spermine, did not prevent or compete against the Con G block. However, the Con G block was significantly enhanced when in the presence of spermine (Donevan and McCabe, 2000).

There is an abundance of research looking into the effect of natural insect toxins on NMDA receptor neurotransmission, leading to the interest in ladybirds and their toxins/alkaloid content. Ladybird beetles are thought to contain over 50 different alkaloids, some of which are anti-inflammatory and anti-microbial (Glisan King and Meinwald, 1996). Harmonine, a ladybird alkaloid, have not been thoroughly researched to date. It is thought to act in the same way as memantine in terms of binding and reducing glutamate neurotransmission.

## 1.6 Ladybird beetles and The Harlequin ladybird, *Harmonia axyridis*

Ladybird beetles (Coleoptera: Coccinellidae) are morphologically and ecologically diverse and there are known to be roughly 6000 species (Seago *et al.*, 2011). Their sizes range from 0.8mm to 18mm and they have a range of specialised predation habits, varying from phytophagy and pollenphagy to fungivory and aphidophagy. Chemical ecologists have shown an interest in coccinellids due many of the species possessing a high concentration of alkaloid-based compounds which are released when exposed to a threat (Holloway *et al.*, 1993).

Ladybirds are often oval or round and possess obvious characteristics such as the brightly-coloured and patterned elytra (wing cases) situated on the upper side of the ladybird. These can be found to be patterned with stripes, band or spots. Brightly coloured elytra are thought to suggest a higher toxicity and concentration of alkaloids. The pronotum can be found between the head and the elytra of the ladybird and can also be patterned although, usually not as brightly as the elytra. It allows the head to retract and extend forward. The type of wing cases which ladybird beetles possess, known as elytra, are often hard and leathery. Secondly, their mouths are different to other beetles in the way that they have evolved to bite as well as suck. Their elytra and mouthparts can help distinguish ladybird beetles from other beetles species (Roy *et al.*, 2013).

The multi-coloured Asian ladybird, *Harmonia axyridis* (*H. axyridis*), also known as the Harlequin or Halloween ladybird is native to south-east Asia. However, it has been introduced to four additional continents in more than 38 difference countries (Brown *et al.*, 2011). They have a length of approximately 5 mm or more. By 1945, over 200 different colour forms were documented across its native regions. However, of those which have migrated to Britain, only 3 colour forms have been documented: *succinea*, *conspicua* and

*spectabilis*. The *succinea* colour form is orange- red, with 0-21 black spots whilst *spectabilis* colour forms are back with four red spots. The *conspicua* form is black with two red spots (Honek *et al.*, 2020) (Figure 1.6.1).

*H. axyridis* demonstrates a high tolerance for other species' alkaloids. This species is able to prey upon a range of other species of ladybirds. This coupled with the haemolymph that is released via their tibio-femoral joints (appears as an orange-yellow liquid with an unpleasant odour and taste), causes it to be very invasive. The larvae also exude haemolymph from the ends of long branched spines along the dorsum (Bayoumy *et al.*, 2019; Sloggett and Davis, 2010). Internally, their eggs contain synthesised alkaloids resulting in egg toxicity and the egg's surface is often coated in a mixture of hydrocarbons which flag internal toxicity. Subsequently, their eggs are somewhat protected from predators due to the unpleasant taste (Magro *et al.*, 2018). *H. axyridis* is also capable of physiological modification of alkaloid which may occur outside of the gut. It cannot digest efficiently the more toxic alkaloid, adaline, extracted from the two-spot ladybird, *Adalia bipunctata*, whereas it can modify other alkaloids such as isopropyleine. Moreover, it has been suggested that the adaptation to modify and store an accumulation of novel alkaloids, makes *H. axyridis* successful in intraguild predation of other ladybird beetles (Sloggett and Davis, 2010). Although alkaloids are present, intraguild predation and cannibalistic behaviours are commonly seen among invasive predatory ladybird species (Kajita *et al.*, 2010).



Figure 1.6.1 – The variation in pattern and colouration of the *H. axyridis* elytra (Bezzarides *et al.*, 2007).

#### 1.6.1 Ladybird alkaloids and chemical defences of ladybirds

Coccinellids are known to release a haemolymph from their tibio-femoral joints when threatened. This haemolymph has been found to be rich in over 50 alkaloids including, acyclic amines, piperidines, pyrrolidines and homotropanes. Coccinelline and precoccinelline (Figure 1.6.1.1) were the first ladybird alkaloids to be isolated through methanol extraction of the seven-spot ladybird, *Coccinella septempunctata* (Glisan King and Meinwald, 1996).

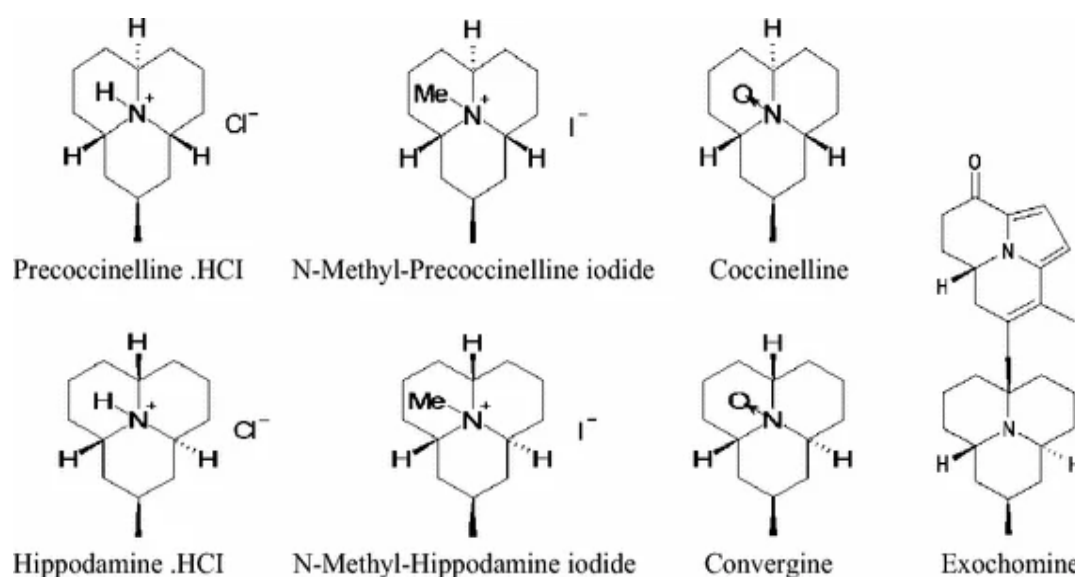


Figure 1.6.1.1 - Examples of the various coccinellid alkaloids with inhibitory effects on nACh receptors (Leong *et al.*, 2015).

The haemolymph of the invasive Harlequin ladybird, *H. axyridis* contains high concentrations of the antimicrobial alkaloid, harmonine. Harmonine is an acyclic amine which has been found to have antimicrobial properties. A study investigating the antimicrobial and anthelmintic properties of harmonine, found that harmonine also reduces acetylcholinesterase activity (Kellershohn *et al.*, 2019; Glisan King and Meinwald, 1996). It has been demonstrated that there is intraspecific variation between *H. axyridis* adults and eggs in terms of harmonine alkaloid content. Furthermore, it has been documented that in adult species, there can be individual and seasonal variation in their harmonine alkaloid content. The chemical defences in the *H. axyridis* species have been found to be more toxic when compared to other species of ladybird beetles, suggesting their protection against invertebrate and vertebrate predators are stronger and more advantageous to their survival. This also translates to the *H. axyridis* eggs as they have also been found to contain more harmonine, resulting in them being less readily eaten by other intraguild predators (Sloggett *et al.*, 2011).

The colouration of elytra of the *H. axyridis* has been found to significantly correlate with alkaloid concentration present and Figure 1.6.1. The area of orange/red sections on red-and-black patterned elytra was found to positively correlate with harmonine alkaloid concentration in this species. Furthermore, carotenoids are vital in the *H. axyridis* diet and are found in many aphids. An aphid-exclusive diet has been linked to a redder pigment in the elytra of *H. axyridis* (Bezzarides *et al.*, 2007).

The reflex bleeding mechanisms and the circumstances in which haemolymph is excreted was initially investigated by Happ and Eisner (1961) (Figure 1.6.1.2). Mexican bean beetles (*Epilachna varivestis*) were attached to a rod and traumatic stimuli were applied in the forms of pinching parts of the body with forceps or applying a hot needle. It was found that upon the application of stimuli on specific legs, the corresponding leg responded by releasing blood from their tibio-femoral joints individually rather than simultaneously. The leg which is closest to the stimulus and injury bled at the knee joint. Furthermore, pinching was found to be ineffective when applied to the abdominal areas of the beetle. Shortly after release, the haemolymph appeared to coagulate rapidly when exposed to air, resulting in forming a crust which did eventually flake off completely. Reflex bleeding was also prevalent in larvae of the Mexican bean beetle, which only occurred when their spines were broken, resulting in the emergence of a droplet of haemolymph. Moreover, the effect of the blood on arthropod predators, such as the *Formica exsectoides* Forel, was investigated by introducing the ants to adult beetles. The ants were attacked immediately and released the haemolymph shortly after, resulting in the droplet spreading on to the assailant. The blood rapidly coagulated and became sticky, leading to the antennae and other ants sticking together, demonstrating the importance of reflex bleeding in terms of survival and protection from predators (Happ and Eisner, 1961).

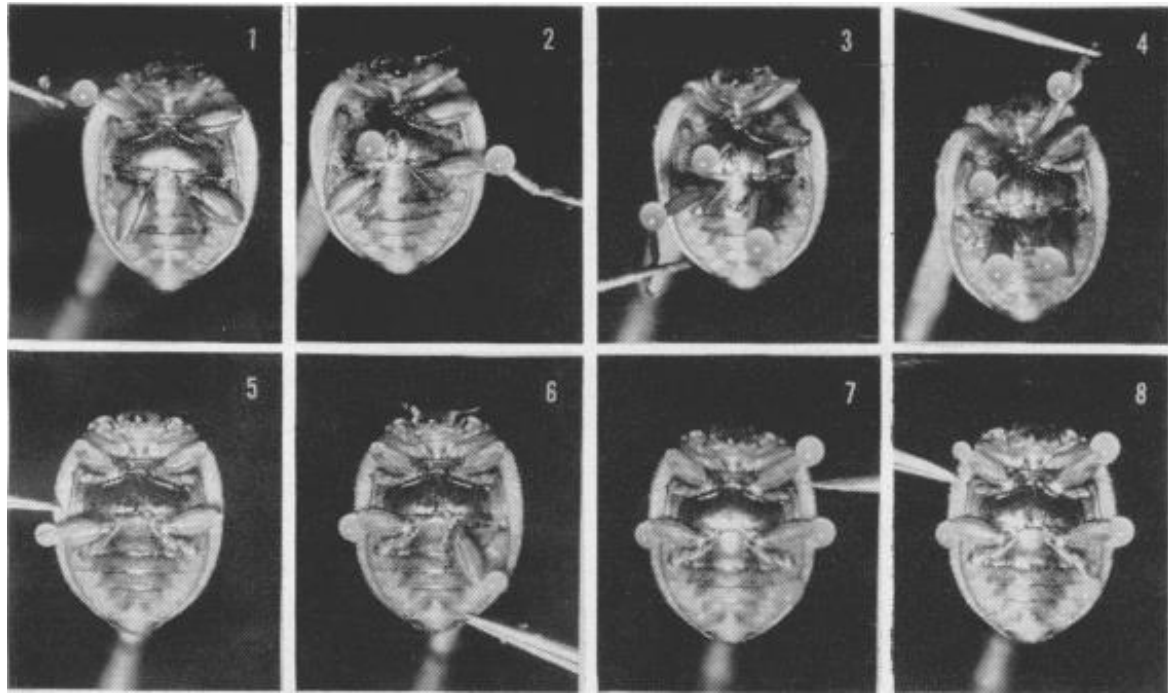


Figure 1.6.1.2 - Examples of reflex bleeding from tibio-femoral joints induced through pinching of the corresponding legs (1-4). Pinching was also applied to the elytra and abdomen in images 5-8, to investigate reflex bleeding around the whole body of a coccinellid beetle (taken from Happ and Eisner, 1961).

The bright colour of the ladybird can indicate the presence of toxic alkaloids. It is believed that these alkaloids are synthesised by ladybird beetles alone rather than gaining them from plants as other insects, such as the monarch butterfly do. However, it has been found that ladybirds do have the ability to retain and store toxic compounds from the consumption of their prey. Many species of the Coccinellinae subfamily produce alkaloids at all stages of development to protect themselves from predation. Eggs are better protected when they have a coating rich in a diversity of hydrocarbons. Furthermore, the strength of the chemical defence is significantly higher when eggs are in groups as opposed to when eggs are deposited in isolation, further deterring intraguild predators such as the *Harmonia axyridis* species of ladybird beetles (Durieux *et al.*, 2010).

However, there are costs of reflex bleeding which have been observed. Some research failed to consider that chemical defences are also used to coat and protect eggs which can lead to transgenerational differences (Bayoumy *et al.*, 2019). It has also been suggested that

reflex bleeding can lead to exhaustion as haematopoiesis processes require an abundance of energy (Knapp *et al.*, 2018). Moreover, reflex bleeding has been known to be costly as ladybirds which were experimentally induced to exude haemolymph were found to have smaller egg sizes, lower egg viability and a short life in comparison to controls (Durieux *et al.*, 2010).

#### 1.6.2. Harmonine

There is evidence of the inhibitory effects of ladybird alkaloids including coccinelline, precoccinelline and hippodamine on the  $\alpha 7$  (nicotinic acetylcholine) nACh receptors. Human  $\alpha 7$  nACh receptors were expressed in *Xenopus* oocytes. Precoccinelline was found to inhibit the expressed nACh receptors non-competitively and in a concentration-dependent manner (Leong *et al.*, 2015). Similarly, harmonine was found to have an inhibitory effect on acetylcholinesterase, prolylendopeptidase and neuraminidase which have been considered to be therapeutic targets of AD (Alam *et al.*, 2002).

Harmonine has the appearance of brownish oil upon isolation from other alkaloids. As well as the protective properties of the toxic alkaloid, harmonine also exhibited inhibitory effects on mycobacterial growth. The *H. axyridis* haemolymph was found to have a large inhibition zone when applied onto agar containing *E. coli*, compared to the haemolymph collected from other species of ladybird beetles from which no inhibition zone was observed (Röhrich *et al.*, 2012).

