Kainate receptor-mediated regulation of chloride homeostasis

by

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A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy

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Abstract

A balance between excitatory and inhibitory activity is needed to maintain proper brain function. At inhibitory synapses, potassium-chloride cotransporter 2 (KCC2) has long been known to be a critical regulator of inhibition through its ability to maintain a low intracellular chloride level. Surprisingly, KCC2 has recently been found to play an important role at the excitatory synapse, where it interacts with several proteins involved in excitatory neurotransmission, including the kainate receptor subunit GluK2.

It is known that independent of kainate receptor activation, the physical interaction between KCC2 and GluK2 is important for KCC2 surface expression, oligomerization and recycling. However, it is unknown whether the activity of kainate receptors can directly influence KCC2 function. My thesis research has revealed a novel functional role for kainate receptors in CA3 pyramidal cells. I show that activating KARs in the hippocampus hyperpolarizes $E_{GABA}$ and increases the driving force for $Cl^-$. This hyperpolarization occurs through both ionotropic and metabotropic KAR signaling. The metabotropic signaling mechanism is dependent on KCC2, but the ionotropic signaling mechanism produces a hyperpolarization of $E_{GABA}$ even in the absence of KCC2 transporter function. These results demonstrate a novel functional interaction between a glutamate receptor and KCC2, a transporter critical for maintaining inhibition, suggesting that the KAR: KCC2 interaction may play an important role in excitatory: inhibitory (E:I) balance in the hippocampus. Additionally, the
ability of KARs to regulate chloride homeostasis independently of KCC2 suggests that KAR signaling can regulate inhibition in multiple ways that may involve other chloride transporters. Activation of kainate-type glutamate receptors could serve as an important mechanism for increasing the strength of inhibition during period of strong glutamatergic activity and a potential target for developing strategies to treat diseases characterized by an imbalance in excitation and inhibition.
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List of Abbreviations

[Cl⁻] Chloride concentration
5HTA+ Serotonin receptor 3A positive
$^{86}$Rb+ Rubidium ion
AChR Acetylcholine receptor
aCSF Artificial cerebrospinal fluid
AP Action potential
AHP Afterhyperpolarization
AMPA $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA Analysis of variance
ATP Adenosine triphosphate
CA Cornus ammonis
CA1 Cornus ammonis 1
CA3 Cornus ammonis 3
CaCC Calcium-activated chloride channel
Ca$^{2+}$ Calcium ion
CCC Cation chloride co-transporter
CCK+ Cholecystokinin positive
Cl⁻ Chloride ion
CIC Chloride channel
DIDS 4,4'-Diisothiocyanato-2,2'-stilbenedisulfonic acid disodium salt
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal cortex</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>Half-maximal effective concentration</td>
</tr>
<tr>
<td>E$_i$</td>
<td>Reversal potential of a given ion (i)</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GluK</td>
<td>Kainate receptor subunit</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HCO$_3^-$</td>
<td>Bicarbonate ion</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>I$_{AHP}$</td>
<td>Afterhyperpolarization current</td>
</tr>
<tr>
<td>IPSC</td>
<td>Inhibitory postsynaptic current</td>
</tr>
<tr>
<td>K$^+$</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>K2P</td>
<td>Two-pore domain potassium channel</td>
</tr>
<tr>
<td>KA</td>
<td>Kainic acid</td>
</tr>
<tr>
<td>KAR</td>
<td>Kainate receptor</td>
</tr>
<tr>
<td>KCC2</td>
<td>Potassium-chloride cotransporter 2</td>
</tr>
<tr>
<td>KCC3</td>
<td>Potassium-chloride cotransporter 3</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ms</td>
<td>Milliseconds</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolts</td>
</tr>
<tr>
<td>mZnR</td>
<td>Metabotropic zinc receptor</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>Na⁺/K⁺ ATPase</td>
<td>Sodium-potassium adenosine triphosphatase</td>
</tr>
<tr>
<td>NCBE</td>
<td>Sodium-chloride-bicarbonate exchanger</td>
</tr>
<tr>
<td>NDAE</td>
<td>Sodium-dependent anion exchanger</td>
</tr>
<tr>
<td>NDCBE</td>
<td>Sodium-dependent chloride bicarbonate exchanger</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylnucleimide</td>
</tr>
<tr>
<td>NKCC1</td>
<td>Sodium potassium chloride co-transporter 1</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>Ammonium ion</td>
</tr>
<tr>
<td>NKCC1</td>
<td>Sodium potassium chloride co-transporter 1</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartic acid receptor</td>
</tr>
<tr>
<td>pA</td>
<td>Picoamperes</td>
</tr>
<tr>
<td>PC</td>
<td>Pyramidal cell</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PPR</td>
<td>Paired pulse ratio</td>
</tr>
<tr>
<td>PV⁺</td>
<td>Parvalbumin positive</td>
</tr>
<tr>
<td>RMP</td>
<td>Resting membrane potential</td>
</tr>
<tr>
<td>Ser940</td>
<td>Serine 940</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>$SLC$</td>
<td>Solute carrier</td>
</tr>
<tr>
<td>SOM+</td>
<td>Somatostatin positive</td>
</tr>
<tr>
<td>$Tl^+$</td>
<td>Thallium ion</td>
</tr>
<tr>
<td>$\mu M$</td>
<td>Micromolar</td>
</tr>
<tr>
<td>$V_{DF}$</td>
<td>Driving force</td>
</tr>
<tr>
<td>$V_{eq}$</td>
<td>Reversal potential</td>
</tr>
<tr>
<td>$V_m$</td>
<td>Membrane potential</td>
</tr>
<tr>
<td>VU</td>
<td>VU 0463271</td>
</tr>
<tr>
<td>WNK</td>
<td>With no lysine kinase</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>Zinc ion</td>
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Chapter 1: Introduction

1 Introduction

1.1 General Introduction

Proper function of the nervous system depends upon a complex balance between excitation and inhibition. Disruption in the excitatory: inhibitory (E: I) balance is a hallmark of many diseases and disorders of the nervous system, including autism (Canitano & Pallagrosi, 2017; Lee, E., Lee, & Kim, 2017; Selten, van Bokhoven, & Nadif Kasri, 2018), schizophrenia (Canitano & Pallagrosi, 2017; Selten et al., 2018), epilepsy (Fukata & Fukata, 2017; Heise et al., 2017; Lopantsev, Both, & Draguhn, 2009; Wong, 2010), and neuropathic pain (Petrou et al., 2012; Potter et al., 2016). This balance is ultimately maintained at the synapse, the structure which allows neurons to communicate with each other via electrical or chemical signaling. At the synapse, neurons influence the activity levels of one another through complex patterns of signaling that broadly, can either facilitate activity (excitation) or prevent it (inhibition). This process is finely regulated by a multitude of proteins expressed at the synapse, including the receptors that detect neurotransmitters as well as the myriad of ion channels, transporters and other regulatory proteins that maintain the necessary electrochemical gradients of the ions involved in cell signaling and the generation of action potentials in the membrane.

Potassium-chloride cotransporter 2 (KCC2) has long been known to be a critical regulator of inhibition through its ability to maintain a low intracellular chloride level, a prerequisite for fast inhibitory GABAergic neurotransmission (Rivera, C. et al., 1999). However, KCC2 has recently been found to play an important role at the excitatory synapse, where it interacts with
several proteins involved in excitatory neurotransmission, including the kainate receptor (KAR) subunit GluK2 (Mahadevan et al., 2014).

It is known that independent of kainate receptor activation, the physical interaction between KCC2 and GluK2 is important for KCC2 surface expression and oligomerization, and recycling (Mahadevan et al., 2014; Pressey et al., 2017). However, it is unknown whether the activity of kainate receptors can directly influence KCC2 function, which could serve as a potential mechanism for excitation to regulate inhibition in a homeostatic manner. Throughout my thesis, I demonstrate that independently activating either the ionotropic or metabotropic KAR signaling pathways produces a hyperpolarization of the reversal potential for GABA ($E_{GABA}$). I also show that hyperpolarization of $E_{GABA}$ occurs through two different mechanisms, one that results in an increase in KCC2 function, and one that occurs independently of KCC2 function. This effect was not observed in GluK1/2$^{-/-}$ CA3-pyramidal cells and is independent of zinc release from mossy fibre (MF) terminals. This represents a novel homeostatic mechanism for glutamatergic activity to modulate the strength of inhibitory synaptic transmission in the hippocampus through KCC2.

1.2 Synaptic Transmission

1.2.1 Electrical and Chemical Neurotransmission

In the central nervous system (CNS), neurons communicate via a unique structure known as the synapse (Sherrington, 1906). There are two main categories of synapses: electrical and chemical. Historically, the debate on whether neurons communicate electrically or chemical was a source of controversy, but it is now recognized that both types of synapse exist and serve important functions in the nervous system (Pereda, 2014).
At chemical synapses, neurons do not physically connect in order to communicate, but instead make the use of neurotransmitters that are released by one cell and detected by the adjacent cell (Connors & Long, 2004; Sheng, Sabatini, & Sudhof, 2012). Broadly, an electrical signal from the presynapse is converted into a chemical signal via the release of neurotransmitters into the synaptic cleft, a region where the presynaptic terminal is in very close proximity (~20nM) to the post-synaptic density (Kandel, Schwartz, & Jessell, 2000). Vesicle release is triggered by an action potential in the presynaptic cell, which activates voltage-gated Ca\(^{2+}\) channels and causes a subsequent influx of Ca\(^{2+}\). This rise of Ca\(^{2+}\) is what triggers the fusion of stored synaptic vesicles with the membrane, releasing neurotransmitters into the synaptic cleft (Lodish et al., 2000). These neurotransmitters diffuse across the cleft and may bind to post-synaptic receptor proteins, which can exert a number of possible effects on the post-synaptic cell (Figure 1.1A), including opening channels to allow the flux of specific ions, or via downstream effects of intracellular signaling cascades (Kennedy, 2000; Sheng & Kim, 2002). Chemical signals can be terminated in several ways, all of which somehow involve removal of the neurotransmitter from the synaptic cleft. This includes enzymatic degradation of neurotransmitters, diffusion away from the synaptic cleft, and reuptake by the presynaptic neuron or nearby glial cells (Lodish et al., 2000).

At electrical synapses, the presynaptic and postsynaptic membranes are physically connected at gap junctions via specialized proteins known as connexons. Connexons form channels between the two neurons, which allows the direct flow of ions and some small molecules from one cell to another (Figure 1.1B). These types of synapses allow for very fast, simple, and bidirectional communication between neurons (Connors & Long, 2004).

Broadly, each type of synapse has advantages and limitations. Electrical synapses allow very fast communication compared to chemical synapses, which are relatively slower due to the
process of chemical diffusion across the synaptic cleft. However, chemical synapses generally allow more complex forms of communication, for example, the ability to convert an excitatory signal into an inhibitory one. Though both types of synapse are present in the mammalian brain, it is likely for this reason that chemical synapses form the majority of synapses.
Figure 1.1 Electrical and chemical synapses.

(A) Schematic of a chemical synapse; neurotransmitters stored in synaptic vesicles fuse to the membrane, releasing neurotransmitters into the synaptic cleft. Neurotransmitters bind to post-synaptic receptors, including ion channels, which convert the chemical signal into an electrical signal by allowing the flux of ions into the cell.

(B) Schematic of an electrical synapse; gap junction channels form a physical connection between the pre and postsynaptic neuron, allowing ions to flow between the two cells.

Image from (Pereda, 2014)
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1.2.2 Neuronal Membrane Properties

All living cells have an electrical gradient across their surface membranes, but neurons make especially sophisticated and dynamic use of this gradient to communicate. The resting electrochemical gradient in neurons is generated by a combination of differing permeability to various ion species, as well as the existence of pumps and transporters that actively move ions across the membrane. This results in a membrane potential ($V_m$) at rest of about -70mV (Kandel, Spencer, & Brinley, 1961; Kandel & Spencer, 1961; Purves et al., 2001; Spruston & Johnston, 1992), meaning that the inside of the cell is more electrically negative than the outside.

Under normal resting conditions in mammalian neurons, the membrane is more permeable to $\text{K}^+$ than to $\text{Na}^+$ due to the presence of abundant potassium leak channels. In addition to this, sodium-potassium adenosine triphosphatase ($\text{Na}^+/\text{K}^+$ ATPase), a pump that actively uses the energy of adenosine triphosphate (ATP) also contributes to establishing the electrochemical gradient of these cations. It moves 2 $\text{K}^+$ ions from the outside to the inside of the cell and 3 $\text{Na}^+$ ions from the inside to the outside of the cell for every molecule of ATP it hydrolyses (Forrest, 2014; Vassalle, 1987). This contributes to a higher concentration of $\text{Na}^+$ on the outside of the cell (compared to the inside), and a higher concentration of $\text{K}^+$ inside the cell (Table 1.1). The inside of the cell is also abundant in large, membrane-impermeable proteins that carry a net negative charge ($\text{A}^-$), which contributes to repelling small anions such as $\text{Cl}^-$ outside of the cell. Altogether, the membrane’s permeability to $\text{K}^+$ and the action of the $\text{Na}^+/\text{K}^+$ ATPase produce an electrical difference across the membrane of about -70mV. Consequently, the cell is considered polarized relative to its external environment (MacKay, 2010).

Any change that results in a less negative membrane potential than the resting potential is referred to as depolarizing, and any change that results in a more negative membrane potential is
referred to as hyperpolarizing. Reversal potential \( (E_i) \) describes the membrane potential at which there is no net flow of a given ion species and can be calculated using the Nernst equation. The difference between \( V_m \) and \( E_i \) is referred to as driving force for that ion. If there is an increase in permeability for an ion species, \( V_m \) will move towards \( E_i \). For example, if at rest \( V_m = -70\text{mV} \), an increase of membrane permeability to sodium \( (E_{Na} = \sim +60\text{mV}) \) will depolarize the membrane. Since the membrane is not permeable to only a single ion species at a time, \( V_m \) is not usually equal to the \( E_i \) of any given ion. \( V_m \) depends on the equilibrium potentials of all ion species in the cell and can be calculated using the Goldman equation, which accounts for the permeability of multiple ion species at a time (MacKay, 2010).

Action potentials can be elicited when the membrane sufficiently depolarizes to the action potential threshold, which on average is about -50mV or greater (MacKay, 2010). This amount of depolarization triggers the opening of voltage-gated \( \text{Na}^+ \) channels, and the subsequent increase in permeability to sodium and influx of \( \text{Na}^+ \) into the cell rapidly depolarizes the membrane towards \( E_{Na} \) (Hodgkin & Katz, 1949; Hodgkin & Huxley, 1952; Huxley, 2002). This represents the rising phase of the action potential. After a brief period, \( \text{Na}^+ \) channels inactivate, and the membrane begins to re-polarize toward its normal resting \( V_m \), representing the falling phase of the action potential. The opening of voltage-gated \( \text{K}^+ \) channels can also be triggered during the AP, causing a characteristic ‘hyperpolarizing spike’ or afterhyperpolarization (AHP) after the falling phase of the action potential (Hodgkin & Huxley, 1952; Huxley, 2002) (Figure 1.2).

The action potential propagates along the cell membrane, eventually reaching the synaptic terminals, where it can trigger the flux of ions through gap junctions at electrical synapses or trigger the release of neurotransmitters into the synaptic cleft at chemical synapses.
Table 1.1 Extracellular and intracellular ion concentrations of mammalian neurons

<table>
<thead>
<tr>
<th>Ion</th>
<th>Intracellular (mM)</th>
<th>Extracellular (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium (K⁺)</td>
<td>140</td>
<td>5</td>
</tr>
<tr>
<td>Sodium (Na⁺)</td>
<td>5–15</td>
<td>145</td>
</tr>
<tr>
<td>Chloride (Cl⁻)</td>
<td>4–30</td>
<td>110</td>
</tr>
<tr>
<td>Calcium (Ca²⁺)</td>
<td>0.0001</td>
<td>1–2</td>
</tr>
<tr>
<td>Protein (A⁻)</td>
<td>126</td>
<td>10</td>
</tr>
</tbody>
</table>

Adapted from (Purves et al., 2001) and (MacKay, 2010)
Figure 1.2 Action potential

A schematic of an action potential. At rest, neurons have an average membrane potential of -70mV (1). A stimulus (2) that causes the membrane to depolarize to the action potential threshold triggers the opening of voltage gated Na\(^+\) channels, which causes a subsequent voltage rise (3). As Na\(^+\) channels deactivate, the voltage begins to fall (4) and may be followed by a subsequent afterhyperpolarization (5) and an eventual return to resting membrane potential (6).
1.2.3 Excitatory and Inhibitory Neurotransmission

Neurotransmitters are often broadly described as ‘excitatory’ or ‘inhibitory’ according to their action on the postsynaptic cell. Excitatory neurotransmitters increase the probability of action potential firing, while inhibitory neurotransmitters decrease this probability (Purves et al., 2001). In general, depolarization is associated with excitatory activity if sufficient to reach the action potential threshold, while hyperpolarization is associated with inhibitory activity.

Whether a given neurotransmitter is excitatory or inhibitory is not a property of the neurotransmitter molecule itself, but a property of the action its receptor exerts on the postsynaptic cell. Many receptors are ligand-gated ion channels, and their effects on the postsynaptic cell depend on which ions they flux and the existing electrochemical gradient of that ion in the cell. Other receptors exert more complex effects, not through directly fluxing ions, but through intracellular signaling cascades that may indirectly lead to changes in ion permeability. In either case, what is a classically considered either an ‘excitatory’ or ‘inhibitory’ neurotransmitter can exert different and sometimes unexpected effects depending on which receptor it binds to and the internal state of the postsynaptic cell. Because of this, it is important to note that classifying a neurotransmitter as ‘excitatory’ or ‘inhibitory’ is helpful as a general rule but does not always hold true under all conditions.

Two of the most prominent neurotransmitters in the brain are glutamate and gamma-aminobutyric acid (GABA). Glutamate is considered the primary excitatory neurotransmitter, while GABA is considered the primary inhibitory neurotransmitter, because under most conditions, these neurotransmitters reliably facilitate excitation or inhibition, respectively.

Glutamate, the primary excitatory neurotransmitter, is the transmitter of the majority of excitatory synapses in the brain (McEntee & Crook, 1993). Glutamate can bind to and activate
several different receptors, the most predominate of which are cation channels such as \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartic acid (NMDA), and kainate receptors (Traynelis et al., 2010). AMPA receptors are ligand-gated cation channels that are permeable to Na\(^+\) and K\(^+\), with some subtypes also being permeable to Ca\(^{2+}\). Under normal resting conditions, activation of this type of receptor will allow Na\(^+\) to flux into the cell, depolarizing the membrane potential and thus facilitating excitation (Hodgkin & Huxley, 1952; Huxley, 2002; Purves et al., 2008).

GABA, the primary inhibitory neurotransmitter, can bind to and activate several different GABA receptors (Figure 1.3A). The GABA\(_A\) receptor is an ion channel that is predominantly permeable to Cl\(^-\) but also is permeable to bicarbonate (HCO\(_3^-\)), with an HCO\(_3^-\) to Cl\(^-\) permeability ratio of 0.2–0.4 (Kaila, Price, Payne, Puskarjov, & Voipio, 2014). At rest in a mature mammalian neuron, intracellular chloride concentration is lower than extracellular chloride concentration. When the GABA\(_A\) receptor is activated, Cl\(^-\) fluxes into the cell, subsequently hyperpolarizing the membrane potential and opposing action potential generation by preventing the membrane from depolarizing to the action potential threshold (Purves et al., 2001).

Because of the GABA\(_A\) receptor’s permeability to Cl\(^-\), the maintenance of the Cl\(^-\) electrochemical gradient is critical for fast hyperpolarizing inhibition. This gradient is maintained by members of the cation chloride co-transporter (CCC) family, including KCC2 and sodium potassium chloride co-transporter 1 (NKCC1), which are a part of a family of secondarily active transporters that utilize the existing cation gradients to move Cl\(^-\) across the membrane (Mercado, Mount, & Gamba, 2004). Their role in chloride regulation will be discussed in more detail in Chapter 1.4: Chloride Regulation.
$E_{GABA}$ is the potential at which there is no net ion flow through the GABA$_A$ receptor, and is used as a measure of GABA function relative to $V_m$. While GABAergic activity is usually hyperpolarizing, GABA can also be excitatory, or can exert inhibitory effects through ‘shunting’ depolarization depending on the Cl$^-$ (and to a lesser extent, HCO$_3^-$) electrochemical gradient. Shunting inhibition occurs when $E_{GABA}$ is depolarized relative to the resting membrane potential but hyperpolarized relative to the action potential threshold, which simultaneously depolarizes the membrane potential and yet opposes AP generation by ‘shunting’ the membrane to a $V_m$ that is hyperpolarized relative the AP threshold (Staley & Mody, 1992; Staley, Soldo, & Proctor, 1995) (Figure 1.3B).

Both excitation and inhibition are critical to normal brain function and an imbalance in their relative contributions is a hallmark of many neurological diseases and disorders, including autism (Rubenstein & Merzenich, 2003), schizophrenia (Canitano & Pallagrosi, 2017), epilepsy (Fukata & Fukata, 2017).
Figure 1.3 GABA$\textsubscript{A}$ receptor

(A) Schematic of the GABA$\textsubscript{A}$ receptor, a Cl$^-$ permeable receptor composed of five subunits, most commonly two $\alpha$ subunits, two $\beta$ subunits, and one $\gamma$ subunit.

(B) Schematic of $E_{\text{GABA}}$ relative to $V_m$ and AP threshold. GABA can be inhibitory, shunting, or excitatory depending on where $E_{\text{GABA}}$ lies relative to AP threshold, which is critically dependent on the Cl$^-$ electrochemical gradient.

