# The Electrophysiology of Ionotropic Glutamate Receptors Expressed in Human Cell Lines, HEK293 and TE671

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## Abstract

Ionotropic glutamate receptors consist of NMDA, AMPA and kainate receptors and are responsible for the conduction of synaptic transmission, long term potentiation, and synaptic plasticity and are implicated in a wide range of neurological diseases. Human models of these receptors are difficult to produce, and the majority of the data gathered is from the expression of these receptors in *Xenopus* oocytes and mice and rat models, which produce data that is less applicable to humans. There is a requirement of human models of these receptors, due to their associations with various human diseases, so it is necessary to produce valid and applicable results. This study demonstrates two potential human *in vitro* models, one for kainate receptors in the human cell line HEK293, and another for potentially all three types of ionotropic glutamate receptor in the human cell line TE671. The kainate receptor subunit GluK2 was expressed in HEK293 cells after transfection with the Escort IV reagent and our evidence suggests that all three ionotropic glutamate receptors are expressed in TE671 cells that have been differentiated by db-cAMP. The differentiated TE671 cells present an altered morphological cell type, that identify more with neuronal cells, rather than muscle cells, which are the usual morphological phenotype of TE671 cells under normal physiological conditions. The response of native nicotinic

acetylcholine receptors in TE671 cells is also altered when the cells are differentiated, to produce a more neuronal nicotinic acetylcholine receptor type response rather than the muscle type. These findings provide potential human models of a variety of ionotropic glutamate receptors, which can be used in future research of these receptors, particularly in the pathophysiology of human disease, including schizophrenia, Alzheimer's disease, and epilepsy. These diseases have previously been associated with a range of ionotropic glutamate receptors, but the associations are not fully understood, and so the models described here provide a valuable resource in future research of how such diseases are impacted by ionotropic glutamate receptors.

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# Chapter 1

# Introduction

## 1.1 Neurons, Synapses and Neurotransmitter Mechanism of Action

### 1.1.1 The General Mechanism of Action of Neurons

The main cells of the central nervous system (CNS), known as neurons, transmit chemical and electrical signals throughout the brain via synaptic clefts, where two neurons meet (Figure 1). The transmission of chemicals (neurotransmitters) between neurons can lead to the opening or closing of ion gated channels, altering the neuronal membrane potential (de Lera Ruiz and Kraus, 2015). Typically, there is a small amount of extracellular neurotransmitter released, which does not result in stimulation of the postsynaptic target cell. However, neurotransmitter is released from the presynaptic neuron in larger amounts when the action potential threshold of the presynaptic neuron is reached. The neurotransmitter binds to receptors on the postsynaptic cell and causes an excitatory or inhibitory response, depending on various factors such as the type of neurotransmitter and the type of receptor that it binds to.

### 1.1.2 How do Excitatory Synapses Function?

Excitatory ion-channel synapses involve ion-gated channels, found in postsynaptic neuroreceptors. During this process, sodium ion channels open and local depolarisation of the postsynaptic neuron occurs. If the voltage threshold (approximately 15 mV less negative than the resting potential

in excitatory neurons (Mackenna and Callander, 1990) is reached, an action potential is initiated. Excitatory postsynaptic potential (EPSP) is a term used to describe the depolarisation of postsynaptic neurons, which depends on the excitatory and inhibitory stimuli of the postsynaptic receptors. If the excitatory stimuli exceed the inhibitory stimuli, an action potential is produced, which can cause the release of neurotransmitters from the presynaptic site of that neuron, thus continuing the action potential. There are multiple known excitatory neurotransmitters including glutamate, acetylcholine, catecholamines, serotonin and histamine.

### 1.1.3 The Mechanism of Action of Inhibitory Synapses

Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter, synthesised from glutamate by the enzyme glutamic acid decarboxylase. When GABA binds to $GABA_A$ receptors, chloride ion channels open allowing influx of these anions, causing a decrease in the membrane potential. This reduces the likelihood of the postsynaptic cell reaching its action potential threshold, thereby inhibiting the cell (Everington, Gibbard, Swinny and Seifi, 2018). $GABA_B$ receptors are metabotropic (G protein-coupled) receptors and when activated by the binding of GABA, they indirectly cause the opening of potassium channels which cause potassium ions to exit the cell, thereby making the cell hyperpolarised and reducing the likelihood of the cell to reach its action potential

(Terunuma, 2018). In every region of the brain, GABA is used to fine-tune neurotransmission and when GABA or its receptors are malfunctional, it can contribute to the expression of several brain conditions such as anxiety disorders, schizophrenia, and autism (Kuffler and Edwards, 1958).

## 1.2 Glutamate Receptor Function in the Central Nervous System

### 1.2.1 What is Glutamate?

L-Glutamate (Figure 2) is a ubiquitous free amino-acid found in the brain and is the most abundant excitatory neurotransmitter in the central nervous system (CNS). Glutamate receptors can be classified into two main types, including metabotropic (mGluR), which are G protein-coupled receptors and ionotropic receptors, which are ligand-gated ion channels. Ionotropic glutamate receptors (iGluRs) can further be differentiated in three more groups including N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid (AMPA) and kainate (KA) receptors, named due to their selective agonists – NMDA, AMPA and KA.

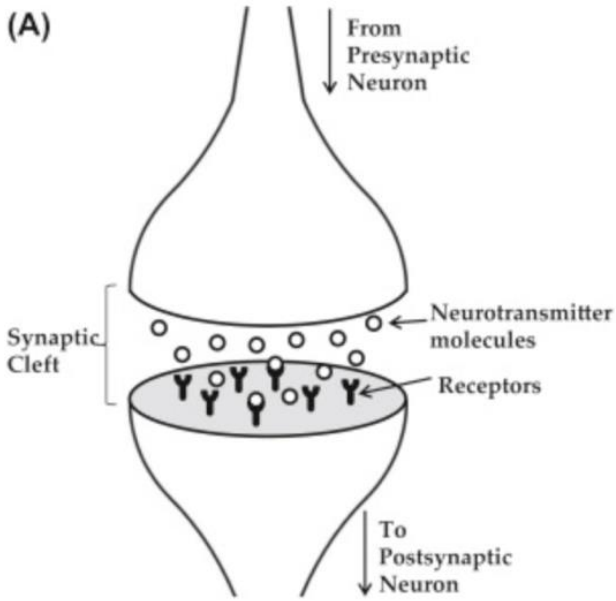



***Figure 1 – Synaptic Transmission.*** *Presynaptic neuron releasing neurotransmitters into a synaptic cleft and binding to receptors in the postsynaptic membrane. Taken from Kozma, 2018.*

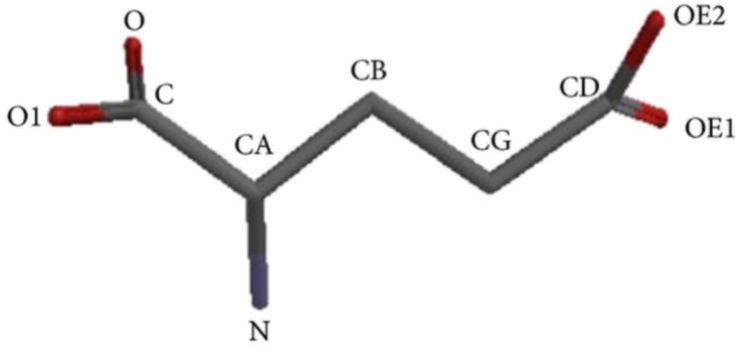



***Figure 2*** – ***Structure of L-glutamate.*** *Taken from Gonzalez and Barreto, 2013.*

### 1.2.2 The Excitatory Pathways of Ionotropic Glutamate Receptors

The general excitatory pathway of glutamate mediated by ionotropic glutamate receptors, involves an action potential in a presynaptic neuron propagating an influx of calcium ions at the nerve terminal. This leads to the release of glutamate into the synaptic cleft, where glutamate then diffuses across and binds to postsynaptic GluRs. If glutamate binds to an iGluR, sodium and/or calcium ion channels in the postsynaptic neuron will open, leading to the depolarisation of the postsynaptic cell. If this causes the membrane potential to reach its threshold, an action potential will be formed and thus, the electrical impulse will continue (Figure 3) (Zhu and Gouaux, 2017). Once the postsynaptic iGluRs are activated, the glutamate in the extracellular space (synaptic cleft) is cleared and taken up by glutamate transporters, such as excitatory amino acid transporters (EAATs) that are located on the surface of glial cells (Magi, 2019). Glutamine synthetase then converts the glutamate to glutamine, which can be released back into the extracellular space as a non-neuroactive species. The glutamine is then taken back up into presynaptic neurons, where it is converted back into glutamate – this is known as the glutamate-glutamine cycle (Bak, Schousboe and Waagepetersen, 2006).

The pathway of glutamate mediated by metabotropic glutamate receptors is different to iGluRs, as the pathway

leads to intracellular signalling cascades in the postsynaptic neuron, rather than the production of depolarising currents in the postsynaptic cell.

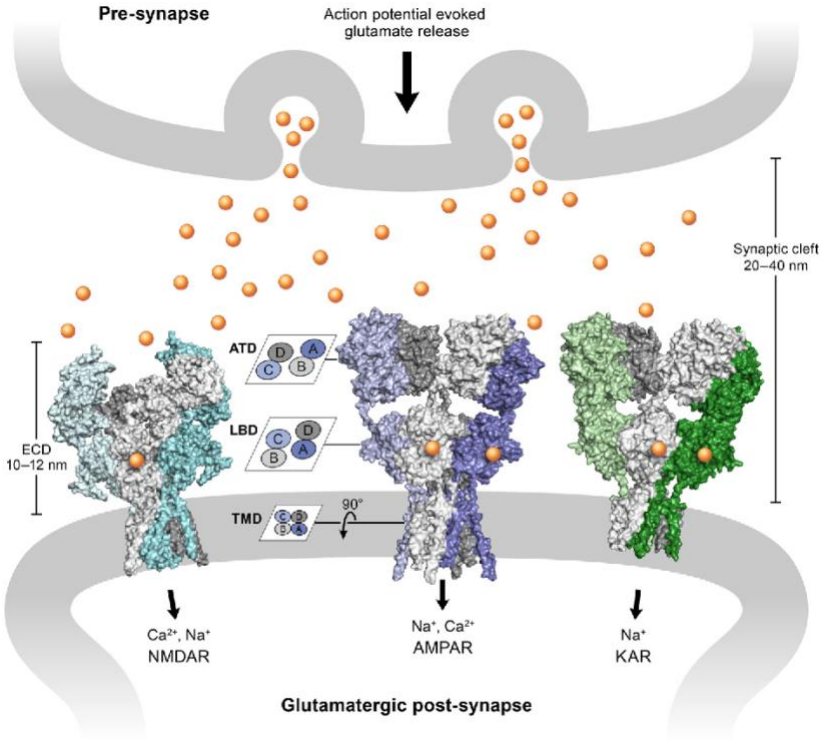



***Figure 3 – Ionotropic Glutamatergic Synaptic Transmission.*** *Presynaptic action potential triggering glutamate release into the synaptic cleft. The glutamate binds to postsynaptic ionotropic glutamate receptors, causing the opening of ion-gated channels, including sodium and calcium ion channels. Taken from Zhu and Gouaux, 2017.*

### 1.2.3 The Functions of Glutamate in the Brain

Glutamate and its different receptors have many different functions across the brain and body. The downstream effects of glutamate activating GluRs and initiating an action potential in postsynaptic neurons, include releasing brain growth factors such as brain-derived neurotrophic factor (BDNF), which modifies the neuroplasticity of the cell, calcium ion homeostasis, and the neuron's sensitivity to glutamate (Martin and Finsterwald, 2011). Glutamate receptors are abundantly found within hippocampal mossy fibres and both iGluRs and mGluRs are known to have a large role in synaptic plasticity, which plays an important part in the learning and memory functions of neurons in the hippocampus.

Dysfunctional glutamate signalling, leading to excessive glutamate excitation, is known to alter brain function. An excess of glutamate excitation is known to be excitotoxic due to the high influx of calcium ions into the cell when iGluRs are activated (Lewerenz and Maher, 2015), which contribute to multiple diseases such as neurocognitive diseases, neuropsychiatric diseases, and amyotrophic lateral sclerosis (ALS) (Foran and Trotti, 2009).

Glutamate is also released in neurons of the mossy fibre (MF) pathway within the hippocampus. It was previously believed that the synapses in this pathway were only glutamatergic however, it has been shown that GABA is also present in this pathway. The hippocampus has multiple functions however,

the most well-known are spatial awareness, learning and memory (Witton, Brown, Jones, and Randall, 2010). When the hippocampus is damaged or has loss of function, it is known to lead to multiple neurodegenerative disorders involving memory loss and learning disabilities such as Alzheimer's disease and Autism (Dhikav and Anand, 2012).

## 1.3 How Glutamate Receptors are Involved in Disease

As mentioned, glutamate receptors have long been associated with CNS disorders, which consist of neurodevelopmental disorders, neurodegenerative disorders, and mood disorders (Bittigau and Ikonomidou, 1997). There are usually multiple factors contributing towards the causes of CNS disorders, typically involving defective excitatory and/or inhibitory neurotransmission. The different types of glutamate receptors have roles in different pathways and different effects on mental processes depending on the area of the brain they are located in. Therefore, the location of certain glutamate receptors and their ability or inability to carry out their function can lead to a wide variation of CNS disorders. There are numerous studies of different CNS disorders that are known to be caused by glutamate receptors (Zhou and Sheng, 2013; Levite, 2014), suggesting that multiple glutamate receptors are simultaneously involved in the pathophysiology of individual diseases however, the locations of the defective glutamate

receptors involved are usually slightly different for each type of disorder (Bittigau and Ikonomidou, 1997).

Glutamate receptor function is involved in many psychiatric disorders including but not limited to, schizophrenia, bipolar disorder (BD) and major depressive disorder (MDP). Schizophrenia is a psychiatric disorder which involves an array of symptoms that differ between patients, some of which include visual and auditory hallucinations, delusions, and disorganised thinking. The causes of schizophrenia are debated and complex, with the major focus on dopamine receptors and their interactions with glutamate receptors. Glutamatergic neurons activate inhibitory GABAergic neurons, which then influence dopamine release from presynaptic neurons (Deng and Dean, 2013). Data show that both mGluRs and iGluRs have roles in the pathophysiology of schizophrenia (Maksymetz, Moran and Conn, 2017; Koromina, 2019). For example, multiple deleterious single base mutations within mGluR genes have been identified in patients with schizophrenia (Saini *et al.*, 2017). Furthermore, multiple studies reported that abnormally expressed iGluRs and mGluRs are present in schizophrenic patients, including a decreased expression of AMPAR and KAR in the hippocampus of schizophrenic brains (Rubio, Drummond, and Meador-Woodruff, 2012).

Bipolar disorder (BD) is another psychiatric disorder impacted by GluRs. BD is identified by major mood fluctuations from extreme highs and extreme depressive low episodes. All three types of iGluR (NMDAR, AMPAR and KAR) are known to be involved in BD, due to a reduced expression of these receptors in the medial temporal cortex (Beneyto, 2007) and a reduced expression in the prefrontal cortex of the NMDAR subunit GluN1 (Beneyto and Meador-Woodruff, 2007). Major depressive disorder also involves reduced expression of NMDAR in the prefrontal cortex however, it is the subunit GluN2 that is less expressed rather than the GluN1 subunit. These disorders and their associated glutamate receptor expression, demonstrate the degree of the impact that the location of GluRs and their adequate or inadequate expression have over disease.