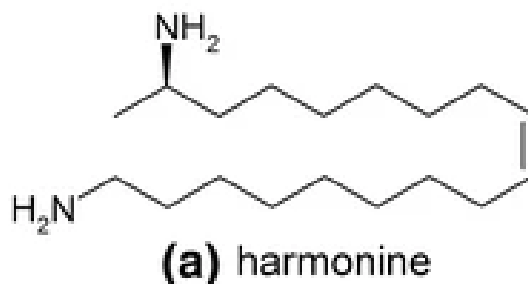


Figure 1.6.2.1 – Molecular structure of the harmonine alkaloid present in the *H. axyridis* and used as a chemical defence (Slogget *et al.*, 2011).

Furthermore, harmonine was also demonstrated to have antibacterial effects and it was equally effective against *Staphylococcus aureus* and the multi-resistant MRSA strain (Röhrich *et al.*, 2012). Moreover, harmonine has been found to have an effect on both mammalian and insect nicotinic acetylcholine receptors. *H. axyridis* extract containing harmonine was found to inhibit the acetylcholine response of TE671 cell and locust neurons, suggesting similar results may be found for other receptors related to AD (Patel, 2018; Patel *et al.*, 2020; Richards, 2011). This further supports the ideas that ladybird alkaloids may be useful in blocking key receptors related to the pathology of AD.

## 1.7 Aims and Objectives

This study has two main components. In the first part, the aim was to conduct a systematic review and descriptive analysis of clinical trials of memantine conducted in humans between the years 2008-2012. This was performed to determine whether memantine is effective as a treatment for AD and to assess the need for new alternatives. In the second part of this study, the aim was to investigate whether the *H. axyridis* alkaloids, particularly harmonine, are able to demonstrate antagonistic effects on NMDA receptor neurotransmission, with comparison to memantine, using electrophysiological methods. TEVC was used to examine the effects of harmonine on NMDA receptors containing either GluN2A or GluN2B subunits, each co-expressed with a GluN1-1a subunit in *Xenopus laevis* oocytes.

## 2 Methods and Materials

### 2.1 Systematic review and descriptive analysis of clinical trials of memantine

#### *Inclusion and Exclusion Criteria*

Clinical trial studies which primarily tested the drug memantine and were catalogued on the HHI, U.S national library of medicine, ClinicalTrials.gov website was assessed.

ClinicalTrials.gov is a database of privately and publicly funded clinical studies conducted around the world <https://clinicaltrials.gov/>. The database stores data relating to 386,745 research studies conducted in all 50 US states and in 219 countries. The clinical trials that will be analysed have been completed with results with the years of completion ranging from 2008-2012.

In addition to memantine studies, controlled clinical trials which compared memantine to another AD treatment were also included. All studies included were completed and were quantitative, i.e., statistical tests were performed to test for significance in difference between primary outcomes. Incomplete studies with the absence of results were excluded in the data analysis.

#### *Search Strategy and Outcome Measures*

Keywords included in the search strategy were, “memantine” OR other memantine trade names (e.g., Namenda, Ebixa, Abixa) AND “Alzheimer’s Disease”. All clinical trial studies reported original findings relating to memantine, Alzheimer’s Disease, other treatment used alongside memantine and other trade names for memantine. The outcome measures and measurement scales varied throughout the clinical trials. As a consequence, studies were grouped according to their outcome measures and the general field they assessed. Groups include ‘cognition and behaviour’, ‘linguistic communication’, ‘molecular biomarkers’, and ‘imaging’.

### *Data Extraction and Data Synthesis*

Data extracted included the study design, age/sample size, intervention (type of drug, dose taken and duration), primary and secondary results and conclusions made by the study authors. Cognitive function and behaviour were measured using ADCS/ADL inventory, MMSE, ADAS-cog, NPI, SIB, CIBIC-plus, SRT-IR, CGIC, ADCS-ADLsev-abv and CAS. Functional outcomes were measured using CIBIC-plus, ADCS-ADLsev-abv, ADCS/ADL, MMSE, ADAS-cog, NPI, CAS. Linguistic communication was measured using FLCI and SBI. Molecular biomarkers were measured by assessing the metabolite ratios of N-acetylaspartate (NAA) to Creatine (Cr) N-acetylaspartate (NAA) was measured with MRS, NAA/Cr ratio changes from pre-to-post treatment. Imaging techniques used BBSI and Hippocampal Volume Slope. Clinical trial studies were also analysed to assess whether memantine has a positive or negative effect on AD patient symptoms and to understand why by comparing outcomes and results.

## 2.2 Production of NMDA receptor subunit cDNA

### 2.2.1 Transformation of *E. coli*

An overnight starter culture of cells was prepared by adding the *E. coli* strain XL-10 to 5 mL of Luria-Bertani (LB) broth and incubating at 37 °C in an orbital shaker at 250 rpm. In a 200 mL conical flask, 50 mL of LB broth and the starter culture was added and incubated for a further 2 hours. The culture was transferred to a 50 mL falcon tube and then placed in ice for 15 mins. Then it was centrifuged at 2000 rpm for 10 mins at room temperature. The supernatant was discarded, the cell pellet was resuspended in 10 mL of 0.1M CaCl<sub>2</sub> (ice cold, filter sterilised) and then left for 15 mins. This was then centrifuged at 2000 rpm for 10 mins at room temperature. The supernatant was discarded, and the pellet was resuspended in 200 mL of 0.1M CaCl<sub>2</sub> (ice cold, filter sterilised).

1  $\mu\text{L}$  of plasmid DNA (pDNA) was added to 200  $\mu\text{L}$  of chemically competent cells and placed in ice for 20 mins. They were then transferred to a water bath at 42 °C for 1 min then put in ice promptly for 2 mins. 1 mL of LB broth with 10 mM glucose was added and cells were placed in a 37 °C water for 30 mins. 100  $\mu\text{L}$  of cells were spread over 95 mm Petri dishes containing LB agar with 50  $\mu\text{g/mL}$  ampicillin and incubated at 37 °C overnight.

### 2.2.2 Isolation of plasmid DNA from transformed *E. coli*

A QIAGEN QIAprep Spin Miniprep kit (70) was used to isolate the plasmids from the culture. The following centrifugation steps were performed at 14,000 rpm at room temperature: 1.5 mL of starter culture was centrifuged for 3 mins then the supernatant was discarded. This step was repeated until all starter culture had been centrifuged. The pellet was thoroughly resuspended in 200  $\mu\text{L}$  Resuspension solution by vortexing and pipetting up and down until homogeneous. Resuspended cells were lysed by adding 200  $\mu\text{L}$  of lysis solution. Contents were gently mixed by inverting until clear and viscous. 350  $\mu\text{L}$  of neutralising/binding solution was added and gently inverted 4-6 times. These were then centrifuged for 10 mins, forming a cloudy, viscous precipitate and a clear lysate. A GenElute Miniprep Binding Column was assembled and prepared by adding 500  $\mu\text{L}$  of Column Preparation Solution to each column and centrifuged for 1 min. Flow-through liquid was discarded and the clear lysate was added to the column and centrifuged for 1 min. 750  $\mu\text{L}$  of diluted Wash Solution was added to the column and centrifuged for 1 min. Flow-through liquid was then discarded and centrifuged again for 2 mins. The column was transferred to a 1.5 mL Eppendorf tube. 100  $\mu\text{L}$  of Elution solution was added to column and centrifuged for 1 min. DNA in eluate was stored at -20 °C and concentration and purity was determined by a Thermo-scientific NanoDrop 1000 spectrophotometer. Concentrations were as follows: 61.0 ng/ $\mu\text{L}$  for GuN1-

1a subunit, 197.10 ng/ $\mu$ L for N2A subunit, 168.9 ng/ $\mu$ L for N2B subunit, 146.0 ng/ $\mu$ L for GluN2A subunit, 109.0 ng/ $\mu$ L for GluN2B subunit.

### 2.3 Electrophysiology and oocyte expression system to study ion channels

The use of *Xenopus laevis* oocytes to study electrophysiological properties of ion channels are common. Most studies investigating the electrophysiology of oocytes use the two-electrode voltage clamp (TEVC) technique (Dascal, 1987) as diagrammatically presented in Figure 2.3.1.

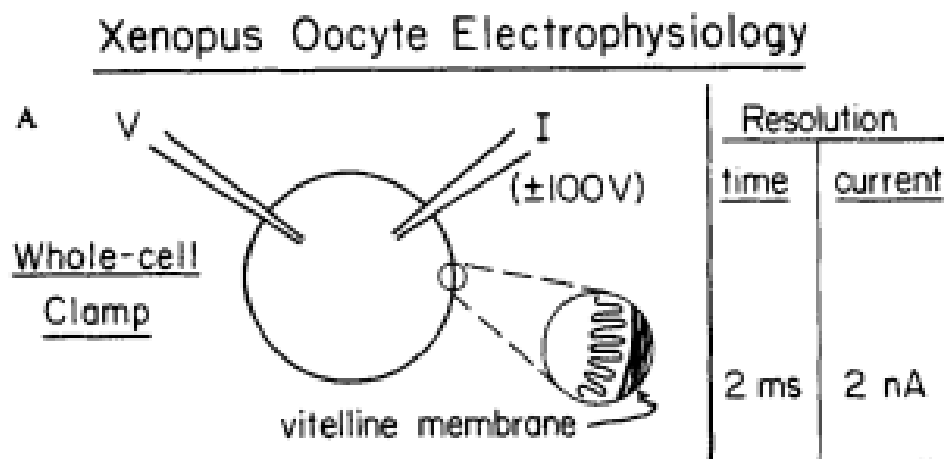


Figure 2.3.1 - Two-electrode Voltage clamp technique using *Xenopus laevis* oocytes (Dascal, 1987).

During TEVC the membrane of the oocyte is impaled with two microelectrodes, one of which measures the membrane potential of the oocyte, while the other injects current to maintain that membrane potential; the injected current is equal and opposite to that flowing through ion channels following their activation. NMDA receptors have been found to be successfully expressed with all the correct properties of their neuronal counterparts using *Xenopus laevis* oocytes (Verdoorn and Dingledine, 1988).

*Xenopus laevis* oocytes, which have been extracted from the ovaries of an adult female South African clawed toad, are commonly used to investigate electrophysiological properties of specific ion channels, receptors, and transporters due to their size and shape (Baumgartner *et al.*, 1999) (Figure 2.3.2a). Oocytes go through 6 maturation stages (I-VI). Oocytes at stages V and VI are usually used for electrophysiological studies, as shown in Figure 2.3.2b. The size of the cells is approximately 1.2 mm in diameter with a white vegetal pole and a darker animal pole, where the nucleus is present. A glycoprotein matrix, known as the vitelline membrane, covers the outside of the oocyte and a layer of follicle cells are also present. Follicle cells are electrically coupled to the oocyte through gap junctions (Theodoulou and Miller, 1995; Bianchi and Driscoll, 2006).

Foreign genetic material is injected into oocyte by microinjection. RNA is microinjected into the vegetal pole of the oocyte or cDNA may be microinjected into the nucleus situated in the animal pole of the oocyte. DNAs that are injected into the oocyte nucleus are assembled into chromatin and transcribed (Theodoulou and Miller, 1995).

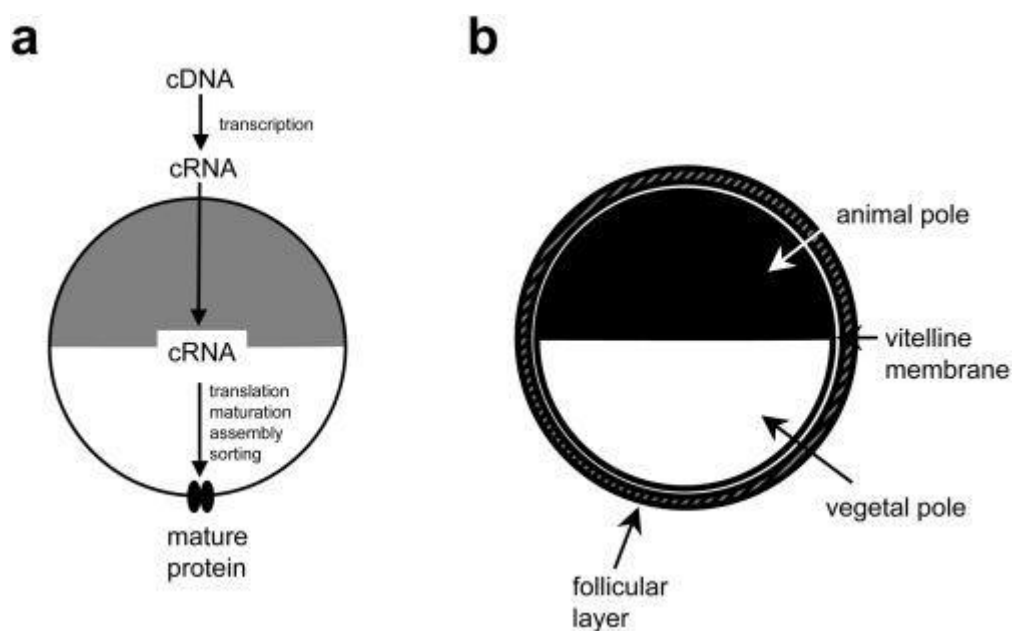


Figure 2.3.2 – (a) *Xenopus laevis* oocyte expression system. (b) Structure of a *Xenopus laevis* oocyte (taken from Bianchi and Driscoll, 2006).

## 2.4 *Xenopus laevis* oocyte preparation and injection

*Xenopus laevis* oocytes were acquired from the European *Xenopus* Resource Centre (University of Portsmouth, UK). Oocytes were treated with 0.1 mg/mL collagenase (type 1A, Sigma) incubated in  $\text{Ca}^{2+}$  free Barth's GTP solution (96 mM NaCl, 2 mM KCl, 5 mM HEPES, 2.5 mM pyruvic acid, 0.5 mM theophylline, at a pH of 7.5 followed by adding 0.05 mg/mL gentamicin) for 50-60 mins to separate them and to remove the follicular layer. This was followed by thorough washing with  $\text{Ca}^{2+}$  free Barth's GTP 3-4 times. Oocytes at stage IV-V were selected, particularly oocytes that had a well-defined divide separating the animal and vegetal poles. Follicular membrane layers were carefully peeled using forceps and then the oocytes were injected with GluN1-1a/GluN2A or GluN1-1a/GluN2B subunit cDNA.