A – From (Vinkers & Olivier, 2012), under Creative Commons Attribution License
B - Modified from (Balena et al., 2010)
1.3 The Hippocampus

The hippocampus is a brain structure that was originally named for its distinctive shape (hippocampus meaning ‘sea horse’), with three of its major histological divisions deriving their name from their curling, ram’s-horn like structure, CA1, CA2, and CA3 (cornus Ammonis 1, 2, and 3). While it was originally thought that the hippocampus served primarily an olfactory function due to its strong connectivity to the olfactory bulbs, it has since been discovered that it serves a number of essential functions in learning and memory, including consolidation of both short and long term memory, as well as the spatial memory required for navigation (Anderson et al., 2007).

The idea that the hippocampus was critical to memory consolidation historically gained traction from a famous study of Henry Molaison (HM), a patient who had a bilateral medial temporal lobectomy, which included the bilateral removal of two-thirds of his hippocampi. The surgery was originally performed in an attempt to alleviate HM’s severe epileptic seizures, which were localized to his temporal lobes. Following the surgery, HM developed severe anterograde amnesia, becoming unable to form new long-term explicit memories, although his working memory and procedural memory remained intact (Scoville and Milner, 1957). This study and the multitude of studies that followed it, highlighted the role of the hippocampus in memory consolidation.

In general, the hippocampus has also historically served as an important model system for many studies of neurophysiology, including seminal studies in neuroplasticity. For example, the hippocampus was where long-term potentiation (LTP) was first described (Bliss and Lomo, 1973). Because of its well-defined circuitry and important role in learning and memory, the
hippocampus is one of the most widely-studies regions of the brain, and it is widely used as a model system to study synaptic transmission (Anderson et al., 2007).

1.3.1 Hippocampal Anatomy

The hippocampal formation is located in the medial temporal lobe of the brain and is composed of several subdivisions including the hippocampus proper, the dentate gyrus, subiculum, presubiculum, parasubiculum, and entorhinal cortex (Anderson et al., 2007).

The hippocampus proper has an organized laminar structure, with relatively simple, well-defined circuitry. It is histologically divided into three main subsections: CA1, CA2, and CA3, which lie in close proximity to the dentate gyrus (Figure 1.4). Several types of neurons exist in the hippocampus, including both the characteristic pyramidal cells (also sometimes referred to as principal neurons), which are excitatory, as well as interneurons, which are usually but not always inhibitory (Anderson et al., 2007).

In terms of connectivity, the entorhinal cortex (EC) is a major source of both inputs and outputs to the hippocampus. The EC provides inputs to the CA1, CA3, and dentate gyrus along a prominent pathway known as the perforant pathway, which perforates the subiculum before reaching the CA fields. Other pathways of note include the Schaffer collaterals, which form a pathway connecting CA3 to CA1, and the mossy fibres, which are a pathway from the granule cells of the dentate gyrus to the CA3 (Figure 1.4), named for the distinctive, ‘mossy’ appearance of its large synaptic terminals. Interneurons are distributed throughout the hippocampus and provide inhibition to nearby pyramidal cells and other interneurons (Anderson et al., 2007).

Each of the CA subfields is organized into stratified layers: the alveus, the stratum oriens, the stratum pyramidale, the stratum lucidum (which exists only in the CA3), the stratum radiatum, and the stratum lacunosum/moleculare. The stratum pyramidale is where the cell
bodies of hippocampal pyramidal cells lie, with their dendrites, axons, and the cell bodies of the majority interneurons distributed throughout the other strata (Anderson et al., 2007).
Figure 1.4 Hippocampal Pathways

(A) Schematic of major pathways within the hippocampal formation. CA1 pyramidal cells receive inputs from the CA3 via the Schaffer collaterals. CA3 pyramidal cells receive inputs from the EC, and from the dentate gyrus (DG) via the mossy fibres. The DG receives inputs from the EC via the perforant pathway.

(B) Schematic of major inputs to the hippocampus from the EC.

Image from (Deng et al., 2010)
Used with permission. Rightslink license #4526570520158.
1.3.2 Hippocampal cell types and their function

Within the hippocampus, there are many different cell types that serve different functional roles depending on their neurotransmitter content and location. Broadly, three major neuron types exist in the hippocampus; pyramidal cells, interneurons, and the granule cells of the dentate gyrus. While pyramidal cells and granule cells typically produce excitatory activity, interneurons usually produce inhibition (Anderson et al., 2007).

Granule cells, which form the dentate gyrus, are much smaller than pyramidal cells and provide a large amount of excitatory input to the CA3 pyramidal cells. They were the first neurons to be shown to exhibit LTP (Bliss and Lomo, 1973).

Pyramidal cells form the major neuron subtype of the stratum pyramidale of the CA subfields and typically provide excitatory input to other pyramidal cells as well as interneurons. They can have both local and long-range projections. Pyramidal cells in the CA subfields are noted for having many dendritic spines, an anatomical substructure that consists of a protrusion from the dendrite that typically receives one synaptic input. Both the CA1 and CA3 contain numerous spines throughout their dendritic tree (Anderson et al., 2007).

Interneurons, which represent only 10-15% of the cell population in the hippocampus, are nonetheless a major determinant of circuit function (Pelkey et al., 2017). They are remarkably diverse in their anatomy and function, and many attempts have been made to organize them into groups based on their anatomy, molecular expression profiles, developmental origins, electrophysiological properties, and postsynaptic targeting (Pelkey et al., 2017). Dozens of different interneuron types have been described using these attributes, but it has recently been proposed that these diverse categorizations can be broken down into three main types of
interneurons: Pavalbumin-positive (PV+), somatostatin positive (SOM+) and serotonin receptor 3A positive (5HTA+) (Rudy et al., 2011).

Interneurons form inhibitory inputs on pyramidal cells as well as other interneurons (including themselves), and receive excitatory inputs from pyramidal cells, forming many complex microcircuits in the hippocampus. Feedback microcircuits between interneurons and pyramidal cells are thought to be responsible for generating the prominent oscillations observed in the hippocampus, such as the theta rhythm (Kullmann, 2011).

The majority of inhibitory synapses are formed outside of dendritic spines, on the soma and the shafts of both apical and proximal dendrites. Interneuronal inputs are segregated in the dendritic tree, with certain types of interneurons preferentially innervating different areas of the tree, the most rapid and strong inhibition coming from interneurons which synapse on more proximal locations (Anderson et al., 2007).

1.3.3 Properties of MF-CA3 synapses

MF-CA3 synapses have several properties that differentiate them from other types of synapses in the hippocampus, including specialized anatomical structures and unique functional properties. The terminals of dentate granule cells form ‘giant boutons’ which synapse onto thorny excrescences, a large, specialized form of dendritic spine located on the apical dendrites of the CA3 pyramidal cell (Figure 1.5A). The giant bouton surrounds the spine on the CA3 dendrite (Figure 1.5B). A single synapse formed between a mossy fibre bouton and a thorny excrescence can have between 3 and 80 active zones that are opposed by a post synaptic density (PSD) on the post-synaptic neuron (Henze et al., 2000). Giant boutons also have filopodial extensions that are thought to exclusively contact GABAergic interneurons (Amaral and Dent, 1981, Rollenhagen and Lübke, 2010).
In addition to their complex anatomy, MF-CA3 synapses exhibit functional features that make them distinct from other synapses in the hippocampus. For example, mossy fibre boutons contain zinc, which is co-released by these synapses along with glutamate during neurotransmission (Qian and Noebels, 2005), and is an important factor in regulating excitability by modulating the action of NMDA receptors (Vogt et al., 2000, Smart et al., 2004). Zinc is also known to modulate the activity of glycine receptors (Madry et al., 2008) and GABA_A receptors (Hosie et al., 2003, Smart et al., 2004). Recently, it was discovered that zinc has its own metabotropic zinc receptor (mZnR), which can regulate inhibition postsynaptically by modulating the activity of KCC2 (Chorin et al., 2011). Zinc’s ability to modulate both excitation and inhibition at the mossy fibre synapse means that Zn^{2+} release is an important factor to consider when studying these synapses.

MF-CA3 synapses are also unique in the hippocampus with respect to kainate receptor function. They are the only synapse in the hippocampus where postsynaptic kainate receptor-mediated currents have been recorded, and there is a kainate-receptor-mediated component in addition to the AMPAR-mediated component of CA3 EPSCs (Castillo et al., 1997). This sets the CA3 apart from the CA1, where the importance of presynaptic KAR activity has been studied, but postsynaptic KAR currents have not been recorded. KAR function within the hippocampus is reviewed in more detail in Chapter 1.7: Kainate Receptors.
Figure 1.5 Anatomical features of MF-CA3 synapses

(C) Schematic of a CA3 pyramidal cell, showing the location of thorny excrescences

(D) Schematic of a giant mossy fiber bouton (MFB) synapsing onto the thorny excrescence of a CA3 pyramidal cell. Insets: A synapse formed by a MFB on a CA3 pyramidal cell (left) and a synapse formed by a filopodial extension of the bouton onto a GABAergic interneuron (right).

A - (Sandi, 2004), used with permission, Rightslink license #4527091317215
B - (Pinheiro, Paulo S. and Mulle, 2008), used with permission, Rightslink license #452710000259
1.4 Chloride Regulation

Because the GABA\textsubscript{A} receptor is permeable to chloride, fast inhibitory synaptic transmission depends on the electrochemical gradient of Cl\textsuperscript{-} across the neuronal surface membrane. This gradient is primarily regulated by a family of proteins known as cation chloride cotransporters (CCCs). CCCs are ubiquitous in the body and normally function to regulate cell volume and osmolarity, but also have several specialized family members that are crucial to regulating Cl\textsuperscript{-} in neurons. Of these, KCC2 and NKCC1 play the most prominent role in regulating the neuronal chloride gradient.

1.4.1 Cation-chloride co-transporters

CCCs are a family of proteins that were originally identified for their volume-regulation properties. As a family they are expressed ubiquitously in all tissues of the body, with certain subtypes expressing in specific tissue types. CCCs differ in their structure, cation specificity, and expression pattern, but all share the characteristic ability to symport both cations and chloride across the cell membrane. They do so in an electroneutral manner, transporting equal numbers of cations and anions and thus producing no current (Mercado et al., 2004, Gamba, Gerardo, 2005).

There are seven members of the CCC family, which include NCC, four isoforms of the KCC subtype (KCC1, KCC2, KCC3, and KCC4) and two isoforms of the NKCC subtype (NKCC1, NKCC2). Of these, KCC2 and NKCC1 play a major role in regulating the chloride gradient in neurons (Kaila et al., 2014). These two CCCs have opposite roles, with NKCC1 typically bringing Cl\textsuperscript{-} into the cell and KCC2 extruding it.
1.4.2 Structure and Function of KCC2

KCC2 is a membrane protein encoded by the gene SLC12A5. It has a size of approximately 140kDa and is predicted to have 12 membrane-spanning domains, with a large extracellular domain that is the site of several N-glycosylations, and an intracellular N-terminus and C-terminus (Figure 1.6). The intracellular regions contain several phosphorylation sites, the majority of which are located on the larger C-tail, and are targeted by several different kinases, including protein kinase C (PKC), Src-family kinases, and with no lysine (WNK)-family kinases (Chamma et al., 2012). The effects of phosphorylation on KCC2 function will be discussed in more detail in Chapter 1.6: KCC2 regulation.

Two isoforms of KCC2 exist: KCC2a and KCC2b, which differ in their N-termini due to alternative first exons of the SLC12A5 gene. The major isoform in the mature brain is KCC2b, and it is this isoform that undergoes a robust upregulation during development (Markkanen et al., 2014). While a knockout of both isoforms at once is fatal at birth due to respiratory failure (Hübner et al., 2001), selective knockout of KCC2b alone results in mice that can survive for several weeks after birth (Woo et al., 2002). While both isoforms are expressed in the mature brain, KCC2a shows low or no expression in the adult cortex, hippocampus, thalamus, and cerebellum, while KCC2b is prominently expressed in these areas (Markkanen et al., 2014). This suggests that KCC2b plays the more prominent role in chloride regulation in the adult hippocampus.

KCC2 is the only member of the CCC family that is exclusively expressed in the nervous system, where it’s expression is neuron-specific (Payne, J. A. et al., 1996, Williams et al., 1999, Karadsheh and Delpire, 2001, Uvarov et al., 2005). KCC2 functions as the main extruder of chloride in mature neurons. KCC2 is not the only member of the CCC family expressed in the brain. Several other CCCs are expressed in the nervous system, including NKCC1. Together,
KCC2 and NKCC1 both serve as important regulators of Cl⁻ homeostasis (Kaila et al., 2014). KCC2 and NKCC1 are unique in that unlike many CCCs, which become active only under the swelling conditions of an osmotic challenge, KCC2 and NKCC1 are active under isotonic conditions (Gamba, Gerardo, 2005). KCC2’s isotonic activity is dependent on the ISO domain located on the C-terminus, as KCC2 lacking this particular region retains swelling-activated transport but lacks isotonic activity (Acton et al., 2012). Both KCC2 and NKCC1 play a prominent role in establishing and maintaining the Cl⁻ gradient in neurons, both during development, and in mature neurons (Kaila et al., 2014).

During early development, NKCC1 is prominently expressed, while KCC2 remains expressed at lower levels. Consequently, the intracellular chloride concentration in immature neurons is relatively high, which renders GABA excitatory, in stark contrast to GABA’s function as the primary inhibitory neurotransmitter in the mature nervous system. All vertebrates examined to date undergo a developmental shift from excitatory to inhibitory GABA (Ben-Ari, 2002). Excitatory GABA is required for a number of developmental processes, including facilitation of the formation of both glutamate and GABA synapses (Ben-Ari, 2002). As development progress, KCC2 undergoes a robust upregulation and continues to increase around the time of birth. The exact time frame of this upregulation is both brain region and species specific, beginning prenatally in humans (Sedmak et al., 2016) and postnatally in rodents (Rivera, C. et al., 1999), and coincides with the developmental switch from excitatory to inhibitory GABA action.

Under normal resting conditions, KCC2 extrudes Cl⁻ and K⁺ from the cell in a 1:1 ratio, due to the strong K⁺ gradient generated by the Na⁺/K⁺ ATPase. However, the transporter is capable of reversing transport direction if the potassium gradient is altered (Thompson and Gähwiler, 1989, Payne, J. A., 1997). In contrast to KCC2, under normal resting conditions
NKCC1 symports Na\(^+\), K\(^+\) and Cl\(^-\) into the cell in a 1:1:2 ratio due to the existing Na\(^+\) gradient generated by the Na\(^+\)/K\(^+\) ATPase (Figure 1.7). Depending on the relative contribution of KCC2 and NKCC1 to the regulation of the Cl\(^-\) gradient, the intracellular [Cl\(^-\)] can be relatively high or relatively low, which can render the action of GABA\(_A\) receptors excitatory or inhibitory, respectively (Figure 1.8)
Figure 1.6 Structure of KCC2
A schematic of a single KCC2 molecule expressed in the cell membrane. Major sites of n-glycosylations as well as known intracellular phosphorylation sites are indicated.

Image from (Chamma et al., 2012), under Creative Commons Attribution License
Figure 1.7 Transporter function of NKCC1 and KCC2

A schematic showing Na/K ATPase, KCC2, NKCC1 and GABA_A receptors in the membrane. The electrochemical gradient of Na+ and K+ partially generated by the Na/K ATPase is utilized by NKCC1 and KCC2 to transport Cl- across the membrane. The chloride electrochemical gradient determines whether GABA_A activation is depolarizing or hyperpolarizing.
Figure 1.8 Function of NKCC1 and KCC2 during development

(A) During development, NKCC1 plays the more prominent role in regulating intracellular [Cl\textsuperscript{-}], transporting Na\textsuperscript{+}, K\textsuperscript{+}, and Cl\textsuperscript{-} into the cell. As a consequence, intracellular [Cl\textsuperscript{-}] is high and this renders GABA depolarizing and therefore excitatory.

(B) In the mature neuron, KCC2 is more prominent, extruding Cl\textsuperscript{-} and K\textsuperscript{+} from the cell and rendering GABA inhibitory.
1.4.3 Role of KCC2 at the Excitatory Synapse

In addition to its well-established role in maintaining the chloride gradient necessary for fast hyperpolarizing inhibition, KCC2 also serves many important functions at the excitatory synapse, where it is required for the formation and maintenance of dendritic spines as well as playing a role in confining AMPA receptors to the synapse. KCC2 is highly expressed at excitatory synapses, including at dendritic spine heads (Gulyás et al., 2001). The time window for the development of dendritic spines closely matches the developmental upregulation of KCC2 (Rivera, C. et al., 1999, Yuste and Bonhoeffer, 2004, Chamma et al., 2012). In mature neurons, KCC2 clusters at both inhibitory and excitatory synapses, but has a longer dwell time at excitatory synapses that is mediated by its interaction for cytoskeletal protein F-actin (Chamma et al., 2013).

Expression of KCC2 is important for dendritic spine formation (Li et al., 2007, Fiumelli et al., 2013). When KCC2 expression is reduced or eliminated during development, dendritic spines form aberrant filopodia-like structures, and there is a reduction in the number of functional excitatory synapses formed, revealing a structural role for KCC2 that is dependent on its interaction with cytoskeletal protein 4.1N (Li et al., 2007). This role is independent of its function as an ion transporter, as mutations of KCC2 that disrupt transporter function but not cytoskeletal interactions still allow normal spine formation (Li et al., 2007, Fiumelli et al., 2013). Interestingly, transfection of a construct that contained just the C-terminal domain of KCC2 increased spine density (Fiumelli et al., 2013).

In mature dendritic spines, KCC2 also plays a role in confining postsynaptic AMPA receptors in the synapse. Interrupting KCC2’s interaction with the cytoskeleton results in increased lateral diffusion of AMPA receptors outside of the synapse, but pharmacological
blockade of its transporter function does not (Gauvain et al., 2011). Suppression of KCC2 after spines have already developed leads to increased spine head volume, likely due to KCC2’s role in osmotic regulation (Gauvain et al., 2011, Chamma et al., 2012).

Taken together, this evidence indicates that in addition to its role in inhibition, KCC2 plays a critical role in excitation by facilitating the normal development of the formation of dendritic spines and thus excitatory synapses. In mature neurons, it remains at the excitatory synapse, where it influences AMPA receptor content and diffusion, and serves an osmoregulatory role in spine maintenance.

1.4.4 KCC2 dysfunction in neurological diseases and disorders

KCC2 dysfunction has been implicated in a wide variety of neurological diseases and disorders, including epilepsy (Rivera, Claudio et al., 2002, Pathak et al., 2007, Puskarjov et al., 2014, Kahle et al., 2014), neuropathic pain (Coull et al., 2003, Lavertu et al., 2014), schizophrenia (Hyde et al., 2011, Tao et al., 2012), autism (Lemonnier et al., 2012), amiotrophic lateral sclerosis (Fuchs et al., 2010) and Huntington’s disease (Dargaei et al., 2018).

In most of these cases, the common theme is that KCC2 function is reduced and the resulting imbalance in chloride regulation contributes to impaired inhibition. This is usually due to a reduced expression and/or reduced function KCC2 rather than mutations in KCC2 itself, though several mutations of KCC2 with reduced transporter ability have been reported and implicated in temporal lobe epilepsy (Kahle and Staley, 2008, Kahle et al., 2014). When KCC2 is impaired, NKCC1 can become the predominant Cl⁻ transporter, and in many neurological diseases and disorders neurons have a phenotype that resembles immature neurons in regards to Cl⁻ homeostasis, with a higher intracellular [Cl⁻] and excitatory GABA action (Kahle et al., 2008).
1.5 Methods for studying KCC2 function

Since KCC2 transports ions in an electroneutral manner, it does not generate a current and thus it’s activity cannot be directly recorded. This has resulted in researchers developing many different methods for assessing KCC2 function that do not directly measure transport activity itself, but rather the consequences of it, such as changes in intracellular ion concentrations or changes in electrophysiology properties of GABA responses (Medina et al., 2014). Although there are numerous ways of assessing KCC2 function, only a few of the common methods will be discussed here, with a focus on electrophysiological methods, which form the basis of the studies presented in this thesis.

1.5.1 Ionic Flux Assays and Chloride Sensors

While KCC2 physiologically transports only K\(^+\) and Cl\(^-\), it is also able to transport numerous other ions, a fact that is taken advantage of by various kinds of ion flux assays, which use traceable ions as a substitute for K\(^+\). One example of this kind of assay is the rubidium flux assay. \(^{86}\text{Rb}^+\) is a radioactive tracer that KCC2 can transport in place of K\(^+\). This technique was used in the early characterization of both NKCC1 (Xu, J. C. et al., 1994, Gamba, G. et al., 1994) and KCC2 (Payne, J. A., 1997) and allows a measure of how much transport activity is present. However, this technique is also difficult to use in tissues with multiple cell types (such as brain slices) and the use of radioactive isotopes also poses a potential health risk to researchers (Medina et al., 2014).

Other examples of ion flux assays include thallium (Tl\(^+\)) and ammonium (NH\(_4^+\)) flux assays. Both techniques utilize traceable ions, which KCC2 transports in place of K\(^+\), which can then be detected with fluorescent dyes in the case of Tl\(^+\), or by changes in pH in the case of NH\(_4^+\). These tracers have an advantage over \(^{86}\text{Rb}^+\) in that they can be used track both cellular and
subcellular changes in KCC2 function in real time. However, they also both involve measuring KCC2 in reverse transport mode, which subsequently increases intracellular $[\text{Cl}^-]$ (Medina et al., 2014). In NH$_4^+$-flux assays, complex changes in pH that follow NH$_4^+$ import into the cell may also change the function of other transporters, channels, and ion exchangers in the neuron (Medina et al., 2014).

Techniques have also been developed to measure the intracellular $[\text{Cl}^-]$ using fluorescent chloride sensors as a measure of KCC2 function. Chloride imaging allows real time measurement of KCC2 function at the cellular level and in subcellular compartments and holds an advantage over other ion flux assays because it does not involve introducing new ions to the neuron or reversing KCC2 transport. Historically this has proven difficult to do, as commercially available chloride sensors have poor accuracy at physiological $[\text{Cl}^-]$ due to their sensitivity to fluctuations in pH. Improvements in chloride sensors and techniques for using them have improved in recent years, and their non-invasiveness and subcellular resolution make them an attractive tool for measuring KCC2 function (Ludwig et al., 2017).