Excitotoxicity of glutamate is another cause of disease in the brain and body. The overstimulation of postsynaptic neurons due to increased iGluR activation, leads to excessive and uncontrolled influx of calcium and/or sodium ions. An excessive influx of sodium ions can lead to swelling of the neuron and then lysis (Dong, Wang, and Qin, 2009), whereas excessive influx of calcium ions leads to endoplasmic reticulum and mitochondrial modifications, which cause the activation of multiple enzymes involved in apoptotic cascades (Mattson, 2008). BDNF, as well as other neurotrophic factors are known to protect neurons against excitotoxicity by triggering the expression of antioxidant and anti-apoptotic enzymes (Tejeda

*et al.,* 2019). Glutamate excitotoxicity has been associated with a wide range of disorders including but not limited to epilepsy, Parkinson's disease, ALS, Alzheimer's disease, Huntington's disease, and psychiatric disorders.

ALS is a motor neuron degenerative disease, where inherited Cu/Zn-superoxide dismutase (SOD) mutations increase motor neuronal vulnerability to excitotoxicity. AMPAR has been implicated as the primary receptor mediating this vulnerability to oxidative stress and calcium homeostasis (Kawahara and Kwak, 2005). Huntington's disease is an inherited degenerative disease, where striatal, cortical and brainstem neurons undergo degeneration and are vulnerable to NMDAR-mediated excitotoxicity (Beal, 1988). This neurodegenerative disease is caused by mutations of the huntingtin protein, which disrupt calcium homeostasis and increase the sensitivity of NMDAR.

iGluRs have a profound impact on many neurological pathways which can lead to a range of disorders and disease, by various mechanisms of action. This provides insight into the importance of understanding the impacts iGluRs have within the human body, and thus why it is so important to develop thorough and reliable models of these receptors and the systems they are involved in.

## 1.4 How do Glutamate Receptors Influence Synaptic Plasticity and Regional Activity?

Synaptic plasticity is the ability of synapses to alter their strength, which plays a role in the formation and retention of memories in the hippocampus, where memories are programmed via the modification of synaptic strength (Takeuchi, Duszkiewicz and Morris, 2014). Hebb's rule describes the effect of the recurrent and persistent stimulation of a postsynaptic neuron by a presynaptic neuron. Under certain circumstances of repetitive stimulation, two neurons can build a type of familiarity which allows for more efficient synaptic transmission between them. Research on this phenomenon is of vital importance, considering that certain glutamate receptors have been shown to be crucial to synaptic plasticity in the cells they are located in (Bortolotto *et al*., 1999).

Long term potentiation (LTP) is a type of synaptic plasticity that has been extensively researched in the Schaffer Collateral Commissural (SCC) pathway, a neuronal pathway located in the hippocampus (Wheal, Lancaster, and Bliss, 1983). The hippocampus has roles in memory, learning, spatial awareness, and navigation and is composed of multiple structures, including but not limited to the dentate gyrus, Cornu Ammonis 1-4 (CA1-4), subiculum, parahippocampal gyrus and collateral sulcus. The dentate gyrus structure contains granule cells, which are neurons with small cell bodies that form part of

the perforant and mossy fibre (MF) pathways. These granule cells have axons that are named mossy fibres. The MF pathway is a component of the trisynaptic circuit in the hippocampus and mossy fibre long term potentiation (MF-LTP) is commonly used as a model for studying the functional roles of glutamate receptors in LTP (Kwon and Castillo, 2008). The dentate gyrus mossy fibres reach the CA3 pyramidal cells, which are the principal excitatory cells used by the CA fields in the hippocampus. The CA3 cells transmit synaptic signals via Schaffer collateral axons in the Schaffer collateral commissural (SCC) pathway and the synapses formed here between CA3 and CA1 neurons, are used frequently to study synaptic plasticity (Bliss, Collingridge and Morris, 2014). LTP is extensively researched between these CA3 and CA1 cells, in which AMPAR and NMDAR are involved in strengthening synapses, between the Schaffer collateral axons of the CA3 neurons and the CA1 pyramidal cells in the hippocampus (Blanke and VanDongen, 2009).

AMPAR and NMDAR are expressed, usually together in a postsynaptic cell, as when AMPAR is activated by the binding of glutamate, they allow an influx of sodium ions into the cell. This causes a small depolarisation of the postsynaptic neuron, which is required to remove a magnesium ion blockade from the NMDAR and allow influx of calcium ions through the unblocked NMDA receptor. LTP describes the requirement of prolonged and recurrent release of glutamate from the Schaffer collaterals to prolong the activation of AMPAR located

in CA1 pyramidal cells, to produce a strong enough postsynaptic depolarisation current to remove the $Mg^{2+}$ block from NMDAR. Once the magnesium blockade is removed and glutamate binds to NMDAR, calcium ion influx begins, which then can further depolarise the cell or act as a secondary messenger by activating various cellular cascades (Seeburg *et al.,* 1995). One study suggests that KAR may be involved in this pathway, due to the discovery that during early development, CA3-CA1 synaptic transmission in the SCC pathway is regulated by a high-affinity, G-protein dependent KAR (Lauri *et al*., 2006).

There are two phases of LTP – early and late-phase, which are both triggered by the influx of calcium ions. Early-phase LTP can last for up to a few hours and is characterised by increased expression of AMPA receptors in the postsynaptic cell membrane. This change initiated by early-phase LTP then allows for more AMPA receptors to become activated by glutamate, and thus, through the cascade of reactions described previously, depolarisation of the postsynaptic cell. This, along with repeated release of glutamate by the presynaptic neuron, leads to late-phase LTP. Late-phase LTP is expressed by an increase of transcription factors that lead to the synthesis of various proteins, including AMPAR, which are then inserted into the postsynaptic cell membrane, thus strengthening the synapse further (Baltaci, Mogulkoc and Baltaci, 2018).

MF-LTP is an NMDA receptor-independent process, with multiple studies suggesting that instead, KA receptors are involved (Lauri *et al.,* 2006). LTP in the SCC pathway is NMDAR-dependent and causes an increase of calcium ions in the postsynaptic neuron whereas, MF-LTP is not only NMDAR-independent, but requires an increase of calcium ions in the presynaptic neuron, rather than the postsynaptic cell. This type of LTP also requires the activation of protein kinase A, which leads to a persistent increase in the release of glutamate from the presynaptic cell (Nicoll and Malenka, 1995). These studies show that not only are NMDAR and AMPAR involved in LTP, but also KAR.

## 1.5 NMDA Receptors

### 1.5.1 Introduction to NMDA Receptors

NMDA receptors (NMDAR) are the most extensively studied iGluR due to the range of CNS disorders they are implicated in. NMDAR has characteristically different properties to the other iGluRs, in that they require two agonists to bind in order to be activated – glutamate and glycine. They also have a high permeability to calcium ions, which is unlike AMPAR and KAR, and they have a voltage-dependent, extracellular magnesium ion blockage (Hansen, 2018). The magnesium blockages are present when the cell is at rest, and to release the magnesium from the NMDAR pore, the cell needs to be slightly

depolarised. This is caused by the activation of AMPAR that produces a rapid response and decay. This depolarisation causes the magnesium to be released from the NMDAR pore, which unblocks the ion channel of the receptor, and upon the binding of glutamate, allows the movement of cations, specifically calcium ions (Blanke and VanDongen, 2009). These receptors are named coincidence receptors, due to the dependence on both presynaptic glutamate release and the depolarisation of the postsynaptic neuron, therefore the response of NMDAR is much slower and longer lasting than AMPAR and KAR (Hestrin, 1990).

The structure of NMDAR is a heteromeric composition of a range of assemblies of its subunits, GluN1, GluN2A-D and GluN3A-B, with 'classical' NMDAR consisting of two GluN1 subunits and two GluN2 subunits. The GluN1 subunit has eight reported isoforms that arise from three different splice events, which produce differences in gating and pharmacological properties. These isoforms are referred to as GluN1-1a – GluN1-4a and GluN1-1b – GluN1-4b (Rumbaugh, 2000). There is a central ion channel pore, in which extracellular $Mg^{2+}$ binds and blocks the ion channel at rest and different isoforms of the subunit GluN2 can cause differences in the permeability to calcium ions and the sensitivity to magnesium block. The GluN2A and GluN2B subunits form receptors that are more sensitive to magnesium block and more permeable to calcium ions than the GluN2C and GluN2D subunits (Monyer, 1992). This is because the GluN2 subunit is responsible in

determining functional roles of the receptor, such as magnesium ion block sensitivity, deactivation kinetics, maximal channel open probability, and the permeability of NMDAR to divalent cations (Paoletti, Ascher and Neyton, 1997). The GluN2 subunit holds the ligand binding domain (LBD) for glutamate, whereas the GluN1 and GluN3 LBDs bind glycine, which is usually found in the extracellular space at a constant concentration. Typically, the NMDA receptors are in an alternating subunit arrangement, where the heterodimers (e.g. GluN1/GluN2) are positioned in a GluN1/GluN2/GluN1/GluN2 conformation, leaving a pore in the middle of the subunits, which has been shown to have structural properties similar to an inverted potassium ion channel (Figure 4) (Salussolia, 2011). The subunits each consist of an extracellular amino terminal domain (ATD), which modulate the assembly and function of NMDAR and an intracellular carboxyl-terminal domain (CTD). A transmembrane domain (TMD) is found in all subunits, consisting of transmembrane helices (M1, M3 and M4) and a re-entrant loop (M2), which is found in the ion channel pore. Q/R/N sites are located in this M2 re-entrant loop, which usually undergo some sort of RNA sequence editing and contribute to the cation permeability of the pore. The agonist-binding domain (ABD) is bilobed, with the agonist binding site located between the two lobes and is formed from the S1 and S2 segments of the protein (Figure 5) (Hansen *et al.,* 2018).

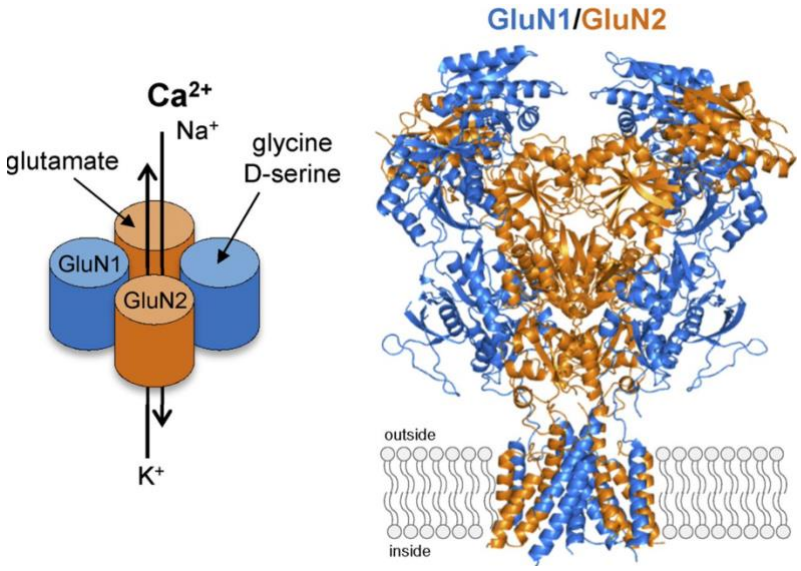



***Figure 4 – Structure of GluN1/GluN2 NMDAR.*** *The crystal structure of an NMDAR comprising two GluN1 and two GluN2 and a diagram demonstrating the formation of the ion channel pore. Taken from Hansen et al., 2018.*

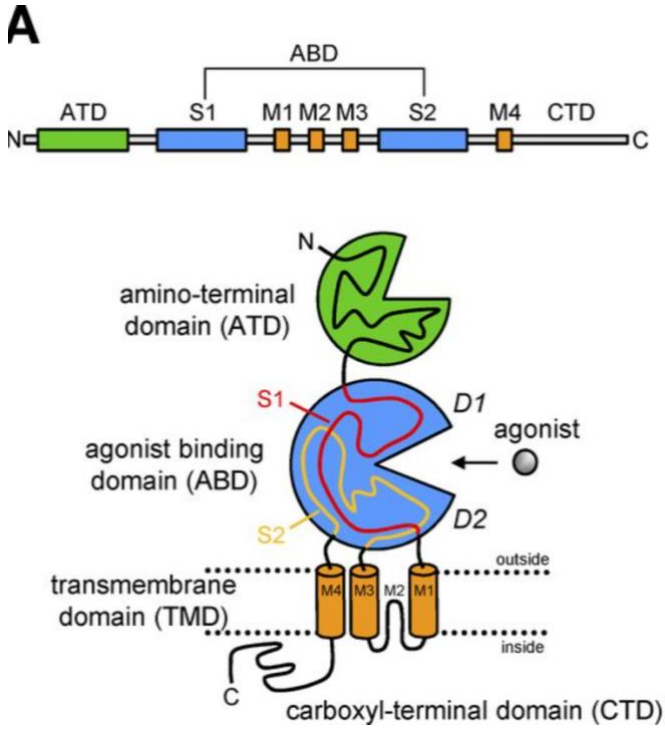



***Figure 5*** – ***Structure of NMDAR Subunit.*** *Polypeptide chain of an NMDAR subunit, including the locations of the ATD, ABD, TMD and CTD in relation to the cell membrane. Taken from Hansen et al., 2018.*

### 1.5.2 Antagonists of NMDAR

Antagonists of NMDAR act upon the different subunits, depending on the antagonist. Ifenprodil is an uncompetitive NMDAR antagonist which highly selectively binds to the GluN2B subunit and is used pharmacologically to detect NMDAR (Perin-Dureau, Rachline, Neyton and Paoletti, 2002). However, a strong, non-competitive antagonist of NMDAR, ketamine, is not selective for any specific subunits. Rather, it is thought that ketamine has interactions with the magnesium ion blocking site in the channel pore (Kotermanski and Johnson, 2009) and has been studied as a potential treatment for major depressive disorder and treatment-resistant depression in the human cell line HEK293S (Zhang *et al*., 2021). This provides a human model of NMDAR and its impact on depression, which successfully builds on previous studies carried out in rat models, that provide evidence of ketamine-induced NMDAR activity reduction (Yang *et al.,* 2018). Another popular NMDAR antagonist, used often in experiments, is memantine, which is currently used as a treatment for Alzheimer's disease. MK-801 is an NMDAR antagonist with a higher affinity for the GluN1-GluN2B subunits than memantine, and therefore it is not used as a clinical treatment due to side effects caused by the long dwell time on the receptor (Song *et al*., 2018). Many NMDAR antagonists that are used clinically, present side-effects depending on the NMDAR-affinity of the antagonist, which can include headache, nausea, nightmares, and fatigue (Jamero, 2011). Methadone is an NMDAR antagonist that has been used in pain reduction and opioid resistance, however a recent

study has shown that methadone does not interact with NMDAR at certain doses and is therefore a relatively weak antagonist of NMDAR (Davis, 2021).

### 1.5.3 The Excitotoxicity of NMDAR Overstimulation

The overstimulation of NMDAR can lead to an excessive increase in the influx of calcium ions into postsynaptic neurons, which can lead to apoptotic cascades. This has been shown to be linked to Alzheimer's disease, stroke, and other traumatic brain injury, as well as alcohol withdrawal (Dong, Wang and Qin, 2009). Hours, up to days after a stroke has occurred, neurons in the brain surrounding the dead brain tissue, will undergo neuronal apoptosis and death. The mechanism of this cell death is due to NMDAR-dependent excitotoxicity, due to an increase in extracellular glutamate. In stroke models, through the activation of NMDAR and thereby, the activation of glutamatergic synapses, cell death can be rescued (Lai *et al*., 2014), providing a target for post-stroke treatment. Treatment targeted at NMDAR applied post-stroke, however, would not give enough time to be effective in preventing or reducing cell death. Therefore, NMDAR antagonists can be used in the at-risk population as a preventative measure and the NMDAR antagonist, memantine when administered in low doses pre-stroke, has been shown to reduce brain damage in patients after a stroke (Trotman *et al*., 2015).