Injection pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, US) using a programmable micropipette puller (P-97 Flaming/Brown, Sutter Instruments Co., US) and the tip broken back to produce a diameter of about 25  $\mu\text{m}$ . The pipette was back filled with filtered paraffin before being inserted into a Nanoliter injector (World Precision Instruments, Inc.). 1  $\mu\text{L}$  of each subunit cDNA (GluN1-1a- 61.0 ng/ $\mu\text{l}$ ; GluN2A- 146.0 ng/ $\mu\text{l}$ ; GluN2B- 109.0 ng/ $\mu\text{l}$ ) was pipetted onto sterile parafilm and the tip of the pipette was manipulated into the solution. The needle was then filled with the cDNA, removing air bubbles by emptying if necessary. Peeled oocytes were then placed into a 35 mm Petri dish with grooved plastic to hold oocytes in place, containing Barth's GTP solution (96 mM NaCl, 2 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 5 mM HEPES, 2.5 mM, pyruvic acid & 0.5mM theophylline, at a pH of 7.5. 5 mL of 10 mg/mL of gentamicin was added after autoclaving).

Each oocyte was injected with 50 nL of cDNA solution into the nucleus contained within the animal pole and then transferred using a Pasteur pipette into a separate Petri dish containing fresh Barth's GTP. Oocytes were incubated for 2-3 days at 18 °C to allow for protein expression. GTP was changed daily, and any damaged oocytes were removed.

## 2.5 Two-electrode Voltage Clamp Recording

Microelectrodes made from borosilicate glass capillaries were pulled using a Sutter P-97 micropipette puller and had a resistance of 0.5-2 MΩ when filled with 3 M KCl.

Microelectrodes were inserted into the outer membrane of oocytes that were placed in a perfusion bath. Oocytes exhibiting a resting membrane potential of at least -15 mV were clamped at -50, -75 or -100 mV using an Axoclamp 2A voltage-clamp amplifier (Axon Instruments Inc., USA). Output currents were recorded and analysed using WinEDR software (John Dempster, Institute of Pharmacy & Biomedical Sciences, University of Strathclyde, UK). Standard Xenopus Ringer (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 5 mM HEPES, with a pH 7.6 using NaOH) was perfused through a gravity fed perfusion system.

NMDA (10<sup>-4</sup> M) and glycine (10<sup>-5</sup> M) solution was applied for approximately 5 seconds to test for a stable response and whether oocytes had successfully expressed the NMDA receptor. Preliminary tests were taken by applying NMDA (10<sup>-4</sup> M) and glycine (10<sup>-5</sup> M) solution at varying holding potentials (-25, -50, -75 and -100 mV). For the purpose of comparison with *H. axyridis* alkaloid extract (HAE), memantine inhibition was tested by co-applying it at varying concentrations (10<sup>-8</sup> to 10<sup>-4</sup> M) with of NMDA (10<sup>-4</sup> M) + glycine (10<sup>-5</sup> M) solution (Memantine: Apexbio with purity 98%). This was done by first applying NMDA (10<sup>-4</sup> M) and glycine (10<sup>-5</sup> M) alone until a stable response was obtained then memantine was introduced starting with the lowest concentration and increasing it at 10-s intervals. Lastly, the HAE (extracted by Dr Mike Birkett at Rothamsted Research: 90% harmonine alkaloid

content) was tested in the same way as memantine using a concentration range of 0.003 µg/mL to 3 µg/mL.

## 2.6 Data Analysis

For each part of the study, i.e. systematic review and electrophysiology, data were inputted into Microsoft Excel to collate findings. The electrophysiology data were normalised as a percentage of the NMDA ( $10^{-4}$  M) + glycine ( $10^{-5}$  M) control response for each NMDA receptor subunit combination at different holding potentials. GraphPad Prism 8 (GraphPad Software, US) was used to generate graphs and trace recordings and concentration-inhibition plots were fitted with curves using the “log(inhibitor) vs. normalised response - Variable slope” non-linear regression equation.

$$\% \text{ Control response} = 100 / (1 + (IC_{50}/B)^S) \quad \text{Equation 1}$$

where **B** is the concentration of the blocker and **S** is the Hill slope. This provided estimates of  $IC_{50}$  values for memantine and HAE.

*Xenopus laevis* oocytes were injected with NMDA receptor subunit cDNA using a combination of GluN2A or GluN2B subunit cDNA with GluN1-1a subunit cDNA. Therefore, functional NMDA receptors were expressed and the effects of NMDA ( $10^{-4}$  M) + glycine ( $10^{-5}$  M) solution, memantine and the *H. axyridis* alkaloid extract (HAE) were directly applied and tested using the TEVC technique.

### 3 Results

#### 3.1 Systematic review of clinical trials of memantine

The pipeline followed and number of studies excluded and included is presented in Figure 3.1.1 below. Seventy-one studies were initially identified. However, after excluding studies with no published or incomplete results, 10 studies were finally included for review.

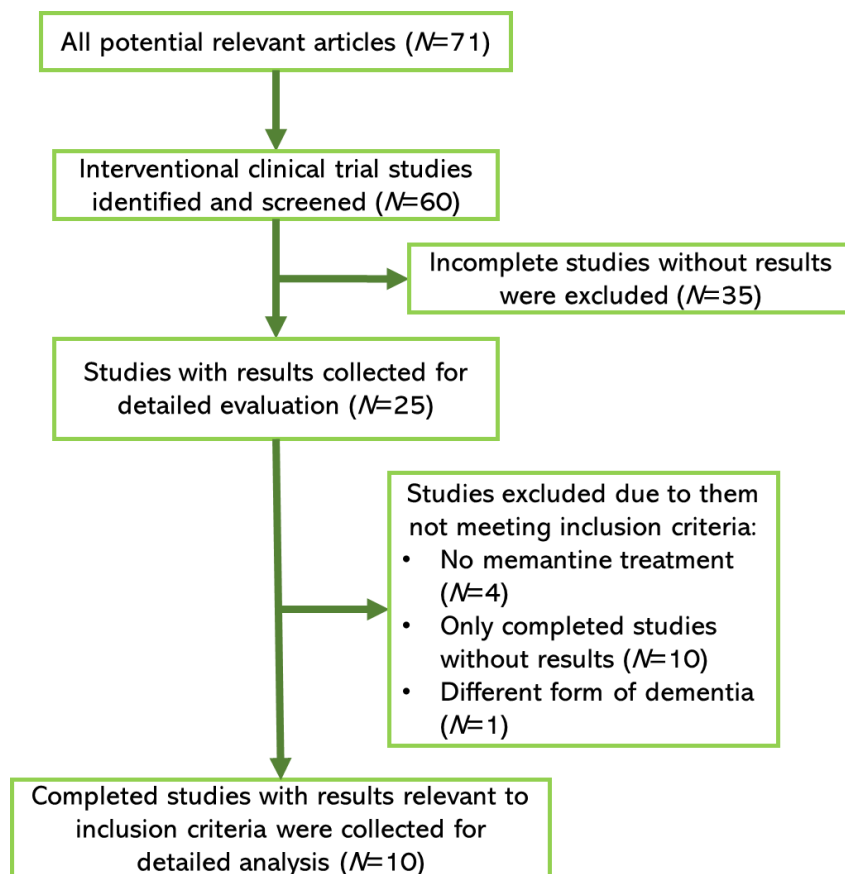


Figure 3.1.1 - Flow diagram demonstrating the process of identification, screening, eligibility, and included articles

### 3.2 The findings of clinical trials of memantine

Of the 10 reviewed clinical trial studies of memantine, five studies used molecular biomarkers or imaging as a primary outcome. These studies are described in Table 3.2.1. Three studies measured molecular biomarkers (Gordon, 2011; Glodzik, 2011; Yesavage 2010). Gordon (2011) conducted a 58-week observation study using 12 participants ranging from 50 years and older. Gordon (2011) used single group assignment and administered all patients with a stable dose of cholinesterase inhibitor over 24 weeks, followed by another 24 weeks on memantine (20mg) in combination with a stable dose of cholinesterase inhibitor as described in Table 3.2.1. The Wilcoxon signed-rank test was used to examine the change between various metabolite ratios pre- and post-treatment, in order to identify whether the rate of change differed while on monotherapy as compared with combination therapy, demonstrating statistical significance ( $p < 0.05$ ). There was a statistical correlation found between the changes in MRS and/or CSF biomarkers and an improvement in cognitive function in patients. Furthermore, NAA ratios were also calculated by MRS. NAA ratios were found to increase after memantine treatment. This increase in NAA within the hippocampus suggests that there is an improvement in neuronal viability, demonstrating that memantine has a positive effect on participants.

Glodzik (2011) recruited 17 participants aged 55-90 years old. The treatment group was given 20 mg/day of memantine per day and the second group were administered 2 placebo tablets of the same dose. Interventions for both groups were carried out for 16 weeks. NAA/Cr ratios in the hippocampus were measured and found to increase by 0.23 from pre-treatment to post-treatment for the memantine treatment group. This suggests that neuronal integrity was improved for participants treated with memantine compared to those in the placebo group, providing further evidence demonstrating that memantine has a positive

effect on AD participants. However, a p-value was not calculated thus, statistical significance cannot be compared with the findings from the Gordon *et al.* (2011) study,

Table 3.2.1 - Molecular Biomarkers and Imaging Studies collected for further analysis. Details of clinical trials used are presented in the following columns: Study/Country, Treatment/Intervention, Outcome, Results/ p-value (<0.05).

| Study/<br>Country                            | Title                                                                                                                                                                                                                                               | Treatment                                                                                                                                                                                                                                                                                                                                                                                                                    | Outcome                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Results/p-<br>value |
|----------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| Gordon<br>M.L.,<br>2011,<br>United<br>States | An Open-label<br>Exploratory Study<br>with Memantine:<br>Correlation Between<br>Proton Magnetic<br>Resonance<br>Spectroscopy,<br>Cerebrospinal Fluid<br>Biomarkers, and<br>Cognition in Patients<br>with Mild to<br>Moderate Alzheimer's<br>Disease | 24-week observational<br>lead-in period, wherein<br>patients already on a stable<br>dose of donepezil,<br>rivastigmine, or<br>galantamine continue on<br>that dose, followed by a 24-<br>week open-label<br>memantine period, wherein<br>patients receive open-label<br>memantine treatment<br>titrated to a dose of 10 mg<br>orally b.i.d., in addition to<br>their ongoing stable<br>cholinesterase inhibitor<br>treatment | <p><b>Ratios</b> of myo-inositol (mI), N-acetylaspartate (NAA), total creatine (Cr), and choline (Cho) by single voxel 1H MRS (proton magnetic resonance spectroscopy).</p> <p><b>Mean (<math>\pm</math> SD) metabolite levels</b> (normalized to T2-corrected water signal intensity) and metabolite ratios for Alzheimer's disease subjects at baseline (t0), after 24 weeks of ongoing monotherapy with stable-dose cholinesterase inhibitor (t1), and after another 24 weeks of combination therapy with memantine in addition to stable-dose cholinesterase inhibitor (t2).</p> <p>The <b>Wilcoxon signed-rank test</b> was used to examine whether the change between t0 and t1 differed from the change between t1 and t2 [(t2 - t1) - (t1 - t0)].</p> | <b>p &lt; 0.05</b>  |

|                                    |                                                                                                                                                               |                                                                                                                                                                                                |                                                                                                                                                                                                                                                                                                          |                                                                                                                                                                     |
|------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Glodzik. L., 2011, United States   | Effects of Memantine on the Magnetic Resonance Spectroscopy (MRS) Measures of Neuronal Integrity in Subjects at Risk for Alzheimer's Disease                  | <p><b>Memantine:</b> Participants will be asked to take memantine (20mg/day) for 16 weeks</p> <p><b>Placebo:</b> participants will be asked to take 2 tablets per day to match active drug</p> | <p>Changes in N-acetylaspartate (NAA) were measured with MRS</p> <p>NAA/Cr ratio changes from pre-to-post treatment were examined.</p>                                                                                                                                                                   | <p><b>NAA/Creatine ratio</b></p> <p>Pre-treatment-Memantine=1.35<br/>Placebo=1.86</p> <p>Post-treatment-Memantine=1.58<br/>Placebo=1.26</p>                         |
| Yesavage J.A., 2010, United States | The Effect of Memantine on Brain Structure and Chemistry in Alzheimer's Disease Patients: A Randomized, Placebo-Controlled, 52-Week Clinical Trial            | <p><b>Memantine:</b> 10mg/day</p> <p><b>Placebo:</b> 10mg/day</p>                                                                                                                              | <p>Changes in N-acetylaspartate (NAA) were measured with MRS</p> <p>NAA/Cr ratio changes from pre-to-post treatment, magnetic resonance spectroscopic imaging (MRS) measures of hippocampal n-acetyl aspartate (NAA) and magnetic resonance imaging volumetric measures (MRI) of hippocampal volume.</p> | <p><b>NAA/Creatine ratio</b></p> <p><b>Pre-treatment-</b><br/>Memantine=1.47<br/>Placebo=1.38</p> <p><b>Post-treatment-</b><br/>Memantine=1.62<br/>Placebo=1.41</p> |
| Lundbeck H., 2009, Denmark         | A 1-year Randomised, Double-blind Placebo-controlled Study to Evaluate the Effects of Memantine on Rate of Brain Atrophy in Patients with Alzheimer's Disease | <p><b>Memantine:</b> 10 mg tablets twice daily</p> <p><b>Placebo:</b> Tablets twice daily</p>                                                                                                  | <p><b>Brain Boundary Shift Integral (BBSI):</b><br/>Measures direct changes in total brain volume per visit interval</p>                                                                                                                                                                                 | <b>p = 0.9754</b>                                                                                                                                                   |

|                                 |                                                      |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                          |                  |
|---------------------------------|------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|------------------|
| Morris, J., 2009, United States | Corticolimbic Degeneration and Treatment of Dementia | <b>Donepezil treatment group:</b> Donepezil (Aricept®): 5mg/day for six weeks and if no serious side-effects increased to 10mg/day.<br><b>Memantine (Namenda) (in combination with Donepezil):</b> Drug treatment will begin with 5 mg/day of donepezil for six weeks. After 6 weeks, memantine will start at 10 mg/day and increased to its full dose only if no serious side-effects are encountered. Memantine (20 mg/day) will be added to the drug treatment regimen after the dose of donepezil has been established (i.e., at six weeks). | Hippocampal Volume Slope | <b>p = 0.095</b> |
|---------------------------------|------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|------------------|

Similarly, Yesavage (2010) also compared the metabolite ratios, pre- and post-treatment with memantine and a placebo drug, of 17 participants at ages 55-95 years. However, the memantine and placebo treatment groups were administered a lower dose of 10 mg/day, while the other two studies (Gordon, 2011; Glodzik, 2011) used 20 mg/day of memantine and/or 20 mg/day of placebo or cholinesterase inhibitors. Memantine was shown to be effective in improving the neuronal integrity of AD patients, although the increase in NAA/Cr ratios after treatment with memantine compared to the placebo group, was found to be smaller and less significant (increase of 0.15) than the improvement demonstrated in Glodzik's study (2011) (increase of 0.23). This suggests that lower doses of memantine (10 mg/day) may be less effective in improving the neuronal integrity and reducing the progression of AD in comparison to higher doses (20 mg/day). Therefore, it is concluded that memantine was found to have a positive effect on participants in all molecular studies, particularly at a dose of 20 mg per day.