### 1.5.2 Electrophysiology

Electrophysiological methods for assessing KCC2 function rely on indirect measures of the consequences of KCC2 transport, typically by assessing GABAergic neurotransmission, which depends on the Cl$^-$ electrochemical gradient. One of the most common methods for assessing KCC2 function is by determining $E_{\text{GABA}}$, the reversal potential for the GABA$_A$ receptor. This method is an indirect measure of KCC2 function, because the current flow through the GABA$_A$ receptor depends on $[\text{Cl}^-]$, which is primarily regulated by KCC2 in mature neurons. While the GABA$_A$ receptor is permeable to Cl$^-$, it is also permeable to HCO$_3^-$, and thus $E_{\text{GABA}}$ will not necessarily be equal to $E_{\text{Cl}}$ but will be deviated to more positive values. Many
Electrophysiologists include buffers for HCO$_3^-$ in their intracellular solutions, minimizing the contribution of HCO$_3^-$ to $E_{\text{GABA}}$. $E_{\text{GABA}}$ can be measured by stimulating GABA release onto the recorded cell in the presence of antagonists to block possible glutamate receptor activation, and measuring the response while voltage-clamping the membrane at a series of different voltages (Figure 1.9A). The voltage at which there is no net current flow through the GABA$_A$ receptor is $E_{\text{GABA}}$. This can be determined by performing a linear regression of the IPSC amplitudes plotted against the membrane voltage (I-V curve). The intercept of this line with the abscissa is taken as $E_{\text{GABA}}$ (Figure 1.9B).

Electrophysiological determination of $E_{\text{GABA}}$ can be made using the gramicidin perforated patch clamp technique. This involves the use of antibiotic gramicidin, which perforates the membrane with pores that allow the researcher to record currents while preserving the intracellular $[\text{Cl}^-]$ (Ebihara et al., 1995, Kyrozis and Reichling, 1995). This technique was originally thought to be advantageous because it allows the measurement of GABA currents without altering the $[\text{Cl}^-]$. However, it has also been shown that it is possible to record changes in $E_{\text{GABA}}$ with whole-cell patch clamp (Ormond and Woodin, 2011, Ormond and Woodin, 2009, Takkala and Woodin, 2013, Mahadevan et al., 2017), which allows the addition of pharmacological agents into the cell via the recording pipette. Whole-cell recordings are also less time-consuming than gramicidin, which require additional time to allow the drug to perforate the membrane. Gramicidin can also be challenging for longer-term recordings because perforation over time can lead to an unintentional whole-cell break-in. The use of the whole-cell technique was necessary in the experiments in this thesis due to the longer-term nature of the recordings, which required the washing-in and -out of pharmacological agents, and the need to include pharmacological agents in the recording pipette.
Figure 1.9 Experimental determination of $E_{\text{GABA}}$

(A) A sample recording of GABA currents recorded by stimulating GABA release onto the neuron at 10mV voltage steps from -90mV to -50mV. (scale bar: 60 pA, 10 ms)

(B) A sample I-V curve generated from the trace in (A). The amplitude of each IPSC is plotted against the holding voltage of the neuron and where the linear regression of this curve crosses the y-axis (I=0) is the reversal potential for GABA ($E_{\text{GABA}}$).
1.6 KCC2 regulation

KCC2 expression and function can be regulated in a number of ways, including phosphorylation (Lee et al., 2007), association with lipid rafts (Watanabe et al., 2009), oligomerization (Blaesse et al., 2006), and numerous protein interactions. KCC2 regulation has been widely studied and because of the implication of impaired KCC2 function in a variety of neurological disease and disorders, targeting KCC2 and its regulatory interactions may provide a potential target for developing therapeutic drugs. Phosphorylation is the most widely-studied and well-characterized forms of KCC2 regulation, and the discovery of many diverse KCC2 protein interactors have emerged in recent years and have been recognized as an increasingly important mechanism of regulating KCC2 function. For this reason, phosphorylation and protein interactions will be covered in more detail in the following sections.

1.6.1 Phosphorylation

KCC2 harbors many phosphorylation sites on both of its intracellular tails, with the largest number of putative phosphorylation sites located on its C-terminal tail. These include phosphorylation sites for 3 main families of kinases and phosphatases, including serine (S), threonine (T), and tyrosine (Y) residues.

KCC2 has several serine sites that are putative phosphorylation sites. The most well-studied of these sites is S940, which has been shown to be directly phosphorylated by protein kinase C (PKC) (Lee et al., 2007). Phosphorylation of this site rapidly increases both the surface stability and transporter efficacy of KCC2. PKC may also serve as a common mechanism by which metabotropic signaling from a wide variety of G-protein coupled receptors (GPCRs) can regulate KCC2 function (Mahadevan and Woodin, 2016). PKC-mediated regulation of KCC2
has been predicted to be a downstream effect of activation of a wide variety of metabotropic receptors, including mGluRs (Banke and Gegelashvili, 2008) and mZnRs (Chorin et al., 2011). S940 is also a prominent target of dephosphorylation by protein phosphatase 1 (PP1). This results in a removal of KCC2 from the membrane and targets KCC2 for a calpain-mediated degradation (Puskarjov et al., 2012, Zhou et al., 2012). This is a mechanism of KCC2 downregulation that is seen in pathophysiological states where an increased Ca\textsuperscript{2+} influx via NMDARs activates this dephosphorylation pathway, downregulating KCC2 and producing depolarizing \textsubscript{A}GABA currents (Lee et al., 2011).

KCC2 can also be phosphorylated at threonine sites by with-no-lysine kinases (WNK). WNK phosphorylation of T906 and T1007 sites decreases KCC2 activity (Inoue et al., 2012, Friedel et al., 2015). Threonine phosphorylation shows an interesting developmental profile, with the majority of KCC2 phosphorylated at T906/1007 during development but only a small proportion of KCC2 phosphorylated in the mature brain (Rinehart et al., 2009). WNK1-regulated phosphorylation has been shown to be critical to maintain depolarizing \textsubscript{A}GABA in immature neurons (Friedel et al., 2015).

Tyrosine phosphorylation of KCC2 is less well-studied and has more complex effects on KCC2 function. When tyrosine phosphorylation was activated by muscarinic acetylcholine receptors (AChRs), it was shown to decrease surface stability of KCC2 and promote degradation (Lee et al., 2010). Other studies have showed the opposite effect, showing that phosphorylation of Y1087 increased KCC2 transport function by increasing both oligomerization and association with lipid rafts (Watanabe et al., 2009). It was also found that in development, dephosphorylation of tyrosine sites resulted in a functional loss of KCC2 transport activity (Wake et al., 2007). Although this evidence is controversial, it shows that tyrosine phosphorylation of KCC2 is important for regulating surface expression and function of KCC2.
1.6.2 Protein interactions

Even prior to the publication of a KCC2 protein interactome (Mahadevan et al., 2017), dozens of protein interactions with KCC2 were identified. These protein-protein interactions have important implications for KCC2 surface expression, function, and KCC2’s more secondary role as a structural protein at the excitatory synapse. In addition to physical interactions, there are also many proteins that have been shown to functionally interact with KCC2 and regulate its transporter function and surface expression in complex ways. While the full range of protein interactors of KCC2 is too extensive to cover in detail in this thesis, the following section will discuss some of the most well-studied interacting protein partners of KCC2, including Na\(^+\)/K\(^+\) ATPase, structural proteins 4.1N and β-pix, protein associated with myc (PAM), cation-chloride cotransporter interacting protein 1 (ClP1), metabotropic glutamate receptors (mGluRs), metabotropic zinc receptor (mZnR), Neto2 and kainate receptor subunit GluK2.

1.6.2.1 Na\(^+\)/K\(^+\) ATPase

One of the first interacting partners of KCC2 to be discovered was Na\(^+\)/K\(^+\) ATPase. This interaction is important both physically and functionally. KCC2 co-immunoprecipitates with the α2 subunit of the Na\(^+\)/K\(^+\) ATPase and animals lacking this subunit display aberrant Cl\(^-\) homeostasis despite having no changes in KCC2 expression, consistent with a reduced function of KCC2 (Ikeda et al., 2004). This interaction makes sense given that KCC2’s transport function relies on the ionic gradients generated by the Na\(^+\)/K\(^+\) ATPase.

1.6.2.2 Structural proteins

KCC2 also interacts with several structural proteins, including 4.1N and β-pix. It’s physical interaction with 4.1N, an actin-interacting structural protein, plays a key role in regulating dendritic spine development (Li et al., 2007). KCC2 also interactions with the b
isoform of β-PIX, a nucleotide exchange factor that regulates the activity of cofilin-1, a major actin-regulatory protein. KCC2-deficient cells display abnormally high levels of phosphorylated and therefore inactivated cofilin-1, which results in a stabilization of the actin cytoskeleton. This results in reduced spine motility and fewer functional excitatory synapses (Llano et al., 2015).

Protein associated with myc (PAM), is a myc-binding protein that is widely expressed in the brain. It was identified in a yeast-two hybrid assay to directly bind to KCC2 and was subsequently shown to cause increased $^{86}$Rb$^+$ uptake when co-expressed in HEK-293 cells (Garbarini and Delpire, 2008). The relevance on this interaction in vivo is not known.

1.6.2.3 CIP1

Cotransporter interacting protein 1 (CIP1) has also been shown to physically interact with KCC2. CIP1 is a transport-inactive member of the CCC family that interacts with NKCC1 and inhibits it’s transport activity (Caron et al., 2000). It also co-immunoprecipitates with KCC2 and was demonstrated to increase KCC2 transport activity in a $^{86}$Rb$^+$ flux assay when co-expressed in HEK-293 cells (Wenz et al., 2009).

1.6.2.4 Metabotropic receptors and zinc

KCC2 has also be shown to interact with several metabotropic receptors, including metabotropic glutamate receptors (mGluRs), metabotropic zinc receptors (mZnRs) and the GluK2-subunit of kainate receptors, which are ionotropic glutamate receptors that also display metabotropic signaling (KAR signaling will reviewed in more detail in Chapter 1.7 Kainate Receptors). Tonically active group I mGluRs alter KCC2 activity via a PKC-dependent pathway that enhances KCC2 function. When group I mGluR function is pharmacologically blocked, intracellular [Cl$^-$] rises and $E_{GABA}$ depolarizes (Banke and Gegelashvili, 2008).
MF-CA3 synapses are rich in zinc that is synaptically released into the extracellular compartment during neuronal activity, and zinc release at these synapses is important factor in KCC2 regulation. Activation of mZnRs at hippocampal MF-CA3 synapses by Zn\(^{2+}\) release enhances KCC2 transport as assessed by a NH\(_4\)^+ flux assay, and consequently hyperpolarizes \(E_{\text{GABA}}\) (Chorin et al., 2011). This is in contrast to the effect of intracellular Zn\(^{2+}\), which has been shown to downregulate KCC2 function (Hershfinkel et al., 2009). Synaptic Zn\(^{2+}\) release also results in an increase in KCC2 surface expression (Chorin et al., 2011). Because of these functional interactions between KCC2, mGluRs, mZnRs, and several other G-protein signaling receptors, it has been proposed that GPCRs may broadly act as KCC2 enhancers (Mahadevan and Woodin, 2016).

1.6.2.5 Kainate receptor subunits

Finally, KCC2 has been shown to physically interact with several KAR subunits – the GluK2 subunit (Mahadevan et al., 2014, Pressey et al., 2017) and Neto2, which is considered to be an auxiliary subunit of KARs (Ivakine et al., 2013). KCC2’s interaction with the GluK2 subunit of the kainate receptor as part of a macromolecular complex that is required for normal KCC2 surface expression and oligomerization (Mahadevan et al., 2014). This interaction is critical to the rationale of this thesis, and will be discussed in greater detail in Chapter 1.8 The KCC2 / GluK2 interaction.

1.7 Kainate Receptors

1.7.1 Overview of Glutamate Receptors

Glutamate is often referred to as the primary excitatory neurotransmitter in the CNS. It binds to several different receptors, most of which are named after the agonists historically used
to activate them. They are divided into two groups: ionotropic glutamate receptors and metabotropic glutamate receptors. AMPAR, NMDAR, KAR are classified under ‘ionotropic glutamate receptors’, and mGluRs are considered ‘metabotropic glutamate receptors’.

AMPA receptors are the principal ionotropic glutamate receptors and are necessary for fast excitatory neurotransmission. AMPA receptors allow the influx of cations, including Na\(^+\), K\(^+\), and Ca\(^{2+}\) depending on subunit composition (Traynelis et al., 2010). Opening of the channel increases the cell’s permeability to these cations, and it is particularly the influx of Na\(^+\) which causes the cell to depolarize and thus mediates excitation.

NMDA receptors similarly allow the influx of cations, most importantly Na\(^+\) and Ca\(^{2+}\). However, NMDA receptors require not only the binding of glutamate to open, but also the binding of glycine (Kleckner and Dingledine, 1988). NMDARs are also sensitive to voltage, because the channel pore is blocked by Mg\(^{2+}\) at a normal membrane resting potential (Mayer et al., 1984, Nowak et al., 1984). Sufficient depolarization of the membrane potential is required to relieve this block and allow a current. When the Mg\(^{2+}\) block is relieved, the subsequent influx of calcium through the NMDAR is critical to some forms of LTP and LTD (Lüscher and Malenka, 2012).

Like AMPA receptors, KARs produce currents that are largely carried by Na\(^+\). They are also permeable to K\(^+\) and Ca\(^{2+}\) depending on subunit composition and RNA editing. Compared to AMPAR and NMDAR currents, ionotropic KAR currents have very slow decay kinetics. These kinetics are largely due to the interaction of KARs with Neto proteins, which are considered an auxiliary subunit of KARs (Zhang et al., 2009, Straub et al., 2011, Tang et al., 2011). Compared to AMPARs, KAR currents are smaller, limited only to certain synapses, and they are sensitive to many of the same agonists and antagonists as AMPARs, which historically made them difficult to study. The development of more specific AMPAR blockers such as GYKI53655 have
been instrumental in the study of KARs (Paternain, A. V. et al., 1995, Wilding and Huettner, 1995, Lerma and Marques, 2013). KAR structure and function will be discussed in more detail in Chapter 1.7.2 KAR structure and function.

Unlike AMPA, NMDA, and KAR receptors, mGluRs are a family of glutamate receptors that do not produce a current when activated. Instead, they are G-protein coupled and signal metabotropically, producing downstream effects that serve an important modulatory role in altering cell excitability and neurotransmitter release (Niswender and Conn, 2010).

However, the distinction between glutamate receptors that signal ionotropically or metabotropically is not as simple as their classification implies. While all three ionotropic glutamate receptors signal ionotropically, there is also evidence to suggest that these receptors have an additional, metabotropic mode of signaling that acts independently of ion flux through the receptor. Metabotropic KAR signaling is the most well-characterized of the three; KARs are known to signal via a mechanism involving G-proteins, and this signaling is independent of ion flux through the receptor (Rozas et al., 2003). This signaling mechanism will be discussed in further detail in Chapter 1.7.4 Metabotropic KAR signaling.

While KAR metabotropic signaling is the most well-established and accepted in the literature, there is also evidence that both AMPARs (Wang et al., 1997, Bai et al., 2002) and NMDARs (Nabavi et al., 2013, Abrahamsson et al., 2017) may have a metabotropic signaling mechanism that acts in the absence of ion flux, though it is less well characterized than metabotropic KAR signaling.

1.7.2 KAR structure and function

KARs are tetrameric glutamate receptor proteins that are important mediators of glutamate action. KAR tetramers are formed from various combinations of the five KAR
subunits: GluK1, GluK2, GluK3, GluK4, and GluK5. The current nomenclature for these subunits is a relatively recent change, and it is important to note that they were previously referred to as GluR5, GluR6, GluR7, KA1, and KA2 respectively (Collingridge *et al.*, 2009), as most of the literature prior to 2009 uses the former names for these subunits (For quick reference between nomenclatures, see Table 1.2). To form a functional KAR tetramer, GluK1-3 can form either homomers or heteromers. GluK4 and GluK5 are unable to form homomers, and instead must form heteromers with the GluK1-3 subunits (Lerma and Marques, 2013).

KAR subunits have the same basic structural layout as AMPA and NMDA receptors (Pinheiro, Paulo and Mulle, 2006), including an extracellular N-terminus, and an extracellular loop between transmembrane segments 3 and 4, which together form the ligand-binding domain. They also have an intracellular C-terminus (Figure 1.10). Additionally, KARs are subject to splice variations and RNA editing in several areas, including numerous splice variations on the C-terminus. For example, GluK1-2 KAR subunits that normally form receptors permeable to Na\(^+\) and K\(^+\) can be rendered Ca\(^{2+}\)-permeable following editing of a conserved glutamine (Q) to an arginine (R) (merged).

KARs are expressed throughout the entire brain, with different subunits showing differing patterns of expression in regard to both brain area and cell type. For example, GluK1 mainly expresses in hippocampal and cortical interneurons, while GluK2 subunits are expressed mostly by principal cells, including in the hippocampus and cerebellum. GluK3 shows less abundant expression, appearing in layer IV of the neocortex and in the dentate gyrus of the hippocampus. GluK4 is expressed in CA3 pyramidal neurons, the dentate gyrus, neocortex, and cerebellum, and GluK5 is expressed abundantly throughout the whole brain (Lerma and Marques, 2013).
Subcellularly, KARs can express both pre-synaptically and post-synaptically, where they can exert different effects depending on their localization (Jaskolski et al., 2005). The best studied example of the different subcellular rules of KARs exists in the hippocampus, where both their pre and post synaptic effects have been well characterized. At both the mossy fibres and Schaffer collaterals, KARs regulate the presynaptic release of neurotransmitters onto pyramidal cells. Additionally, KARs contribute to postsynaptic currents in CA3 pyramidal cells at mossy fibre synapses (Figure 1.11).

The diversity in the functional effects of KAR activation is partially due to the diversity in the types of signaling KARs can perform. While they are classed as an ionotropic glutamate receptor, they possess two modes of signaling: canonical (ionotropic) and noncanonical (metabotropic) (Lerma and Marques, 2013). Canonical signaling involves the opening of the ion channel that is permeable to Na⁺, K⁺, and Ca²⁺ depending on subunit configuration and RNA editing. This ionotropic action has a smaller amplitude than an AMPA current but also has slower decay kinetics and can directly contribute to membrane depolarization. Noncanonical signaling can occur independent of ion channel activation and involves a G-protein signaling pathway, which is known to activate phospholipase C (PLC) and PKC downstream, and can contribute to membrane excitability in several ways, such as regulating the Iₐ₃ (Figure 1.12). Both modes of signaling will be covered in more detail in the following sections.
### Table 1.2 KAR nomenclature

<table>
<thead>
<tr>
<th>Gene</th>
<th>Current Subunit Nomenclature</th>
<th>Previous Subunit Nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRIK1</td>
<td>GluK1</td>
<td>GluR5</td>
</tr>
<tr>
<td>GRIK2</td>
<td>GluK2</td>
<td>GluR6</td>
</tr>
<tr>
<td>GRIK3</td>
<td>GluK3</td>
<td>GluR7</td>
</tr>
<tr>
<td>GRIK4</td>
<td>GluK4</td>
<td>KA1</td>
</tr>
<tr>
<td>GRIK5</td>
<td>GluK5</td>
<td>KA2</td>
</tr>
</tbody>
</table>

Modified from *(Collingridge et al., 2009)*
Figure 1.10 Structure of a KAR subunit

A schematic showing showing the basic structure of a KAR subunit, including the four transmembrane segments, the extracellular N-terminus, extracellular loop that forms the ligand binding domain along with the N-terminus, and the intracellular C-terminus. This structure is conserved across ionotropic glutamate receptors.

Image from (Pinheiro, Paulo and Mulle, 2006)
Used with permission, Rightslink license # 4526550534257
Figure 1.11 Subcellular KAR localization in the hippocampus

In the hippocampus, KARs have both presynaptic and postsynaptic effects. At mossy fibre, perforant pathway, and commissural synapses onto CA3 pyramidal cells, and Schaffer collateral synapses onto CA1 pyramidal cells, KARs regulate presynaptic neurotransmitter release. On CA3 pyramidal cells, they are expressed postsynaptically, and produce a current.

Image from (Jaskolski et al., 2005).
Used with permission, Rightslink license #4526540909303.
Figure 1.12 KAR signaling modes

A schematic showing the two modes of signaling of KARs. The canonical (ionotropic) pathway involves cation flux through the receptor, which can directly contribute to membrane depolarization by producing a current. The non-canonical (metabotropic) pathway does not require channel opening, and signals through a metabotropic pathway that involves G-proteins, PLC, and PKC.

Image from (Lerma and Marques, 2013)
Used with permission. Rightslink license #4526550731765
1.7.3 Ionotropic KAR signaling

Kainate receptors form an ion channel that is permeable to Na\(^+\), K\(^+\), with some subtypes also being permeable to Ca\(^{2+}\). In the absence of auxiliary subunits, KAR currents resemble AMPA currents, as they share similar ion affinities and kinetics. *In vivo*, however, KAR currents are characteristically smaller in amplitude and slower to decay than those of AMPA currents, instead bearing a close resemblance NMDA currents (*Figure 1.13A*). This characteristic small, slow KAR current observed under normal conditions is due to the KAR’s interaction with auxiliary subunits, namely, the Neto proteins (Zhang *et al.*, 2009, Straub *et al.*, 2011, Tang *et al.*, 2011). In the Neto1 KO, KAR currents display a rapid decay similar to an AMPAR current (Straub *et al.*, 2011, Lerma and Marques, 2013) (*Figure 1.13B*). Ionotropic KAR signaling also differs from AMPARs and NMDARs in that the gating of the channel is gated by external monovalent cations and anions. KAR subunits possess an ion binding pocket and the opening of the channel requires these binding pockets be occupied in order for the channel to open (Paternain *et al.*, 2003, Bowie, 2002, Bowie, 2010).