### 1.5.4 NMDA Receptors and Disease

NMDA receptors have been shown to be important in the pathophysiology of Alzheimer's disease (AD), via glutamate excitotoxicity. AD is a neurodegenerative disease that is the major cause of dementia and involves loss of memory, lucidity, changes in behaviour and eventually loss of bodily function. The genetic mutations associated with AD can ultimately lead to the excessive production of amyloid-beta (Aβ). The influence of this on NMDAR is widely misunderstood, with what seems like various impacts of Aβ on NMDAR expression and function. For example, one study showed that an increase in Aβ production, decreased the expression of NMDAR (Hynd, Scott and Dodd, 2001), whereas other studies show that the direct application of Aβ onto NMDAR-containing cortical neurons gives a reduction in NMDA-evoked currents (Snyder *et al.,* 2005). Further data suggests that Aβ has a direct impact on the function of NMDAR (Texidó *et al*., 2011), by causing an increase in extracellular glutamate (Brito-Moreira *et al.,* 2011) and thus leading to an excessive exposure of NMDAR to glutamate, resulting in excitotoxicity.

In Huntington's disease, the overstimulation and therefore overactivity of NMDAR is a supposed mechanism of the neuronal death that occurs in the brain in Huntington's disease. This has been implicated in multiple studies which use the quinolinic acid agonist of NMDAR to replicate the neurological characteristics of Huntington's disease in rats

(Beal *et al.,* 1986), as well as in baboons (Hantraye, Riche, Maziere and Isacson, 1990). This strengthens the applicability of the claims from the study in rats, as baboons are non-human primates and therefore closer in genetic relation to humans. Human studies have involved clinical trials of NMDAR antagonists as treatment for Huntington's disease, however, they were not successful due to side effects caused by the blockage of NMDAR activity (Lipton, 2004).

### 1.5.5 Limitations in NMDAR Studies

Many NMDAR studies are carried out in rats, mice, and *Xenopus* oocytes rather than in human models. There is a prevalent lack of NMDAR research in human cell lines, suggesting a difficulty in expressing functional receptors successfully in human cells. There are, however, a few studies that have successfully transiently transfected NMDAR subunits into human cell lines such as the HEK293 cell line (Domingues *et al.,* 2006). Experiments carried out in *Xenopus* oocytes and non-human mammalian cells are not strongly accurate or reliable when aiming to apply the data to human disease. This can be due to multiple factors, including but not limited to the differences in cell body size, cell membrane composition, and the post-translational processing of RNA. It is therefore important and necessary to increase the number of available human NMDAR models.

## 1.6 AMPA Receptors

### 1.6.1 AMPA Receptor Structure, Agonists and Antagonists

The name AMPA receptor comes from the synthetic glutamate analogue, AMPA which has a high affinity for AMPA receptors, however, also has a low affinity to kainate receptors (Shi, Lu, Milstein, and Nicoll, 2009). Its endogenous agonist is glutamate, alike other iGluRs. AMPAR has characteristically rapid response kinetics and like NMDAR, are crucial for NMDAR-dependent LTP, synaptic plasticity, and neurotransmission (Greger, Watson and Cull-Candy, 2017). AMPAR usually forms heterotetrametric receptors consisting of a range of combinations of GluA1-4 subunits and the functional properties such as ion selectivity, channel kinetics and receptor trafficking properties of the receptor depends on the combination of these subunits. The four subunits can each bind an agonist, and as the agonist glutamate binds to a subunit, the channel pore opens and continues to open with more glutamate that is bound to the receptor. Like NMDAR, each AMPAR subunit consists of a TMD, comprising of M1-M4 segments, which have similar locations and functions to the TMD segments in the NMDAR subunits, as well as the LBD and CTD (Figure 6). AMPAR subunits have an extracellular N-terminal domain (NTD), which contributes to the assembly and localisation of AMPAR (Kamalova and Nakagawa, 2020). These subunit mRNAs can undergo alternative splicing and

RNA editing to produce isoforms of the subunits, which further increase the diversity of their functional properties. The GluA2 subunit widely determines the functional properties of AMPAR. An RNA edit (Q607R) in the pore loop tip causes a change of glutamine (Q) to arginine (R), which is positively charged, in contrast to glutamine which is uncharged (Hollmann, Hartley and Heinemann, 1991). This leads to a change in calcium ion permeability, with the unedited Q/R site forming a calcium ion permeable pore and the edited Q/R site forming a calcium ion impermeable pore. In AMPA receptors that do not contain GluA2, the receptor is permeable to calcium ions (Cull-Candy, Kelly and Farrant, 2006), however most of the AMPA receptors in the CNS contain GluA2 subunits, so are mostly calcium ion impermeable and the main ions gated by AMPAR are sodium ions. This is theorised to be for the intent of protection against excitotoxicity (Kim *et al*., 2001), as the prevention of calcium ions entering the cell by AMPAR, work against the NMDAR calcium ion influx that is triggered by glutamate. It is also suggested that AMPAR not containing GluA2 and therefore permeable to calcium ions, can assist in synaptic plasticity and a type of NMDAR-independent LTP. The AMPA receptors have a variety of auxiliary subunits, which impact their expression and trafficking. Transmembrane AMPAR regulatory proteins (TARPs) are among the most studied auxiliary subunits of AMPAR and have a role in synaptic plasticity and neuronal physiology, by modifying the kinetics and conductance of the receptors (Miguez-Cabello *et al*., 2020). There are a range of antagonists of AMPAR, including a

competitive anagonist called becampanel, which has been researched as a potential treatment for epilepsy, pain, and cerebral ischemia (Citraro *et al*., 2014). Fanapanel is another competitive AMPAR antagonist that was tested in trials for the treatment of cerebral ischemia, however trials were ended due to side effects including neurological deterioration and sedation (Elting *et al.,* 2002).

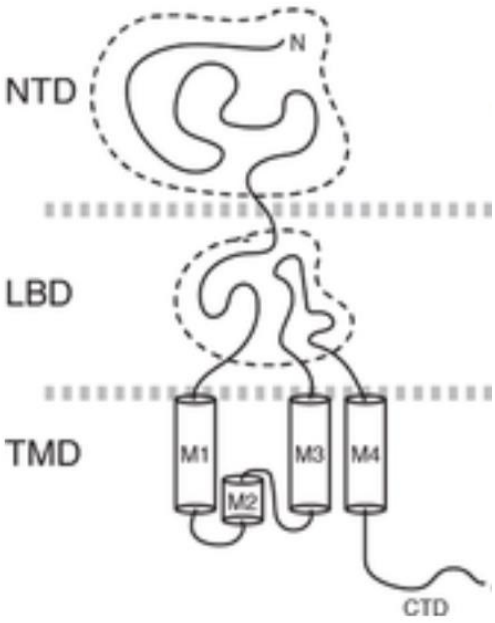



***Figure 6 – Structure of AMPAR Subunit.*** *The structure of an AMPAR subunit, with its domains in the cell membrane. Taken from Kamalova and Nakagawa, 2020.*

### 1.6.2 AMPAR and Disease

iGluRs have similar roles in disease and usually present themselves in the same diseases. AMPAR, like NMDAR, has a role in AD with a reduction in AMPAR expression, that leads to impairments in LTP and synaptic plasticity, thereby reducing memory and cognitive function. The increased Aβ production, which is associated with AD mutations, has a direct impact on AMPAR trafficking by binding close to the GluA2 subunit (Zhao *et al.,* 2010).

Other diseases are associated with a reduction in GluA2 containing AMPAR, as this causes an increase in intracellular calcium ions, which ultimately leads to excitotoxicity and cell death. In ALS patients, the Q/R editing of GluA2 subunits is dysfunctional in motor neurons due to a reduction in ADAR2 expression, which is an RNA editing enzyme (Yamashita and Kwak, 2014). This reduction in GluA2 editing leads to the production of calcium permeable AMPAR, thus promoting death of these neurons. Studies have shown that following traumatic brain injury and ischemia, the GluA2 subunit is downregulated in the hippocampus and may also be unedited (Pellegrini-Giampietro *et al*., 1992). These calcium ion permeable AMPA receptors, lead to cell death in the ischemic brain.

In a thorough study of the GluA2 subunit and its implications in disease, multiple mutations in the GluA2 gene were identified

in intellectually disabled patients, and in patients who are on the autism spectrum disorder (Salpietro *et al.,* 2019). The study identified mutations in the GluA2 subunit in patients with a range of intellectual disabilities. The mutated GluA2 subunits were expressed in the HEK human cell line, which provides reliable data in relation to gaining insight into the electrophysiology of human AMPAR. It was found that the mutations that were identified had a range of implications, and it was concluded that the GluA2 mutations led to a decrease in the glutamate response. It would then be a logical assumption that this reduction in glutamate response (e.g., pore opening), leads to a reduction in NMDAR-dependent LTP, which is associated with learning and memory.

The common contributing factor to disease involving AMPAR, is the GluA2 subunit and whether it is edited or not and thus, whether the AMPAR is calcium ion permeable or impermeable. This can lead to a conclusion that the majority of diseases associated with AMPAR, are implicated by the excitotoxicity and neuronal cell death that occurs when AMPAR is converted from calcium ion impermeable to calcium ion permeable.

## 1.7 Kainate Receptors

### 1.7.1 Kainate Receptor Structure, Agonists and Antagonists

Kainate receptors have the native biological agonist, L-glutamate and have a high affinity for kainate (Shi, Lu, Milstein and Nicoll, 2009) – a compound isolated from the algae *Digenea simplex.* There is known cross-reactivity between AMPAR and KAR, as KAR is known to be susceptible to activation by AMPA and vice versa. Another challenge among KAR studies, is the lack of specific compounds that can block or activate KAR. This means that there is limited data on the specificity of KAR, as well as a lack of structural data, however, it is known that the structure of KAR subunits is similar to both NMDAR and AMPAR subunits (see Figures 4, 5 and 6), with a TMD consisting of M1-M4 segments, and an extracellular LBD and ATD. Regardless of the limitations of the research, there have been major developments in the understanding of KAR, including the localisation of KAR subunits in the brain. GluK4 is present in most neurons across all human brain regions that have been studied, specifically in the dentate gyrus and CA3 of the hippocampus, as well as the neocortex (Bahn *et al*., 1994). Similar to GluK4, GluK5 is also abundantly present throughout the brain (Bahn *et al*., 1994). GluK2 subunits are mainly expressed in the hippocampus, cerebellum, and cerebrum. The GluK1 subunit is also expressed in the hippocampus and cerebrum (Rozas *et al*.,

2003) and the GluK3 subunit is found in the hippocampus dentate gyrus and the neocortex (Wisden *et al.,* 1993).

There are numerous known antagonists of KAR, however many if not all, are also antagonists of AMPAR, which poses difficulties in research attempting to differentiate between the two. Quinoxalinediones are competitive AMPAR and KAR antagonists and the quinoxalinedione antagonist of both AMPAR and KAR, 6,7-dinitroquinoxaline-2,3-dione (DNQX), induces partial domain closure and is usually used to reduce excitatory postsynaptic currents (EPSCs) in neurons where these receptors are located (Fisahn, 2004). KAR-mediated EPSCs (KAR-ESPCs) have irregularly slow kinetics and the glutamate response of KAR is relatively small compared to NMDAR and AMPAR, due to the fast glutamate desensitisation of KAR (Bannister, 2005).

### 1.7.2 KAR Structure, Functions and Editing

KAR is distributed pre- and post-synaptically throughout the brain and are formed by tetrameric combinations of KAR subunits GluK1-5. The subunits GluK1-3 can form either homomeric or heteromeric functional KAR, whereas the GluK4 and GluK5 subunits can only form functional receptors when they are combined with any of the GluK1-3 subunits. GluK4 and GluK5 have a stronger affinity for glutamate than the other three subunits (Schiffer, Swanson and Heinemann, 1997). The four subunits of the kainate receptor must each bind a

glutamate molecule to activate KAR, which triggers an increase in permeability to monovalent cations such as sodium ions, by the opening of the ion-channel pore, which leads to cell depolarisation.

The pre-mRNAs of GluK1 and GluK2 can undertake post-transcriptional editing at the Q/R site, which leads to a modification in calcium ion permeability. GRIK2 can have further editing at the I/V and Y/C sites, in the transmembrane 1 domain (TM1), which causes modifications of the ionic properties of TM1 (Köhler, Burnashev, Sakmann and Seeburg, 1993). These post-translational alterations cause changes in codons and splice sites, allowing for a range of functionally diverse proteins to be produced (Barbon and Barlati, 2011). The functions of KAR can span from the modulation of synaptic transmission, neuronal excitation, neuronal development and LTP, and neurodegeneration and cell death. KAR, like AMPAR is known to have auxiliary proteins, including NETO1 and NETO2, which regulate a variety of the receptor functions, including binding affinity, localisation, deactivation, and desensitisation kinetics (Wyeth *et al.,* 2017).

### 1.7.3 KAR Mutations and Associated Diseases

KAR dysfunction can have a wide range of impact on neurological processes, which can lead to a variety of disorders and disease, such as intellectual disabilities, autistic spectrum disorder, AD, schizophrenia, BD, and epilepsy. KAR

have also been shown to have a role in the transmission of pain signals in the CNS, specifically in dorsal root ganglia neurons (Rozas, Paternain and Lerma, 2003). In addition, a phase II trial has shown that the nonselective KAR antagonist, NGX424, was able to reduce pain from migraines (Swanson, 2008). The subunits involved in pain transmission are GluK1 and GluK5. GluK1 is also known to be involved in epilepsy, as well as the GluK2 subunit (Fritsch *et al.,* 2014). The amygdala and hippocampus are brain structures that are associated with specific types of epilepsy and seizures, which are areas where a range of all KAR subunits are expressed. The selective activation of GluK1 has been shown to induce myoclonic seizures and proconvulsant effects (Fritsch *et al.,* 2014) however, it has also been shown that GluK1 activation can lead to anticonvulsant effects, by inhibiting principal cells (Braga, Aroniadou-Anderjaska, Xie and Li, 2003).

The GRIK4 gene (encoding GluK4) has been reported to show chromosomal breakpoints identified in a patient affected by both schizophrenia and mild learning disabilities. This finding led to an association between variants across GRIK4 and susceptibility for psychiatric disorders (Pickard *et al.,* 2006). A subsequent study identified a deletion mutation in GRIK4 which reduces the risk of developing BD and cognitive abilities (Koromina, Flitton, Mellor and Knight, 2018; Knight *et al.,* 2011). This mutation leads to an increase in expressed GluK4, suggesting that the GluK4 subunit has protective properties against this disorder. It has also been suggested that GluK4

has a role in the modulation of MF-LTP (Catches, Xu and Contractor, 2012). Although it is not known how this function affects behavioural changes that were seen in GRIK4 knock-out mice, such behavioural changes included decreased anxiety and depression. This may lead to the conclusion that GRIK4 mutations, and thus increased expression of GluK4 subunits, is likely to impact on increases in anxiety, depression, and other mood defects.

### 1.7.4 GluK2 and Disease

The GluK2 subunit is associated with disease more than any of the other KAR subunits, possibly due to its high expression and generalised localisation throughout the brain. GluK2 has been identified as having a role in intellectual disability, through the identification of a deletion edit in the GRIK2 gene (Lerma and Marques, 2013). This edit produced a truncated, non-functional GluK2 subunit that led to a reduction in KAR-dependent synaptic transmission and glutamate signalling, which may impact KAR-dependent MF-LTP, and cognitive function. Temporal lobe epilepsy (TLE) is another disorder linked to the reduction in activity of GluK2, and these subunits are associated with TLE due to their abundant expression in neurons in the CA3 region of the hippocampus (Werner *et al*., 1991). In studies that block or knock-out GluK2, seizures and the sensitivity to seizures are reduced (Mulle *et al*., 1998), demonstrating this association between GluK2 and TLE.