Only 2 brain imaging studies were included (Lundbeck, 2009; Morris 2009), as shown in Table 3.2.1. Lundbeck (2009) used a large sample of 277 participants aged 50 years and older and administered 10 mg of memantine per day for the treatment group. The second group was given a placebo drug at the same dose. The rate of brain atrophy was examined by Brain Boundary Shift Integral (BBSI) and Magnetic Resonance Imaging (MRI) performed when cognitive tasks were given, was used to assess cognition as a secondary outcome. To further assess the direct changes in total brain volume per visit per interval, statistical analysis was conducted ( $p = 0.975$ ), suggesting that memantine did not significantly affect total brain volume per visit. Secondary outcome measures included imaging (Total Hippocampal Volume (HCV), cognitive and behavioural measures (Controlled Oral Word Association (COWAT), MMSE). Cognitive and behavioural symptoms were significantly improved in AD patients who were administered memantine for the outcome measure, COWAT ( $p = 0.035$ ). Results from other secondary outcome measures (HCV and MMSE) found no significant difference in cognitive and behavioural performance ( $p = 0.602$ ) and HCV ( $p = 0.842$ ). Therefore, memantine was not found to be effective in treating AD patients for these particular measures.

The duration of the Lundbeck (2009) study was carried out over 1 year, which was considerably longer compared to the second imaging study by Morris (2009), which was conducted over 28 weeks. Morris (2009) observed a smaller sample of 39 participants at the ages of 50 to 95 years of age and measured hippocampal slope and administered memantine alongside donepezil, a cholinesterase inhibitor. However, Lundbeck's (2009) study only administered memantine alone which is the key treatment of interest. In the Morris study, Group 1 received 5 mg/day of donepezil for 6 weeks, which increased to 10 mg/day if there were no serious side effects. Group 2 was compared and given 5 mg/day of memantine alongside donepezil for 6 weeks. Memantine was increased to 10 mg/day and then increased to 20 mg/day if there were no serious side effects after 6 weeks. Donepezil

alone was compared with the combined treatment of memantine and donepezil. The safety and tolerability of the combined treatments with memantine was statistically analysed. Memantine alone was not significantly effective and tolerable in treating AD patients safely ( $p = 0.975$ ) (Lundbeck, 2009). The rate of brain atrophy was not reduced in AD patients given memantine when compared to the placebo group. Therefore, memantine did not have a positive effect on AD patients by slowing the rate of brain atrophy. Similarly, it was not effective in conjunction with Donepezil (Morris, 2009).

Cognition and behavioural symptoms were assessed in 4 out of the 10 included studies. Dysken (2012) compared the effects of memantine, vitamin E in the form of dl-alpha-tocopheryl acetate and the combination of both memantine and vitamin E. The last group was given matching placebos for dl-alpha-tocopherol and memantine. The other three cognitive and behavioural outcome studies recruited a sample aged 50 years or above and used a wide age range of 40 years old and older ( $N = 613$ ). In the Dysken (2012) study, the effect of vitamin E, memantine or the combination on the clinical progression of cognitive and behavioural symptoms in AD patients was assessed and compared to the placebo group. The results found that memantine in combination with Vitamin E or alone did not significantly delay the progression of clinical symptoms of AD as the p-values were not found to be significant when comparing treatment groups to the placebo. The p-values for differences in each outcome measure, MMSE, ADAS-cog and NPI, are presented in Table 3.2.3. Therefore, this study suggests that memantine does not improve or treat cognitive or behavioural symptoms in AD patients.

Table 3.2.2 - Cognition and Behavioural studies collected for further analysis. Details of clinical trials used are presented in the following columns: Study/Country, Treatment/Intervention, Outcome, Results/ p-value (<0.05).

| Study/<br>Country                                                               | Title                                                                                                                                                                                               | Treatment                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | Outcome                                                                                                                                                                                   | Results<br>/p-value                                                                                                                                                                                                         |
|---------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Dysken M.,<br>2012,<br>United<br>States,<br>Puerto Rico                         | A Randomized,<br>Clinical Trial of Vitamin<br>E<br>and Memantine in Alzh<br>eimer's<br>Disease (TEAM-AD)                                                                                            | <p><b>dl-alpha-tocopherol:</b><br/>Alpha-tocopherol<br/>(vitamin E in the form of<br/>dl-alpha-tocopheryl<br/>acetate), 2000<br/>international units (IU)<br/>per day, taken as one<br/>1000 IU hard-gelatin,<br/>liquid-filled capsules<br/>twice a day plus<br/>matching memantine<br/>placebos</p> <p><b>Memantine:</b> Memantine<br/>20 mg per day, titrated<br/>over four weeks to a<br/>maintenance dosage of<br/>10 mg pills taken twice a<br/>day plus matching<br/>vitamin E placebo</p> <p><b>Vitamin E+</b><br/><b>Memantine:</b> 2000 IU of<br/>Alpha-tocopherol<br/>(vitamin E) per day plus<br/>20 mg memantine per<br/>day.</p> <p><b>Placebo:</b> Matching<br/>placebos for dl-alpha-<br/>tocopherol and<br/>memantine</p> | <p>Mini-Mental State<br/>Examination (MMSE)</p> <p>Alzheimer's Disease<br/>Assessment Scale -<br/>Cognitive (ADAS-cog)</p> <p>Neuropsychiatric<br/>Inventory Change from<br/>Baseline</p> | <p><b>MMSE:</b> p =<br/>0.84</p> <p><b>ADAS-cog:</b><br/>Vitamin E,<br/>Placebo: p =<br/>0.10<br/>Memantine,<br/>Placebo: p =<br/>0.25 Vitamin E<br/>+ Memantine,<br/>Placebo: p =<br/>0.14</p> <p><b>NPI:</b> p = 0.94</p> |
| (a)Graham<br>S.M., 2008,<br>United<br>States,<br>Argentina,<br>Chile,<br>Mexico | A Randomized,<br>Double-Blind, Placebo-<br>Controlled Evaluation<br>of the Safety and<br>Efficacy<br>of Memantine in<br>Patients with<br>Moderate-to-<br>Severe Dementia of<br>the Alzheimer's Type | <p><b>Memantine:</b> 28mg (7mg<br/>capsule) once daily and<br/>oral administration for<br/>24 weeks.</p> <p><b>Placebo:</b> Matching<br/>placebo oral<br/>administration once<br/>daily.</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | <p>Severe Impairment<br/>Battery (SIB)</p> <p>Clinician's Interview-<br/>Based Impression of<br/>Change with Caregiver<br/>Input (CIBIC-plus)</p>                                         | <p><b>SIB:</b> p &lt; 0.001</p> <p><b>CIBIC-Plus:</b><br/>p = 0.008</p>                                                                                                                                                     |

|                                  |                                                                                                                                       |                                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                  |                                         |
|----------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------|
| Reisberg B., 2011, United States | Memantine and Comprehensive, Individualized, Patient Centred Management of Alzheimer's Disease: A Randomized Controlled Trial         | <p><b>Behavioural:</b> individualised management of AD including caregiver training. Activity therapy, cognitive simulation therapy, exercise, caregiver support, AD, Alzheimer's Disease</p> <p><b>Only Memantine:</b> 10mg twice daily</p>                                                                                 | <p>Clinician Interview-Based Assessment of Change Plus Caregiver Input (CIBIC-Plus)</p> <p>The Alzheimer's Disease Cooperative Study Activities of Daily Living Inventory Modified for Severe Dementia Abbreviated Version (ADCS-ADLsev-abv)</p> | No results-standard deviation presented |
| Pelton G., 2010, United States   | Effects of Combined Memantine (Namenda) Plus Escitalopram (Lexapro) Treatment in Elderly Depressed Patients with Cognitive Impairment | <p><b>Escitalopram:</b> 10mg/day will be given for the first week and 20mg/ day starting at week 2. <b>Memantine:</b> After two weeks on Lexapro, Memantine 5mg will be added. The dose will increase to 10mg for the second week and will be increased at a rate of 5mg per week. Memantine dosage will not exceed 20mg</p> | Change in Selective Reminding Test - Total Immediate Recall (SRT-IR)                                                                                                                                                                             | No results-standard deviation presented |

Table 3.2.3 – p-values of statistical tests calculated for three treatment groups compared to the placebo in Dysken study (2012).

| Outcome Measure | p-values per treatment group |                    |                              |
|-----------------|------------------------------|--------------------|------------------------------|
|                 | Vitamin E, Placebo           | Memantine, Placebo | Vitamin + Memantine, Placebo |
| <b>MMSE</b>     | 0.84                         | 0.84               | 0.84                         |
| <b>ADAS-cog</b> | 0.10                         | 0.25               | 0.14                         |
| <b>NPI</b>      | 0.94                         | 0.94               | 0.94                         |

Graham *et al.*, (2008)<sup>(a)</sup> used a large sample of 677 participants aged 50 and older, all of which had moderate-to-severe AD and were treated by acetylcholinesterase inhibitors. For this particular intervention, the memantine treatment group were administered 28 mg per day of memantine for 24 weeks. The placebo treatment group was given a matching placebo once daily for the same duration. The safety and efficacy of using memantine alongside acetylcholinesterase inhibitors was assessed to examine its effects on linguistic communication (Table 3.2.2). Statistical analysis suggested that memantine, particularly at a high dose of 28 mg, had a positive effect on linguistic communication and was safe to use alongside other treatments ( $p < 0.001$ - SIB;  $p = 0.008$ - CIBIC-Plus) (Table 3.2.3).

In contrast, Reisberg *et al.*, (2011) did not present p-values for statistical tests to assess differences in outcomes. However, they did quantitatively assess the effects of memantine and individualised management on the behavioural symptoms of moderate-to-severe AD patients ( $N = 20$ ) aged 50 years and older. One group was treated by individualised management of AD including activity therapy, cognitive simulation therapy, exercise, and caregiver support. The treatment group was administered 10 mg twice daily which was a common dose used in most studies discussed.

The effects of memantine on the functional symptoms of AD patients were examined in 2 out of 10 of the eligible studies (Table 3.2.4). Dysken (2012) compared the effects of memantine, vitamin E and the combination of both on progression of functional symptoms in AD patients to the placebo group. Vitamin E ( $p = 0.03$ ) was found to significantly improved functional capabilities when compared to the memantine treatment group ( $p = 0.40$ ) and placebo group ( $p = 0.49$ ) which were not found to be significantly effective. Moreover, memantine was not found to have a positive effect on AD patients and their functional capabilities.

Table 3.2.4 - Functional studies collected for further analysis. Details of clinical trials used are presented in the following columns: Study/Country, Treatment/Intervention, Outcome, Results/ p-value.

| Study/<br>Country                                       | Title                                                                                                                                                                                                                         | Treatment                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | Outcome                                                                                                 | Results/<br>p-value                                                                                                                                         |
|---------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Dysken M.,<br>2012,<br>United<br>States,<br>Puerto Rico | A Randomized,<br>Clinical Trial of<br>Vitamin E<br>and Memantine i<br>n Alzheimer's<br>Disease (TEAM-<br>AD)<br>Delaying the<br>Progression of<br>Driving<br>Impairment in<br>Individuals with<br>Mild Alzheimer's<br>Disease | <b>dl-alpha-tocopherol:</b> Alpha-tocopherol (vitamin E in the form of dl-alpha-tocopheryl acetate), 2000 international units (IU) per day, taken as one 1000 IU hard-gelatin, liquid-filled capsules twice a day plus matching memantine placebo<br><b>Memantine:</b> Memantine 20 mg per day, titrated over four weeks to a maintenance dosage of 10 mg pills taken twice a day plus matching vitamin E placebo<br><b>Vitamin E+ Memantine:</b> 2000 IU of Alpha-tocopherol (vitamin E) per day plus 20 mg memantine per day.<br><b>Placebo:</b> Matching placebos for dl-alpha-tocopherol and memantine | Alzheimer's<br>Disease<br>Cooperative<br>Study/Activities of<br>Daily Living<br>(ADCS/ADL)<br>Inventory | <b>Vitamin E,<br/>Placebo:</b> $p = 0.03$<br><br><b>Memantine,<br/>Placebo:</b> $p = 0.40$<br><br><b>Vitamin E +<br/>Memantine,<br/>Placebo:</b> $p = 0.49$ |

|                                  |                                                                                                                               |                                                                                                                                                                                                                                   |                                                                                                                                                   |                                               |
|----------------------------------|-------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------|
| Reisberg B., 2011, United States | Memantine and Comprehensive, Individualized, Patient Centred Management of Alzheimer's Disease: A Randomized Controlled Trial | <b>Behavioural:</b> individualised management of AD including caregiver training. Activity therapy, cognitive simulation therapy, exercise, caregiver support, AD, Alzheimer's Disease<br><b>Only Memantine:</b> 10mg twice daily | The Alzheimer's Disease Cooperative Study Activities of Daily Living Inventory Modified for Severe Dementia Abbreviated Version (ADCS-ADLsev-abv) | No p-value presented- used standard deviation |
|----------------------------------|-------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------|

Table 3.2.5 - Linguistic Communication studies collected for further analysis. Details of clinical trials used are presented in the following columns: Study/Country, Treatment/Intervention, Outcome, Results/ p-value (<0.05).

| Study/<br>Country                                            | Title                                                                                                                                                                  | Treatment                                                                                                                                                 | Outcome                                                                                                                   | Results/p-value                                              |
|--------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------|
| (a)<br>Graham, 2008, United States, Argentina, Chile, Mexico | A Randomized, Double-Blind, Placebo-Controlled Evaluation of the Safety and Efficacy of Memantine in Patients with Moderate-to-Severe Dementia of the Alzheimer's Type | <b>Memantine:</b> 28mg (7mg capsule) once daily and oral administration for 24 weeks.<br><b>Placebo:</b> Matching placebo oral administration once daily. | Severe Impairment Battery (SIB)<br><br>Clinician's Interview-Based Impression of Change with Caregiver Input (CIBIC-plus) | <b>SIB:</b> p < 0.001<br><br><b>CIBIC-Plus:</b><br>p = 0.008 |
| (b)<br>Graham, 2008, Australia, New Zealand, South Africa    | A Randomized, Double-Blind, Placebo-Controlled Evaluation of the Efficacy of Memantine on Functional Communication in Patients                                         | <b>Memantine:</b> 20mg (10mg twice daily) oral administration for 12 weeks<br><b>Placebo:</b> oral administration twice daily for 12 weeks                | Functional Linguistic Communication Inventory (FLCI)                                                                      | p < 0.070                                                    |

|  |                               |  |  |  |
|--|-------------------------------|--|--|--|
|  | with Alzheimer's Disease (AD) |  |  |  |
|--|-------------------------------|--|--|--|

Linguistic communication was also examined in AD patients and only 2 out of the 10 studies were relevant (Table 3.2.5). As mentioned earlier, Graham (2008)<sup>a</sup> administered to the memantine treatment group 28 mg per day of memantine for 24 weeks. The placebo treatment group was given a matching placebo once daily for the same duration. Memantine was observed to have a positive effect on linguistic communication and was safe to use alongside other treatments ( $p = 0.001$ - SIB;  $p = 0.008$ - CIBIC-Plus).