Although KARs are abundantly expressed in the brain, synaptic KAR currents have only been detected in a limited number of synapses. In the hippocampus, KAR currents have been recorded in pyramidal cells only at MF-CA3 synapses (Castillo *et al.*, 1997, Vignes and Collingridge, 1997). KAR currents have also been recorded in interneurons in the CA1 (Cossart *et al.*, 1998, Frerking *et al.*, 1998). Outside of the hippocampus, KAR currents have been recorded in specific synapses in the cerebellum, amygdala, spinal cord, and at thalamocortical connections (Lerma and Marques, 2013).

Historically, it was unclear what the purpose of postsynaptic KAR currents was, since they were smaller, slower, and less prevalent than AMPA currents. It is now clear that
postsynaptic ionotropic KAR signaling serves an important modulatory role in neurotransmission. For example, in hippocampal interneurons, KAR currents may encode different kinds of information than AMPA currents and mediate the characteristic theta frequency oscillations that are known to be relevant to behavior (Frerking and Ohlinger-Frerking, 2002, Goldin et al., 2007). At hippocampal MF-CA3 synapses, postsynaptic KAR currents play a critical role in regulating excitability, amplifying spike transmission and contributing to sustained depolarization (Sachidhanandam et al., 2009, Pinheiro et al., 2013). However, there may be additional functions of KAR currents still to be revealed, such as the regulation of KCC2-mediated inhibition studied in this thesis.

KARs also have a well-studied role in regulation presynaptic release of neurotransmitters at many different synapses. While many of these effects are likely mediated by metabotropic signaling (reviewed below), there is evidence that the ionotropic action of Ca^{2+}-permeable KARs facilitates glutamate release at MF-CA3 synapses, contributing to a form of LTP that is NMDAR-independent. Ca^{2+} influx through the KAR is thought to induce further Ca^{2+} release from internal stores (Lauri et al., 2003, Pinheiro et al., 2007, Scott et al., 2008, Andrade-Talavera et al., 2012). The normal synaptic facilitation observed at these synapses is reduced when antagonists against calcium-permeable KARs are applied.
Figure 1.13 Kinetic characteristics of KAR currents

(A) A sample of scaled responses from AMPARs, NMDARs, and KARs. Under normal conditions, KAR currents display slower kinetics that resemble those of NMDA receptors.

(B) The difference in KAR-mediated currents seen in WT mice and NETO1 KO mice. In the absence of NETO1, KAR currents display faster kinetics.

Image from (Lerma and Marques, 2013)
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1.7.4 Metabotropic KAR signaling

In addition to the ability to signal ionotropically, KARs have a second, non-canonical signaling mechanism that can be activated independently of the ion channel. Non-canonical KAR signaling is now known to involve at least two kinds of mechanisms. One form may be G-protein independent and involves the activation of adenylate cyclase and PKA, which is involved in mediating presynaptic glutamate release. The second form is metabotropic and is known to involve G-proteins and sometimes also the downstream activation of several different second messenger cascades, which involve PLC, PKA and/or PKC, which is involved in decreasing neurotransmitter release (Negrete-Díaz et al., 2018). There is some controversy in the field as to which KAR subunit(s) and which G-protein(s) are responsible for mediating non-canonical signaling (Lerma and Marques, 2013).

G-proteins are subcategorized into several families based on their structures and the signaling cascades they activate. For example, G_q proteins stimulate PLC which generates a second messenger cascade involving inositol triphosphate (IP_3) and diacylglycerol (DAG), which can then activate PKC. G_\text{i/o} proteins, which are pertussis toxin (PTx) sensitive, inhibit adenylyl cyclase, which lowers the level of cyclic AMP (cAMP) in the cell (Wettschureck and Offermanns, 2005). There is some controversy regarding which of these G-protein families mediates metabotropic KAR signaling (Lerma and Marques, 2013). The downstream activation of PLC suggests that noncanonical KAR signaling may involve G_q proteins. However, KAR signaling is also PTx-sensitive (Rodríguez-Moreno and Lerma, 1998, Cunha et al., 2000, Rodrigues and Lerma, 2012), suggesting that G_\text{i/o} proteins may be involved, and a recent proteomic analysis revealed that GluK1 subunits interact with G_o proteins (Rutkowska-Wlodarczyk et al., 2015). This evidence suggests that the metabotropic effects of KAR signaling
may be mediated by several different G-proteins, or that some of these effects may be activated indirectly as a secondary effect of KAR activation. To further highlight the complexity of this mode of KAR signaling, it should be noted that metabotropic KAR signaling does not necessarily always involve kinases as second messengers, and instead may involve G_{i/o} proteins directly inhibiting Ca^{2+} channels (Frerking et al., 2001, Negrete-Díaz et al., 2018).

The effects of metabotropic KAR signaling are diverse but can be broken down into a few subcategories: postsynaptic regulation of the I_{AHP}, and presynaptic regulation of neurotransmitter release. These actions allow KARs to influence neuronal excitability in different ways.

The afterhyperpolarization current (I_{AHP}) is a current that, when activated upon bursts of action potentials, hyperpolarizes the membrane for a short period, inhibiting the cell’s ability to fire another action potential immediately after the previous action potential. The decay time of the I_{AHP} varies, but in some situations it may last for several seconds (Lancaster and Adams, 1986, Lerma and Marques, 2013). Activation of KARs reduces the I_{AHP} and involves the activation of G_{i/o} proteins and PKC (Melyan et al., 2002, Melyan et al., 2004). This regulates excitability of the cell, increasing firing frequency and enhancing circuit excitability (Fisahn et al., 2005, Ruiz et al., 2005).

KARs have also been shown to regulate both glutamate and GABA release at specific synapses. At MF-CA3 synapses glutamate release can be modulated in a biphasic fashion depending on the amount of KAR activation. When relatively high concentrations of KA (>100nM) are applied, synaptic release of glutamate is inhibited (Negrete-Díaz et al., 2006, Lauri et al., 2006), and when relatively low concentrations of KA are applied (<100nM), synaptic release of glutamate is facilitated (Schmitz et al., 2001, Pinheiro et al., 2007, Scott et al., 2008, Fernandes et al., 2009, Negrete-Díaz et al., 2018). This bimodal effect has been observed at MF-
CA3 synapse, but has not yet been investigated at CA3-CA1 synapses (Negrete-Díaz et al., 2018). Both depression (Rodríguez-Moreno and Lerma, 1998, Vignes et al., 1998, Rodríguez-Moreno et al., 2000) and facilitation (Frerking et al., 1998, Cossart et al., 1998, Jiang et al., 2001) of GABA release have also been reported, though facilitation of GABA release is more consistent with and ionotropic signaling mechanism (Rodrigues and Lerma, 2012).

Taken together, this evidence suggests that KARs are powerful modulators of neuronal activity, exerting their effects both on presynaptic neurotransmitter release in a complex, bimodal fashion, as well as directly regulating cell excitability by modulating the I_{AHP}.

1.7.5 Kainate receptor knockout mice

To study KAR function in the brain, there are a number of valuable tools, including genetic knockout mice for each of the GluK1-5 subunits as well as many crossbred strains that have been produced to knock out combinations of multiple subunits. Total kainate receptor knockout is not lethal, but produces a mouse with abnormal compulsive behaviors, motor issues, and impaired corticostriatal synaptic function (Xu, Jian et al., 2017). Knockouts of individual subunits produce less severe phenotypes, with fewer or no behavioral effects and more nuanced physiological effects depending on the cell type and brain area being studied.

Knockout of either of the high-affinity kainate receptor subunits produces different effects in the hippocampus. GluK4−/− mice display reduced anxiety-like behavior and impairment in LTP at MF-CA3 synapses (Catches et al., 2012). In GluK5−/− mice, KAR-mediated EPSCs are present in CA3 pyramidal cells, but the normal KAR-mediated inhibition of the I_{AHP} is absent (Ruiz et al., 2005). Ablation of both GluK4 and GluK5 results in a complete loss of kainate receptor currents and alters subcellular localization of the remaining principal subunits, including a reduced expression of GluK2/3 subunits on dendritic spines (Fernandes et al., 2009),
highlighting the importance of the high-affinity subunits for function and expression of kainate receptors.

Similarly, knockout of any of the principal kainate subunits produces different effects depending on the subunit knocked out, the cell type, and the brain area. GluK3Δ/Δ mice have intact KAR-mediated currents, but display reduced short and long-term synaptic potentiation at mossy fibres (Pinheiro, Paulo S. et al., 2007). In contrast, GluK2Δ/Δ mice lack mossy fibre -CA3 kainate currents and have a reduced susceptibility to kainate-induced seizures, but display no differences in motor function or learning and memory when compared to controls (Mulle et al., 1998). At mossy fibre synapses, bath application of kainate normally reduces EPSCs via the activation of presynaptic kainate receptors that depress synaptic transmission (Vignes et al., 1998, Bortolotto et al., 1999, Kamiya and Ozawa, 2000), an effect that is preserved in the GluK1Δ/Δ mouse but is absent in the GluK2Δ/Δ mouse (Contractor et al., 2000). GluK1Δ/Δ mice and GluK2Δ/Δ mice are both susceptible to kainate inhibition of evoked IPSCs in hippocampal interneurons, but mice deficient for both subunits are not (Mulle et al., 2000). Taken together this evidence suggests that the principal subunits serve different physiological roles depending on where they are expressed, but there is no single subunit that is absolutely required for KARs in general to be expressed and perform at least some of their functions. One possible explanation for the relatively minor effects observed in any of the single-subunit knockout mice compared to the total knockout could result from a developmental compensation for the loss of a particular subunit. However, this is considered unlikely, as in these studies mRNA levels of other KAR subunits remains unchanged in both GluK1Δ/Δ (Mulle et al., 2000) and GluK2Δ/Δ mice (Mulle et al., 1998).

Taken together, the evidence from various KAR subunit knockout mice suggests that kainate receptors play complex, modulatory roles in synaptic transmission that differ depending on the subunit expression in different brain areas and neuron types.
1.8 The KCC2 / GluK2 interaction

As a glutamate receptor, KARs are typically associated with excitatory synaptic transmission. However, in addition to their ability to contribute to postsynaptic currents during excitatory neurotransmission, they serve many diverse roles in the hippocampus, including the regulation of both glutamate and GABA release. Their role in the hippocampus is made even more complex by their numerous protein-protein interactions, including interactions with traditionally ‘inhibitory’ proteins such as KCC2.

The identification of KCC2 as an interactor of Neto2, a KAR auxiliary subunit (Ivakine et al., 2013) led to further studies of whether KCC2 might also interact with other KAR subunits. It was found that KCC2 interacts with the GluK2 subunit, and that this interaction is important for the formation and stabilization of KCC2 oligomers, which are believed to be the functional form of KCC2 (Mahadevan et al., 2014). The presence of GluK2 is required for KCC2 to properly traffic to the cell membrane, where it can perform its transport function and regulate Cl⁻ homeostasis. GluK2 also contributes to KCC2 recycling, increasing the amount of KCC2 that is recycled back to the membrane (Pressey et al., 2017). This GluK2-mediated regulation of KCC2 function is independent of the activation of GluK2-containing receptors, because when KCC2 and GluK2 were overexpressed in non-neuronal HEK293 cells, GluK2 interacted with and regulated KCC2 oligomers in the absence of KAR activation (Mahadevan et al., 2014). This suggests that a physical interaction between GluK2 and KCC2 is required for KCC2 function, however, whether a functional interaction exists between KCC2 and GluK2 remains unknown.

The physical interaction between these two proteins suggests that they may also interact functionally in the membrane. Given KCC2’s prominent role in regulating inhibition, and the kainate receptor’s role in excitatory neurotransmission, a functional interaction between these
proteins could potentially serve as a mechanism for regulating the balance between excitatory and inhibitory activity.

1.9 Objectives & aims

The MAIN OBJECTIVE of my thesis was to characterize the functional interaction between GluK2-containing kainate receptors and KCC2. Specifically, I aimed to determine whether KAR activation could regulate KCC2 function, and if so, determine whether this regulation was mediated by ionotropic or metabotropic KAR signaling. To address this objective, I completed three specific aims:

(1) **Determine if postsynaptic KAR activation at MF-CA3 synapses can regulate KCC2 function.**

    KAR signaling occurs via both the ionotropic and metabotropic mechanisms. I hypothesized that either or both signaling pathways could stimulate KCC2-mediated Cl⁻ extrusion, which would result in a decreased neuronal [Cl⁻] and a subsequent hyperpolarization of E\textsubscript{GABA}. To determine if activation of KARs in general could regulate KCC2 function, I bath applied 1μM kainic acid to acute hippocampal slices while recording from CA3 pyramidal cells in order to stimulate both ionotropic and metabotropic KAR signaling. This hypothesis was tested in Chapter 3.

(2) **Determine if selective activation of ionotrophic KAR signaling regulates KCC2 function.**

    I hypothesized that activation of ionotrophic KAR signaling could regulate KCC2 function independent of metabotropic signaling. To test this, I bath applied 1μM kainic acid to acute hippocampal slices while recording from CA3 pyramidal cells in the presence of
metabotropic signaling blockers NEM or GDP-β-S to isolate ionotropic signaling from metabotropic signaling. This hypothesis was tested in Chapter 4.

(3) **Determine if selective activation of metabotropic KAR signaling regulates KCC2 function.**

I hypothesized that activation of metabotropic KAR signaling could regulate KCC2 function independent of metabotropic signaling. To test this, I bath applied 0.1uM kainic acid to acute hippocampal slices while recording from CA3 pyramidal cells. This lower concentration of kainic acid is sufficient to activate metabotropic signaling while reducing ionotropic effects. This hypothesis was tested in Chapter 5.
Chapter 2 : Materials and Methods

2 Materials and Methods

2.1 Experimental animals

Experiments were performed on male and female mice. C57Bl/6 mice were obtained from a colony in the Faculty of Arts and Sciences Biosciences Facility (originally obtained from Jackson Laboratories). GluK1/2−/− mice were originally obtained from Dr. Chris McBain; NIH, Bethesda (Mulle et al., 1998, Mulle et al., 2000, Contractor et al., 2000), and were used to establish a colony in the Faculty of Arts and Sciences Biosciences Facility. All mice were housed under a 12-h/12-h light/dark cycle with ad libitum access to food and water.

All animal procedures were approved by the University of Toronto Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Chemicals

The following agonists and antagonists were used in experiments: kainic acid (1uM or 0.1uM, Sigma Aldrich), NEM (50 μM; Sigma Aldrich), GDP-β-S (300 μM; Sigma Aldrich), DL-APV (50μM; Tocris Bioscience), GYKI 52466 (10μM; Tocris Bioscience), CGP55845 (3μM; Tocris Bioscience), DL-AP3 (300μM; Tocris Bioscience), ZX1 (100μM; Strem Chemicals), and VU 0463271 (1μM; Tocris Bioscience). All chemicals were diluted in artificial cerebrospinal fluid (aCSF) with the exception of GDP-β-S, which was added to pipette internal solution, CGP55845, which was diluted in dimethyl sulfoxide (DMSO), and VU 0463271, which was also
diluted in DMSO resulting in a concentration 0.0002% DMSO in aCSF (all experiments) and 0.0003% (VU experiments) used for electrophysiological recordings.

All recording were made in the presence of the following glutamate and GABA<sub>B</sub> receptor inhibitors to isolate GABAergic currents: DL-APV (50μM), GYKI52466(10μM) CGP55845 (3μM), and DL-AP3 (300μM); control recordings included these receptor inhibitors alone. KARs were activated using kainic acid (Sigma Aldrich); 1μM to activate both ionotropic and metabotropic signaling and 0.1μM to activate metabotropic signaling (Table 2.1).

### 2.3 Acute hippocampal slice preparation

P19-P26 C57Bl/6 mice were anesthetized with isoflurane and brains were rapidly removed after decapitation and placed into a cutting solution containing the following (in mM): 205 sucrose, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 25 glucose, 0.4 ascorbic acid, 1 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, and 3 sodium pyruvate, pH 7.4, osmolality ~295 mOsm/kg. Coronal slices (300 μm) containing the hippocampus were prepared and recovered at 32°C in a 50:50 mixture composed of cutting saline aCSF for 30 min and then placed in aCSF alone for 30 min. During experimentation slices were perfused at a rate of ~ 2 ml/min in aCSF. The aCSF solution consisted of the following (in mM): 123 NaCl, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 25 glucose, 2 CaCl<sub>2</sub>, and 1 MgCl<sub>2</sub> in double-distilled water and saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub>, pH 7.4, osmolarity ~295mOsm.
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2.4 Patch clamp electrophysiology

Recording pipettes were pulled from thin-walled borosilicate (TW-150 F, World Precision Industries; Sarasota, Florida) to resistances of 5–10 MΩ with a Sutter Instruments P-87 (Novato, CA, USA). Pipettes were filled with an internal solution containing the following (in mM): 130 potassium gluconate, 10 KCl, 10 HEPES, 0.2 EGTA, 4 ATP, 0.3 GTP, and 10 phosphocreatine, pH 7.4, osmolality ~285 mOsm/kg.

Whole-cell patch-clamp recordings were obtained from putative pyramidal cells in the CA3. All experiments were performed at 22–24°C. Recordings began 5 minutes after entering whole-cell configuration, this time point is taken as t=0. \( E_{\text{GABA}} \) was determined in voltage clamp mode by evoking inhibitory postsynaptic currents (IPSCs) while step depolarizing the membrane potential. During each current step an IPSC was evoked by a stimulation electrode placed in the stratum lucidum/radiatum (Figure 2.1). A linear regression of the IPSC amplitudes was then used and the intercept of this line with the abscissa was taken as \( E_{\text{GABA}} \).

Stimulus intensity was controlled using an A.M.P.I. ISO-FLEX stimulus isolator (IBIS Instrumentation Canada Inc., Ottawa, ON, CA), and stimulus duration was controlled using Multiclamp 700B Commander software and pClamp 9.2 software (Axon Instruments Inc., Union City, CA, USA). For \( E_{\text{GABA}} \) recordings, 50 milliseconds after the current clamp step was initiated, a 20-200\( \mu \)A, 0.3ms extracellular stimulus was triggered. Stimulus intensity was adjusted for each cell before recordings began to ensure a robust PSC was visible without directly evoking an action potential in the patched cell. Stimulus intensity was not changed for each individual cell once recording began.
Figure 2.1 Placement of recording and stimulating electrodes in the hippocampus

Schematic diagram of the hippocampus and regional connections. Inhibitory interneurons in the striatum lucidum/radiatum were stimulated, while recordings were made from CA3 pyramidal neurons, which are innervated by inhibitory GABAergic interneurons.

Image modified from (Kullmann and Lamsa, 2007)
2.5 Statistical analysis

All population data is expressed as mean ± SD, unless otherwise specified. Full time course data was analyzed using two-way repeated measures ANOVA. All other data was analyzed using a paired Student’s t-test to examine the statistical significance of the differences. Each n value represents an individual neuron.

Only one n value was obtained from each acute slice. Multiple n values were obtained from the same animal.

The number of cells (n) and animals (N) used in each figure are detailed in Table 2.2.
Table 2.2 Number of replicates in electrophysiology experiments

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Chapter 3: KAR activation regulates KCC2 function

3 KAR activation regulates KCC2 function

3.1 Statement of Authorship

The results from this chapter have been published in the *Journal of Physiology* (Garand *et al.*, 2018). All data presented in this thesis were from experiments I performed.

3.2 Introduction, Objectives, and Hypothesis

While KCC2 requires a physical interaction with the GluK2 subunit of the kainate receptor to express in the membrane, form functional oligomers, and undergo normal recycling (Mahadevan *et al.*, 2014, Pressey *et al.*, 2017), it is not known whether the functional activation of a KAR regulates KCC2 function. This is an important question to address because the presence of KCC2 and KARs in a macromolecular complex would place these two proteins in close physical proximity, which could allow them to easily exert functional influence on each other. A functional interaction between postsynaptic KARs, which regulate membrane excitability and are associated with excitatory activity, and KCC2, which is critical for maintaining fast hyperpolarizing inhibition, could also serve as a potential regulatory mechanism for excitatory: inhibitory balance. My **objective** was to determine if postsynaptic KAR activation at MF-CA3 synapses can regulate KCC2 function.