There is emerging evidence that KAR, specifically the GluK2 subunit, has a role in glucose homeostasis, where in GluK2 knock-out mice, there was an improved tolerance to glucose, suggesting that the GluK2 subunit impacts glucose homeostasis negatively (Abarkan *et al*., 2019). The functions of glucose homeostasis are known to decline naturally with age, such as insulin sensitivity, insulin secretion and glucagon secretion (De Tata, 2014). This age-related decline in functions was reduced in the GluK2 knock-out mice, suggesting that the impact that GluK2 has on glucose homeostasis, is also impactful on the age-related deterioration of glucose homeostasis.

Autosomal recessive juvenile Parkinson's (ARJP) is a rare neurodegenerative disease characterised by the dysfunction of dopamine receptors and neuronal death of dopamine containing neurons. It is most commonly caused by mutations in the PARK2 gene that encodes the Parkin enzyme, which functions in regulating and trafficking ubiquitin (Zhang, Hang, Yao and Lim, 2016). The Parkin enzyme interacts with the GluK2 subunit and ubiquinates it however, when Parkin is mutated, GluK2 accumulates in the cell (Regoni *et al*., 2020) and it has been suggested that this is due to the mutated Parkin protein, slowing down the turnover of GluK2. This is a rare disease which is poorly researched and understood however, the involvement of GluK2 and KAR may suggest brain function alterations, contributing to symptoms of the disease, such as slow movements.

A recent genetic study identified mutations in both GRIK2 and GRIK4 that are associated with schizophrenia, autism, and intellectual disabilities (Koromina *et al*., 2019), and showed the functional effects of some of the mutations. Of particular interest, was a damaging missense mutation found in GluK2 (GluK2 K525E), which is located in the LBD of GluK2, and therefore could have an impact on a range of functions, such as binding affinity and ion permeability. The mutant did not form functional homomeric KAR and had to be expressed with a wild-type GluK2 subunit to form a functional receptor. The study showed that the mutation led to a general decrease in the response to glutamate, suggesting that the K525E mutation forms functional KAR that responds to the glutamate agonist, but are less active than wild-type GluK2 homologous KAR. The glutamate $EC_{50}$ in the mutated GluK2 KAR was around 4-fold larger than the wild-type, which may suggest a weaker binding affinity for glutamate, which would be expected as the mutation is located in the LBD. This study used *Xenopus* oocytes expressing the KAR subunits to gather electrophysiological data, which brings some physiological differences when compared to human cells. Such differences of mammalian cells and *Xenopus* oocytes include the cell membrane composition and post-translational processing. The oocytes are also larger in size than human cells and limited perfusion techniques leads to a slower solution exchange than when using human cells, so the response kinetics data gathered from *Xenopus* oocytes are less accurate. Replicating

this data using transfected HEK293 cells to measure the change in membrane voltage upon KAR activation, would provide a more valid measure of this than using *Xenopus* oocyte expression. HEK293 cells are a popular human cell line for use in iGluR expression and electrophysiological studies (Domingues *et al*., 2006). GRIK2 DNA has been successfully transfected into HEK293 cells recently, where electrophysiological data was gathered to define the role of GluK2 in early childhood development disorders (Stolz *et al.,* 2021).

## 1.8 The TE671 Cell Line and Acetylcholine Receptors

### 1.8.1 What are TE671 Cells?

The TE671 human cell line was originally classified as medulloblastoma, however it has since been shown that this cell line is a derivative of the rhabdomyosarcoma TE32 cell line (a rare type of malignant muscle tumour), due to TE671 expressing a muscle type nicotinic acetylcholine receptor (Schoepfer, Luther and Lindstrom, 1988). The TE671 cells are of a round, elongated morphology with spindle-type extensions. They grow in a monolayer, which is useful in experimental procedures such as patch clamping, as it allows for focus on a singular cell. The TE671 cell line is often used

for expressing proteins via transfection and transduction. The KAR GluK5 subunit has been successfully expressed in these cells previously, along with AMPAR and NMDAR subunits (Luksch *et al.,* 2011).

There are two different types of ACh receptor, each having different functions – nicotinic and muscarinic. The TE671 cells express both nicotinic acetylcholine receptors (nAChR) and muscarinic ACh receptors (mAChRs) (Shao *et al.*, 1998). ACh has various functions depending on its location, such as acting as a neurotransmitter in the nervous system and at neuromuscular junctions (NMJs), smooth muscle contraction, heart rate modulation and regulation of bodily fluid secretion (Sam & Bordoni, 2021).

### 1.8.2 Nicotinic Acetylcholine Receptors

Nicotinic ACh receptors (nAChR) are ionotropic receptors that can be stimulated by nicotine and are expressed in presynaptic neurons, to modulate neurotransmitter release (Dani, 2001). They are also expressed post-synaptically, where they have a major role in rapid synaptic transmission throughout the nervous system. nAChRs can be categorised as either neuronal ($N_N$) type, found throughout the nervous system, or muscle ($N_M$) type, found in voluntary skeletal muscles at neuromuscular junctions (NMJ). All nAChRs are formed from five subunits in the formation of a pore and all AChR subunits have the same structure, consisting of an extracellular NTD, four TMDs (M1-M4) and a cytoplasmic loop domain (Cetin,

Beeson, Vincent and Webster, 2020) (Figure 7). $N_M$-AChRs are formed from two $\alpha$1 subunits, one $\beta$1 subunit, one $\delta$ and one $\varepsilon$ subunit, that make up a central pore and the ACh binding sites are found at the $\alpha$-$\delta$, or α-$\varepsilon$ subunit interfaces (Cetin, Beeson, Vincent and Webster, 2020). In contrast, neuronal type nAChRs, are constructed from at least 2 $\alpha$ subunits ($\alpha$2-$\alpha$10) and β subunits (β2-β4) in numerous combinations that can be homomeric or heteromeric (Hendrickson, Guildford and Tapper, 2013). The ACh binding sites are between either two $\alpha$ subunits or an $\alpha$ and $\beta$ subunit. Two ACh molecules are required to open the channel pore, which then allows the influx of sodium ions and causes depolarisation of the cell (Olsen *et al.* 1999). Like iGluRs, nAChRs are widely distributed and involved in human disease. The subunits $\alpha$4, $\alpha$7, and $\beta$2 are highly expressed in the brain and are involved in numerous neuropathological disorders, including AD, Parkinson's disease and schizophrenia (Aráoz *et al.,* 2015).

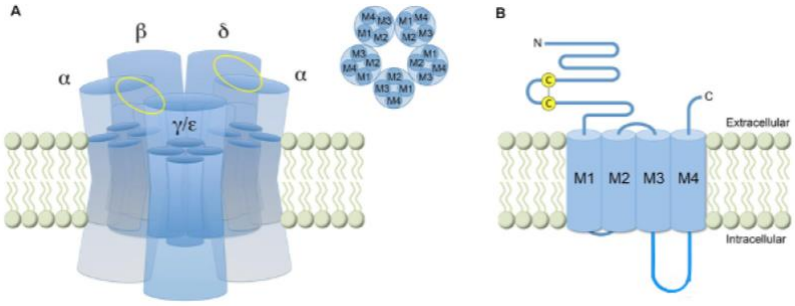



***Figure 7 – Structure of Muscle Type nAChR. (A)*** *Ion-channel pore formation of five subunits. (**B)** Segments of an AChR subunit. Taken from Cetin, Beeson, Vincent and Webster, 2020.*

### 1.8.3 Muscarinic Acetylcholine Receptors

Muscarinic ACh receptors (mAChR) are G protein-coupled receptors that can be stimulated by muscarine and are expressed in peripheral tissues such as the eye, heart, and gastrointestinal tract. There are 5 subtypes of mAChRs, $M_1$-$M_5$ (Eglen, 2005). Once ACh binds to these receptors, the intracellular G protein is activated. There are two different G proteins associated with mAChRs – $G_q$ which is associated with $M_1$, $M_3$ and $M_5$ subtypes of mAChR, and $G_i$ which is associated with $M_2$ and $M_4$ subtypes. Once the $G_q$ coupled protein is activated, it triggers a signalling cascade leading to an influx of calcium ions which triggers the contraction of the smooth muscle (Kudlak and Tadi, 2021).

### 1.8.4 Differentiation of TE671 cells

Cellular differentiation describes a process in which one subtype of a cell converts to another, usually becoming more specialised. Differentiation occurs naturally during growth and repair but can also be induced artificially using certain reagents.

The $N_M$ subtype of nAChRs is typically expressed in TE671 cells under normal physiological conditions, however a more neuronal morphological subtype has been reported (Syapin, Salvaterra and Engelhardt, 1982), with the potential expression of the $N_N$ subtype. This would imply the expression of $\alpha$2-$\alpha$10 and β2-β4 subunits that are found in $N_N$-AChRs, as well as $\alpha$1

and β1 subunits which are found in $N_M$-AChRs. This has been demonstrated in multiple studies, one of particular interest uses dibutyryl cyclic adenosine monophosphate (db-cAMP) to instigate cell differentiation (Siegel and Lukas, 1988). Cell differentiation can cause changes in the cell's morphology, membrane potential, sensitivity to signals, and metabolic activity and are usually due to gene expression alterations.

TE671 cells can undergo cellular differentiation and have at least five different morphological cell types, including the neuronal cell type, which can be initiated through the application of dibutyryl c-AMP (db-cAMP) (Siegel and Lukas, 1988). The db-cAMP reagent activates the cAMP signalling pathway, which leads to a change in morphology, including the expansion of the spindle-shaped extensions and extension of the cell body (Siegel and Lukas, 1988). These neuronal morphological changes make TE671 cells an attractive target in researching the presence of synaptic receptors, such as glutamate receptors.

## 1.9 Aims of the Study

The overriding aim of this study is to investigate the electrophysiology of glutamate receptors in human cell lines in

order to try and develop convenient models for their study. The specific objectives were:

1. To provide a comprehensive literature review of glutamate receptor structure, function, distribution, and role in nervous system diseases.
2. To measure the membrane ionic currents in homomeric GluK2 KAR expressing cells when the receptors are activated by glutamate and compare this to a mutated GluK2(K252E) KAR that was identified by Koromina *et al.* 2019.
3. To gain insight into the type and characteristics of glutamate receptors expressed in db-cAMP-differentiated TE671 cells.

# Chapter 2

# Materials and Methods

## 2.1 Materials

**Reagents and Solutions:**

Escort™ IV Transfection reagent (SIGMA)

Dibutyryl-cAMP (db-cAMP) (SIGMA)

Ampicillin sodium salt (10 mg/mL) (SIGMA)

L-glutamate salt 147.13 mg (SIGMA)

Eagle's Minimum Essential Medium (SIGMA)

L-Glutamine (200 mM) (Gibco™)

Penicillin-Streptomycin (10,000 U/mL) (Gibco™)

Trypsin-EDTA (0.25%) (Gibco™)

**Plasmids:**

Human cDNA KAR subunit GRIK2 (GenScript) in pcDNA3.1 (Accession number: NM_021956)

Human cDNA KAR subunit GRIK2(K525E) in pcDNA3.1 (obtained from Dr. Maria Koromina, University of Nottingham (Koromina, 2019))

**DNA Isolation:**

GenElute™ Plasmid Miniprep Kit (SIGMA)

## 2.2 Culture of HEK293 and TE671 Cells

HEK293 and TE671 cells were cultured and maintained in the same conditions. Cells were taken from stock solutions in the liquid nitrogen vapour phase and were thawed rapidly by gentle agitation in a 37 ºC water bath for 2 minutes. The

thawed cells were then added to 10 mL of cell culture medium containing Eagle's Minimum Essential Medium (EMEM), 10% Fetal Bovine Serum (FBS), 2 mM L-Glutamine and 1% Penicillin-Streptomycin (5,000 U/mL). The cells were centrifuged at 1000 rpm (Centaur 2) for 10 minutes and then the supernatant was removed. The cell pellet was then resuspended in 5 mL of cell culture medium, and this was added to a 25 $cm^2$ Corning cell culture flask and placed in an incubator at 36.5 °C and 5 % $CO_2$. After 24 hours, the cells were approximately 60% confluent and at 48 hours, 80% confluent. A week later, the cells were 100 % confluent. After a week the cells were passaged; the media was aspirated from the flasks, leaving behind cells attached to the bottom of the flasks and the cells were dissociated by addition of 3 mL of Trypsin-EDTA (0.05%) for 2-3 minutes followed by pipetting up and down. Then the Trypsin-cell mixture was added to 7 mL cell culture medium and centrifuged at 1000 rpm for 10 minutes. The supernatant was aspirated, and cells were resuspended in 6 mL of cell culture medium. 0.5 mL of the resuspended cells was added to a 25 $cm^2$ Corning cell culture flask with 4.5 mL of the cell medium. At this stage, cells were also plated onto cover slips (~5 × 22mm) were placed into 35 mm dishes in 1-1.5 mL of cell medium and 60 uL of cell suspension was added and the dishes were gently swirled. The passaging process was repeated every 4-7 days, using the cells from the flasks. Cells were taken from stocks to start this process from the beginning again, only once the cells were growing more slowly than usual. The cells were passaged from

stocks for up to 20 times until they began growing too slowly, at which point new stocks were used.

## 2.3 Differentiation of TE671 Cells with db-cAMP

TE671 cells were passaged and plated onto cover slips as described above and incubated at 37 °C for 24 hours. The medium in the dishes was removed and 750 uL of serum-free cell culture medium was added to each dish, immersing the cover slips, then 7.5 uL of db-cAMP (40 mM) was added to each dish and the dishes were swirled gently to mix. The cells were incubated again at 37 $^{o}$C for 24-72 hours before use in patch clamp experiments.

## 2.4 Transformation of XL10-Gold Supercompetent *E. coli*

XL10-Gold Ultracompetent *E. coli* cells (obtained from Agilent) were transformed using the heat shock method, to produce pcDNA to be later used in HEK-293 cell transfection. The XL10-Gold Supercompetent *E. coli* cells were removed from the -70 $^{o}$C freezer and were thawed on ice for 10 minutes, then 200 µL of the cells and 2 µL of β-mercaptoethanol were added to an Eppendorf tube and were gently shaken. 200 µL of this mixture was aliquoted into a pre-chilled 14 mL BD Falcon tube and this was incubated on ice for 10 minutes (swirling every 2 minutes). 1.5 µL of the pcDNA to be transformed (GRIK2 and

mutated GRIK2-K525E) was added and the mixture was incubated on ice for 30 minutes (swirling every 10 minutes). After this, the mixture was heated at 42 $^{o}$C in a water bath for 45 seconds and then left to cool on ice for 2 minutes. 950 µL of super optimal catabolite repression medium (SOC) (1% tryptone, 0.5% yeast extract, 1% NaCl, 10 mM glucose, pH 7.5) was preheated to 42 $^{o}$C and this preheated SOC was added to the mixture and incubated at 37 $^{o}$C with shaking at 250 RPM for 1 hour. 250 µL of this mixture was added to a Luria broth (LB) (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agarose, pH 7.5) with 50 µg/mL ampicillin agar plate (15 g Agar (Sigma) added to 500 mL LB broth) and was incubated at 37 $^{o}$C overnight. A single colony was taken and inoculated in 30 mL LB broth 100 µg/mL ampicillin and incubated at 37 $^{o}$C with shaking overnight.