In a second study by Graham *et al.*, Graham (2008)<sup>b</sup> (Table 3.2.5) investigated the effects of memantine on linguistic communication of AD patients were examined. 264 participants were recruited and the memantine treatment group and were given a smaller dose of 20 mg/day (placebo group administered 20 mg/day of placebo) for 12 weeks, compared to Graham (2008)<sup>a</sup> who administered 28 mg per day. The Functional Linguistic Communication Inventory was used as an outcome measure, and baseline information was to compared patients' linguistic capabilities after treatment. Statistical analysis suggested a stronger dose of 28 mg a day had a better effect on the language of participants with AD. However, the results were not found to be statistically significant ( $p = 0.070$ ). Therefore, memantine at a lower dose may have not been as effective as using a higher dose to improve functional linguistic communication in AD patients. This was also a pattern found for other studies. For example, Yesavage (2010) prescribed AD patients only 10 mg per day and Lundbeck (2009) administered AD patients 10 mg twice daily.

Furthermore, Graham (2008)<sup>b</sup> conducted the study for 12 weeks compared to Graham (2008)<sup>a</sup> who conducted it for 24 weeks, therefore observations may be more thorough and statistical tests used larger data sets. Although, other studies such as Yesavage (2010) and

Lundbeck (2009) both carried out research over the duration of 1 year, suggesting a more in-depth and wider data set. Furthermore, the results may be difficult to compare and analyse as the intervention of these two studies were slightly different in that Graham (2008)<sup>a</sup> administered memantine to participants already being treated with acetylcholinesterase inhibitors.

### 3.3 Electrophysiological analysis of NMDA receptors expressed in *Xenopus laevis* oocytes

*Xenopus laevis* oocytes were injected with NMDA receptor subunit cDNA using a combination of GluN2A or GluN2B subunit cDNA with GluN1-1a subunit cDNA. Functional NMDA receptors were expressed and the effects of NMDA ( $10^{-4}$  M) + glycine ( $10^{-5}$  M) solution, memantine and the *H. axyridis* extract (HAE) were directly assessed using the TEVC technique. The current-voltage relationship of NMDA receptors expressed in *Xenopus laevis* oocytes are displayed in Figure 3.3.1.

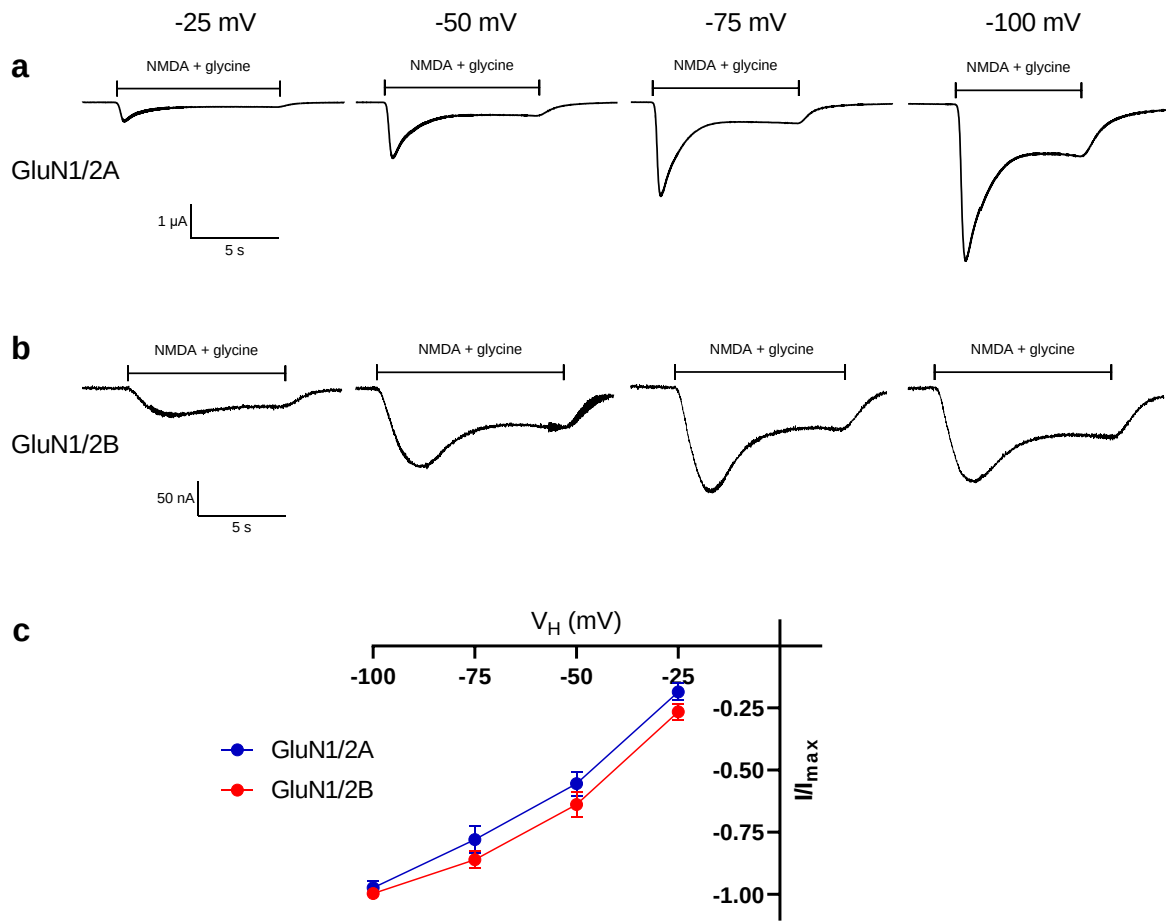


Figure 3.3.1 - Responses to application of NMDA ( $10^{-4}$  M) + glycine ( $10^{-5}$  M) solution of NMDA receptors expressed in *Xenopus laevis* oocytes. Top traces (a) show the NMDA receptor response for the GluN1-1a/GluN2A subunit combination. Bottom traces (b) show the NMDA receptor response for the GluN1-1a/GluN2B subunit combination. Responses were recorded at varying holding potentials of -25 mV, -50 mV, -75 mV and -100 mV. (c) Mean current-voltage relationships ( $n = 9-11$ ) for both subunit combinations.

Oocytes that demonstrated an NMDA ( $10^{-4}$  M) + glycine ( $10^{-5}$  M) response were recorded at various holding potentials to assess the changes in the size of response and, hence, their current-voltage (I-V) relationship. Responses which were recorded at higher holding potentials, such as -75 and -100 mV, were found to have larger peak responses compared to lower holding potentials of -25 and -50 mV (Figure 3.3.1a-b). The GluN1-1a/GluN2B receptor subunit demonstrated a smaller difference in inhibition from -75 mV to -100 mV compared to the GluN1-1a/GluN2A receptor subunit as peaks were found to be similar Figure 3.3.1b). As shown in Figure 3.3.1c, the mean current-voltage relationship

demonstrated an increase in current as the voltage became more negative but with a characteristic (to NMDA receptors) flattening of the curve at more negative holding potential.

### 3.4 The concentration-dependent effect of memantine on NMDA receptors expressed in *Xenopus laevis* oocytes using the TEVC

The application of memantine at different concentrations resulted in inhibition of the NMDA receptor response from NMDA receptors expressed in *Xenopus* oocyte in a concentration-dependent manner. As shown in Figure 3.4.1, as the concentration of memantine increased, the NMDA receptor response was reduced with the highest concentration of  $10^{-4}$  M completely inhibiting the receptor response for both subunit combinations (Figure 3.4.1). Both GluN1-1a/GluN2A and GluN1-1a/GluN2B subunit combinations have shown to respond in similar ways to memantine. This concentration dependent effect is further evident when combining data from repeated experiments using 9-13 oocytes to generate mean concentration-inhibition curves (Figure 3.4.2). Fitting Equation 1 to the data revealed  $IC_{50}$  values that were dependent on the holding potential of the oocyte (Table 3.4.1). Memantine was shown to have a greater inhibitory effect (lower  $IC_{50}$ ) on the GluN1-1a/GluN2A subunit

combination at -75 mV ( $p < 0.0001$ ) in comparison to the GluN1-1a/GluN2B subunit combination, but otherwise the  $IC_{50}$ s were similar ( $p > 0.05$ ) at -50 mV and -100

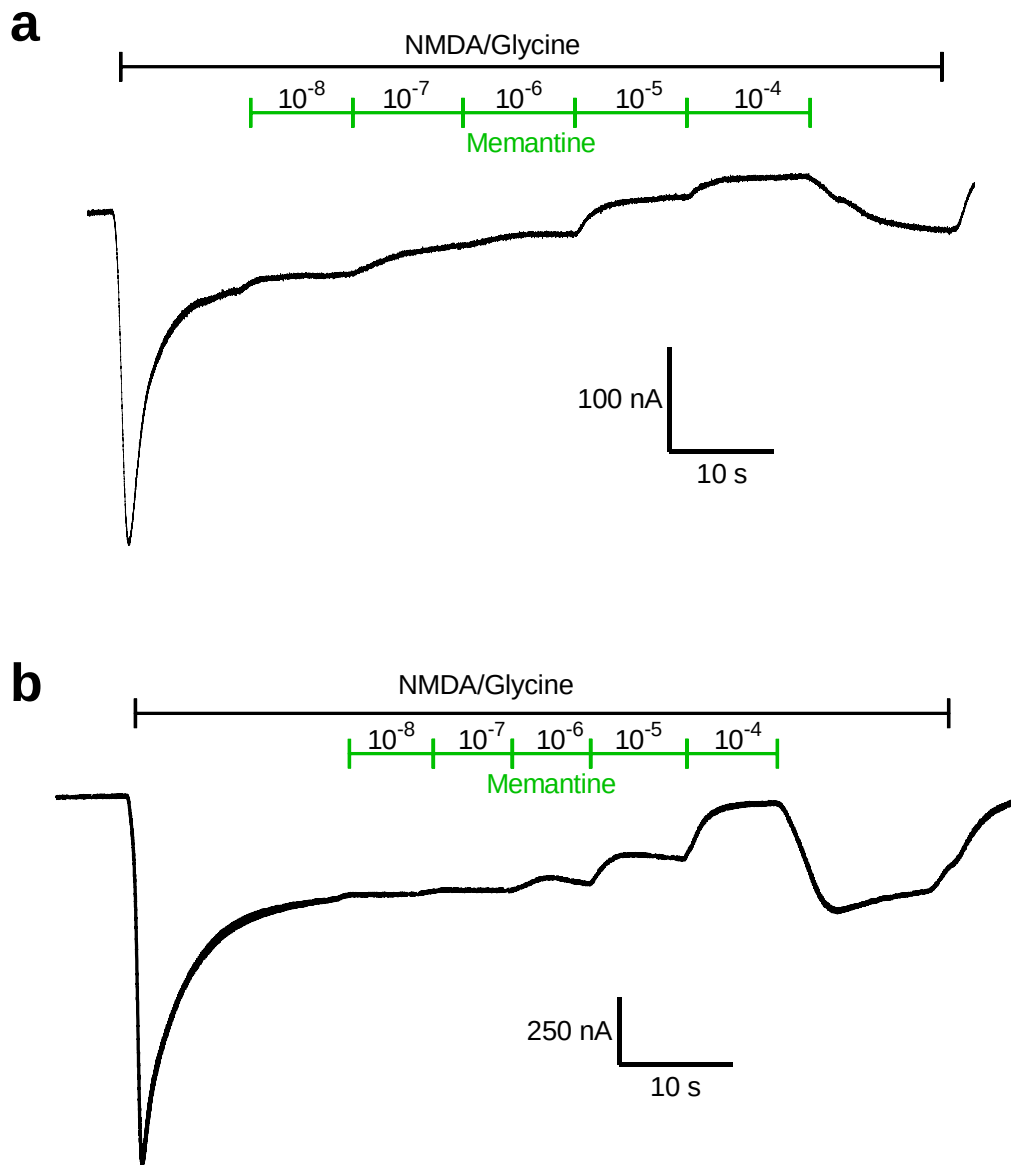


Figure 3.4.1 - Recorded responses of NMDA receptor GluN1-1a/GluN2A (a) and GluN1-1a/GluN2B subunit combinations (b) expressed in *Xenopus* oocytes to NMDA (10<sup>-4</sup> M) + glycine (10<sup>-5</sup> M) solution (black bar) followed by the application of 10<sup>-8</sup> to 10<sup>-4</sup> M memantine (green bars) for 10 secs per concentration at the holding potential of -75 mV. Each trace demonstrated a change in current once NMDA (10<sup>-4</sup> M) + glycine (10<sup>-5</sup> M) solution was applied and a reduction in current as memantine was applied at increasing concentrations.