Because KAR signaling occurs via both the ionotropic and metabotropic mechanisms, it is possible that either or both pathways could regulate KCC2 independently. Ionotropic glutamate receptor activation causes an influx of Na⁺ and sometimes Ca⁺, and in excitotoxic
conditions a large influx of cations causes a passive influx of both Cl⁻ and water into the cell (Rungta et al., 2015). KCC2 is predicted to extrude 500 water molecules from the cell for each molecule of Cl⁻ and K⁺ it transports (MacAulay and Zeuthen, 2010) and increases transport in response to cell swelling (Lauf and Bauer, 1987, Flatman, 2008, Acton et al., 2012). KCC2’s role in regulating cell volume and osmolarity may come into play during periods of intense excitatory activity, including ionotropic KAR activation, if the influx of cations also leads to osmotic changes. Additionally, metabotropic KAR activation could possibly enhance KCC2 function because metabotropic KAR signaling involves the downstream activation of PKC (Melyan et al., 2002, Melyan et al., 2004). PKC is known to phosphorylate KCC2 at S940 and increase transporter function (Lee et al., 2007). Therefore I hypothesized that both of these signaling pathways could stimulate KCC2-mediated Cl⁻ extrusion through independent mechanisms, which would result in a decreased neuronal [Cl⁻] and a subsequent hyperpolarization of E_GABA (Figure 3.1). I first performed experiments to determine if activation of KARs in general could regulate KCC2 function. I activated both ionotropic and metabotropic KAR signaling using bath application of 1μM kainic acid (KA) to acute hippocampal slices while recording from CA3 pyramidal cells. This concentration of KA is sufficient to produce an ionotropic current in the CA3 (Castillo et al., 1997), and would also able to activate metabotropic signaling, as the metabotropic effects of KARs have been studied using nanomolar concentrations of kainic acid (Melyan et al., 2002, Fernandes et al., 2009). All experiments were performed on CA3 pyramidal cells in the hippocampus, because this is the only area in the hippocampus where postsynaptic KAR currents have been recorded in a pyramidal cell. In the CA3 there is a kainate-receptor-mediated component in addition to the AMPAR-mediated component of EPSCs during excitatory neurotransmission (Castillo et al., 1997).
Figure 3.1 Schematic of hypothesis for Chapter 3

Schematic diagram showing how KAR activation could potentially regulate KCC2 function. Metabotropic activation leads to the activation of G-proteins and PKC, which could then phosphorylate KCC2 and S940 and increase ion transport, lowering intracellular [Cl⁻]. Ionotropic activation leads to an influx of cations and water, which could activate KCC2 by activating swelling-activated transport and therefore lowering intracellular [Cl⁻].
3.3 Results

3.3.1 KA application modulates IPSCs in CA3 pyramidal cells

To determine the effects of KAR activation on KCC2 function, I bath applied the KAR agonist kainic acid (KA, 1μM) for 5 minutes while recording from CA3 pyramidal cells in whole-cell mode. KA was bath perfused in conjunction with a cocktail of antagonists to prevent the activation of other ionotropic glutamate receptors (10μM GYKI52466 to block AMPA, 50μM DL-APV to block NMDA), GABA_B receptors (CGP55845 3μM), and mGluRs (300μM DL-AP3; see Table 2.1). It was particularly important to prevent AMPAR activation because although KA has a higher affinity for KARs, it also has a lower affinity for AMPARs and can cause AMPAR activation depending on KA concentration and AMPAR subunit composition (Hampson and Manalo, 1998). In addition to this, KA was bath applied to the whole slice and therefore able to cause depolarization in all cells, and the stimulating electrode could potentially non-specifically stimulate any cells in its vicinity, either of which could potentially cause secondary activation of other glutamate receptors via subsequent glutamate release. mGluRs were also blocked because mGluR activation is already known to increase KCC2 function (Banke and Gegelashvili, 2008).

E_GABA was taken as a measure of KCC2 function and was recorded every 5 minutes by stimulating the stratum radiatum/lucidum to cause GABA release from interneurons onto the recorded cell. To confirm that the currents recorded were due to GABA_A receptors, I briefly recorded E_GABA using the above antagonist cocktail before and after the addition of gabazine (10μM), a GABA_A antagonist. Gabazine application abolished the currents produced after stimulation.
Figure 3.2 GABAA-receptor mediated currents in CA3 pyramidal cells

Effect of gabazine (10μM) on GABAergic PSCs in a CA3 pyramidal cell at different holding potentials, evoked by electrical stimulation of the stratum lucidum/radiatum (↑) in the presence of GYKI52466 (10uM), DL-APV (50μM), CGP55845 (3μM), and DL-AP3 (300μM). Scale bars: 40 pA, 20 ms.
For KA application experiments, all measurements taken at t = 0 are values obtained immediately prior to bath application of KA, 5 minutes after whole cell configuration was achieved. After 5 minutes of KA application (t=0 to t=5), KA was washed out using aCSF containing only the previously mentioned antagonists (t=5 to t=15). Control cells were perfused with the same antagonist cocktail as experimental cells for the course of the experiment but did not have KA applied.

KA application produced a significant hyperpolarization of $E_{\text{GABA}}$ (t=5 min), compared to control $E_{\text{GABA}}$ measurements that were made in aCSF containing only inhibitors (Figure 3.3). Upon KA application (t=5), $E_{\text{GABA}}$ hyperpolarized from $-70.1 \pm 6.3$ mV at t=0 to $-75.9 \pm 5.7$ mV at t=5. This was a statistically significant difference when compared to controls using a two-way repeated measure ANOVA (Figure 3.3A) and when $E_{\text{GABA}}$ at t=0 and t=5 were compared within the KA group using a paired Student’s t test (Figure 3.3B). $E_{\text{GABA}}$ of control cells did not significantly change over the course of the experiment. Following 5 minutes of KA washout, $E_{\text{GABA}}$ transiently depolarized back to baseline values and was not significantly different from controls at this time point (t=10 min) (Figure 3.3A). However, after 10 minutes of washout (t=15 min), $E_{\text{GABA}}$ again hyperpolarized to $-76.3 \pm 5.8$ mV. This was a statistically significant difference when compared to controls using a two-way repeated measure ANOVA (Figure 3.3A) and when $E_{\text{GABA}}$ at t=0 and t=15 were compared within the KA group using a paired Student’s t-test (Figure 3.3D).
Figure 3.3 KARs activation with 1µM kainic acid modulates IPSCs in CA3 pyramidal cells.

(A) Plot of group data showing the effect of 1µM kainic acid application on $E_{\text{GABA}}$ over time.
(B) Group data comparing $E_{\text{GABA}}$ at t=0 and t=5.
(C) Plot of voltage-current curve for a cell at t=0 ($E_{\text{GABA}}=-75.1$ mV) and during KA perfusion $t=5$ ($E_{\text{GABA}}=-86.2$ mV). Inset, examples of evoked IPSCs.
(D) Group data for the same cells as (B) comparing $E_{\text{GABA}}$ at t=0 and t=15.
(E) Plot of voltage-current curve for the same cells as (C) at t=0 ($E_{\text{GABA}}=-75.1$ mV) and during KA washout at $t=15$ ($E_{\text{GABA}}=-86.2$ mV). Inset, examples of evoked IPSCs

Scale bars: 60 pA, 10 ms. A,B,D n=11 control, n=8 KA. Error bars indicate SEM.

* = p < 0.05
In addition to monitoring $E_{\text{GABA}}$, the resting membrane potential (RMP), conductance, and paired pulse ratio (PPR) were monitored every 5 minutes. RMP was monitored because the effects of GABA$_A$ receptor activation not only depend on the Cl$^-$ gradient, but also the electrical gradient across the membrane, which together contribute to the electrochemical gradient for Cl$^-$. Driving force ($V_{DF}$), the total forces acting upon a given ion in the membrane, can be computed with the following equation: $V_{DF} = V_m - V_{eq}$, where $V_m =$ the resting membrane potential and $V_{eq} =$ the reversal potential for the ion or receptor, in this case, $V_{eq} = E_{\text{GABA}}$. Driving force also indicates the direction of Cl$^-$ flux across the membrane when the GABA$_A$ receptor is opened. In addition to determining the RMP and driving forces, GABA conductance and PPR were monitored to determine if changes in presynaptic neurotransmitter release contributed to the observed effects of KA application. This is especially important because KARs are well known for their ability to regulate presynaptic release of both glutamate and GABA in the hippocampus.

The observed change in $E_{\text{GABA}}$ at $t=5$ was accompanied by a significant depolarization of the resting membrane potential from $-78.3 \pm 11.0\text{mV}$ to $-51.7 \pm 18.8\text{mV}$ (Figure 3.5A), which resulted in a significant change in the driving force for Cl$^-$ from $-8.2 \pm 11.9\text{mV}$ to $+24.2 \pm 22.0\text{mV}$ (Figure 3.5B). This effect was accompanied by a significant decrease in synaptic conductance from $4.1 \pm 2.6\text{pS}$ to $3.0 \pm 3.0\text{pS}$ in KA-treated cells but not in controls. There were no significant differences paired pulse ratio for either group (Figure 3.5C,D).

Similarly, the change in $E_{\text{GABA}}$ at $t=15$ was accompanied by a significant depolarization of the resting membrane potential from $-78.3 \pm 11.0\text{mV}$ to $-64.4 \pm 13.2\text{mV}$ (Figure 3.5A), which resulted in a significant change in the driving force for Cl$^-$ from $-8.2 \pm 11.9\text{mV}$ to $+11.3 \pm 17.0\text{mV}$ (Figure 3.5B). This effect was not accompanied by a significant change in conductance or paired pulse ratio compared to $t=0$ (Figure 3.5C,D).
Figure 3.4 KAR activation with 1μM kainic acid increases the driving force for chloride.

(A) Group data showing resting membrane potential at t=0 and t=5
(B) Group data showing driving force for Cl⁻ at t=0 and t=5
(C) Group data showing Cl⁻ conductance at t=0 and t=5
(D) Group data showing paired pulse ratio at t=0 and t=5

A,B,C, n=11 control, n=8 KA. D, n=9 control, n=8 KA. Error bars indicate SEM.
* = p < 0.05, ** = p < 0.01
Figure 3.5 1μM KA application increases the driving force for chloride after KA washout.

(A) Group data showing resting membrane potential at t=0 and t=15
(B) Group data showing driving force for Cl⁻ at t=0 and t=15
(C) Group data showing Cl⁻ conductance at t=0 and t=15
(D) Group data showing paired pulse ratio at t=0 and t=15

A,B,C, n=11 control, n=8 KA. D, n=9 control, n=8 KA. Error bars indicate SEM.
* = p < 0.05, ** = p < 0.01
Next, I sought to determine whether the observed effects were due to a change in KCC2 transporter function or due to some other cause, such as another Cl⁻ transporter. At the age this study was performed (postnatal day 19-25) E$_{\text{GABA}}$ prior to KA application was variable and often more depolarized than the resting membrane potential, which may indicate that not all neurons recorded from had adult expression levels of KCC2. Furthermore, even in adult neurons, KCC2 is not the only transporter than regulates the Cl⁻ gradient, as NKCC1 is also expressed in these cells (Kaila et al., 2014). The hyperpolarization of E$_{\text{GABA}}$ that I observed could be due to either an increase in KCC2 function, a decrease in NKCC1 function, a combination of both, or some other factor such as influxes of water and changes in osmolarity.

To determine if KCC2 is required for the hyperpolarization of E$_{\text{GABA}}$ I observed in previous experiments, I repeated the previous experiments with the addition of the specific KCC2 antagonist, VU0463271 (VU). VU antagonists displays a 100-fold selectivity for KCC2 over NKCC1 (Delpire et al., 2012), allowing me to parse out the specific contribution of KCC2 to the observed effects of KA application on E$_{\text{GABA}}$. VU (1μM) was applied in combination with the same antagonist cocktail used previously including: 10μM GYKI52466 to block AMPA, 50μM DL-APV to block NMDA, 3μM CGP55845 3μM to block GABA$_B$ receptors, and 300μM DL-AP3 to block mGluRs (Table 2.1). Cells were washed with VU and inhibitors thorough the experiment, during KA wash-in KA was added as an additional component to the aCSF containing the VU and inhibitors. Control cells had VU and other antagonists perfused throughout the experiment but were not perfused with KA.

With the addition of VU, 1μM KA application again produced a significant hyperpolarization of E$_{\text{GABA}}$ (t=5 min), compared to control E$_{\text{GABA}}$ measurements that were made in aCSF containing only VU and inhibitors (Figure 3.6). During KA application (t=5), E$_{\text{GABA}}$ hyperpolarized from $-63.5 \pm 3.6$ mV at t=0 to $-68.9 \pm 4.2$ mV at t=5. This was a statistically
significant difference when compared to controls using a two-way repeated measure ANOVA (Figure 3.6A) and when E_{GABA} at t=0 and t=5 were compared within the KA group using a paired Student’s t test (Figure 3.6B). Following 5 minutes of KA washout, E_{GABA} depolarized back to baseline values and was not significantly different from controls at this time point (t=10 min). In contrast to previous results, after 10 minutes of KA washout (t=15 min), E_{GABA} again depolarized to $-59.7 \pm 4.1$ mV. This was a not statistically significant difference when compared to controls using a two-way repeated measure ANOVA (Figure 3.6A), or when E_{GABA} at t=0 and t=15 was compared within the KA group using a paired Student’s t-test (Figure 3.6D).

The observed change in E_{GABA} at t=5 was accompanied by a significant depolarization of the resting membrane potential and driving force (Figure 3.6A,B). This effect was accompanied by a significant decrease in synaptic conductance from $7.0 \pm 2.9$ pS to $2.1 \pm 1.6$ pS in KA-treated cells but not in controls. There were no significant differences paired pulse ratio in either group.
Figure 3.6 KAR-mediated modulation of IPSCs has a KCC2-independent component

(A) Plot of group data showing the effect of 1 μM kainic acid application on $E_{\text{GABA}}$ over time in the presence of VU 0463271.

(B) Group data comparing $E_{\text{GABA}}$ at t=0 and t=5.

(C) Plot of voltage-current curve for a cell at t=0 ($E_{\text{GABA}}$ = −68.6 mV) and during KA perfusion t = 5 ($E_{\text{GABA}}$ = −71.9 mV). Inset, examples of evoked IPSCs.

(D) Group data for the same cells as (B) comparing $E_{\text{GABA}}$ at t=0 and t=15.

(E) Plot of voltage-current curve for the same cell as (C) at t=0 ($E_{\text{GABA}}$ = −68.6 mV) and during KA washout at t = 15 ($E_{\text{GABA}}$ = −59.2 mV). Inset, examples of evoked IPSCs

Scale bars: 100 pA, 20 ms. A,B,D n=7 control, n=7 KA. Error bars indicate SEM. * = p < 0.05.
Figure 3.7 KAR-mediated modulation of IPSCs in CA3 increase the driving force for chloride independent of KCC2.

(A) Group data showing resting membrane potential at t=0 and t=5  
(B) Group data showing driving force for Cl⁻ at t=0 and t=5  
(C) Group data showing Cl⁻ conductance at t=0 and t=5  
(D) Group data showing paired pulse ratio at t=0 and t=5

n=7 control, n=7 KA. Error bars indicate SEM. *** = p < 0.001.
3.3.2 KA application does not modulate IPSCs in GluK2<sup>−/−</sup> CA3 PCs

Next, I aimed to determine whether the hyperpolarization of \( E_{\text{GABA}} \) that was observed upon KA application was due to GluK1/GluK2-containing kainate receptors, I performed recordings on brain slices taken from a GluK1/2<sup>+/−</sup> mouse (Mulle <i>et al.</i>, 2000, Mulle <i>et al.</i>, 1998, Contractor <i>et al.</i>, 2000). The GluK1/2<sup>+/−</sup> mouse line is completely deficient of GluK1 and GluK2 containing kainate receptors, but theoretically could still contain GluK3, GluK4, and GluK5-containing receptors. However, expression of GluK3 is mostly restricted to the dentate gyrus in the hippocampus, showing very little expression in other hippocampal areas (Darstein <i>et al.</i>, 2003). Additionally, GluK4 and GluK5 require the presence of GluK1, GluK2, or GluK3 to form a functional oligomeric receptor, so it is expected that CA3 pyramidal cells in the GluK1/2<sup>+/−</sup> would contain few to no kainate receptors. It is also important to note while these mice still express KCC2, KCC2 surface membrane expression is significantly reduced, resulting in depolarized \( E_{\text{GABA}} \) in GluK1/2<sup>+/−</sup> cultured neurons (Mahadevan <i>et al.</i>, 2014).

When GluK1/2<sup>+/−</sup> brain slices were perfused with 1μM KA, there were no significant changes in \( E_{\text{GABA}} \) (<b>Figure 3.8</b>), RMP, driving force, conductance, or PPR (<b>Figure 3.9</b>) compared to GluK1/2<sup>+/−</sup> neurons which were only perfused with antagonists. Both controls and KA-perfused cells displayed a significant hyperpolarization of \( E_{\text{GABA}} \) at \( t = 15 \) compared within-group to \( t = 0 \), but there was no significant difference between the two groups (<b>Figure 3.8A</b>).
Figure 3.8 KA application does not modulate IPSCs in GluK1/2−/− neurons

(A) Plot of group data showing the effect of 1μM kainic acid application on $E_{\text{GABA}}$ over time in GluK1/2−/− neurons.

(B) Group data showing $E_{\text{GABA}}$ at t=0 and t=5, and t=15.

(C) Plot of voltage-current curve for a cell at t=0 ($E_{\text{GABA}}$=−75.1mV) and perfused with KA at t = 5 ($E_{\text{GABA}}$=−86.2mV). Inset, examples of evoked IPSCs (scale bar: 200 pA, 10 ms)

A,B n=6 control, n=8 KA. Error bars indicate SEM. * = p < 0.05
Figure 3.9 KA application does not alter other membrane properties in GluK1/2-/ neurons.

(A) Group data showing resting membrane potential at t=0 and t=5.
(B) Group data showing driving force for Cl- at t=0 and t=5.
(C) Group data showing Cl- conductance at t=0 and t=5.
(D) Group data showing paired pulse ratio at t=0 and t=5.

A,B,C n=6 control, n=8 KA. D n=6 control, n=6 KA. Error bars indicate SEM. * = p < 0.05, ** = p < 0.01.
3.3.3 KA application modulates IPSCs independent of zinc release

Finally, to determine if synaptically released Zn$^{2+}$ contributes to the effects of KA application on CA3 pyramidal cells, I performed a set of experiments with the addition of the rapid, specific, membrane-impermeable zinc chelator ZX1 (Pan et al., 2011) to the perfusion in addition to all previously used inhibitors. It has been previously shown that at MF-CA3 synapses, extracellular zinc release and subsequent metabotropic zinc receptor (mZnR) activation can enhance KCC2 function (Chorin et al., 2011). Furthermore, kainic acid application has been shown to enhance both KCC2 surface expression and transport activity via mZnR activation in hippocampal neurons in a seizure model (Gilad et al., 2015). Therefore, it was important to determine if zinc release contributed to the observed effects of KA application on $E_{\text{GABA}}$, because all experiments were performed in the CA3 of the hippocampus, where mossy fibre terminals could potentially release zinc onto CA3 pyramidal cells during my experiments. Even though AMPA, NMDA, and mGlu receptors were pharmacologically blocked, it is still possible that KA application and/or the stimulating electrode used to stimulate interneuronal GABA release could have also activated MF terminals, thus causing release of Zn$^{2+}$ and activation of mZnRs.

ZX1 (100μM) was applied in combination with the same antagonist cocktail used previously including: 10μM GYKI52466 to block AMPA, 50μM DL-APV to block NMDA, 3μM CGP55845 3μM to block GABA$_B$ receptors, and 300μM DL-AP3 to block mGluRs (Table 2.1). Cells were washed with ZX1 and inhibitors thorough the experiment, during KA wash-in KA was added as an additional component to the aCSF containing the ZX1 and inhibitors. Control cells had ZX1 and other antagonists perfused throughout the experiment but were not perfused with KA.
Upon 1μM KA application (t=5 min) in the presence of inhibitors and ZX1, $E_{\text{GABA}}$ significantly hyperpolarized, compared to control $E_{\text{GABA}}$ measurements that were made in aCSF containing only inhibitors and ZX1. Upon KA application (t=5), $E_{\text{GABA}}$ hyperpolarized from $-63.3 \pm 7.4$ mV at $t=0$ to $-72.4 \pm 11.9$ mV at $t=5$. This was a statistically significant difference when compared to controls using a two-way repeated measure ANOVA (Figure 3.10A) and when $E_{\text{GABA}}$ at $t=0$ and $t=5$ were compared within the KA group but not the control group using a paired Student’s $t$ test (Figure 3.10B). Following 5 minutes of KA washout (t=10 min), $E_{\text{GABA}}$ depolarized compared to $t=5$, but remained significantly hyperpolarized compared to $t=0$, with a value of $-70.5 \pm 9.8$ mV. This effect persisted after 10 minutes of washout (t=15 min), at which point $E_{\text{GABA}}$ was significantly hyperpolarized compared to $t=0$, with a mean of $-73.3 \pm 9.2$ mV. This difference was significant when compared to controls both with a two way repeated measures ANOVA (Figure 3.10A) and when compared within the KA group but not the control group to $t=0$ with a paired Student’s $t$-test (Figure 3.10D).

The observed change in $E_{\text{GABA}}$ at $t=5$ was accompanied by a significant depolarization of the resting membrane potential and driving force (Figure 3.11A,B). Like earlier experiments with 1μM KA application, a significant decrease in conductance from 7.6 ± 4.9pS to 1.9 ± 1.2pS was observed in KA-treated but not control cells (Figure 3.11C). There was no significant differences in paired pulse ratio (Figure 3.11D).
Figure 3.10 KAR-mediated modulation of IPSCs occurs independent of Zn$^{2+}$ release

(A) Plot of group data showing the effect of 1μM kainic acid application on $E_{\text{GABA}}$ over time in the presence of ZX1.

(B) Group data showing $E_{\text{GABA}}$ at $t=0$ and $t=5$ in the presence of ZX1.

(C) Plot of voltage-current curve for a cell at $t=0$ ($E_{\text{GABA}}=-68.2\text{mV}$) and during KA perfusion $t=5$ ($E_{\text{GABA}}=-72.7\text{mV}$) in the presence of ZX1. Inset, examples of evoked IPSCs.

(D) Group data showing $E_{\text{GABA}}$ at $t=0$ and $t=5$, and $t=15$ in the presence of ZX1.

(E) Plot of voltage-current curve for the same cell at $t=0$ ($E_{\text{GABA}}=-68.2\text{mV}$) and during KA washout at $t=15$ ($E_{\text{GABA}}=-75.1\text{mV}$) in the presence of ZX1. Inset, examples of evoked IPSCs.

Scale bars: 100 pA, 10 ms. A,B n=6 control, n=6 KA. Error bars indicate SEM.