The GenElute$^{TM}$ Plasmid Miniprep Kit (SIGMA) was used to isolate the bacterially replicated pcDNA (the solutions mentioned are included in this kit). 1.5 mL of the culture from the last step was aliquoted into an Eppendorf tube and centrifuged at room temperature at 12,000 ×g for 5 minutes. The supernatant was removed, and another 1.5 mL of the culture was added and centrifuged again under the same conditions. The supernatant was removed again, and this was repeated one more time with another 1.5 mL of the culture. The pellet was resuspended in 200 µL of resuspension solution using the 200 µL pipette tip to resuspend the cells until a homogeneous mixture was formed. Then, 200 µL of the lysis

solution was added and the tube was gently inverted to mix. The mixture was left for 5 minutes and then 350 µL of neutralisation solution was added and the tube was inverted again 4-6 times. The mixture was centrifuged (MSE Microcentaur microcentrifuge) at the maximum speed for 10 minutes until the solution was clear. Then 500 µL of the column preparation solution was added to the binding column in a collection tube and this was centrifuged at 12,000 ×g for 1 minute. The flow-through was removed and the cleared lysate was transferred to the binding column. This was then centrifuged at 12,000 ×g for 30-60 seconds and the flow-through was again removed. 750 µL of the wash solution was added and centrifuged at 12,000 ×g for 30-60 seconds and the flow-through was removed. The column was again centrifuged at 12,000 ×g to dry it and then the column was transferred to a new collection tube. 100 µL of the elution solution was added and centrifuged at 12,000 ×g for 1 minute to elute the pcDNA. The resulting pcDNA samples were stored on ice and the pcDNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific).

## 2.5 Transfection of HEK293 Cells with GRIK2 pcDNA

HEK293 cells were passaged and plated into 25 $cm^3$ flasks as described above and incubated for 24 hours to achieve a confluence of 60-80%. The GRIK2 pcDNA was diluted in

EMEM (+ 2 mM L-Glutamine) to give 100 μL of 1 μg/100 μL final concentration. In a separate tube, 10 μL Escort™ IV was diluted to a final volume of 100 μL with EMEM (+ 2 mM L-Glutamine). The pcDNA solution was added directly to the reagent solution and was gently mixed by pipetting up and down. DNA/liposome complexes were left to form for 45 minutes at room temperature.

The cells were washed twice with EMEM (+ 2 mM L-Glutamine), then 2 mL of EMEM (+ 2 mM L-Glutamine) were added to each flask. The Escort IV/pcDNA complex solution (200 μL) was added to the flask and the flasks were swirled gently to mix. The cells were incubated at 37 °C for 8 hours, then 2.5 mL of EMEM, 20% Fetal Bovine Serum (FBS), 2 mM L-Glutamine and 2% Penicillin-Streptomycin (5,000 U/mL) was added to each dish to terminate transfection. Cells were incubated at 37 °C for 18 hours, then passaged and plated onto cover slips as described above. The cells were incubated for a further 24 hours before use in patch clamp experiments.

## 2.6 Whole-Cell Patch Clamp

The whole-cell patch clamp technique was used to measure membrane ion currents of HEK293 cells and TE671 cells. Borosilicate glass capillary tubes were heated and pulled using a horizontal programmable micropipette puller (Sutter P-97) to give pipette tip resistance of 4-7 MΩ. The micropipettes were

filled to 50% capacity with pipette solution (CsCl (140 mM), NaCl (5 mM), EGTA (11 mM), HEPES (5 mM), $CaCl_2$ (1 mM), pH 7.2 adjusted with CsOH (1 M)) and attached to a pipette holder connected to a micromanipulator, which was used to allow intricate movement of the pipette tip to establish a whole-cell seal with the cell. The coverslips carrying cells were placed into a bath containing Mammalian Ringer (MR) solution (NaCl (135 mM), KCl (5.4 mM), CaCl (1 M), HEPES (5 mM), D-Glucose (10 mM), pH 7.4 adjusted with NaOH (1 M)) that was continuously perfused. An inverted microscope was used to view the cells, pipette tip and perfusion tip. The pipette tip was positioned and lowered until the tip was just touching the surface of the bath. At this point, positive pressure was applied via a syringe attached to the pipette and the pipette offset was corrected to 0 mV, via a patch clamp amplifier (Axopatch 200). The pipette was then lowered and positioned adjacent to the target cell, then the micromanipulator was used to move the pipette tip towards the cell and movement was halted when a slight decrease in the current in response to a 5 mV voltage pulse was seen on the digital oscilloscope (HITACHI VC-6523), which confirmed contact with the cell membrane. Negative pressure was applied via a syringe to seal the pipette tip to the cell membrane and to generate a Giga-Ohm seal. Then further negative pressure was applied via the syringe until a whole-cell seal was formed, with a minimum holding resistance of ~200 MΩ.

Membrane currents of the cells were recorded under the whole-cell state with the patch clamp amplifier, which was connected to a digital interface (National Instruments BNC-2110), linked to a desktop computer, where data was viewed in the software WinWCP Strathclyde Electrophysiology Software (Dr John Dempster, University of Strathclyde, Scotland, UK).

All test solutions were applied to cells as 1 second pulses using an ALA DAD-VC superfusion system that provides rapid focal application (about 50 ms exchange time) under pressure from a micromanifold tip of 100 μm diameter. A rest period of at least 30 s was allowed between each test solution application. The non-transfected HEK293 cells were tested under the application of L-glutamate (0.1 mM) and L-glycine (0.1 mM). The transfected HEK293 cells were exposed to a range of concentrations of L-glutamate ($10^{-7}$ M – $10^{-3}$ M) with a constant concentration of L-glycine (0.1 mM).

For undifferentiated TE671 cells, the agonist used was acetylcholine (ACh) diluted in MR, with ranging concentrations of $10^{-8}$ M – $10^{-3}$ M. A solution of L-glutamate (0.1 mM) and L-glycine (0.1 mM) was also tested on undifferentiated TE671 cells.

The differentiated TE671 cells were tested with a range of agonists and antagonist samples. A range of L-glutamate concentrations ($10^{-7}$ M – $10^{-3}$ M) with a constant concentration of glycine (0.1 mM) in each sample was used to test the

abundance of cells expressing glutamate receptors and the sensitivity of responsiveness of these cells to L-glutamate. A range of ACh concentrations ($10^{-8}$ M – $10^{-3}$ M) were applied to test whether there was a change in the response of AChRs to this agonist. A mixture of glycine (0.1 mM) and NMDA (0.1 mM) was used to test for the presence of NMDA receptors and a mixture of glycine (0.1 mM), L-glutamate (0.1 mM) and $MgCl_2$ (1 mM) was used, as magnesium is a known blocker of NMDA receptors. A mixture of memantine hydrochloride (0.1 mM), glycine (0.1 mM) and L-glutamate (0.1 mM) was used, as memantine hydrochloride is a known antagonist of NMDA receptors. AMPA (0.1 mM) and kainic acid (0.1 mM) were tested separately to test for the presence of non-NMDAR iGluRs and a mixture of 6,7-dinitroquinoxaline-2, 3-dione (DNQX) (0.1 mM) and L-glutamate (0.1 mM) was used to test for the presence of these receptors as DNXQ is a known antagonist of non-NMDA glutamate receptors.

## 2.7 Data Measurement and Analysis

Data was gathered in the WinWCP Strathclyde Electrophysiology Software, where each trace (amplitude of whole-cell current (pA) measured against time (ms)) was individually analysed. The software was then used to calculate the mean negative peak in the given timeframe using 6-15 averaged points. The resulting means were then taken as the largest response to the certain sample that was being tested

and this data was further analysed in GraphPad Prism 9 for normalisation, graph plotting, curve fitting and statistical analysis. Concentration-response curves were fit with the equation:

$$\% \text{ maximum response} = 100/(1 + 10^{(LogEC50-X)S})$$

Equation 1 where *X* is Log[agonist] and *S* is the Hill slope.

# Chapter 3

# Results

## 3.1 Expression of GluK2 Receptors in HEK293 Cells

### 3.1.1 Bacterial Transformation and DNA Purification

Bacterial transformation via the freeze-shock method, followed by DNA isolation and purification using miniprep kits was utilised to produce copies of the wild-type and mutated (K525E) GRIK2 pcDNA. This method was first attempted with the wild-type GRIK2 DNA and the first two attempts yielded low concentrations of pcDNA (56.95 ng/μL and 57.60 ng/μL) and were of insufficient purity, with A60/A80 values higher than 1.8, which is the standard value of a pure sample of DNA (Table 1). The high A60/A80 values suggested the presence of RNA, which could be caused by multiple steps during the purification process. The transformation method was adjusted after this, so that the colonies picked were inoculated in 30 mL of LB broth (100 μg/mL ampicillin) instead of only 5 mL, thus providing greater opportunity for bacterial replication. After this alteration, the concentration of the DNA yield was higher and there were less impurities (Table 1); the A60/A80 values were still higher than 1.8 but were much smaller than previously, so this DNA was used for the transfection of HEK293 cells. When the GRIK2-K525E DNA was used, colonies were successfully formed on the LB and ampicillin agar plate, suggesting successful uptake of the exogenous DNA, however, there was no visible growth of the overnight cultures of these colonies, suggesting that the colonies seen were non-viable, resulting in no yield of DNA.

***Table 1 – Wild-Type GRIK2 DNA Concentration and Purity.***

*The concentration (ng/μl), A260, A280 and A60/A80 values of transformed and purified GRIK2 DNA.*

| **DNA** | **Concentration (ng/μL)** | **A260** | **A280** | **A60/A80** |
|---|---|---|---|---|
| GRIK2 1st attempt | 56.95 | 1.847 | 1.162 | 2.146 |
| GRIK2 2nd attempt | 57.60 | 1.677 | 1.275 | 2.138 |
| GRIK2 3rd attempt | 101.75 | 1.990 | 1.030 | 1.932 |
| GRIK2 4th attempt | 102.25 | 2.041 | 1.075 | 1.900 |

### 3.1.2 Non-Transfected HEK293 Cells and Glutamate

Non-transfected HEK293 cells were patch clamped and treated with a single concentration of glutamate (0.1 mM) and glycine (0.1 mM) to provide evidence that HEK293 cells do not endogenously express glutamate receptors. Twenty-one different HEK293 cells were tested, ten from one passage and eleven from a different passage. For each cell, an average was taken of ten points from the most negative peak in the trace and then these values were averaged to give a mean value of -5.1 pA (SD. 16.5). A one sample t and Wilcoxon test with a hypothesis value of 0 pA, gave a P value of 0.17, showing that this was not a significant response. A trace of one of the readings is provided in Figure 8A. This trace and the mean response show that there is little to no response to glutamate in non-transfected HEK293 cells.

### 3.1.3 Transfection of HEK293 Cells with GRIK2

The HEK293 cells that had undergone the transfection process with the GRIK2 pcDNA were patch clamped and treated with a range of glutamate concentrations ($10^{-7}$ M – $10^{-3}$ M), to measure whether there was a glutamate response and therefore whether the transfection was successful. It is important to mention that these cells proved difficult to sustain a stable whole-cell seal and over sixty cells, across three different passages and transfections were attempted to form a whole-cell seal. However, only eleven cells were able to form a stable seal that lasted through all five applications of glutamate

concentrations and out of these eleven cells that were tested, only four responded to glutamate. The data collected was normalised, where the largest response from each cell was 100% and the zero response being 0%. The % response at each concentration from the cells were averaged and this data was then plotted in a concentration-response graph and a curve corresponding to Equation 1 fitted (Figure 9), which shows a weak sigmoidal relationship, and large error bars, likely due to the small sample size of four. The $EC_{50}$ from this curve is 79 μM (95% CI 18.6 - 276.5 μM). A trace of a glutamate response has been shown in Figure 8B at a glutamate concentration of 0.01 mM ($10^{-5}$ M) and the desensitisation tau value (581 ± 21.74 (sd) ms) was taken from an exponential curve fitted to the decay of the response.

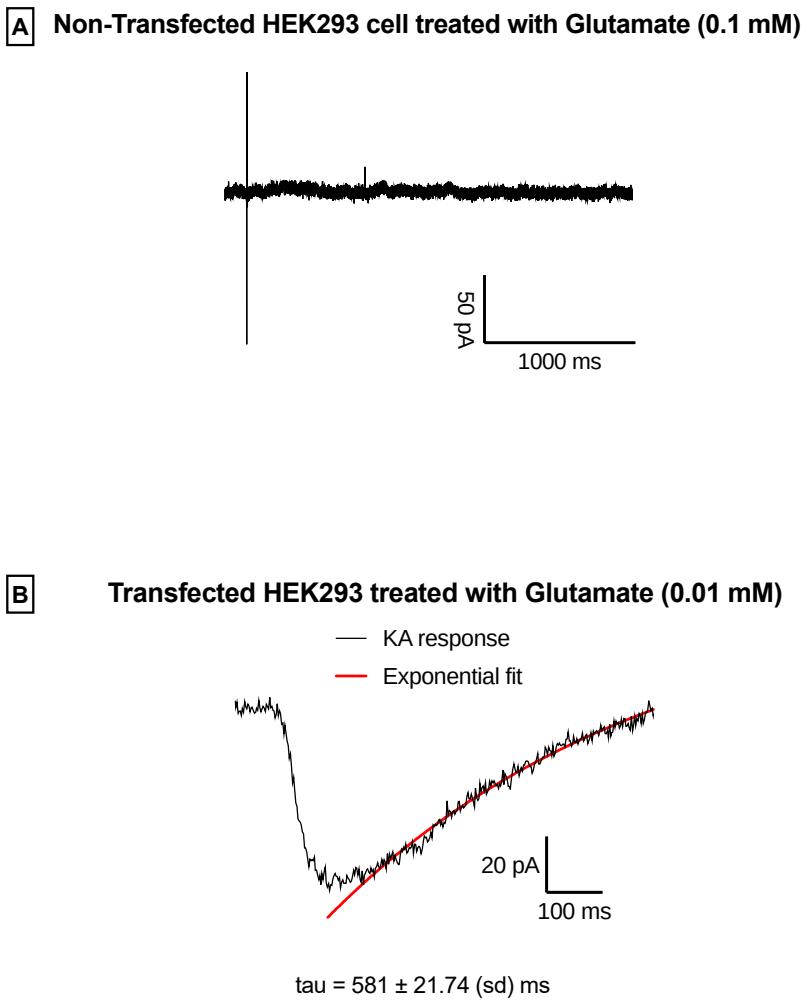



***Figure 8 – Responses of HEK293 Cells to Glutamate. (A)*** *Trace of non-transfected HEK293 cell in response to glutamate (0.1 mM).* ***(B)*** *Trace of wild-type GRIK2 transfected HEK293 cell in response to glutamate (0.01 mM), with a red line of fitted exponential decay.*

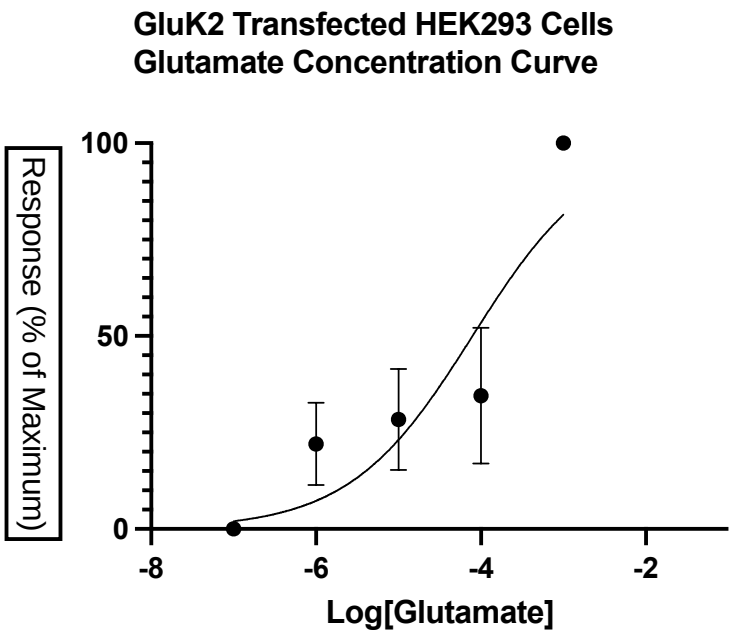



***Figure 9 – Transfected HEK293 Glutamate Concentration Curve.*** *Glutamate concentration-response curve of wild-type GRIK2 transfected HEK293 cells, with SEM error bars and fitted curve of Equation 1 (n=4).*

## 3.2 Ionotropic Receptor Expression in Undifferentiated and Differentiated TE671 Cells

### 3.2.1 Morphology of Differentiated TE671 Cells

Differentiated TE671 cells showed differences in morphology compared to undifferentiated TE671 cells. The undifferentiated TE671 cells typically presented as elongated, round cells with spindle-shaped extensions which grow close to other cells whereas, the differentiated cells showed more of a neuronal morphology with longer extensions which appear similar to dendrites. The differentiated cells grow much slower, suggesting they are less successful in this process than undifferentiated cells. The differentiated cells are more difficult to patch clamp than undifferentiated cells as there was a less successful rate of forming a whole-cell seal in the differentiated cells, and out of the 26 cells that were tested, only 10 responded to glutamate, suggesting that not all of the cells differentiated.