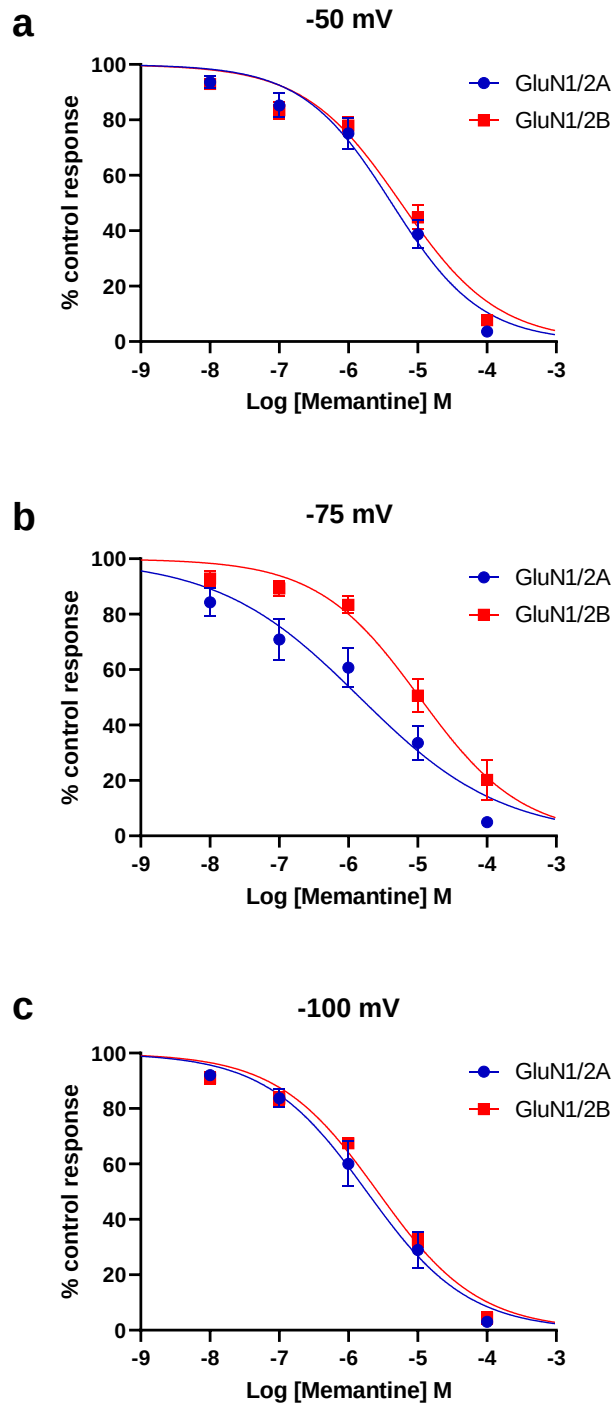


Figure 3.4.2 - Memantine concentration-inhibition curves demonstrating the concentration-dependent effect of memantine on NMDA receptors expressed in the *Xenopus* oocytes. Recordings were taken at different holding potentials: -50 mV (a), -75 mV (b) and -100 mV (c) for both GluN1-1a/GluN2A and GluN1-1a/GluN2B NMDA receptor subunit combinations. Responses were measured after applying varying concentrations of memantine from  $10^{-8}$ - $10^{-4}$  M for 10 seconds each concentration prior to the application of NMDA ( $10^{-4}$  M) + glycine ( $10^{-2}$  M) solution alone (control). The plots represent the mean  $\pm$ SEM of up to 15 replicates per NMDA receptor subunit and expressed as a percentage (%) of the control response to (NMDA ( $10^{-4}$  M) + glycine ( $10^{-2}$  M) solution). Curves were generated by fitting Equation 1.

Table 3.4.1 - IC<sub>50</sub> values and 95% Confidence Intervals for inhibition of GluN1-1a/GluN2A and GluN1-1a/GluN2B receptors by memantine. Values were estimated from curve fits to data in Figure 3.4.2.

| Subunit<br>combination  | IC <sub>50</sub> (95% CI) ( $\mu\text{g/mL}$ ) |                  |                  |
|-------------------------|------------------------------------------------|------------------|------------------|
|                         | -50mV                                          | -75mV            | -100mV           |
| <b>GluN1-1a/ GluN2A</b> | 4.19 (2.80-6.19)                               | 1.45 (0.71-2.90) | 1.84 (1.21-2.96) |
| <b>GluN1-1a/ GluN2B</b> | 5.72 (4.18-7.79)                               | 10.6 (6.63-17.2) | 2.64 (2.05-3.38) |

For GluN1-1a/GluN2A, the IC<sub>50</sub> value was lowest (Table 3.4.1) at the holding potential of -75mV, suggesting that there was a higher potency at mid-range holding potentials compared to -50 and -100mV. The holding potential of -50 mV, memantine was the least potent to GluN1-1a/GluN2A receptor subunit, also suggesting less negative membrane potential results in less inhibition and a lower potency compared to mid-range potentials of -75 mV. However, for GluN1-1a/GluN2B, unlike GluN1-1a/ GluN2A, the IC<sub>50</sub> value was lowest at -100 mV, suggesting that the optimum for GluN1-1a/ GluN2B inhibition is at a more depolarised membrane potential in comparison to GluN1-1a/ GluN2A receptors. Analysing the voltage dependence of inhibition at individual memantine concentrations supports the increase in inhibition at more negative membrane potentials. The optimum inhibition for the GluN1-1a/GluN2B receptor subunit shifted in a more negative direction, suggesting a difference in the pore region and binding site. Figure 3.4.3 demonstrates the inhibition of NMDA receptor response vs. voltage. It generally demonstrates a voltage-dependent inhibition to NMDA receptor response when holding potentials are more negative. As expected, the mid-range concentrations of memantine demonstrated the greatest voltage-dependence of inhibition. Higher concentrations caused saturation of the inhibitory effect whereas lower concentrations resulted in weak inhibition at all holding potentials.

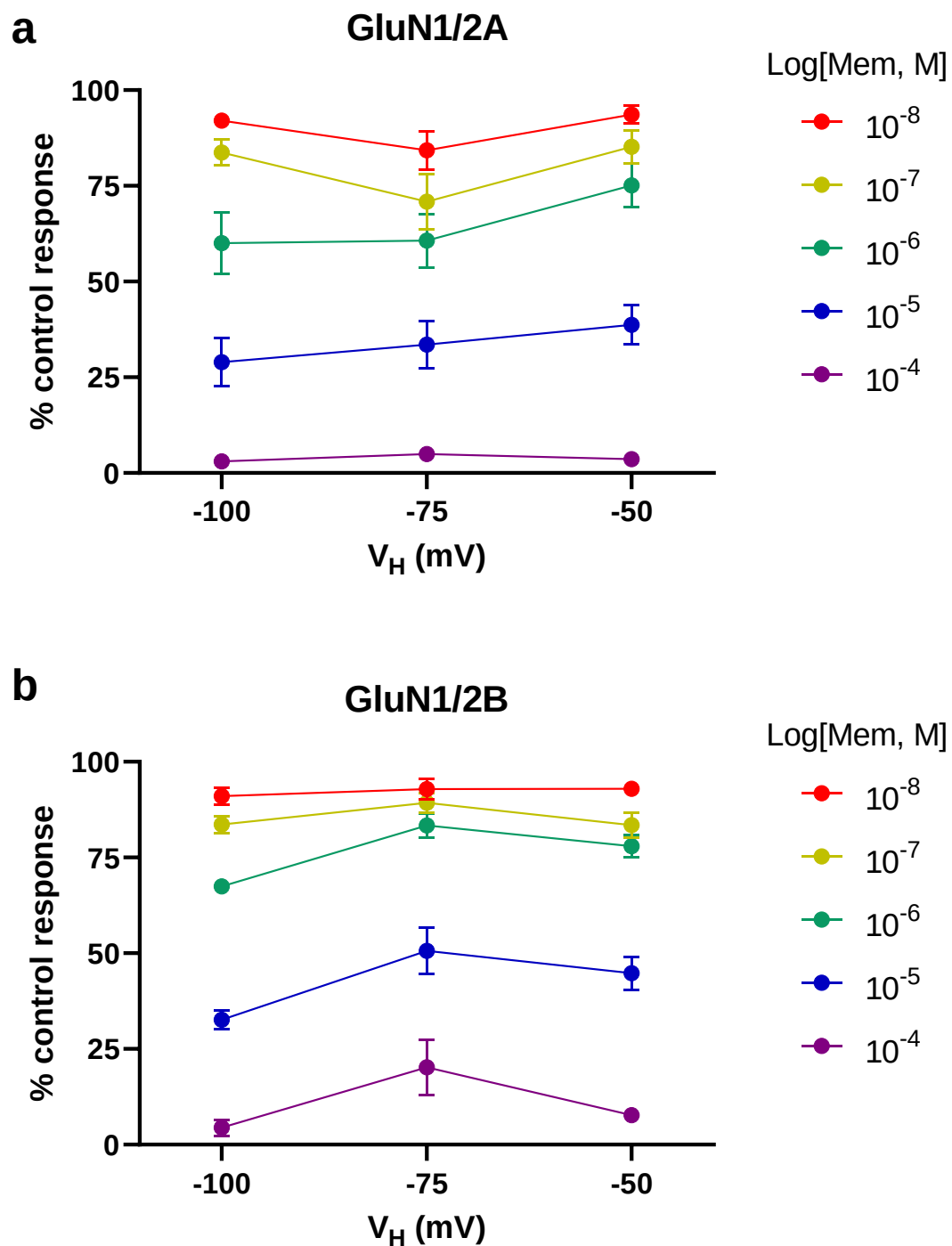


Figure 3.4.3 - Memantine inhibition (as % control response) vs. voltage plotted for each concentration of memantine tested. Recordings were taken at different holding potentials: -50 mV, -75 mV and -100 mV for both GluN1-1a/GluN2A (a) and GluN1-1a/GluN2B (b) NMDA receptor subunit combinations. Responses were measured after applying varying concentrations of memantine from  $10^{-8}$  to  $10^{-4}$  M for 10 seconds each prior to the application of NMDA ( $10^{-4}$  M) + glycine ( $10^{-5}$  M) solution alone (control = 100%).

### 3.5 The concentration-dependent effect of *H. axyridis* alkaloid extract (HAE) on NMDA receptors expressed in *Xenopus laevis* oocytes using the TEVC.

The application of different concentrations of HAE resulted in a concentration-dependent inhibition of the NMDA ( $10^{-4}$  M) + glycine ( $10^{-5}$  M) solution response. The higher concentrations of HAE (0.3 and 3  $\mu\text{g/mL}$ ) were shown to cause an increase in that saturation of inhibitory effect of NMDA receptor response, whilst lower concentrations result in weaker inhibition at all holding potentials. In both receptor subtypes, but much more so for GluN1-1a/GluN2A, the 0.03  $\mu\text{g/mL}$  concentration was observed to cause a small positive modulation of the expressed NMDA receptors instead of inhibiting them, however, this effect was reduced at the lowest concentration of 0.003  $\mu\text{g/mL}$ . In terms of the differences between the GluN1-1a/GluN2A subunit and GluN1-1a/GluN2B subunit, the stronger concentration shows to have a stronger inhibitory effect on the GluN1-1a/GluN2A subunit (shown as trace a in Figure 3.5.1) compared to the GluN1-1a/GluN2B (as shown as trace b in Figure 3.5.1) as the GluN1-1a/GluN2A was found to become almost completely blocked unlike the GluN1-1a/GluN2B.

Combining data from multiple oocytes allowed for the plotting and fitting of concentration-inhibition curves (Figure 3.5.2) in order to generate  $\text{IC}_{50}$  values (Table 3.5.1). The higher concentrations of HAE (0.3 and 3  $\mu\text{g/mL}$ ) demonstrated the most inhibition of the NMDA receptor response, which was found for both subunit combinations, however, receptors were not completely inhibited at the highest concentration. The potentiation of responses by up to 36% by 0.03  $\mu\text{g/mL}$  HAE, and to a lesser extent by 0.003  $\mu\text{g/mL}$  HAE, is also clearly demonstrated by these plots. The GluN1-1a/GluN2A subunit combination had lower  $\text{IC}_{50}$  values at -50 mV and -75 mV, but at -100 mV GluN1-1a/GluN2B had a lower  $\text{IC}_{50}$ . This suggests that HAE inhibition is more strongly voltage-dependent on GluN1-1a/GluN2B with the  $\text{IC}_{50}$  at -100 mV almost 10-fold lower than at -50 mV.