* = $p < 0.05$, *** = $p < 0.001$. 
Figure 3.11 KAR activation increases the driving force for Cl⁻ independent of Zn²⁺ release

(A) Group data showing resting membrane potential at t=0 and t=5.
(B) Group data showing driving force for Cl⁻ at t=0 and t=5.
(C) Group data showing Cl⁻ conductance at t=0 and t=5.
(D) Group data showing paired pulse ratio at t=0 and t=5.

n=6 control, n=6 KA. Error bars indicate SEM. * = p < 0.05, ** = p < 0.01.
3.4 Conclusions

In this study, I have shown that KA application at a concentration sufficient to activate both metabotropic and ionotropic KAR signaling is able to modulate IPSCs in hippocampal CA3 pyramidal cells. KA application produces a significant hyperpolarization of $E_{\text{GABA}}$ and combined with an expected depolarization of the membrane due to ionotropic KAR signaling, this produces a significant increase in the driving force for chloride. KA application also resulted in a decreased Cl$^-$ conductance, likely due to decreased presynaptic GABA release, which is a well-documented effect of KAR activation in the hippocampus (Rodríguez-Moreno et al., 1997, Vignes et al., 1998). This effect is absent in GluK1/2$^{-/-}$ neurons, suggesting that GluK1 and/or GluK2-containing KARs are critical for KAR-mediated regulation of IPSCs. KAR-mediated hyperpolarization of $E_{\text{GABA}}$ also occurred in the presence of specific Zn$^{2+}$ chelator ZX1, suggesting that mZnRs, which have been previously demonstrated to regulate KCC2 transporter function and surface expression (Chorin et al., 2011, Gilad et al., 2015), are not required for this process. Taken together these results suggest that GluK1/2-containing KAR activation can modulate IPSCs and therefore regulate inhibition in the hippocampus.

I originally hypothesized that both ionotropic and metabotropic KAR activation would lead to increased KCC2 function and therefore a hyperpolarized $E_{\text{GABA}}$, through different two different mechanisms that correspond to ionotropic and metabotropic KAR signaling. However, when KARs were activated in the presence of VU0463271, a specific KCC2 antagonist, I observed an unexpected effect. $E_{\text{GABA}}$ hyperpolarized during KA application at $t=5$, but did not transiently return to baseline during KA washout at $t=10$ or depolarize after 10 minutes of KA washout at $t=15$, as observed in previous experiments without the inclusion of VU. This suggests that there may be two mechanisms to KAR-mediated regulation of $E_{\text{GABA}}$, one that occurs
immediately during KA application and is able to modulate $E_{\text{GABA}}$ independently of KCC2 transport (at t=5) and one that occurs after washout that depends on KCC2 transport (at t=15).

KCC2 is the primary extruder of Cl$^-$ in mature neurons (Rivera et al., 1999, Blaesse et al., 2009, Acton et al., 2012), and a hyperpolarization of $E_{\text{GABA}}$ often corresponds to an increase in KCC2 function. This suggests that the hyperpolarization of $E_{\text{GABA}}$ observed during KA washout at t=15, which is absent when KCC2 transport is blocked with VU, is likely due to an increase in KCC2 function. However, the hyperpolarization of $E_{\text{GABA}}$ that is present during KA application at t=5 is still present when KCC2 transport is blocked, suggesting that KARs can somehow regulate [Cl$^-$] by a mechanism that does not involve KCC2 transporter function. The mechanism of action for how KAR signaling could regulate intracellular [Cl$^-$] remains to be determined.

While KCC2 is the primary extruder of Cl$^-$ in mature neurons, NKCC1 also plays a role in Cl$^-$ homeostasis, and while it’s action is critical for depolarizing GABA in early development (Yamada et al., 2004, Sipila et al., 2006), it is also upregulated during development and is present in mature neurons (Clayton et al., 1998, Wang et al., 2002, Balakrishnan et al., 2003, Hyde et al., 2011, Kaila et al., 2014). NKCC1 transports Cl$^-$ in the opposite direction of KCC2, importing Cl$^-$ into the cell under normal resting conditions. Therefore, while a hyperpolarization of $E_{\text{GABA}}$ could possibly indicate an increase in KCC2 transport function, it could also indicate a decrease in NKCC1 transporter function. A decrease in NKCC1 function may account for the KCC2-independent hyperpolarization of $E_{\text{GABA}}$ during KA application. However, it is unlikely that KA application would cause a decrease in NKCC1 function, given that in previous studies, KA application has been associated with increased NKCC1 expression and function in models of epilepsy, which use similar concentrations of kainate to generate epileptiform activity in the hippocampus (Dzhala et al., 2005, Dzhala et al., 2010, Nogueira et al., 2015). Further
experiments involving pharmacological blockade of NKCC1 with bumetanide would be a simple way to explore the possibility of NKCC1’s involvement in KAR-mediated regulation of $[\text{Cl}^-]$.

A second possible explanation for KCC2-independent KAR-mediated regulation of $E_{\text{GABA}}$ could involve osmotic changes brought on by cation influx through the KAR during ionotropic signaling. Activation of ionotropic glutamate receptors, including KARs causes an influx of $\text{Na}^+$ and sometimes $\text{Ca}^+$, and under excitotoxic conditions, this can lead to a passive influx of other ions, including $\text{Cl}^-$, as well as water into the cell (Rungta et al., 2015). It is therefore possible that ionotropic KAR activity may transiently change $E_{\text{GABA}}$, which is mostly dependent on $[\text{Cl}^-]$, due to local changes in intracellular cation concentrations and subsequent water influx. A relative reduction in intracellular $[\text{Cl}^-]$ could occur due to water influx, and a therefore $E_{\text{GABA}}$ would appear to be hyperpolarized based on osmolarity changes (and therefore changes to the electrochemical gradients) during ionotropic KAR activation, a mechanism which would not necessarily require KCC2 transport activity.

It would be interesting to determine if the KCC2-dependent and KCC2-independent mechanisms of KAR-mediated modulation of IPSCs correspond to the two modes of KAR signaling. It is possible that the effects observed immediately upon KA application are due to ionotropic signaling and subsequent osmotic changes in the cell brought on by cation influx, while effects observed after washout may be due to a longer-lasting metabotropic effect in which metabotropic KAR signaling increases KCC2 transporter function. This hypothesis is explored further in Chapter 4 and Chapter 5, in which I attempt to isolate ionotropic and metabotropic KAR signaling, respectively, and observe the effects of independent activation of both of these pathways on IPSCs and $E_{\text{GABA}}$. 
Chapter 4: Ionotropic KAR activation regulates KCC2 function

4 Ionotropic KAR activation regulates KCC2 function

4.1 Statement of Authorship

The results from this chapter have been published in the Journal of Physiology (Garand et al., 2018). All data presented in this thesis were from experiments I performed.

4.2 Introduction, Objectives, and Hypothesis

In Chapter 3, I demonstrated that kainic acid application in the hippocampus modulated IPSCs and produced a hyperpolarization of $EG_{ABA}$ in CA3 pyramidal cells. Hyperpolarization of $EG_{ABA}$ was observed at two different time points in the experiment: during KA application at $t=5$, and 10 minutes after KA washout at $t=15$. The effect seen at $t=5$ was still present when KCC2 ion transport was blocked with VU0463271, suggesting a KCC2-independent mechanism, but the effect seen at $t=15$ was abolished when KCC2 ion transport was blocked with VU0463271, suggesting a KCC2-dependent mechanism. I hypothesized that the hyperpolarization of $EG_{ABA}$ observed at the two different time points was due to two different mechanisms that corresponded to the two independent modes of KARs signaling; ionotropic and metabotropic.

Specifically, I hypothesized that the effect observed during KA application ($t=5$) was due to ionotropic KAR signaling. The effect observed during washout ($t=15$) was more likely to be due to metabotropic KAR signaling for several reasons. Firstly, hyperpolarization of $EG_{ABA}$ at $t=15$ was demonstrated to depend on KCC2, and metabotropic KAR signaling is known to activate PKC (Melyan et al., 2002, Melyan et al., 2004), which is known to be able to
phosphorylate KCC2 at serine 940 and increase KCC2 transport function (Lee et al., 2007). Additionally, PKC phosphorylation of KCC2 at Ser940 both increases KCC2 transport function and increases KCC2 expression in the membrane by decreasing the rate of internalization, a process which can occur on a timescale of minutes. Because of the immediacy of the effects observed at t=5, an effect which did not require KCC2 transport, an ionotropic mechanism would be more likely. Activation of ionotropic glutamate receptors, including KARs, results in an immediate cation influx, which could account for the rapid hyperpolarization of $E_{GABA}$ at t=5. Therefore, my **objective** was to determine if selective activation of ionotropic KAR signaling at MF-CA3 synapses could modulate IPSCs and hyperpolarize $E_{GABA}$ independent of metabotropic KAR signaling.

In order to selectively activate ionotropic KAR signaling without also activating metabotropic KAR signaling, I used a pharmacological approach to prevent G-protein activation by KARs. There are several different G-protein antagonists that are commonly used to study metabotropic KAR function in the literature. One commonly used G-protein antagonist is N-ethylmaleimide (NEM), which has been demonstrated to inhibit KAR-mediated metabotropic signaling and prevent KAR-mediated regulation of $I_{AHP}$ (Frerking, M. et al., 2001, Melyan et al., 2002). NEM is a membrane-permeable sulfhydryl alkylating agent that interferes with G-protein function by alkylating cysteine residues in the G-protein that are critical for coupling to receptors in the membrane (Jakobs et al., 1982, Winslow et al., 1987, Shapiro et al., 1994). However, while NEM is effective at preventing G-protein signaling, it also has several other effects in neurons, including the ability to increase KCC2 transport by increasing phosphorylation of Ser940 (Payne, 1997, Conway et al., 2017). For this reason, I performed two separate sets of experiments, one with NEM and one with another pharmacological agent, GDP-β-S, to isolate the ionotropic signaling pathway and mitigate any potential concerns regarding known effects of
NEM on KCC2. GDP-β-S has a different mechanism of action than NEM for preventing G-protein signaling. GDP-β-S is a GDP analogue that interferes with G-protein binding. Similar to NEM, GDP-β-S has been found to inhibit KARs ability to reduce the amplitude of the I_{SAHP} (Ruiz et al., 2005, Segerstråle et al., 2010).

As previously described, all experiments were performed on CA3 pyramidal cells in the hippocampus. To determine the effects of ionotropic KAR activation on KCC2 function, I bath applied the KAR agonist kainic acid (KA, 1μM) for 5 minutes while recording from CA3 pyramidal cells in whole-cell mode. In all experiments, KA was bath perfused in conjunction with a cocktail of antagonists to prevent the activation of other ionotropic glutamate receptors (10μM GYKI52466 to block AMPA, 50μM DL-APV to block NMDA), GABA_B receptors (CGP55845 3μM), and mGluRs (300μM DL-AP3; see Table 2.1). G-protein signaling antagonists NEM (50μM) was bath applied in conjunction with these inhibitors where described. GDP-B-S (300μM) was added to the internal solution in the patch pipette where described.

4.3 Results

4.3.1 Ionotropic KAR signaling modulates IPSCs in CA3 pyramidal cells.

To determine the effects of ionotropic KAR activation on E_{GABA}, I bath applied the KAR agonist kainic acid (KA, 1μM) for 5 minutes while recording from CA3 pyramidal cells in whole-cell mode. KA was bath perfused in conjunction with a cocktail of antagonists to prevent the activation of other ionotropic glutamate receptors (10μM GYKI52466 to block AMPA, 50μM DL-APV to block NMDA), GABA_B receptors (CGP55845 3μM), and mGluRs (300μM DL-AP3; see Table 2.1). All antagonists were bath applied throughout the experiment, from the time the cell was patched until the end of the experiment. In addition to these antagonists, the G-
protein antagonist NEM (50μM) was bath applied to the slices throughout the entire experiment to isolate ionotropic KAR signaling from G-protein mediated metabotropic KAR signaling.

As described previously E\text{GABA} was recorded every 5 minutes by stimulating the \textit{stratum radiatum/lucidum} to cause GABA release from interneurons onto the recorded cell. All measurements taken at t = 0 are values obtained immediately prior to bath application of KA, 5 minutes after whole cell configuration was achieved. After 5 minutes of KA application (t=0 to t=5), KA was washed out using aCSF containing only the previously mentioned antagonists and NEM for 10 minutes (t=5 to t=15). Control cells were perfused with the same antagonist cocktail as experimental cells (including NEM) for the course of the experiment but did not have KA applied.

KA application in the presence of NEM produced a significant hyperpolarization of E\text{GABA} (t=5 min), compared to control E\text{GABA} measurements that were made in aCSF containing only inhibitors (\textbf{Figure 4.1}). Upon KA application (t=5), E\text{GABA} hyperpolarized from $-60.5 \pm 6.1$ mV at t=0 to $-70.5 \pm 3.6$ mV at t=5. This was a statistically significant difference when compared to controls using a two-way repeated measure ANOVA (\textbf{Figure 4.1A}) and when E\text{GABA} at t=0 and t=5 were compared within the KA group using a paired Student’s t test (\textbf{Figure 4.1B}). E\text{GABA} of control cells did not significantly change over the course of the experiment.

During KA washout, E\text{GABA} depolarized and was not significantly different from control cells at t=10 or at t=15 when compared to controls using a two-way repeated measures ANOVA (\textbf{Figure 4.1A}) or when t=15 was compared to t=0 within the KA group using a paired Student’s t-test (\textbf{Figure 4.1D}).

During KA application (t=5), the hyperpolarization of E\text{GABA} was accompanied by a significant depolarization of the membrane potential (\textbf{Figure 4.2A}), which together resulted in a
significant increase in the driving force for Cl⁻ (Figure 4.2B). This was not accompanied by any statistically significant changes in conductance or paired pulse ratio (Figure 4.2C,D).

After 10 minutes of KA washout (t=15), there was no significant differences within either the control or KA groups in resting membrane potential, driving force, or paired pulse ratios compared to baseline values (t=0; Figure 4.3A,B,D). However, at t=15 there was a significant decrease in conductance from 5.1 ± 2.8pS to 2.5 ± 1.3pS in KA-treated cells, but not in controls (Figure 4.3C).
Figure 4.1 Ionotropic KAR signaling modulates IPSCs in CA3 pyramidal cells

(A) Plot of group data showing the effect of 1μM kainic acid application in the presence of G-protein antagonist NEM on \( E_{\text{GABA}} \) over time.

(B) Group data comparing \( E_{\text{GABA}} \) at t=0 and t=5.

(C) Plot of voltage-current curve for a cell at t=0 (\( E_{\text{GABA}} = -59.2 \text{mV} \)) and during KA perfusion t = 5 (\( E_{\text{GABA}} = -73.5 \text{mV} \)). Inset, examples of evoked IPSCs.

(D) Group data for the same cells as (B) comparing \( E_{\text{GABA}} \) at t=0 and t=15.

(E) Plot of voltage-current curve for the same cells as (C) at t=0 (\( E_{\text{GABA}} = -59.2 \text{mV} \)) and during KA washout at t = 15 (\( E_{\text{GABA}} = -56.6 \text{mV} \)). Inset, examples of evoked IPSCs.

Scale bars: 60 pA, 10 ms. A,B,D n=13 control, n=6 KA. Error bars indicate SEM.
* = p < 0.05
Figure 4.2 Ionotropic KAR signaling increases the driving force for Cl\textsuperscript{-} in CA3 pyramidal cells.

(A) Group data showing resting membrane potential at t=0 and t=5.
(B) Group data showing driving force for Cl\textsuperscript{-} at t=0 and t=5.
(C) Group data showing Cl\textsuperscript{-} conductance at t=0 and t=5.
(D) Group data showing paired pulse ratio at t=0 and t=5.

A-C, n=13 control, n=6 KA. D, n=7 control, n=6 KA. Error bars indicate SEM. * = p < 0.05.
Figure 4.3 Ionotropic KAR signaling does not increase the driving force for Cl⁻ in CA3 pyramidal cells after KA washout.

(A) Group data showing resting membrane potential at t=0 and t=5.
(B) Group data showing driving force for Cl⁻ at t=0 and t=5.
(C) Group data showing Cl⁻ conductance at t=0 and t=5.
(D) Group data showing paired pulse ratio at t=0 and t=5.

A-C, n=13 control, n=6 KA. D, n=7 control, n=6 KA. Error bars indicate SEM. * = p < 0.05.
4.3.2 Ionotropic KAR signaling isolated with GDP-β-S modulates IPSCs

While NEM is a commonly used antagonist for metabotropic KAR signaling, it has also long been known to enhance KCC2 function (Payne, 1997, Conway et al., 2017) and increase KCC2 surface expression through an interaction with a WNK kinase that alters KCC2 phosphorylation (Conway et al., 2017). Although the control cells in our NEM experiments did not show any changes in $E_{\text{GABA}}$ in response to bath application of NEM in the absence of KA over the course of the experiments, we repeated the above experiments using an alternate G-protein blocker to mitigate potential concerns with NEM confounding results by regulating KCC2. For these experiments, we choose to use GDP-β-S to isolate the ionotropic signaling pathway of KARs. GDP-β-S is a GDP analogue that interferes with G-protein binding, and has been found to inhibit the KAR’s ability to reduce the amplitude of the $I_{\text{SAHP}}$, which is recognized as a downstream effect of metabotropic KAR signaling (Ruiz et al., 2005, Segerstråle et al., 2010).

To confirm that the effects of ionotropic KAR activation on $E_{\text{GABA}}$, I again bath applied the KAR agonist kainic acid (KA, 1μM) for 5 minutes while recording from CA3 pyramidal cells in whole-cell mode. KA was bath perfused in conjunction with a cocktail of antagonists to prevent the activation of other ionotropic glutamate receptors (10μM GYKI52466 to block AMPA, 50μM DL-APV to block NMDA), GABA$_B$ receptors (CGP55845 3μM), and mGluRs (300μM DL-AP3; see Table 2.1). For these experiments, the G-protein antagonist GDP-β-S (300μM) was also added to the pipette internal solution to isolate ionotropic KAR signaling from metabotropic KAR signaling, exposing cells to GDP-β-S once whole-cell mode was achieved and throughout the rest of the experiment. All other antagonists were bath applied throughout the experiment, from the time the cell was patched until the end of the experiment.
As described previously $E_{\text{GABA}}$ was recorded every 5 minutes by stimulating the *stratum radiatum/lucidum* to cause GABA release from interneurons onto the recorded cell. All measurements taken at $t = 0$ are values obtained immediately prior to bath application of KA, 5 minutes after whole cell configuration was achieved. After 5 minutes of KA application ($t=0$ to $t=5$), KA was washed out using aCSF containing only the previously mentioned antagonists and NEM for 10 minutes ($t=5$ to $t=15$). Control cells were also patched with pipettes filled with internal solution containing GDP-$\beta$-S (300$\mu$M) and perfused with the same antagonist cocktail as experimental cells for the course of the experiment but did not have KA applied.

Similar to results obtained with NEM, KA application in the presence of GDP-$\beta$-S produced a significant hyperpolarization of $E_{\text{GABA}}$ ($t=5$ min), compared to control $E_{\text{GABA}}$ measurements that were made in aCSF containing only inhibitors and GDP-$\beta$-S (Figure 4.4). Upon KA application ($t=5$), $E_{\text{GABA}}$ hyperpolarized from $-61.8 \pm 3.7$ mV at $t=0$ to $-70.1 \pm 4.7$ mV at $t=5$. This was a statistically significant difference when compared to controls using a two-way repeated measure ANOVA (Figure 4.4A) and when $E_{\text{GABA}}$ at $t=0$ and $t=5$ were compared within the KA group using a paired Student’s t test (Figure 4.4B). $E_{\text{GABA}}$ of control cells did not significantly change over the course of the experiment. During KA washout, $E_{\text{GABA}}$ depolarized back towards baseline values, but remained significantly different from control cells at $t=10$ and at $t=15$ when compared to controls using a two-way repeated measures ANOVA (Figure 4.4A) and when $t=15$ was compared to $t=0$ within the KA group using a paired Student’s t-test (Figure 4.4D).

During KA application ($t=5$), the hyperpolarization of $E_{\text{GABA}}$ was accompanied by a significant depolarization of the membrane potential (Figure 4.5A), which together resulted in a significant increase in the driving force for Cl$^-$ (Figure 4.5B). This was not accompanied by any statistically significant changes in conductance or paired pulse ratio (Figure 4.5C,D).
After 10 minutes of KA washout (t=15), there was no significant differences within either the control or KA groups conductance or paired pulse ratios compared to baseline values (t=0; Figure 4.6C,D). However, at t=15 there was a significant depolarization of the resting membrane potential and an increase in Cl⁻ driving force, both in KA-treated cells as well as in controls (Figure 4.6A,B).
Figure 4.4 Ionotropic KAR signaling modulates IPSCs in the presence of GDP-β-S.

(A) Plot of group data showing the effect of 1μM kainic acid application on $E_{\text{GABA}}$ over time in the presence of G-protein antagonist GDP-β-S.

(B) Group data comparing $E_{\text{GABA}}$ at t=0 and t=5.

(C) Plot of voltage-current curve for a cell at t=0 ($E_{\text{GABA}}=-68.6\text{mV}$) and during KA perfusion t = 5 ($E_{\text{GABA}}=-74.2\text{mV}$). Inset, examples of evoked IPSCs.

(D) Group data for the same cells as (B) comparing $E_{\text{GABA}}$ at t=0 and t=15.

(E) Plot of voltage-current curve for the same cells as (C) at t=0 ($E_{\text{GABA}}=-68.6\text{mV}$) and during KA washout at t = 15 ($E_{\text{GABA}}=-68.0\text{mV}$). Inset, examples of evoked IPSCs.

Scale bars: 60 pA, 10 ms. A,B,D n=7 control, n=6 KA. Error bars indicate SEM.

* = p < 0.05, ** = p < 0.01, *** = p < 0.001.
Figure 4.5 Ionotropic KAR signaling increases driving force for Cl− in the presence of GDP-β-S.

(A) Group data showing resting membrane potential at t=0 and t=5.
(B) Group data showing driving force for Cl− at t=0 and t=5.
(C) Group data showing Cl− conductance at t=0 and t=5.
(D) Group data showing paired pulse ratio at t=0 and t=5.

n=7 control, n=8 KA.
Error bars indicate SEM. * = p < 0.05, ** = p < 0.01.
Figure 4.6 Ionotropic KAR signaling increases the driving force for Cl⁻ in the presence of GDP-β-S throughout washout.