### 3.2.2 TE671 Cells and Acetylcholine Response

Undifferentiated TE671 cells were patch clamped and treated with a range of acetylcholine concentrations ranging from $10^{-8}$ M to $10^{-3}$ M. The traces of one cell's responses to the concentrations $10^{-6}$ M to $10^{-3}$ M are shown in Figure 10, where the response intensity increases in with the concentration. Nineteen cells were patch clamped, and the data was normalised and averaged, plotted in a concentration-response graph and fitted by a curve according to Equation 1 (Figure

11A). This demonstrated a sigmoidal relationship, with an estimated $EC_{50}$ of 6.7 μM (95% CI 3.6 - 11 μM).

The differentiated TE671 cells proved to be more difficult to patch clamp in comparison to undifferentiated TE671 cells, meaning that there were small sample sizes for tests on these cells. Only four differentiated TE671 cells were tested with a range of ACh concentrations ($10^{-8}$ M to $10^{-3}$ M), because of time restraints. The largest response (-1049 pA) obtained was at a concentration of 1 mM ($10^{-3}$ M) (Figure 12B) and is shown in comparison to an undifferentiated TE671 cell in response to the same concentration of ACh (Figure 12A). It can be seen when comparing these traces, that the differentiated cell has a much smaller and less intense response compared to the undifferentiated cell. The mean response of the undifferentiated TE671 cells in response to acetylcholine (1 mM) was -929.2 mV, whereas the mean response of differentiated TE671 cells was -29.3 mV. A Welch's t test was used to compare these mean responses, which gave a P value of 0.029 showing that there is a statistical difference between the two. The concentration-response curve of the differentiated TE671 cells (Figure 11B) shows a sigmoidal relationship which, compared to the undifferentiated TE671 cells (Figure 11C), is slightly right shifted. The undifferentiated cell response increases more steeply as the concentration increases whereas, the differentiated cell response increases more gradually as the concentration increases. The ACh $EC_{50}$ of the

differentiated cells was 24.1 μM (95% CI 12.4 – 46.3 μM) and therefore, the $EC_{50}$ of the undifferentiated cells was about 4-fold smaller. The ACh $EC_{50}$ values of the undifferentiated and differentiated cells underwent an unpaired t test, giving a P value of <0.0001, showing there is a significant difference between the two $EC_{50}$ values.

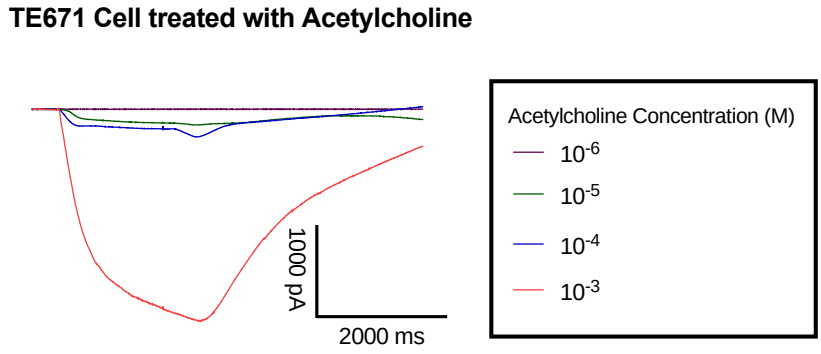



***Figure 10 – Responses of TE671 Cell to Acetylcholine.***

*Traces of a singular undifferentiated TE671 cell in response to a range of acetylcholine concentrations ($10^{-6}$ M to $10^{-3}$ M).*

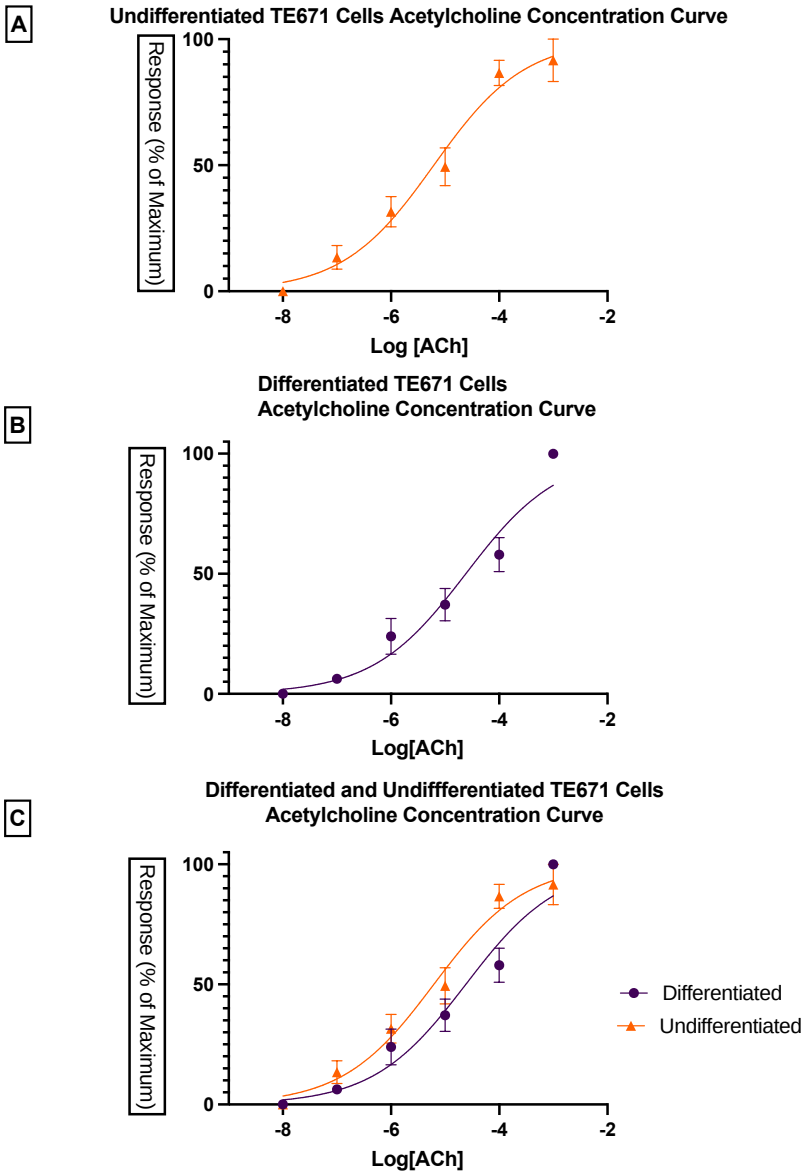



***Figure 11 – Acetylcholine Concentration Curves of TE671 Cells. (A)*** *Concentration-response curve of undifferentiated TE671 cells in response to ACh ($10^{-8}$ M to $10^{-3}$ M) with SEM error bars.* ***(B)*** *Concentration-response curve of differentiated cells in response to ACh ($10^{-8}$ M to $10^{-3}$ M) with SEM error bars.* ***(C)*** *Curves A and B plotted in the same graph with SEM error bars.*

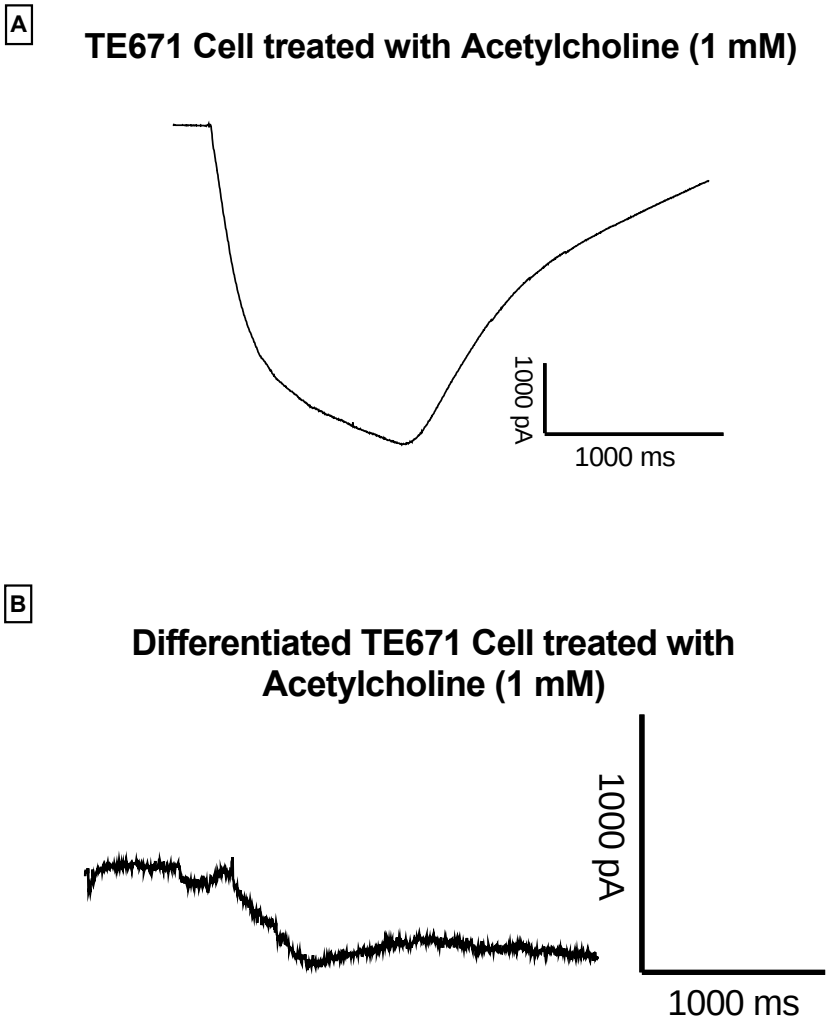



***Figure 12 – Acetylcholine Responses of TE671 Cells. (A)*** *Trace of an undifferentiated TE671 cell in response to ACh (1 mM).* ***(B)*** *Trace of differentiated TE671 cell in response to ACh (1 mM).*

### 3.2.3 TE671 Cells and Glutamate

Undifferentiated TE671 cells were patch clamped and tested with a single high concentration of glutamate (0.1 mM) and glycine (0.1 mM) to test for the presence of glutamate receptors, including NMDA receptors, where glycine is needed for activation. The mean of the responses of six cells was calculated as -3.4 pA (SD 5.1) and tested with a one sample t and Wilcoxon test with a hypothesis value of 0 pA, giving a P value of 0.16 showing no statistically significant response. A trace of one of these cells shows that there is little to no response to glutamate and glycine (Figure 13A), which was expected as undifferentiated TE671 cells have not been reported to endogenously express any glutamate receptors.

TE671 cells were differentiated, and patch clamped to test for responses to a range of glutamate concentrations ($10^{-7}$ M to $10^{-3}$ M), with a constant concentration of glycine (0.1 mM) to allow for potential NMDA receptors to be activated if present. The largest response of a differentiated TE671 cell to glutamate was at a concentration of 0.1 mM (Figure 13B) and can be seen in comparison to the undifferentiated cell in Figure 13A. The sample size of the differentiated cells used in this experiment was ten, as this was the number of differentiated cells that responded however, there were over thirty differentiated cells that were tested but did not respond to glutamate and glycine. The data was analysed and plotted in a concentration curve (Figure 14), showing a sigmoidal curve with no plateau, suggesting the plateau would be at a

concentration higher than $10^{-3}$ M. The glutamate $EC_{50}$ was 38.3 μM (95% CI 21.1 - 68.2 μM). The comparison of the undifferentiated TE671 cells in response to a high dose of glutamate (0.1 mM) with the differentiated TE671 cells shows that the differentiation by db-cAMP produces the expression of glutamate receptors.

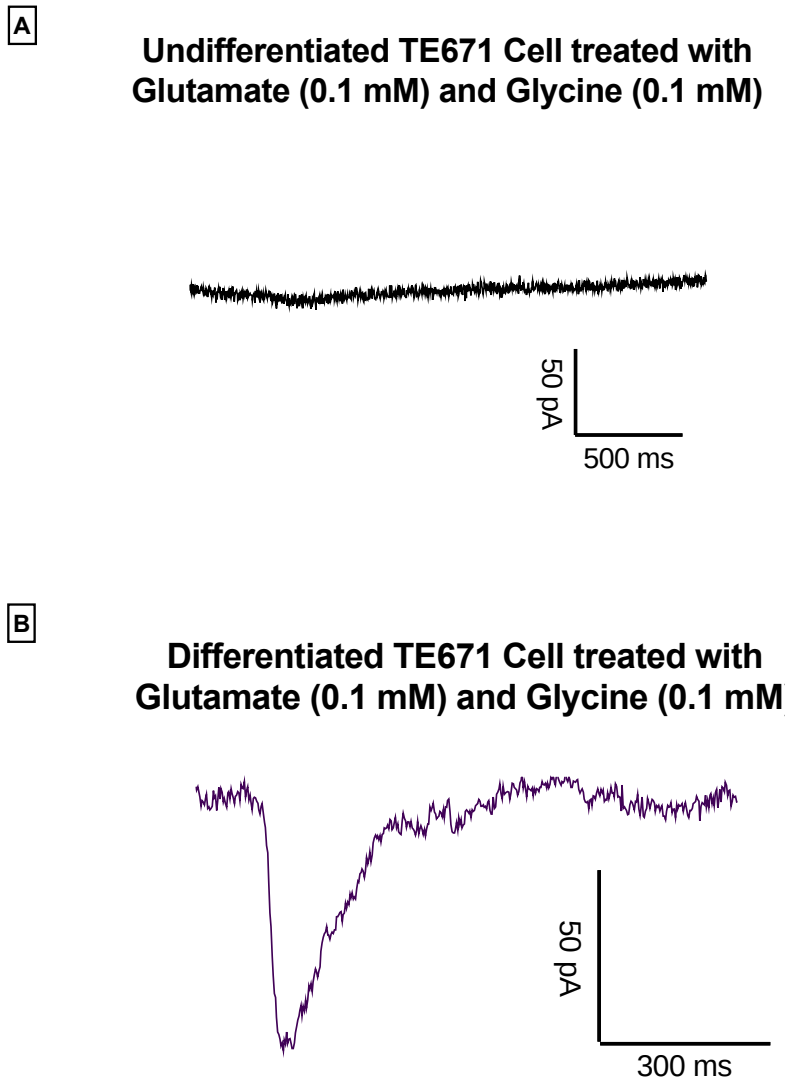



***Figure 13 – Glutamate Responses of TE671 Cells. (A)*** *Trace of an undifferentiated TE671 cell in response to glutamate (0.1 mM) and glycine (0.1 mM).* ***(B)*** *Trace of a differentiated TE671 cell in response to glutamate (0.1 mM) and glycine (0.1 mM).*