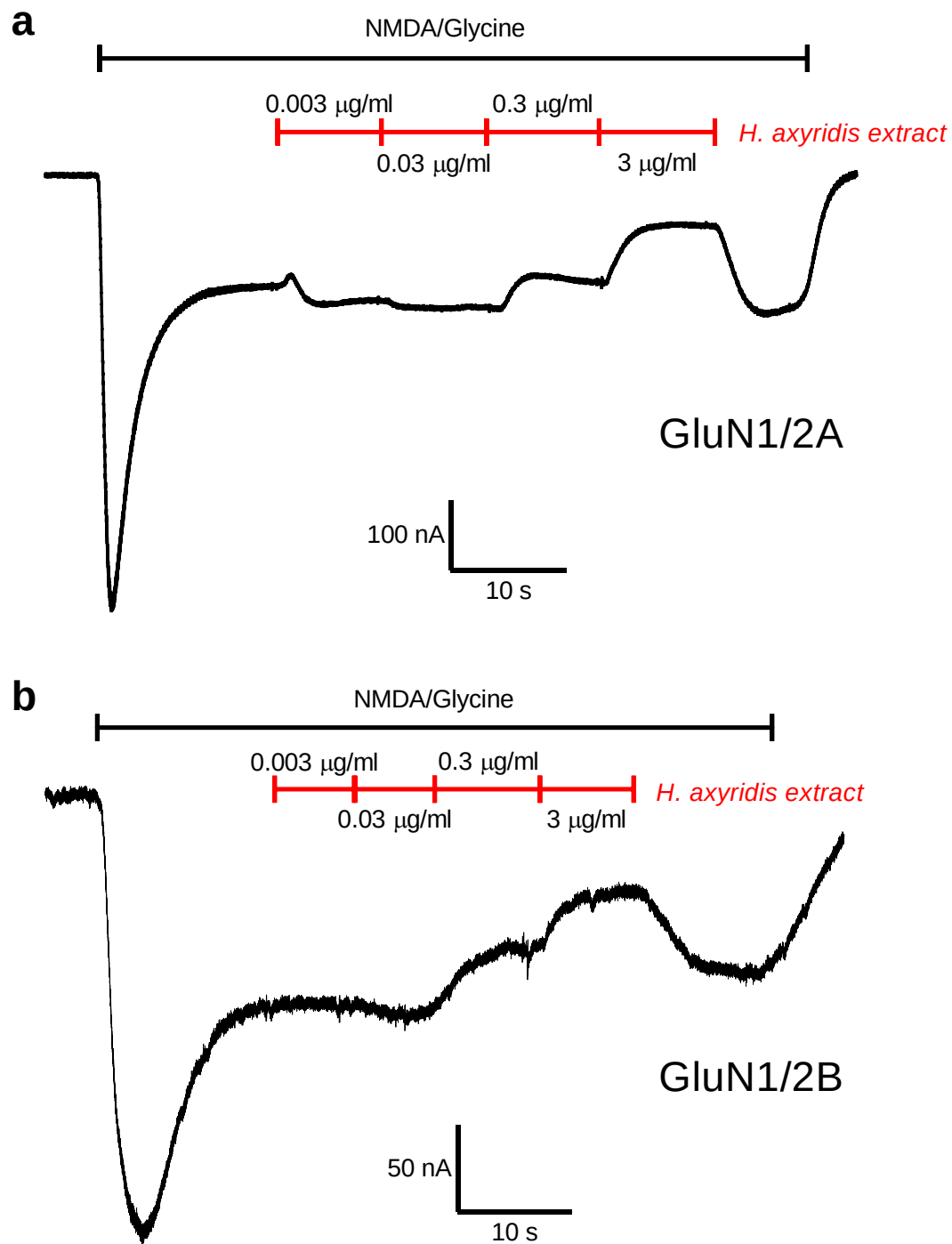
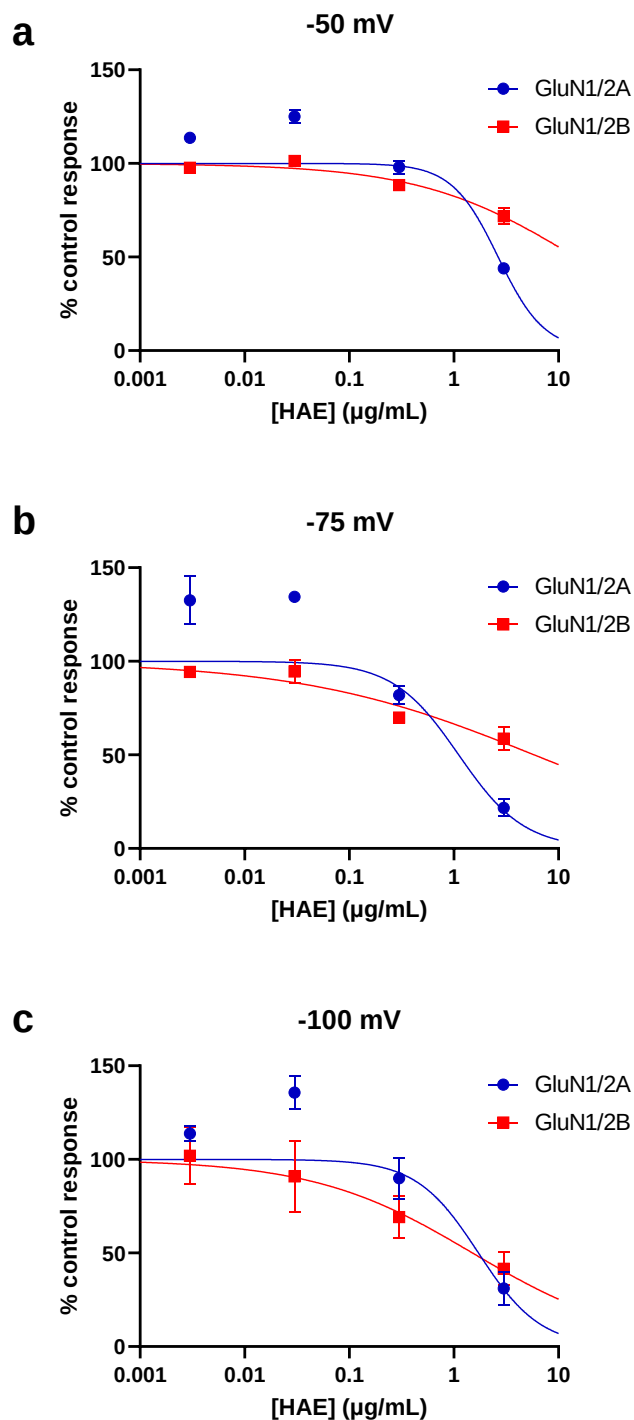


Figure 3.5.1 - Recorded responses of NMDA receptors containing GluN1-1a/GluN2A (a) or GluN1-1a/GluN2B subunits (b) expressed in *Xenopus* oocytes to NMDA ( $10^{-4}$  M) + glycine ( $10^{-5}$  M) solution (black bar) ( $10^{-4}$  M) followed by the application of HAE at 0.003, 0.03, 0.3 and 3  $\mu\text{g/ml}$  (red bars) for 10 secs per concentration at the holding potential of -75 mV.



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Figure 3.5.2 - *H. axyridis* alkaloid extract (HAE) concentration-inhibition curves demonstrating the concentration-dependent effect of HAE on NMDA receptors expressed in the *Xenopus* oocytes. Recordings were taken at different holding potentials: -50 mV, -75 mV and -100 mV for both GluN2A and GluN2B NMDA receptor subunits. Responses were measured after applying varying concentrations of memantine from 0.003, 0.03, 0.3 and 3 μg/mL for 10 seconds each concentration prior to the application of NMDA ( $10^{-4}$  M) + glycine ( $10^{-5}$  M) solution alone (control). The plots represent the mean  $\pm$ SEM of up to 15 replicates per NMDA receptor subunit and expressed as a percentage (%) of the control response to NMDA ( $10^{-4}$  M) + glycine ( $10^{-5}$  M) solution. Curves were generated by fitting Equation 1.

Table 3.5.1 - IC<sub>50</sub> values and 95% Confidence Intervals for inhibition of GluN1-1a/ GluN2A and GluN1-1a/ GluN2B receptor responses by HAE. Values were estimated from curve fits to data in Figure 3.5.2.

| Subunit<br>combination  | IC <sub>50</sub> (95% CI) (μg/mL) |                   |                  |
|-------------------------|-----------------------------------|-------------------|------------------|
|                         | -50 mV                            | -75 mV            | -100 mV          |
| <b>GluN1-1a/ GluN2A</b> | 2.65 (1.37-5.47)                  | 1.11              | 1.72             |
| <b>GluN1-1a/ GluN2B</b> | 14. 6 (6.67-68.7)                 | 5.87 (2.15-49. 7) | 1.51 (0.34-69.2) |

The inhibition of NMDA receptor response vs. voltage was plotted to assess the voltage dependence in more detail at each concentration of HAE. Figure 3.5.1 demonstrates a voltage-dependent inhibition of GluN1-1a/GluN2B receptor responses as the more negative holding potentials such as -100 and -75 mV showed a higher level of inhibition of NMDA receptor response. This pattern was most obvious for higher concentrations of HAE, such as 0.3 and 3 μg/mL (Figure 3.5.3b). For stronger concentrations of HAE, the holding potential of -75 mv was reported to have the greatest effect on the GluN1-1a/ GluN2A subunit combination (Figure 3.5.3a). This is shown for both highest concentrations of HAE, 0.3 and 3 μg/mL (Figure 3.5.3a). Therefore, HAE is more potent at mid-range holding potential compared to more negative potentials of -100 mV.

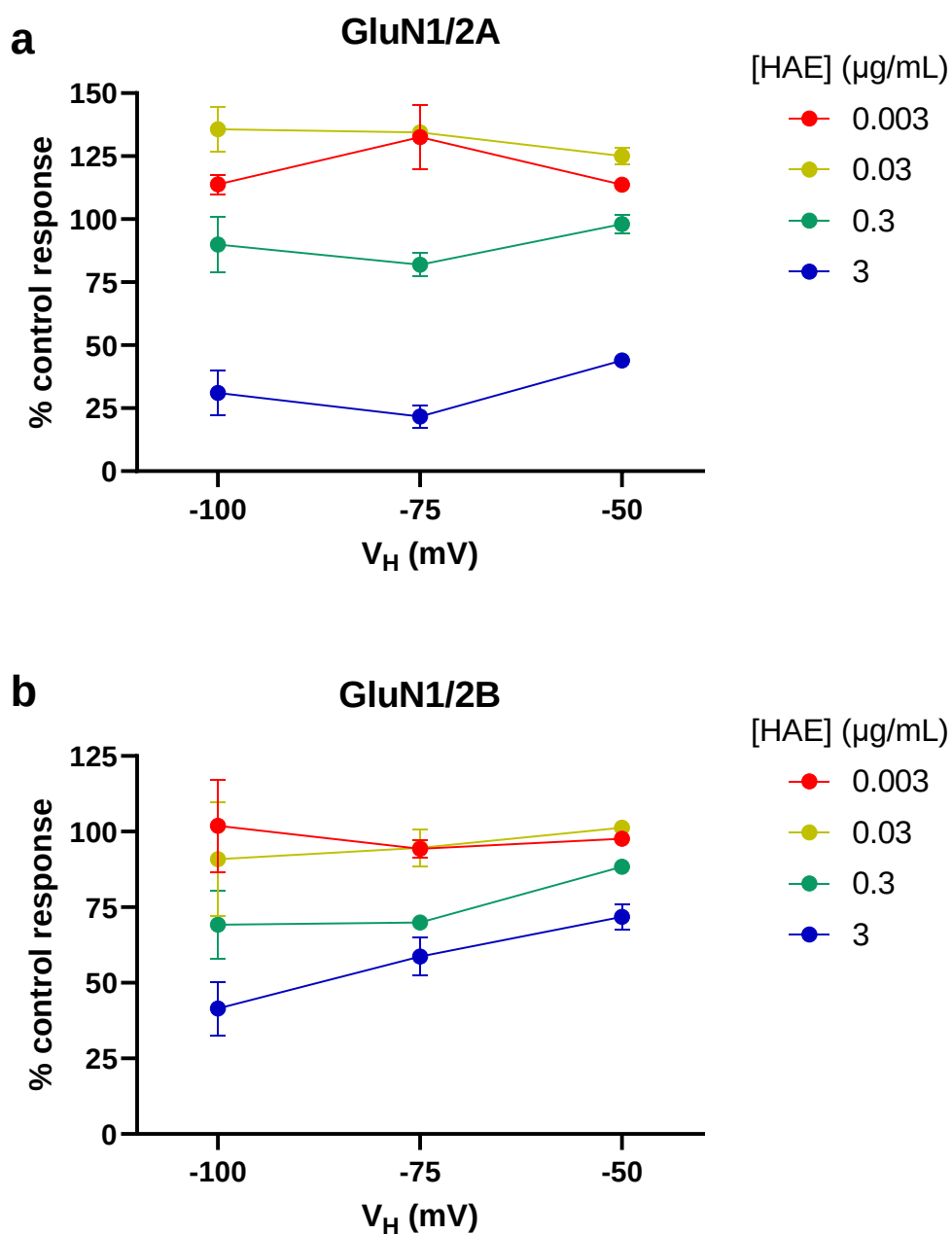


Figure 3.5.3 - HAE inhibition (as % control response) vs. voltage plotted for each concentration of HAE. Recordings were taken at different holding potentials: -50 mV, -75 mV and -100 mV for both GluN1-1a/GluN2A (a) and GluN1-1a/GluN2B (b) NMDA receptor subunit combinations. Responses were measured after applying varying concentrations of memantine from 0.003, 0.03, 0.3 and 3  $\mu\text{g/mL}$  for 10 seconds each concentration prior to the application of NMDA ( $10^{-4}$  M) + glycine ( $10^{-5}$  M) solution alone (control).

## 4 Discussion

### 4.1 Memantine Clinical Trials

The main findings of the systematic review of clinical trials of memantine were that memantine did have a positive effect on participants with Alzheimer's disease in terms of improving or delaying pathological AD symptoms. One key observation was that the most common dose of memantine that was administered to AD patients was 20 mg per day. Memantine was administered differently *per* intervention whether the full dose was given to treatment groups or began at 5 mg/day and then increased to analyse the side effects at different doses. However, most studies did administer a final dose of 20 mg per day of memantine. Higher doses of 28 mg were given in Graham's (2008)<sup>a</sup> study (Table 3.2.2), which demonstrated a more positive effect on patients compared to studies which use only 10 mg per day (e.g., Yesavage, 2010). This suggests that a higher dose of memantine may be more appropriate.

Another variation between the clinical trial studies reviewed was the age range of participants recruited. Dysken (2012) (Table 3.2.2) used a sample with the widest age range (40 years+), making it more representative of younger AD patients. However, all other studies used a sample aged 50 and older. Therefore, younger AD patients were not included, and the results are only representative of aged late onset AD populations.

Similarly, the length of interventions varied between studies. For example, Lundbeck (2009) measured brain boundary shift using MRI technology to measure the rate of brain atrophy over a 1-year period (Table 3.2.1). The large sample size of 277 participants over 1-year intervention means this study is very representative of the larger AD population. In contrast, Gordon (2011) only conducted a 24-week study looking at metabolite ratios using MRS.

Therefore, a smaller data set was used and changes in symptoms over longer period of time cannot be assessed accurately, as with longer clinical trial studies.

There were a number of limitations with the clinical trial studies which were chosen for analysis. For instance, some studies did not use a statistical test or present a p-value. Such, studies are harder to compare with other studies in terms of observed results. For example, Glodzik (2011) and Yesavage (2010) (Table 3.2.1) did not use a statistical test alongside the metabolite ratios that were presented. Furthermore, some studies also used other drugs in combination with memantine to investigate the combined effects, such as Vitamin E or cholinesterase inhibitors, hence studies are not directly comparable. Studies which combined memantine with other NMDA receptor antagonists (e.g., Dysken 2012) did not find combination treatment groups to be as positively effective when compared to those of which used parallel group assignment and administered treatment group with memantine alone.

Furthermore, all molecular biomarker studies, detailed in Figure 3.2.1, used a very small sample size with only 12-17 participants, resulting in less representative results for the AD patient population. There is clearly an ongoing need for more trials and research into molecular biomarkers with longer interventions and larger sample sizes to increase its representability.

In terms of the systematic review, an in-depth analysis of memantine and its effect on AD patients was researched using a wide range of tests and outcome measures, which assessed different manifestations of AD symptoms (e.g., cognition, behaviour, functional, linguistic). The range of symptoms assessed presents a broader perspective on how memantine can improve various pathological processes related to AD. Mainly participants suffering from moderate-to-severe AD were treated and can be argued are the most appropriate sample to use for the chosen clinical trials. Molecular markers and imaging

studies also give a molecular perspective of how memantine directly affects the brain in terms of metabolite ratios and brain volumes. Thus, a relationship between metabolite ratios and how they affect the delay or improvement, or symptoms can be made.