(A) Group data showing resting membrane potential at t=0 and t=15.
(B) Group data showing driving force for Cl⁻ at t=0 and t=15.
(C) Group data showing Cl⁻ conductance at t=0 and t=15.
(D) Group data showing paired pulse ratio at t=0 and t=15.

n=7 control, n=8 KA.
Error bars indicate SEM. * = p < 0.05, ** = p < 0.01.
4.4 Conclusions

In this chapter, I have shown that activating ionotropic KAR signaling while blocking metabotropic KAR signaling with G-protein antagonists is able to modulate IPSCs, hyperpolarize $E_{\text{GABA}}$, and due to a combined resting membrane potential depolarization and a hyperpolarization of $E_{\text{GABA}}$, increase the driving force for $\text{Cl}^-$.

This effect was observed in two separate sets of experiments using two different G-protein antagonists with different mechanisms of action, NEM and GDP-β-S. These results suggest that ionotropic KAR signaling alone can regulate chloride homeostasis.

Blockade of G-protein mediated KAR signaling with NEM produced a significant hyperpolarization of $E_{\text{GABA}}$ and combined with an expected depolarization of the membrane due to ionotrophic KAR signaling. This produced a significant increase in the driving force for chloride. KA application also resulted in a decreased $\text{Cl}^-$ conductance, which is expected to occur because KAR activation is known to decrease presynaptic GABA release (Rodríguez-Moreno et al., 1997, Vignes et al., 1998). In contrast to previous experiments activating both ionotropic and metabotropic KAR signaling, hyperpolarization of $E_{\text{GABA}}$ was observed during KA application ($t=5$) in conjunction with NEM but was no longer significantly different from controls after 10 minutes of KA washout ($t=15$). These results suggest that ionotropic KAR activation alone regulates IPSCs and $E_{\text{GABA}}$ in the CA3 pyramidal cells, and that the effect is temporary, returning to baseline values after KA washout.

Because NEM is known to be able to directly regulate KCC2 function (Payne, 1997, Conway et al., 2017), I performed a second set of experiments with a second G-protein antagonist, GDP-β-S. Similar to what I observed when I applied NEM, the addition of GDP-β-S to the pipette solution in combination with 1μM KA application produced a significant
hyperpolarization of $E_{GABA}$ during KA application ($t=5$), and combined with an expected depolarization of the membrane due to ionotropic KAR signaling, this produced a significant increase in the driving force for chloride. However, in contrast to the NEM experiments, application GDP-β-S along with KA also produced a significant hyperpolarization of $E_{GABA}$ after 10 minutes of KA washout ($t=15$). This was accompanied by a significant depolarization of the membrane and an increase in Cl- conductance. Notably, at $t=15$, controls also displayed a significant depolarization of the RMP and an increase in Cl- driving force, suggesting that GDP-β-S alone can depolarize the RMP and therefore increase Cl- driving force independent of any changes in $E_{GABA}$.

What might account for the observation that $E_{GABA}$ at $t=15$ was not significantly different from controls in NEM experiments, but was significantly different from controls in GDP-β-S experiments? There are several possible explanations. Firstly, because of the different mechanism of action and different delivery methods for the two antagonists, it is possible that there may be differences in how effective the G-protein blockade was in my experiments. While NEM is membrane-permeable, it was delivered by bath application to all cells in the slice, whereas GDP-β-S was directly delivered to only the cell I recorded from in the pipette internal solution, which may have resulted in different levels of G-protein blockade between the two sets experiments. The differences between the two antagonists would be difficult to quantify, but this is an important consideration not only in reference to the ability of these compounds to block metabotropic KAR signaling, but is also critical because both compounds nonspecifically inhibit all G-proteins in the cell and not just those involved in KAR signaling. This could confound results because there may be complex, secondary effects caused by blocking other G-proteins, which in combination with KA application could produce differences between the NEM and GDP-β-S groups. The difference in $E_{GABA}$ at $t=15$ between the two experiments may also be
partially accounted for by a difference in time course of the effect. While $E_{\text{GABA}}$ remained significantly hyperpolarized in GDP-β-S experiments throughout washout, it showed the same trend as $E_{\text{GABA}}$ in the NEM experiments, becoming less hyperpolarized at washout ($t=15$) compared to during KA application ($t=5$). If a longer washout period was employed, it is possible that $E_{\text{GABA}}$ would eventually return to values that were not significantly different from controls, suggesting that G-protein blockade with GDP-β-S produces a stronger and/or longer lasting ionotropic KAR-mediated regulation of $E_{\text{GABA}}$ than when G-proteins are blocked with NEM.

Finally, the differences in $E_{\text{GABA}}$ at $t=15$ between the NEM and GDP-β-S groups may be further complicated by the observation that at $t=15$, NEM control cells with no KA applied did not show any changes in RMP, but GDP-β-S controls and KA-treated cells showed a significantly depolarized membrane potential and increased Cl- driving force at $t=15$. A depolarization of the membrane potential due to GDP-β-S application, to my knowledge, has not been reported in previous literature using GDP-β-S to study metabotropic KAR signaling. However, it is not completely unexpected that a broad inhibition of all G-protein signaling in the cell could affect resting membrane potential, because G-protein coupled receptors are known to regulate many ion channels in the membrane, including several types of calcium and potassium channels (Inanobe and Kurachi, 2014). For example, one subtype of potassium leak channel that contributes to maintenance of the resting membrane potential in neurons, TREK-1, a member of the two-pore domain potassium (K2P) channel family, can be regulated by GPCRs, and the association of TREK-1 with Gβγ activates TREK-1 (Woo et al., 2012). By blocking G-protein activity in general, it is possible that GDP-β-S depolarized the membrane by preventing normal functioning of potassium leak channels.
I originally hypothesized that ionotropic KAR signaling would account for the KCC2-independent regulation of $E_{\text{GABA}}$ that I observed in Chapter 3 during KA application at $t=5$. In both the NEM and GDP-β-S experiments, the largest hyperpolarization of $E_{\text{GABA}}$ temporally corresponded with the KCC2-independent hyperpolarization of $E_{\text{GABA}}$ observed in Chapter 3. In the NEM experiments, this effect was no longer present after 10 minutes of washout ($t=15$), but in the GDP-β-S experiments $E_{\text{GABA}}$ was still significantly hyperpolarized compared to controls at this time point. The potential explanations for the discrepancy between the two G-protein antagonists is discussed in detail above, but it should also be noted that the presence of a significant hyperpolarization of $E_{\text{GABA}}$ at $t=15$ in the GDP-β-S experiments temporally corresponds to the KCC2-dependent hyperpolarization of $E_{\text{GABA}}$ observed in Chapter 3. Taken together, these results suggest that ionotopic KAR signaling can regulate IPSCs and $E_{\text{GABA}}$ independently from metabotropic KAR signaling, and that ionotropic KAR signaling may contribute in whole or part to both KCC2-independent and KCC2-dependant KAR-mediated regulation of $E_{\text{GABA}}$. 
Chapter 5 : Metabotropic KAR activation regulates KCC2 function

5 Metabotropic KAR activation regulates KCC2 function

5.1 Statement of Authorship

The results from this chapter have been published in the Journal of Physiology (Garand et al., 2018). All data presented in this thesis were from experiments I performed.

5.2 Introduction, Objectives, and Hypothesis

I previously demonstrated in Chapter 3 that KAR activation could modulate IPSCs, hyperpolarize \( E_{\text{GABA}} \), and increase the driving force for Cl\(^-\) in CA3 pyramidal cells. When I activated both ionotropic and metabotropic KAR signaling using 1μM KA, I observed a hyperpolarization of \( E_{\text{GABA}} \) 10 minutes after KA washout (t=15), which was abolished when KCC2 ion transport was blocked with VU0463271, suggesting a KCC2-dependent mechanism. I also observed a hyperpolarization of \( E_{\text{GABA}} \) upon KA application at t=5, but this effect was preserved when KCC2 transport was blocked, suggesting a second, KCC2-independent mechanism for KAR-mediated modulation of IPSCs. In Chapter 4, using 2 different methods to block G-protein signaling, I demonstrated that ionotropic KAR signaling alone could modulate IPSCs, hyperpolarized \( E_{\text{GABA}} \), and increase the driving force for Cl\(^-\). Both G-protein antagonists, NEM and GDP-\( \beta \)-S, resulted in a significant hyperpolarization of \( E_{\text{GABA}} \) immediately upon KA application at t=5. This effect was no longer significant after 10 minutes of KA washout at t=15 when NEM was used to isolate ionotrophic signaling. When GDP-\( \beta \)-S was used to isolate ionotropic signaling, \( E_{\text{GABA}} \) depolarized back towards baseline (t=0) values but remained
significantly different from controls even at t=15. Taken together these results suggest that while
ionotropic signaling alone can modulate IPSCs, ionotropic signaling may not account for the
KCC2-dependent hyperpolarization that I observed after KA washout when I activated both
ionotropic and metabotropic KAR signaling in Chapter 3.

Therefore my final objective was to determine if selective activation of metabotropic
KAR signaling at MF-CA3 synapses could modulate IPSCs and hyperpolarize E_{GABA}
independent of ionotropic KAR signaling. I hypothesized that metabotropic KAR signaling
would hyperpolarize E_{GABA} and increase the driving force for Cl\(^-\) (Figure 5.1). Furthermore, I
hypothesized that metabotropic KAR-mediated regulation of E_{GABA} would account for the
KCC2 transport-dependent hyperpolarization of E_{GABA} after KA washout that I observed in
Chapter 3.

I hypothesized that the hyperpolarization of E_{GABA} at t=15 was due to metabotropic KAR
signaling for several reasons. Firstly, this hyperpolarization of E_{GABA} after KA washout was
absent when I selectable activated ionotropic KAR signaling using NEM to prevent G-protein
signaling, so ionotropic KAR signaling is unlikely to fully account for this effect. Secondly,
metabotropic KAR signaling is known to activate PKC (Melyan et al., 2002, Melyan et al.,
2004), which is known to be able to phosphorylate KCC2 (Lee et al., 2007). KAR activation of
PKC could serve as a potential mechanism for KAR-mediated regulation of KCC2 function.
Additionally, PKC phosphorylation of KCC2 at Ser940 both increase transport function and
prevents membrane internalization, which are longer-lasting processes which can persist over a
timescale of minutes (Lee et al., 2007) which may account for the effect occurring 10 minutes
after beginning KA washout. Finally, several other metabotropic receptors have also been
demonstrated to increase KCC2 function, including mGluRs (Banke and Gegelashvili, 2008) and
mZnRs, (Chorin et al., 2011, Gilad et al., 2015) and it has been suggested that GPCR signaling
in general may serve as a conserved mechanism for regulating chloride homeostasis (Mahadevan and Woodin, 2016). As one form of metabotropic KAR signaling is known to involve G-proteins, KCC2-dependent KAR-mediated regulation of $E_{\text{GABA}}$ may work to regulate chloride homeostasis through a similar mechanism as other GPCRs.

The ionotropic response of KARs to KA application is both dose and subunit composition dependent, with lower doses of kainate producing smaller currents. In the hippocampus, the half-maximal effective concentration ($EC_{50}$) for kainate is about 22μM (Lerma et al., 1993, Wilding and Huettner, 1997, Lerma et al., 2001), and in general KARs in CA3 have larger ionotropic responses to similar concentrations of KA than KARs in CA1 (Castillo et al., 1997). Nanomolar concentrations of KA are sufficient to activate metabotropic signaling (Fernandes et al., 2009) while producing much smaller or no kainate receptor currents compared to higher concentrations of KA (Castillo et al., 1997). In order to activate metabotropic signaling while avoiding ionotropic activation, I used a lower concentration of KA (0.1μM) to study the effect of metabotropic signaling on Cl⁻ homeostasis.

As previously described, all experiments were performed on CA3 pyramidal cells in the hippocampus. To determine the effects of metabotropic KAR activation on KCC2 function, I bath applied the KAR agonist kainic acid (KA, 0.1μM) for 5 minutes while recording from CA3 pyramidal cells in whole-cell mode. In all experiments, KA was bath perfused in conjunction with a cocktail of antagonists to prevent the activation of other ionotropic glutamate receptors (10μM GYKI52466, 50μM DL-APV), GABA B receptors (CGP55845), and mGluRs (300μM DL-AP3; see Table 2.1). In a subset of experiments, ZX1 (100μM) or VU0463271 (1μM) were added to the antagonist cocktail to chelate Zn²⁺ and block KCC2 transport function, respectively. As a control, G-protein signaling antagonist NEM (50μM) was bath applied in conjunction with
these inhibitors where described and alternate G-protein signaling antagonist GDP-B-S (300μM) was added to the internal solution in the patch pipette where described.
Figure 5.1 Schematic of hypothesis for Chapter 5

Schematic diagram showing how KAR activation could potentially regulate KCC2 function. Metabotropic activation leads to the activation of G-proteins and PKC, which could then phosphorylate KCC2 and S940 and increase ion transport, lowering intracellular [Cl⁻].
5.3 Results

5.3.1 Metabotropic KAR signaling modulates IPSCs in CA3 pyramidal cells

To determine the effects of KAR activation on KCC2 function, I bath applied the KAR agonist kainic acid (KA, 0.1μM) for 5 minutes while recording from CA3 pyramidal cells in whole-cell mode in conjunction with the antagonist cocktail described above. As described previously, $E_{\text{GABA}}$ was taken as a measure of KCC2 function and was recorded every 5 minutes by stimulating the *stratum radiatum/lucidum* to cause GABA release from interneurons onto the recorded cell. All measurements taken at $t = 0$ are values obtained immediately prior to bath application of KA, 5 minutes after whole cell configuration was achieved. After 5 minutes of KA application ($t=0$ to $t=5$), KA was washed out using aCSF containing only the previously mentioned antagonists ($t=5$ to $t=15$). Control cells were perfused with the same antagonist cocktail as experimental cells for the course of the experiment but did not have KA applied.

In contrast to previous experiments using a higher concentration of KA (1μM), 0.1μM KA application did not produce a significant hyperpolarization of $E_{\text{GABA}}$ at $t=5$ min compared to control $E_{\text{GABA}}$ measurements that were made in aCSF containing only inhibitors (*Figure 5.2*). However, a significant depolarization of the membrane was observed at $t=5$ (*Figure 5.3A*) which resulted in a significant change in driving force for Cl- (*Figure 5.3B*), suggesting that there was still some ionotropic KAR activation at this concentration of KA.

After 10 minutes of KA washout ($t=15$ min), $E_{\text{GABA}}$ became significantly hyperpolarized from $-61.3 \pm 8.7$ mV to $-66.8 \pm 6.0$ mV. This was a statistically significant difference when compared to controls using a two-way repeated measure ANOVA (*Figure 5.2A*) and when
E_{GABA} at t=0 and t=15 were compared within the KA group using a paired Student’s t-test (Figure 5.2D).

The observed change in E_{GABA} at t=15 was not accompanied by a significant change in resting membrane potential (Figure 5.4A), however, there was a significant change in the driving force for Cl\(^-\) from −11.5 ± 9.0mV to −3.5 ± 7.5mV (Figure 5.4B). There were no significant differences in either conductance or paired pulse ratio for either group (Figure 5.4C,D).
Figure 5.2 Metabotropic KAR signaling modulates IPSCs in CA3 pyramidal cells

(A) Plot of group data showing the effect of 0.1μM kainic acid application on $E_{\text{GABA}}$ over time. 
(B) Group data comparing $E_{\text{GABA}}$ at t=0 and t=5. 
(C) Plot of voltage-current curve for a cell at t=0 ($E_{\text{GABA}}=-65.6\text{mV}$) and during KA perfusion at t=5 ($E_{\text{GABA}}=-70.2\text{mV}$). Inset, examples of evoked IPSCs. 
(D) Group data for the same cells as (B) comparing $E_{\text{GABA}}$ at t=0 and t=15. 
(E) Plot of voltage-current curve for the same cells as (C) at t=0 ($E_{\text{GABA}}=-65.6\text{mV}$) and during KA washout at t=15 ($E_{\text{GABA}}=-74.6\text{mV}$). Inset, examples of evoked IPSCs.

Scale bars: 60 pA, 10 ms. n=11 control, n=10 KA. Error bars indicate SEM. * = p < 0.05.
Figure 5.3 0.1μM KA application depolarizes the membrane and increases the driving force for Cl⁻ in CA3 pyramidal cells

(A) Group data showing resting membrane potential at t=0 and t=15.
(B) Group data showing driving force for Cl⁻ at t=0 and t=15.
(C) Group data showing Cl⁻ conductance at t=0 and t=15.
(D) Group data showing paired pulse ratio at t=0 and t=15.

n=11 control, n=10 KA. Error bars indicate SEM. *** = p < 0.001.
Figure 5.4 Metabotropic KAR signaling increases the driving force for Cl$^-$ in CA3 pyramidal cells

(A) Group data showing resting membrane potential at t=0 and t=15.
(B) Group data showing driving force for Cl$^-$ at t=0 and t=15.
(C) Group data showing Cl$^-$ conductance at t=0 and t=15.
(D) Group data showing paired pulse ratio at t=0 and t=15.

A-C, n=11 control, n=10 KA. D, n=9 control, n=10 KA.
Error bars indicate SEM. * = p < 0.05.
5.3.2 Metabotropic KAR-mediated modulation of IPSCs is dependent on Gluk1/2-containing KARs and KCC2 transport

Next, I aimed to determine whether the hyperpolarization of $E_{\text{GABA}}$ that was observed after washout of 0.1μM KA was due to Gluk1/GluK2-containing kainate receptors. I again performed recordings on brain slices taken from GluK1/2−/− mice (Mulle et al., 2000, Mulle et al., 1998, Contractor et al., 2000) as previously described. I applied 0.1μM KA for 5 minutes while recording from CA3 pyramidal cells in whole-cell mode in conjunction with the antagonist cocktail described above. Again, $E_{\text{GABA}}$ was taken as a measure of KCC2 function and was recorded every 5 minutes by stimulating the stratum radiatum/lucidum to cause GABA release from interneurons onto the recorded cell. All measurements taken at $t = 0$ are values obtained immediately prior to bath application of KA, 5 minutes after whole cell configuration was achieved. After 5 minutes of KA application ($t=0$ to $t=5$), KA was washed out using aCSF containing only the previously mentioned antagonists ($t=5$ to $t=15$).

When GluK1/2−/− brain slices were perfused with 0.1μM KA, there were no significant changes in $E_{\text{GABA}}$ compared to controls, or compared within-group to baseline values at during KA application at $t=5$ (Figure 5.5). After 10 minutes of KA washout, both controls and KA-perfused cells displayed a significant hyperpolarization of $E_{\text{GABA}}$ at $t = 15$ compared within-group to $t = 0$, but there was no significant difference between the two groups (Figure 3.8A). At $t=15$, KA-perfused cells had no changes in RMP, driving force, conductance, or PPR (Figure 5.6), however, GluK1/2−/− neurons which were only perfused with antagonists displayed a significant change in driving force, a result of the significant change in $E_{\text{GABA}}$ at $t=15$ since there were no significant changes in RMP (Figure 5.6B). It should be noted that KA-perfused cells showed a similar trend towards and increased Cl- driving force, but it was not statistically significant (Figure 5.6B).
Figure 5.5 0.1μM KA application does not modulate IPSCs in GluK1/2−/− cells.

(A) Plot of group data showing the effect of 0.1μM kainic acid application on $E_{GABA}$ over time in GluK1/2−/− cells.

(B) Group data comparing $E_{GABA}$ at t=0 and t=5.

(C) Plot of voltage-current curve for a cell at t=0 ($E_{GABA} = -80.7\, mV$) and during KA perfusion ($E_{GABA} = -83.1\, mV$). Inset, examples of evoked IPSCs.

(D) Group data for the same cells as (B) comparing $E_{GABA}$ at t=0 and t=15.

(E) Plot of voltage-current curve for the same cells as (C) at t=0 ($E_{GABA} = -80.7\, mV$) and during KA washout at t = 5 ($E_{GABA} = -82.3\, mV$). Inset, examples of evoked IPSCs.

Scale bars: 60 pA, 10 ms. A,B,D n=6 control, n=5 KA. Error bars indicate SEM. * = p < 0.05
Figure 5.6 0.1μM KA application does not change the driving force for Cl⁻ in GluK1/2⁻/⁻ cells.

(A) Group data showing resting membrane potential at t=0 and t=15.
(B) Group data showing driving force for Cl⁻ at t=0 and t=15.
(C) Group data showing Cl⁻ conductance at t=0 and t=15.
(D) Group data showing paired pulse ratio at t=0 and t=15.

A-C, n=6 control, n=5 KA. Error bars indicate SEM. ** = p < 0.01.
Next, I aimed to determine whether metabotropic KAR-mediated regulation of $E_{\text{GABA}}$ was due to a change in KCC2 transporter function, which could account for the KCC2-dependent change in $E_{\text{GABA}}$ that I observed in Chapter 3. To determine if KCC2 is required for the hyperpolarization of $E_{\text{GABA}}$ observed in previous experiments, I repeated the previous experiments in wild type slices with the addition of the specific KCC2 blocker, VU0463271 (VU). VU (1μM) was applied in combination with the same antagonist cocktail used previously including: 10μM GYKI52466 to block AMPA, 50μM DL-APV to block NMDA, 3μM CGP55845 3μM to block GABA$\beta$ receptors, and 300μM DL-AP3 to block mGluRs (Table 2.1). Cells were washed with VU and inhibitors thorough the experiment, during KA wash-in KA was added as an additional component to the aCSF containing the VU and inhibitors. Control cells had VU and other antagonists perfused throughout the experiment, but were not perfused with KA.

With the addition of VU, 0.1μM KA application did not produce any significant changes in $E_{\text{GABA}}$ over the time course of the experiment (Figure 5.7). Similarly, at t=15, there were no changes in RMP, driving force for Cl$, or PPR (Figure 5.8A,D,D). KA-perfused cells displayed a statistically significant decrease in conductance compared to baseline values from 7.3 ± 2.5pS to 4.3 ± 1.7pS (Figure 5.8C). Control cells did not show any significant changes in RMP, driving force for Cl$, conductance, or PPR throughout the experiment.
Figure 5.7 Metabotropic KAR signaling does not modulate IPSCs during KCC2 blockade

(A) Plot of group data showing the effect of 0.1μM kainic acid application on $E_{\text{GABA}}$ over time in the presence of KCC2 antagonist VU 0463271.