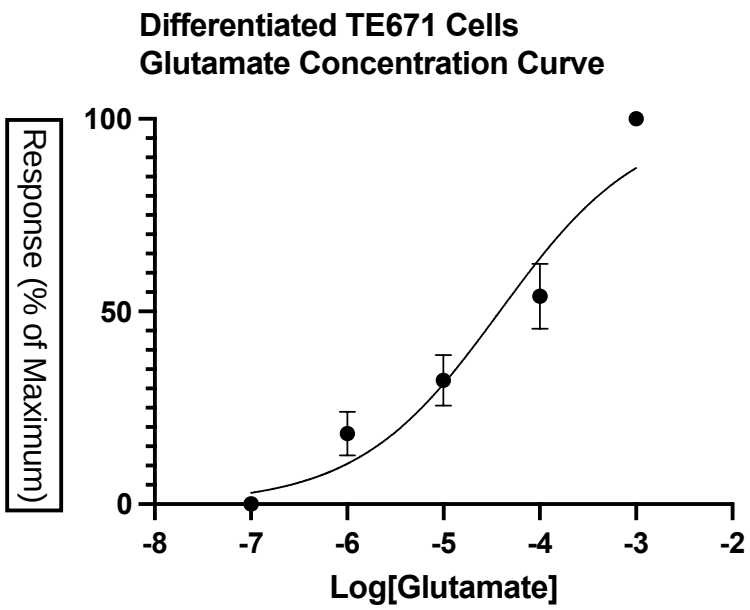



***Figure 14 – Glutamate Concentration Curve for Differentiated TE671 Cells.*** *Concentration-response curve of differentiated TE671 cells in response to a range of glutamate concentrations ($10^{-7}$ M to $10^{-3}$ M) with a constant concentration of glycine (0.1 mM) with SEM error bars. The curve is a fit of Equation 1.*

### 3.2.4 Agonists and Antagonists of iGluRs in Differentiated TE671 Cells

After data was gathered showing that the differentiation of TE671 cells resulted in the presence of a glutamate response and therefore, by assumption, the presence of glutamate receptors, different known agonists and antagonists of ionotropic glutamate receptors were tested on the differentiated cells to gather data on what type of glutamate receptors are being expressed in differentiated TE671 cells. A single concentration of each agonist and antagonist was used to allow for the testing of a wide range of samples, as after a certain amount of time the seals on the cells break, therefore it was important to efficiently utilise the time available. The samples tested were glutamate (0.1 mM) with glycine (0.1 mM), NMDA (0.1 mM) with glycine (0.1 mM), AMPA (0.1 mM), kainic acid (KA) (0.1 mM), memantine (MH) (0.1 mM) with glycine (0.1 mM) and glutamate (0.1 mM), DNQX (0.1 mM) with glutamate (0.1 mM) and the last sample was $MgCl_2$ (1 mM) with glutamate (0.1 mM) and glycine (0.1 mM). Each cell was treated with all of the samples and a total of sixteen cells responded to glutamate, out of twenty-five cells that were tested. The data were normalised against the response to glutamate and glycine and is displayed in Figure 15. The % response of NMDA and glycine was 58.5%, AMPA was 25.5% and KA was 27.5%, suggesting that all the agonists produced responses, with the highest response to NMDA which suggests that all three types of iGluR are expressed, but with

more NMDA receptors than non-NMDA receptors. The data suggests there is a strong presence of NMDA receptors in differentiated TE671 cells, as NMDA gives the largest response in comparison to the other agonists, and in the presence of the NMDAR antagonist, memantine, the response was 34.1%, ($P < 0.0001$ when comparing to the glu/gly response). The response traces of a differentiated TE671 to glutamate (0.1 mM) and glycine (0.1 mM), with and without memantine (0.1 mM), are shown in Figure 16. The traces show a large and short-lived response to glu/gly, without memantine and a smaller response in the presence of memantine. Assuming that the memantine completely blocked all NMDA receptors, the resulting response should come from non-NMDA receptors AMPAR and KAR, which gave responses similar to the response given in the presence of memantine. There is also a small percentage response (14.2%, $P < 0.0001$) in the presence of 1 mM $MgCl_2$, supporting the presence of NMDAR, as $Mg^{2+}$ ions are a known inhibitor of these specific receptors. The data also indicates the presence of non-NMDA iGluRs such as AMPAR and KAR, as responses to both AMPA and KA were obtained, but their % of the glutamate response was smaller than for NMDA. The response to glutamate in the presence of DNQX (15.4%, $P < 0.0001$) was also strongly reduced, which further supports that there is a presence of these receptors, as DNQX is an antagonist of non-NMDA receptors.

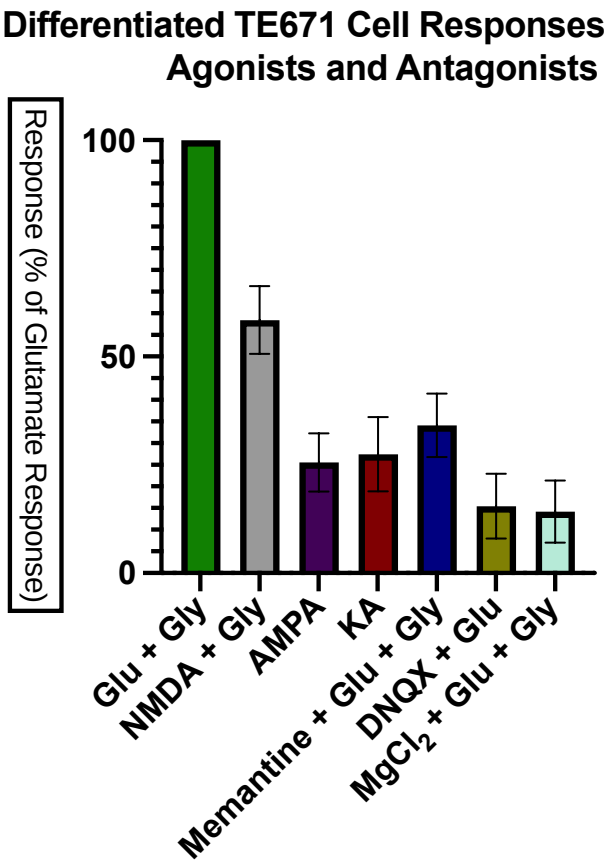



***Figure 15 – Responses to Range of iGluR Agonists and Antagonists in Differentiated TE671 Cells.*** *Differentiated TE671 cells mean percentage responses to each agonist and to glutamate in the presence of each antagonist with SEM error bars.*

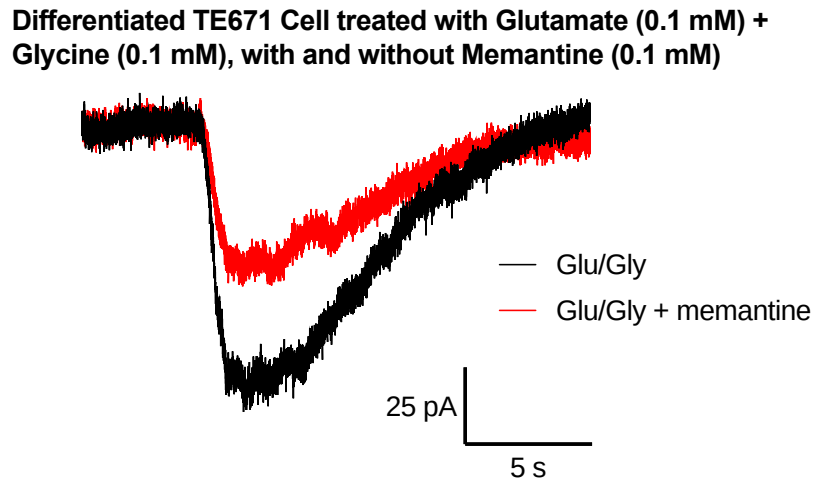



***Figure 16 – Responses of Differentiated TE671 Cells in the Presence of Glutamate and Glycine, with and without Memantine.*** *Traces of differentiated TE671 cell in response to glutamate (0.1 mM) and glycine (0.1 mM), with (red) and without memantine (0.1 mM) (black).*

# Chapter 4

# Discussion

## 4.1 GluK2 Expression in HEK293 Cells and Glutamate Response

The data gathered on non-transfected HEK293 cells show that there was no significant response to glutamate, with the sample size of twenty-one being sufficient to give confidence that HEK293 cells do not endogenously express ionotropic glutamate receptors. These data provide ample evidence that HEK293 cells that have not undergone transfection, do not respond to glutamate and therefore do not natively express glutamate receptors. This is also supported by studies carried out on HEK293 cells, specifically one study that investigated the presence of G protein-coupled receptors and related proteins in HEK293 cells, mentioned the lack of presence of mGluRs and iGluRs in these cells (Atwood *et al*., 2011) and a further study showed that embryonic stem cells, including the HEK293 cell line, did not express functional iGluRs (Pachernegg *et al*., 2013).

The HEK293 cells that underwent transient transfection with GRIK2 pcDNA, using the Escort IV reagent, were difficult to form whole-cell seals with. As mentioned above, over sixty cells were tested and attempted to be patch clamped over three different passages/transfection attempts, and only eleven cells formed seals that remained stable throughout all five sample applications of glutamate ($10^{-7}$ M to $10^{-3}$ M). Out of these eleven cells that were tested, only four responded to glutamate. This suggests that less than half of the cells

successfully expressed functioning GluK2-containing KAR. The difficulty of transient transfection with glutamate receptor DNA, specifically kainate receptor DNA, has been documented before in previous studies (Jones, Wilding, Huettner and Costa, 1997) and is also demonstrated by the fact that there are such few studies investigating glutamate receptor electrophysiology in this expression system. There have however, been a few studies that have successfully managed to express GluK2 in HEK293 cells (Raymond, Blackstone and Huganir, 1993; *Stolz et al*., 2021). This may be due to the usage of a thoroughly optimised transfection technique, which time did not allow for in this study. Nevertheless, the number of studies documenting successful expression of GluK2 in human cells is low, relative to studies presenting the successful expression of NMDAR and other non-native proteins in human cells. This suggests that transfection of such DNA and expression of these receptors (GluK2-containing KAR) is difficult to produce *in vitro*, and particularly in HEK293 cells. The data was used to form a concentration-response curve (Figure 9), which illustrated a weak sigmoidal curve and large error bars, showing low accuracy of this experiment, which is to be expected from a small sample size of four. To compare the glutamate $EC_{50}$ of this experiment with $EC_{50}$ values from other studies would be difficult, as mentioned before, there are few studies on cells that natively express KAR. However, a study that managed to successfully express GluK2 in HEK293 cells, gave a glutamate $EC_{50}$ value of 273 μM (Raymond, Blackstone and Huganir, 1993), which is around 3-fold higher

than the glutamate $EC_{50}$ of this study (79 μM). Interestingly, a study using *Xenopus laevis* oocyes expressing GluK2, provides a glutamate $EC_{50}$ value of 42 μm (Table 2) (*Koromina M. (2018)),* which is much closer to the value given in this study. This may suggest the glutamate $EC_{50}$ result found in this study is more reliable than the one previously found in transfected HEK293 cells. However, it may also demonstrate low accuracy and thus, reliability of the data gathered in this experiment. This is most likely due to the small sample size and lack of time, which led to a restriction in the ability to optimise the transfection technique using Escort IV.

***Table 2 – Glu $EC_{50}$ Values of GluK2 Receptor Subunits.***

*Values reported by Koromina (2018) showing the glutamate $EC_{50}$ of homogenous GluK2 KAR, expressed in Xenopus oocytes.*

| KAR subtypes | Glu $EC_{50}$ (μM), 95%CI and (N) |
|---|---|
| **h.GluK2** | 42.0<br>24.3 – 68.1<br>(9 – 13) |

The response of a transfected HEK293 cell to glutamate (0.01 mM) (Figure 8B), shows a fast desensitisation rate with a τ value of 581 ms. Previous studies typically show smaller τ values for the desensitisation of GluK2 KAR of 4.9 ± 0.2 ms and 3.56 ± 0.4 ms (Stolz *et al*., 2021; Meyerson *et al.,* 2016), however these values were taken from responses at higher concentrations of glutamate (10 mM), which is 1000-fold larger than the concentration used in this study (0.01 mM), suggesting a reason for the desensitisation τ value being different in this experiment. Desensitisation rates depend strongly on agonist concentration (Heckmann and Dudel, 1997), so this may explain the differences.

Regardless of the weak sigmoidal curve and large errors in the concentration-response curve of the transfected cells (Figure 9), the results show clearly that there is a response to glutamate in these transfected cells, however, the cells are temperamental, struggle to stay adhered to glass cover slips, and are difficult to form stable whole-cell seals with. The strongest evidence of the ability of these cells to express GluK2, is the clear response to glutamate at 0.01 mM (Figure 8B), which shows a strong negative peak. The temperamental behaviour of these cells and the length of time needed for the transfection process (4 days from start to patch clamping), influenced the decision to end the study of KAR expression in HEK293 cells here, as time was a main limiting factor of this project. The struggle to yield fresh pcDNA using the freeze-

shock method was also a factor of this decision. Therefore, the conclusion of this investigation is that it is achievable to transiently transfect HEK293 cells with the wild-type kainate receptor subunit GRIK2 DNA, with Escort IV reagent and produce expression of functioning KAR in these transfected cells.

It would be valuable to continue these experiments with more time and resources, to optimise the transfection technique using the Escort IV reagent, as it is stated in the instruction manual that the optimal volumes of pcDNA, reagent, and time needed for different stages of the transfection process differ depending on the type of cell and the pcDNA being transfected. The optimisation of the transfection technique would then perhaps produce cells that are easier to patch clamp and yield data from. The data gained from such experiments would be useful, as there are very few models for glutamate receptors, specifically KAR in human cell lines. A model such as this would be valuable to provide data on the kinetics of KAR and mutated KAR, of which mutations are known to contribute to neurological disorders such as autism, schizophrenia (Koromina *et al*., 2019) and epilepsy (Lerma and Marques, 2013).

## 4.2 Differentiated TE671 Cells and Acetylcholine Response

TE671 cells natively express nicotinic acetylcholine receptors (nAChR) (Schoepfer, Luther and Lindstrom, 1988), so this is a cell line guaranteed to produce a measurable response when treated with ACh and therefore, provided an adequate system to learn and practice the whole-cell patch clamp technique on. The undifferentiated TE671 cells gave an ACh $EC_{50}$ value of 6.7 μM and the traces show expected characteristics of the TE671 nAChR in response to ACh. This ACh $EC_{50}$ value for TE671 cells is similar to values found in the literature; one study shows an ACh $EC_{50}$ of 7.8 μM (Shao Z., *et al.*, 1998) and another with an ACh $EC_{50}$ of 2.7 μM (Jacobsen, n.d.), suggesting that the data is accurate.