A disadvantage of this review is that only 10 studies were found to be useful in specifically looking at AD patients and the effect memantine has on the progression or delay of AD symptoms. Some studies also crossed over in terms of outcome measures and so may have been used more than once in a certain group. Furthermore, all of the studies were published between 2008-2012 and the use of more recent studies would give the latest outlook on memantine and its effect. In future, more clinical trial databases could be utilised to collect more recent trials to improve the quality of the systematic review.

#### 4.2 Memantine inhibits NMDA receptor response in *Xenopus laevis* oocytes

Memantine was shown to inhibit NMDA receptor responses in both a concentration- and voltage- dependent manner (Figure 3.4.2 and 3.4.3). The higher concentrations of memantine ( $10^{-4}$  and  $10^{-5}$  M) resulted in almost complete inhibition of both receptor subtype (GluN1-1a/GluN2A and GluN1-1a/GluN2B) NMDA responses, showing that memantine is concentration dependent as would be expected. Lower levels of inhibition of NMDA receptor response resulted from weaker concentrations of memantine ( $10^{-6}$  and  $10^{-7}$ ). For the GluN1-1a/GluN2A receptor subunit, memantine was shown to have an  $IC_{50}$  value of 1.45  $\mu$ M at -75 mV. Similar research looking into memantine also found similar  $IC_{50}$  values of 1.25, 1.4 and 2.5  $\mu$ M at membrane potentials of around -70 mV, supporting the idea that memantine is highly potent, particularly to the GluN1-1a/GluN2A NMDA receptor subtype and was hypothesised to work in a similar way (Kotermanski *et al.*, 2009; Kashiwagi *et al.*, 2002; Parsons *et al.*, 1995, Blanpied *et al.*, 1997). Although different electrophysiological methods may have been used, such as patch clamp, concentrations and holding potentials were

similar and demonstrated the same concentration-dependent pattern shown in the data (Figure 3.4.2).

A common pattern found, in terms of  $IC_{50}$  values, was that they were lower at the holding potential of -75 mV ( $IC_{50}$  value of 1.45  $\mu$ M) (Table 3.4.1) compared to the more negative potential of -100 mV ( $IC_{50}$  value of 1.84  $\mu$ M) for the GluN1-1a/GluN2A subunit. Memantine was shown to have a lower potency at a more positive membrane potential of -50 mV as the  $IC_{50}$  value was the highest (4.19  $\mu$ M) for the GluN1-1a/GluN2A subunit. In conjunction, there is the phenomenon or idea of 'open channel blockers' which means that they bind to the pore when it has been opened (Johnson and Kotermanski, 2006). It is suggested that these act across a certain range, making the voltage more negative up to a certain limit, the  $IC_{50}$  values are lower suggesting a higher potency. When the binding molecule reaches its maximum inhibition, the voltage driving force acts strongly on blocking molecules, pushing them through the pore, reducing some of their inhibitory effect. This results in the permeation and leakage through the pore; thus, more negative voltages may result in higher  $IC_{50}$  values. This can be observed in the data reported in Table 3.4.1. This may explain why for higher concentrations of memantine, mid-range holding potentials of -75 mV were reported to have a lower  $IC_{50}$  value, suggesting a higher potency at more negative membrane potentials. Furthermore, the optimum inhibition shown for the GluN1-1a/GluN2B receptor subunit shifted in a more negative direction compared to the GluN1-1a/GluN2A receptor subunit suggesting differences in binding. Although the protein sequence at the pore regions is very similar, there may be small difference in receptor structure and shape. For instance, the pore region may be smaller, effecting the binding action of memantine in terms of dissociation and recovery rates.

Memantine demonstrated a concentration-dependent effect on the GluN1-1a/GluN2B receptor response, the more negative the membrane potential, the higher the levels of

inhibition. At -100 mV, the IC<sub>50</sub> value was calculated to be the lowest at 2.64  $\mu$ M, which is similar to values found in literature (Parsons *et al.*, 1995) (Table 3.4.1). Therefore, there was a difference found in the binding action of memantine between different receptor subtypes. Memantine was most potent at -100 mV for GluN1-1a/GluN2B and not mid-range potentials of -75 mV as for the GluN1-1a/GluN2A subtype.

Another key finding was that memantine demonstrated voltage-dependent inhibition (Figure 3.4.3), found for the mid to higher concentrations of memantine. Memantine bound to both receptor subunit combinations in this way, supporting the idea that memantine primarily acts upon the Mg<sup>2+</sup> ion blocking sites located at the selectivity filter, i.e., it selectively bound to the N-site of the GluN1-1a and GluN2 subunits. The electrophysiological traces (Figure 3.4.1) combined with the concentration-inhibition graphs (Figure 3.4.2) correlate with the finding that binding at the asparagine residues forming the selectivity filter of either GluN1 or GluN2 subunits, results in the moderate voltage dependence of memantine blockade and action (Chen and Lipton, 2005).

#### 4.3 *H. axyridis* alkaloid extract (HAE) inhibits NMDA receptor response in *Xenopus laevis* oocytes

Interestingly, HAE has also been shown to act in a similar manner to memantine. HAE also has antagonistic inhibitory effects on the NMDA receptors expressed in *Xenopus laevis* oocytes (Fig. 3.5.1). As shown in Figure 3.5.2, the higher concentrations of HAE responded in a similar way to the higher concentrations of memantine, suggesting their actions of binding to the NMDA receptor are similar. The GluN1-1a/GluN2A subtype was almost completely blocked by the highest concentrations of HAE unlike the GluN1-1a/GluN2B subtype, suggesting it may bind to the GluN2A subunit differently, due to differences in the pore regions situated at the N-site of the GluN1-1a and GluN2 subunits, which memantine

primarily binds to. These differences may occur around the pore regions of the receptor subunits in terms of structure (since amino acid sequence is identical in this receptor resulting in different binding actions with *H. axyridis* alkaloid compounds. GluN1-1a/GluN2A receptor subtypes may have a better selectivity filter than GluN1-1a/GluN2B receptor subtypes, for the process of binding to HAE and other NMDA receptor antagonists that work similarly.

In comparison to memantine, HAE was observed to inhibit NMDA receptor response only partially at the highest concentration (3 µg/mL), whilst the highest concentration of memantine ( $10^{-4}$  M) demonstrated almost complete inhibition of NMDA receptor response, although, the highest concentration of HAE was approximately 10 times lower the memantine (see conversion of concentrations in Ch2.6, pg 43, Equation 1). Nevertheless, HAE was shown to act in a similar way to memantine in terms of mechanism of action. Both binding molecules demonstrated concentration- and voltage-dependent inhibition, suggesting that HAE binds to the NMDA receptor subunit at the pore in the same way as memantine. This is further supported by the similar voltage dependence profiles of HAE and memantine, in particular for the GluN1-1a/GluN2A subtype as HAE was found to be more potent at -75 mV ( $IC_{50}$  value = 1.11 µg/mL) compared to the more negative membrane potential of -100 mV ( $IC_{50}$  value = 1.72 µg/mL) (Table 3.5.1), like memantine. Thus, HAE (harmonine) may also permeate the NMDA receptor pore at highly negative membrane potentials.

However, there were differences between GluN1-1a/GluN2A and the GluN1-1a/GluN2B subunit combinations as the highest level of inhibition was observed at different holding potentials. At -75 mV holding potential, GluN1-1a/GluN2A NMDA receptor response was inhibited the most by HAE, whereas GluN1-1a/GluN2B NMDA receptor response was reduced at the most negative holding potential of -100 mV. This suggests there may be

differences between subunit combinations in terms of structure and binding. Concentration-inhibition curves for HAE (Figure 3.5.2) show differences between subunit combinations. The curves for the GluN1-1a/GluN2A subunit combination show that at lower concentrations there is a positive modulation of the responses to NMDA/glycine while this was negligible for GluN1-1a/GluN2B. This means that GluN1-1a/GluN2A mediated synaptic transmission may be enhanced while the pathological extra-synaptic activation of GluN1-1a/GluN2B is inhibited. This may be a therapeutically beneficial feature of harmonine, assuming that this is the major active component of HAE. Another therapeutic advantage of harmonine is that it has been demonstrated to also inhibit acetylcholinesterase, the target of other AD medications. This gives the molecule multi-target properties that could tackle AD by more than one mechanism. Indeed, much research has focussed on this very promising approach to address numerous diseases including AD.

For the latter, molecules have been designed to incorporate multiple moieties that individually target aspects of AD such as antioxidants, acetylcholinesterase inhibitors, A $\beta$  peptide disaggregators and NMDA receptor inhibitors. Smaller multi-target molecules were designed by combining memantine with the chemical features of neuroprotectants which act via alternative mechanism of action. Examples include anti-oxidant and anti-aggregating activity or acetylcholinesterase inhibition (Marotta *et al.*, 2020).

HAE demonstrated voltage-dependent inhibition in both receptor subunit combinations (Figure 3.5.3), as the more negative the holding potential, the greater the inhibition of NMDA receptor response was observed. This implies that HAE may act as an open channel blocker and acts in a similar way to memantine, forming a case for the harmonine alkaloid to be researched further. Furthermore, HAE has an inhibitory effect on the acetylcholine receptor response in TE671 cells and locust neurons. Different methods were used rather than TEVC, however, HAE was shown to inhibit other receptors related to AD (Patel *et al.*, 2020).

Harmonine was also found to have an inhibitory effect on acetylcholinesterase, prolylendopeptidase and neuraminidase, which have been considered to be therapeutic targets of AD, suggesting harmonine has therapeutic potential in treating AD (Alam *et al.*, 2002).

#### 4.4 Comparison between memantine and HAE inhibition

Although the concentrations used for memantine and HAE demonstrated similar concentration-dependent inhibition, the highest concentration of the HAE (3 µg/mL) (Figure 3.5.1) does not completely inhibit the NMDA receptor as does with the highest concentration of memantine (Figure 3.4.1). This suggests that using higher HAE concentrations in future research may be more effective in demonstrating effects on the NMDA receptor. For GluN1-1a/GluN2A this could be, in part, due to the positive modulation of the receptor by HAE which is not seen with memantine. However, the biggest problem with comparison between memantine and HAE potency is that the latter is an extract which is likely to contain several alkaloid compounds present, hence the discrepancy in concentration units. Since HAE is reported to be approximately 90% harmonine, a better comparison could be made by converting the HAE concentration to M based on the molecular weight of harmonine (293 Da) (Richards, 2015). This means that 3 µg/mL, the highest HAE concentration used) equates to 10.2 µM. Comparing inhibition of both NMDA receptor subtypes at 10 µM (memantine) and 3 µg/mL (HAE) suggests that it is not dissimilar.

#### 4.5 Alkaloids contribution to future research in NMDA antagonist treatment for neurodegenerative and psychiatric diseases

Alkaloids and other substances extracted from insects can widen the field of research into treatments for neurological and psychiatric disorders, including AD. The main positive attribute is that extracted compounds from insects are more natural and are less likely to cause side effects in the way that memantine can. However, there is no evidence to suggest that there are no side effects when treated with extracted compounds from insects as of yet. Spider and wasp venoms have also been shown to have similar inhibitory effects on the NMDA receptor, associated with high glutamate excitotoxicity, however, there is still a need for more research and evidence to support this.

If the research conducted is reproduced in the future, it is suggested that using a larger range of concentrations of HAE, particularly stronger concentrations would be worth investigating. Higher concentrations of HAE may result in complete inhibition in the same way as demonstrated by  $10^{-4}$  memantine. Furthermore, analysing the onset times, dissociation rates and recovery times could be researched in future projects, as these are key to gaining a more in-depth understanding of the binding of memantine to the NMDA receptor and its different subunit combinations. Memantine is currently therapeutically tolerated as it is voltage-dependent and has the appropriate dissociation rates and recovery times. This should be considered and when investigating naturally occurring experimental compounds, found in insects, that target NMDA receptors (e.g. harmonine).

Although not highly potent, harmonine has been shown to affect the neurotransmission mediated by nicotinic acetylcholine receptors, suggesting it may have other therapeutic and antagonistic properties (Patel *et al.*, 2020; Alam *et al.*, 2002). HAE was found to be more potent when applied to both NMDA receptor subtypes, GluN1-1a/GluN2A and GluN1-

1a/GluN2B at a stronger concentration (3 µg/mL), as there was a clear voltage dependent inhibition of the NMDA response. Weaker concentrations of HAE had less of an inhibitory effect and demonstrated modulatory effects by increasing NMDA receptor response. These factors could be researched further.

#### 4.6 Future work

There are multiple alkaloids present in the haemolymph of ladybird beetles, which still have the potential to be explored and researched in all fields of Neuroscience. There is potential for harmonine to be investigated further to examine its inhibitory effects on the NMDA receptor and other receptors involved in the pathology of neurological diseases. Higher concentrations of HAE could be investigated to assess the possibility of complete inhibition of the NMDA receptor as only partial inhibition was observed in this study. Different analogues of harmonine have been examined to assess their effects on acetylcholine receptors and were found to have an inhibitory effect (Nagel, 1987). Further research on other analogues of harmonine could investigate their effect on NMDA receptor response. Different concentrations and analogues of HAE could potentially result in complete NMDA receptor response as observed by memantine. This would further support the notion that harmonine has many therapeutic inhibitory properties for multiple receptors related to disease.

It is proposed that there is a need for more clinical trials for Alzheimer's disease, and the testing of the effects of memantine on all key symptoms of AD. Molecular biomarkers and imaging result in accurate and reproducible results. They also give a deeper molecular perspective of memantine's effect on metabolites in AD patients. In addition, a wider range

of databases containing clinical trials for AD should be explored, which will no doubt result in the comparison and discussion of more recent trials.

## **5 Conclusions**

The systematic review found that memantine was effective in delaying or improving the symptoms of AD patients assessed in clinical trials, particularly for higher doses of memantine and when administered alone rather than in combination with other NMDA receptor antagonists. The electrophysiology study confirmed that memantine is a concentration- and voltage-dependent inhibitor of NMDA receptor responses in two different receptor subtypes. Interestingly, HAE demonstrated very similar effects on both receptor subunits and acted in the same way as memantine. This research has created a case that harmonine (and possibly other HAE components) have therapeutic inhibitory effects on NMDA receptors with the added benefit that it targets other receptors related to the pathology of AD such as acetylcholine receptors, as well as being an acetylcholinesterase inhibitor.

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