The TE671 cells that were differentiated by db-cAMP (40 mM), were tested under the same conditions and this data is shown in Figure 11B in a concentration-response curve. The two data sets (undifferentiated and differentiated TE671 cells) can be compared in Figure 11C, showing that both the undifferentiated and differentiated cells show a typical curve, expected from an agonist concentration-response curve. The difference in ACh $EC_{50}$ values of the undifferentiated ($EC_{50}$ 6.7 μM) and differentiated cells ($EC_{50}$ 24.1 μM), suggests that the differentiated cells require higher concentrations of ACh to produce half of the maximum response. This deduction is also visualised in the comparison of traces of an undifferentiated

cell and differentiated cell, responding to the same concentration of ACh (1 mM) (Figure 12). These images show that the response of the differentiated cell has a much shorter response time and smaller current in comparison to the undifferentiated cell, suggesting that the muscle type nAChRs that are expressed in TE671 cells under normal physiological conditions (Syapin, Salvaterra and Engelhardt, 1982), are changed to a different type in the differentiated cells. It is possible that a neuronal nAChR subtype is expressed instead, as the morphology of the differentiated cells suggests a neuronal phenotype. This suggestion is supported by studies showing that a neuronal nAChR morphological subtype is expressed in response to db-cAMP (Siegel and Lukas, 1988). Neuronal nAChRs consist of a range of subtypes that are comprised of varying subunit combinations; known neuronal subunits are $\alpha$2-$\alpha$10 and $\beta$2-$\beta$4 (Dani, 2001). In a study of *Xenopus* oocytes expressing rat nAChRs, the ACh $EC_{50}$s in some of these neuronal subunits (Table 3) (Papke, Dwoskin and Crooks, 2007) are closer in value to the ACh $EC_{50}$ in the differentiated TE671 cells in this study. A similar study in *Xenopus* oocytes gives ACh $EC_{50}$ values also similar to the value produced in this study (Table 4) (Kachel, Patel, Franzyk and Mellor, 2016), with the subunits being of the neuronal type. The similarities in ACh $EC_{50}$ values, may also support the assumption that the nAChRs expressed in differentiated TE671 cells are of a neuronal subtype. The comparison to ACh $EC_{50}$ values from the literature, suggest that there may be a range of different types of neuronal nAChR present, as

multiple types of receptors give ACh $EC_{50}$ values that are similar to the one found in this study, specifically receptors containing subunits $\alpha$2, $\alpha$3, $\alpha$4 and $\beta$2 (refer to Table 3 and Table 4). Further research may involve Western blots to detect the specific types of neuronal nAChR subunits that are present in differentiated TE671 cells.

The data gathered from the differentiated cells was from a small sample size of four cells, meaning that the data gathered is not as reliable as the undifferentiated cell data. This was due to time limitations, thus with additional time and more repeats of this data, a more reliable picture of the differences between the nAChRs of undifferentiated and differentiated TE671 cells could be gathered.

***Table 3 – ACh $EC_{50}$ Values of nACh Receptor Subunits.***

*Values reported by Papke et al. (2007) showing the ACh $EC_{50}$ values of nAChRs consisting of various neuronal subunits.*

| Receptor | Agonist | |
|---|---|---|
| | ACh | |
| | $EC_{50}$ µmol/L | $I_{max}$ |
| α7 | 68 ± 9 | 1* |
| α4β2 | 37 ± 7.4 | 1* |
| α3β4 | 130 ± 19 | 1* |
| α3β2 | 72 ± 15 | 1* |
| α3β2hα5 | 21 ± 4 | 1* |
| α3β2β3 | 87 ± 22 | 1* |
| α6/3β2β3 | 16 ± 2.4 | 1* |

***Table 4 – ACh $EC_{50}$ Values of nACh Receptor Subunits.***

*Values reported by Kachel et al. (2016) showing the ACh $EC_{50}$ values of nAChRs consisting of various neuronal subunits.*

| | n | ACh $EC_{50}$ (95% CI), µM |
|---|---|---|
| α4β2 | 10 | 11.9 (9.3-15.2) |
| α4β4 | 12 | 9.4 (7.6-11.6) |
| α3β4 | 13 | 103 (96-112) |
| α3β2 | 14 | 17.4 (14.2-21.3) |
| α7 | 13 | 178 (155-205) |
| α1β1γδ | 10 | 16.0 (13.9-18.3) |

## 4.3 Differentiated TE671 Cells and Glutamate Response

The undifferentiated TE671 cells were tested with glutamate and no evidence of a response to glutamate was obtained. After the confirmation that the TE671 cells being used did not natively express glutamate receptors, differentiated TE671 cells were tested with a range of glutamate concentrations ($10^{-7}$ M to $10^{-3}$ M), each sample with a constant concentration of glycine (0.1 mM), which was needed to activate NMDA receptors (Cummings and Popescu, 2015). A trace of one of these cells in response to 0.1 mM glutamate is shown in Figure 13B, where the response is fast and transient. In comparison to the undifferentiated TE671 cell (Figure 13A), it can be concluded that the undifferentiated cell does not give a response to glutamate, whereas the differentiated cell does. The concentration-response curve (Figure 14) of the differentiated TE671 cells responding to glutamate, show the expected sigmoidal curve, with a glutamate $EC_{50}$ of 38.3 μM, however, an upper plateau cannot be seen in this curve suggesting that the maximum response was not reached and would be reached at a higher concentration of glutamate, perhaps at $10^{-2}$ M or $10^{-1}$ M. Therefore, it would be valuable to carry out these experiments with higher concentrations of glutamate and with a larger sample size, as the sample size used was ten cells. The next step was to gather data on the type(s) of glutamate receptors that were being expressed in the differentiated TE671 cells, so a range of agonists for each

iGluR were used, along with known antagonists of these receptors.

## 4.4 Differentiated TE671 Cells and iGluR Agonists and Antagonists

The differentiated TE671 cells were treated with a range of known agonists and antagonists of different iGluRs (NMDAR, AMPAR and KAR), as shown in Figure 15. The largest response given in comparison to glutamate and glycine, was the response to NMDA and glycine with a 58.5% response of the maximum that was given by glutamate and glycine (100%). This provides evidence of the presence of NMDA receptors in differentiated TE671 cells. This evidence is also supported by the reduced response to glu/gly in the presence of $Mg^{2+}$ (response percentage = 14.2%), which was expected to be low, as $Mg^{2+}$ ions block NMDAR in a voltage-dependent manner (Johnson and Kotermanski, 2006). Alongside this, further evidence of the presence of NMDAR, includes the reduction in the response given to glutamate and glycine in the presence of memantine (response percentage = 34.1%), which is a known antagonist of NMDAR. The traces of a differentiated TE671 cell in response to glu/gly, with and without memantine (Figure 16), shows this reduced response, however, still shows a small response in the presence of memantine. This may suggest that the concentration of the antagonist was not high enough to inhibit all NMDA receptors. However, the concentration of memantine used (0.1 mM), was

a concentration high enough to be expected to completely inhibit the NMDAR component of the response. Therefore, the smaller response to memantine that is seen can be assumed to be due to any non-NMDA receptors that may be present in the cells. This hypothesis is supported by the presence of a response to glu/gly, with memantine and $Mg^{2+}$ and the size of the response in the presence of memantine being a similar size of the responses to AMPA (25.5%) and KA (27.5%). These conclusions are presented clearly in Figure 15 and provide sufficient evidence to conclude the presence of NMDAR in differentiated TE671 cells. A limitation of resources and time meant that further research exploring the effects of a range of concentrations of NMDA, memantine and $Mg^{2+}$ could not be carried out, however it would be valuable to continue this research, as it would provide more insight into the behaviours of the NMDA receptors expressed in these differentiated cells, including an $EC_{50}$ of NMDA and $IC_{50}$s of memantine and of $Mg^{2+}$. More data on the response in the presence of $Mg^{2+}$ could provide insight into the type of NMDA receptor being produced, as NMDAR has subunit-dependent properties, specifically properties depending on the sensitivity to extracellular $Mg^{2+}$ (Cull-Candy, Brickley and Farrant, 2001). NMDAR comprising the GluN2A or GluN2B are more sensitive to blockade by $Mg^{2+}$ than NMDAR comprising GluN2C or GluN2D (Zhou and Sheng, 2013). The $Mg^{2+}$ $IC_{50}$ values are different for the different subunit combinations with 2 μM for GluN1/2A and GluN1/2B, 14 μM for GluN1/2C, and 10 μM for

GluN1/2D (Kuner and Schoepfer, 1996), giving a potential research route for the types of NMDAR expressed in differentiated TE671 cells.

Another antagonist of NMDAR is ifenprodil, which selectively blocks the GluN1 subunit that is required for functional expression, as well as the GluN2B subunit. Experiments investigating the response of differentiated TE671 cells in response to glu/gly in the presence of ifenprodil could provide further insights into the type of NMDAR subunits that are expressed. Further research could involve measuring the length of time that these receptors are expressed for after the db-cAMP is applied to the cells. This would be useful if the cells were to be used as a model for NDMARs and related diseases such as Alzheimer's disease. This would be a valuable discovery as there is a lack of iGluR models in human cell lines, either natively expressed or via transient transfection.

The presence of non-NMDA iGluRs in the differentiated TE671 cells is supported by the data gathered. There are responses for both AMPA and KA, however, at much lower percentages than for NMDA. AMPA has a response of 25.5% and KA with a response of 27.5%. The non-NMDAR antagonist, DNQX, which acts as an antagonist for both AMPAR and KAR, reduced the glutamate response to 15.4%. This is a strong sign that there is a presence of these receptors in the differentiated cells. The traces of a cell responding to these

agonists and antagonist shows a visible response to glutamate and AMPA but does not show a strong response to KA. This could suggest that different differentiated cells express different or varying quantities of iGluRs, however, AMPAR and KAR both react to AMPA and KA to varying degrees, therefore there may be both receptors expressed, or just one. It would be expected that AMPA receptors are expressed, as they are usually expressed alongside NMDAR, as they are required to produce a small depolarisation event in order to trigger the magnesium ion block release from NMDAR. Cyclothiazide has been shown to strongly potentiate AMPAR, with no effect on KAR, whilst concanavalin A potentiates KAR, but only weakly affects the response of AMPAR (Partin *et al*., 1993). Testing the differentiated cells with these compounds could provide insight into the type(s) of non-NMDA iGluRs that are expressed in differentiated TE671 cells.

The sample size for these experiments was sixteen, and the error bars are relatively small, suggesting that the data has reliability. The reliability of this data would be stronger with a larger sample size, however time limitations affected this variable negatively. This study demonstrates the first documented research of GluR subunits in the differentiated and non-differentiated TE671 cell line. The presence of non-NMDA receptors in the differentiated TE671 cells would provide a useful model of iGluRs in a human cell line, for diseases such as autism affected by KAR disfunction and

Alzheimer's disease affected by decreased AMPA activation (Chang, Verbich and McKinney, 2012).

## 4.5 Limitations

The most prevalent limitations of this study were time and resources, as the project was set out over a year, with a large portion of that time needed for learning, understanding, and practicing the patch clamp technique. The transfection of HEK293 cells was a process that would have benefitted from increased time availability, as the optimisation of the technique may have improved the number of cooperative cells – referring to the number of cells able to remain in a stable seal throughout sample application. More time would have allowed for fresh GRIK2-K525E mutant DNA to be produced via bacterial transformation and DNA purification, to be used in HEK293 cell transfection, as with the wild-type GRIK2 DNA. This would then provide further data and insight into the kinetics and characteristics of the GluK2 subunit and the mutated GluK2(K525E) subunit of KAR, which was the original aim of this study. A limitation of the patch clamp method is lack of control over the ambient temperature and pressure, which can cause air bubbles in the fine bore tubes of the fast perfusion system, leading to disruption in the resistance being measured; the complexity and difficulty of the patch clamp procedure, leading to small sample sizes over long time

periods, which thereby produce higher errors than desired and thus, lower reliability in the data.

## 4.6 Findings and Implications

The findings from this study provide evidence of the ability to use human cell lines to research the kinetic properties and other characteristics of iGluRs. The transient transfection of the GRIK2 DNA can be used as a human model of an endogenous GluK2 KAR, which would be useful for research in diseases that are known to be implicated by GluK2-containing KAR dysfunction, where the direct link between the KAR dysfunction and pathology of the disease is not fully understood. For example, in autosomal recessive juvenile Parkinson's (ARJP) disease, the mutation in the Parkin enzyme is known to have an impact on GluK2 turnover, however it is not understood how this affects glutamatergic neurons and whether this has a direct impact on brain function. A human *in vivo* model of a GluK2 containing KAR would provide a valuable tool in research, such as investigating the impact of GluK2 on the pathophysiology of ARJP. This concept also applies to the findings that differentiated TE671 cells produce a neuronal cell subtype that express iGluRs, as the difficulty in expressing iGluRs in human cells leads to a restriction in data of the pathophysiological role of such receptors. The differentiated TE671 cells could provide a human cellular model of neurons affected by Alzheimer's

disease, which is impacted by dysfunction of all three types of iGluR.

# Chapter 5

# Conclusions

This study was focused on ionotropic glutamate receptors and the current lack of models of these receptors and their associated diseases in human cell lines. It is established that there is a lack of research in kainate receptors including their kinetics, structure, function, and involvement with disorders such as autism, schizophrenia, bipolar disorder and more. This lack of research is influenced by the difficulty of KAR subunit DNA transfection into human cell lines and the unavailability of human cell lines that natively express KAR. This study presents a new method of transfecting GRIK2 DNA into the HEK293 human cell line successfully, using Escort IV to produce cells that transiently express the wild-type homogenous GluK2 KAR. These transfected cells express functioning KAR, which have been whole-cell patch clamped to measure the electrophysiology of the cells and from which, it can be concluded that the cells produce a response to glutamate, the naturally occurring biological agonist of KAR. This is a valuable discovery, as models of glutamate receptors in human cell lines give a more valid and accurate representation of the function of the receptors, as opposed to using non-human cells. This is due to the differences between human and non-human cells, for example, *Xenopus* oocytes are commonly used in electrophysiology when studying glutamate receptors, which have an array of differences such as a different cell membrane composition, differences in post-translational processing and the size of oocytes, as they are larger than human cells and so the receptor kinetics are more difficult to investigate. These differences would not give as

accurate an insight into what occurs with such receptors in their natural environment (the human body), as using human cell lines would, therefore the finding that the human cell line HEK293 can be transfected with functioning GRIK2 DNA, will allow for more accurate data if using this method rather than using non-human cells.

Another potential model of glutamate receptors was also found in this study, in the human TE671 cell line, differentiated with db-cAMP. The results of this study show that there is a presence of iGluRs in these differentiated cells and specifically, a presence of NMDA receptors, in addition to non-NMDA receptors. The differentiated cells produced responses to glutamate at varying concentrations, and responses to NMDA, AMPA and KA. These responses allow a confident assumption that once TE671 cells are differentiated via db-cAMP, they express NMDA and non-NMDA receptors. The cells also change in morphology and present more physically as neuronal cells, along with changes in the response to acetylcholine, which suggest the presence of neuronal nAChR subtypes. Like the KAR receptors transfected in HEK293 cells, the differentiated TE671 cells have a potential to act as a model for these certain iGluRs that are expressed. Further investigation would have to be carried out to conclude the exact type of receptors that are expressed, as there are different subunit combinations of the receptors and it is not known whether both AMPAR and KAR are expressed, or only one, due to the cross-reactivity of AMPA and KA in these

receptors. Using the differentiated cells for a model of NMDAR would be valuable because of the implications of NMDAR in various diseases such as Alzheimer's disease, which is known to be directly affected by NMDAR. This link to disease reinforces the importance and need for a valid model of NMDAR and other iGluRs and therefore, displays the value of this study in regard to increasing and strengthening the knowledge of such receptors.

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