(B) Group data comparing $E_{\text{GABA}}$ at t=0 and t=5.

(C) Plot of voltage-current curve for a cell at t=0 ($E_{\text{GABA}}=-62.8\text{mV}$) and during KA perfusion at t=5 ($E_{\text{GABA}}=-63.1\text{mV}$). Inset, examples of evoked IPSCs.

(D) Group data for the same cells as (B) comparing $E_{\text{GABA}}$ at t=0 and t=15.

(E) Plot of voltage-current curve for the same cell as (C) at t=0 ($E_{\text{GABA}}=-62.8\text{mV}$) and during KA washout at t=15 ($E_{\text{GABA}}=-63.1\text{mV}$). Inset, examples of evoked IPSCs.

Scale bars: 60 pA, 10 ms. A,B,D n=7 control, n=5 KA. Error bars indicate SEM.
Figure 5.8 Metabotropic KAR signaling does not alter the driving force for Cl⁻ during KCC2 blockade

(A) Group data showing resting membrane potential at t=0 and t=15.
(B) Group data showing driving force for Cl⁻ at t=0 and t=15.
(C) Group data showing Cl⁻ conductance at t=0 and t=15.
(D) Group data showing paired pulse ratio at t=0 and t=15.

n=7 control, n=5 KA. Error bars indicate SEM.
5.3.3 Metabotropic KAR-mediated modulation of IPSCs requires G-protein signaling.

Next, I aimed to determine if the observed changes in $E_{\text{GABA}}$ and Cl$^-$ driving force were due to G-protein mediated metabotropic KAR signaling. This was an important control to perform for several reasons. Firstly, as shown in Figure 5.3, despite using a reduced concentration of KA, I still observed a membrane depolarization upon KA application, suggesting that there may still be some ionotropic KAR signaling occurring. Secondly, it has been proposed that there are several types of metabotropic KAR signaling pathways, including a G-protein independent pathway (Negrete-Díaz et al., 2018), so blockade of G-proteins may provide further insight into the mechanism of the changes in $E_{\text{GABA}}$ after KA washout observed in Figure 5.2.

To test the hypothesis that metabotropic KAR signaling regulates $E_{\text{GABA}}$ through a G-protein mediated mechanism, I repeated the experiment using the two different G-Protein blockers, NEM and GDP-$\beta$-S, while applying the lower concentration of KA (0.1μM). By both using the lower concentration of KA and applying G-protein blockers, I prevented G-protein mediated metabotropic KAR signaling. This leaves the potential sources of KAR signaling as either small amounts of ionotropic signaling, which may account for the RMP depolarization observed using 0.1μM KA, or possibly G-protein independent KAR signaling.

In all experiments I bath applied the KAR agonist kainic acid (KA, 0.1μM) for 5 minutes while recording from CA3 pyramidal cells in whole-cell mode. KA was bath perfused in conjunction with a cocktail of antagonists to prevent the activation of other ionotropic glutamate receptors (10μM GYKI52466 to block AMPA, 50μM DL-APV to block NMDA), GABA$_B$ receptors (CGP55845 3μM), and mGlurS (300μM DL-AP3; see Table 2.1). All antagonists were bath applied throughout the experiment, from the time the cell was patched until the end of the experiment. In addition to these antagonists, the G-protein antagonist NEM (50μM) was bath
applied to the slices, or GDP-β-S (300μM) was added to the pipette internal solution where indicated. $E_{GABA}$ was recorded every 5 minutes and all measurements taken at $t = 0$ are values obtained immediately prior to bath application of KA, 5 minutes after whole cell configuration was achieved. After 5 minutes of KA application ($t=0$ to $t=5$), KA was washed out using aCSF containing only the previously mentioned antagonists for 10 minutes ($t=5$ to $t=15$). Control cells were perfused with the same antagonist cocktail as experimental cells (including NEM) for the course of the experiment but did not have KA applied.

In the presence of NEM, 0.1μM KA application did not produce any significant changes in $E_{GABA}$ over the time course of the experiment (Figure 5.9). However, after 10 minutes of KA washout at $t=15$, there was a significant depolarization of the RMP and a subsequent increase in the driving force for Cl⁻ in KA-perfused cells only (Figure 5.10A,B). There were no changes in conductance or PPR in either group (Figure 5.10C,D). Control cells did not show any significant changes in RMP, driving force for Cl⁻, conductance, or PPR throughout the experiment.
Figure 5.9 0.1μM KA application does not modulate IPSCs in the presence of NEM

(A) Plot of group data showing the effect of 0.1μM kainic acid application on EGABA over time in the presence of NEM.

(B) Group data comparing EGABA at t=0 and t=5.

(C) Plot of voltage-current curve for a cell at t=0 (EGABA=−61.2mV) and during KA perfusion t = 5 (EGABA=−63.8mV). Inset, examples of evoked IPSCs.

(D) Group data for the same cells as (B) comparing EGABA at t=0 and t=15.

(E) Plot of voltage-current curve for the same cell as (C) at t=0 (EGABA=−61.2mV) and during KA perfusion at t = 5 (EGABA=−64.7mV). Inset, examples of evoked IPSCs

Scale bars: 200 pA, 10 ms. A,B,D n=13 control, n=8 KA. Error bars indicate SEM.
Figure 5.10 0.1μM KA application depolarizes the resting membrane potential and increases the driving force for Cl⁻ in the presence of NEM

(A) Group data showing resting membrane potential at t=0 and t=15.
(B) Group data showing driving force for Cl⁻ at t=0 and t=15.
(C) Group data showing Cl⁻ conductance at t=0 and t=15.
(D) Group data showing paired pulse ratio at t=0 and t=15.

A-C n=13 control, n=8 KA, D n=7 control, n=7 KA. Error bars indicate SEM. * = p < 0.05, ** = p < 0.01
With the addition of GDP-β-S (300µM) in the patch pipette, 0.1µM KA application did not produce any significant changes in $E_{\text{GABA}}$ over the time course of the experiment (Figure 5.11). However, after 10 minutes of KA washout at $t=15$, there was a significant depolarization of the RMP and a subsequent increase in the driving force for Cl⁻ in both KA-perfused and control groups (Figure 5.12A,B), an effect that was also observed in both control and 1µM KA-perfused groups in Chapter 4. There were no changes in conductance or PPR in the control group, but KA-perfused cells showed a significant decrease in Cl⁻ conductance from $9.6 \pm 5.9$pS to $6.9 \pm 4.2$pS (Figure 5.12 C,D).
Figure 5.11 0.1μM KA application does not modulate IPSCs in the presence of GDP-β-S

(A) Plot of group data showing the effect of 0.1μM kainic acid application on $E_{\text{GABA}}$ over time in the presence of GDP-β-S.

(B) Group data comparing $E_{\text{GABA}}$ at t=0 and t=5.

(C) Plot of voltage-current curve for a cell at t=0 ($E_{\text{GABA}}=-64.5\text{mV}$) and during KA perfusion t = 5 ($E_{\text{GABA}}=-65.3\text{mV}$). Inset, examples of evoked IPSCs.

(D) Group data for the same cells as (B) comparing $E_{\text{GABA}}$ at t=0 and t=15.

(E) Plot of voltage-current curve for the same cells as (C) at t=0 ($E_{\text{GABA}}=-64.5\text{mV}$) and during KA perfusion at t = 5 ($E_{\text{GABA}}=-62.2\text{mV}$). Inset, examples of evoked IPSCs

Scale bars: 200 pA, 10 ms. A,B,D n=7 control, n=17 KA. Error bars indicate SEM.
Figure 5.12 0.1μM KA application depolarizes the resting membrane potential and increase Cl- driving force in the presence of GDP-β-S.

(A) Group data showing resting membrane potential at t=0 and t=15.
(B) Group data showing driving force for Cl- at t=0 and t=15.
(C) Group data showing Cl- conductance at t=0 and t=15.
(D) Group data showing paired pulse ratio at t=0 and t=15.

A-C, n=7 control, n=17 KA. Error bars indicate SEM. ** = p < 0.01, *** = p < 0.001.
5.3.4 Metabotropic KAR signaling modulates IPSCs independent of zinc release

Finally, to determine if metabotropic KAR signaling could regulate $E_{\text{GABA}}$ independent from zinc release, I performed a set of experiments using the lower concentration of KA (0.1μM) in combination with specific zinc chelator ZX1 (Pan et al., 2011). It has been previously shown that at MF-CA3 synapses, zinc release and subsequent metabotropic zinc receptor (mZnR) activation can enhance KCC2 function (Chorin et al., 2011) and that KCC2 surface expression and transport activity can be increased by via mZnR activation (Gilad et al., 2015), and therefore it is possible that zinc release could account for some of my previous results.

ZX1 (100μM) was added to the aCSF with the same cocktail of antagonists as all other experiments (10μM GYKI52466, 50μM DL-APV, 3μM CGP55845 and 300μM DL-AP3; see Table 2.1) and cells were perfused with this inhibitor-containing aCSF throughout the experiment. KA (0.1μM) was added as an additional component to the aCSF containing the ZX1 and inhibitors for a 5-minute wash in period (t=0 to t=5). Control cells had ZX1 and other antagonists perfused throughout the experiment but were not perfused with KA.

Upon 0.1μM KA application (t=5 min) in the presence of inhibitors and ZX1, $E_{\text{GABA}}$ showed no significant different compared to control $E_{\text{GABA}}$ measurements that were made in aCSF containing only inhibitors and ZX1 (Figure 5.13). After 10 minutes of KA washout, $E_{\text{GABA}}$ hyperpolarized from $-57.7 \pm 3.7$ mV at t=0 to $-62.3 \pm 6.0$ mV at t=15. This was a statistically significant difference when compared to controls using a two-way repeated measure ANOVA (Figure 5.13A) and when $E_{\text{GABA}}$ at t=0 and t=15 were compared within the KA group but not the control group using a paired Student’s t test (Figure 5.13B).
The observed change in $E_{\text{GABA}}$ at $t=15$ was not accompanied by any changes in resting membrane potential, but there was significant increase in the driving force for Cl⁻ (Figure 5.14A,B). A significant decrease in conductance was observed in both KA-treated (6.3 ±4.2pS to 3.8 ± 2.2pS) and control cells (4.3 ± 1.4pS to 3.0 ± 1.4pS) (Figure 5.14C), and there was a significant increase in paired pulse ratio in KA-treated cells only (Figure 5.14D).
Figure 5.13 Metabotropic KAR signaling modulates IPSCs independent of zinc release.

(A) Plot of group data showing the effect of 0.1μM kainic acid application on $E_{\text{GABA}}$ over time in the presence of ZX1.

(B) Group data comparing $E_{\text{GABA}}$ at t=0 and t=5.

(C) Plot of voltage-current curve for a cell at t=0 ($E_{\text{GABA}}$=−53.8mV) and during KA perfusion t = 5 ($E_{\text{GABA}}$ =−56.8mV). Inset, examples of evoked IPSCs.

(D) Group data for the same cells as (B) comparing $E_{\text{GABA}}$ at t=0 and t=15.

(E) Plot of voltage-current curve for the same cells as (C) at t=0 ($E_{\text{GABA}}$=−53.8mV) and during KA washout t=15 ($E_{\text{GABA}}$ =−64.1mV). Inset, examples of evoked IPSCs.

Scale bars: 100 pA, 5 ms. A,B,D n=6 control, n=11 KA. Error bars indicate SEM. * = p < 0.05
Figure 5.14 Metabotropic KAR signaling increase Cl⁻ driving force independent of zinc release.

(A) Group data showing resting membrane potential at t=0 and t=5.
(B) Group data showing driving force for Cl⁻ at t=0 and t=5.
(C) Group data showing Cl⁻ conductance at t=0 and t=5.
(D) Group data showing paired pulse ratio at t=0 and t=5.

A-C, n=6 control, n=11 KA. Error bars indicate SEM. * = p < 0.05, ** = p < 0.01.
5.4 Conclusions

In this chapter, I have shown that activation of metabotropic KAR signaling can modulate IPSCs, hyperpolarize E\textsubscript{GABA}, and increase Cl\textsuperscript{-} driving force in CA3 pyramidal cells using 0.1μM KA application. This modulation is not present during KA application at t=5, but becomes significant 10 minutes after KA washout. I have also shown that this KAR-mediated modulation of E\textsubscript{GABA} is dependent on GluK1/2 containing KARs, because it was not present in GluK1/2\textsuperscript{−/−} slices. It is also abolished when KCC2 transport is blocked with VU, suggesting a KCC2-transport dependent mechanism. At this concentration of KA, there was still a moderate membrane depolarization during KA application, suggesting that activation of metabotropic KAR signaling was not selective and that there may still be some ionotropic KAR activity. Additionally, the decrease in conductance observed in KA-treated cells in VU and GDP-β-S experiments also suggests that there may still be ionotropic KAR activity that causes a decrease in GABA release presynaptically. However, the hyperpolarization of E\textsubscript{GABA} and increase in Cl\textsuperscript{-} driving force was shown to be dependent on G-protein signaling, as it was abolished in the presence of G-protein blockers NEM and GDP-β-S. The NEM and GDP-β-S experiments support a metabotropic mechanism for these changes in E\textsubscript{GABA} and Cl\textsuperscript{-} driving force, despite ionotropic activity likely being present at this lower concentration of KA.

Similar to my experiments using 1μM KA in conjunction with GDP-β-S in Chapter 4, I observed a depolarization of the membrane potential and an increase in the Cl\textsuperscript{-} driving force in both KA-treated and control cells at t=15 when I included GDP-β-S in the pipette internal solution and applied 0.1μM KA. However, this was not accompanied by a significant change in E\textsubscript{GABA}, and therefore the change in Cl\textsuperscript{-} driving force was dependent on the change in membrane potential. Potential explanations for how GDP-β-S could cause a membrane depolarization in
both control and KA-perfused cells were discussed in more detail in Chapter 4.4, but it is likely that nonspecific block of all G-protein signaling could alter the function of potassium and/or calcium channels in the membrane that are normally regulated by G-protein signaling, causing changes in membrane permeability to these ions and subsequent changes in resting membrane potential. Despite the changes in membrane potential and Cl⁻ driving force, 0.1μM KA application in conjunction with GDP-β-S did not produce any significant changes in $E_{\text{GABA}}$, suggesting that G-protein mediated metabotropic KAR signaling is responsible for the hyperpolarization of $E_{\text{GABA}}$ that I observed at $t=15$ with 0.1μM KA application in the absence of GDP-β-S.

Finally, metabotropic KAR-mediated regulation of $E_{\text{GABA}}$ was shown to occur during zinc chelation to determine if zinc release from mossy fibre terminals was involved in the effect. $E_{\text{GABA}}$ hyperpolarize upon KA application in the presence of ZX1, suggesting that mZnR activation is not required for KAR activity to regulate $E_{\text{GABA}}$. However, I also showed that zinc chelation with ZX1 resulted in a decreased conductance in both KA-treated and controlled cells. Because this occurred in control cells that were not treated with KA, this effect cannot be completely accounted for by KAR-mediated regulation of presynaptic GABA release. In some cases, this made $E_{\text{GABA}}$ difficult to determine because the IPSC amplitudes were small and thus the signal-to-noise ratio was lower, making it difficult to completely rule out the involvement of mZnRs. Zinc release in the hippocampus has been shown to regulate presynaptic release of both glutamate and GABA, and perfusion of ZnCl₂ into the CA3 region increases presynaptic GABA release (Takeda et al., 2004). Therefore, the decreased conductance I observed may be a result of zinc chelation preventing the normal zinc-mediated regulation of GABA release. This may also account for some of the changes in paired pulse ratio I observed at $t=15$ with ZX1 zinc chelation.
in KA-treated cells, as changes in paired pulse ratio may indicate changes in presynaptic neurotransmitter release.

Taken together, these results suggest that metabotropic KAR signaling by GluK1/GluK2-containing KARs can modulate IPSCs and regulate $E_{\text{GABA}}$ and Cl- driving force through a KCC2-dependent mechanism. This supports my original hypothesis that the KCC2-dependent component of KAR-mediated modulation of IPSCs, $E_{\text{GABA}}$, and Cl- driving force depends metabotropic signaling by KARs. This also further supports my hypothesis that ionotropic and metabotropic KAR signaling can regulate chloride homeostasis by two independent mechanisms; an ionotropic mechanism that is not dependent in changes in KCC2 function, and a metabotropic mechanism that requires increased KCC2 transport. The results of these experiments reveal a novel mechanism by which KCC2 function can be upregulated by kainate receptor activation, via a metabotropic, G-protein mediated signaling pathway.
Chapter 6: Discussion

6 Discussion

6.1 Thesis Summary

Since its discovery, KCC2 has largely been considered an inhibitory protein (Payne et al., 1996, Rivera et al., 1999). This is because KCC2 is critical for maintaining the low intracellular chloride levels required for fast synaptic inhibition in the mature nervous system (Rivera et al., 1999). However, KCC2 is also an important protein at the excitatory synapse where it regulates spine structure as well as receptor content (Gulyás et al., 2001, Li et al., 2007, Gauvain et al., 2011, Chamma et al., 2012, Chamma et al., 2013, Fiumelli et al., 2013). Recently, a growing number of proteins have been discovered to regulate KCC2 function, including some proteins that are classically associated with excitatory neurotransmission, such as Neto2 (Ivakine et al., 2013) the GluK2 subunit of kainate receptors (Mahadevan et al., 2014, Pressey et al., 2017), and metabotropic glutamate receptors (Banke and Gegelashvili, 2008). KCC2’s ability to interact with and be regulated by proteins associated with excitatory synaptic transmission place KCC2 in the position to be a link between excitatory activity and the regulation of inhibition, potentially serving as a mechanistic link to regulate excitatory: inhibitory balance.

GluK2 has been established as an important protein interactor of KCC2, and their physical interaction is important for KCC2 to express and oligomerize in the membrane (Mahadevan et al., 2014), which is required for KCC2 to transport Cl- out of the cell which maintains a low intracellular [Cl-]. GluK2 also contributes to KCC2 recycling, increasing the amount of KCC2 that is recycled back to the membrane (Pressey et al., 2017). While the
significance of this physical interaction has been explored, it is unknown whether there is a functional interaction between KARs and KCC2. The aim of my research was to address the fundamental question of whether KAR activity could regulate KCC2 function.

The first major finding of my work was the discovery that activation of KARs could regulate IPSCs and hyperpolarize $E_{GABA}$ in the CA3 of the hippocampus. I showed that KAR-mediated regulation of $E_{GABA}$ required Gluk1/Gluk2 containing KARs and did not depend on zinc release at hippocampal mossy fibre terminals. Surprisingly, I found that this effect had two different components with two different time courses; an immediate, KCC2-independent hyperpolarization of $E_{GABA}$ and a delayed, KCC2-dependent hyperpolarization of $E_{GABA}$.

The second major discovery of my research was that KARs could regulate $E_{GABA}$ by both ionotropic and metabotropic signaling, independently of each other. I further established that the immediate, KCC2-independent effect was mediated by ionotropic KAR signaling, and that the delayed, KCC2-dependent effect was mediated by metabotropic KAR signaling and depended on a G-protein mediated mechanism. These findings place Gluk1/Gluk2-containing KARs on a growing list of metabotropic, G-protein signaling receptors that positively regulate KCC2 function, further supporting the idea that G-proteins may be a conserved mechanism for regulating chloride homeostasis. My findings also establish an additional functional role for postsynaptic KARs in the CA3 of the hippocampus. In both cases, ionotropic and metabotropic KAR signaling acted in a homeostatic manner – an increase in postsynaptic KAR activity results in a hyperpolarization of $E_{GABA}$ well below the resting membrane potential, which would ensure robust inhibition upon activation of the $GABA_A$ receptor. This contrasts with pre-synaptic KARs, which have been shown in most cases to inhibit GABA release from presynaptic terminals (Sihra and Rodríguez-Moreno, 2011). Postsynaptic KARs that exist as part of a larger macromolecular complex that includes KCC2 (Mahadevan et al., 2014) would be well positioned
to influence inhibitory activity during periods of high glutamate activity, and the delayed onset of the metabotropic KAR-mediated hyperpolarization of $E_{\text{GABA}}$ could serve to strengthen an ‘inhibitory brake’ after periods of intense glutamatergic activity.

Taken together my findings characterize a novel functional interaction between KCC2 and kainate receptors, an interaction that may serve as a potential mechanism for excitatory: inhibitory balance, as well as reveal a KCC2-independent mechanism by which KARs can regulate chloride homeostasis. Because KCC2 disfunction is implicated in many neurological diseases and disorders, the mechanistic details of the KAR: KCC2 interaction may serve as foundational knowledge that may point to future therapeutic targets that could potentially be utilized to develop strategies to restore aberrant chloride homeostasis.

6.2 Ionotropic KAR signaling regulates $E_{\text{GABA}}$ independent of KCC2 transport function.

I have shown that KAR activation is able to modulate IPSCs, hyperpolarize $E_{\text{GABA}}$, and increase the driving force for $Cl^-$. This effect occurs independently from zinc-release regulation of $E_{\text{GABA}}$, and appears to be mediated by ionotropic KAR signaling, as it occurs when G-protein signaling is blocked. I have also shown that this effect may depend on GluK1/GluK2 containing KARs, but it should be noted that changes in $E_{\text{GABA}}$ mediated by KCC2 could be difficult to discern in GluK1/2<sup>−/−</sup> mice, as these mice also display a reduced surface expression of KCC2 (Mahadevan et al., 2014). However, ionotropic KAR-mediated regulation of $E_{\text{GABA}}$ appears to be KCC2-independent because my initial experiments demonstrated that blockade of KCC2 signaling with VU did not abolish the hyperpolarization of $E_{\text{GABA}}$ with KA application at $t=5$ in WT neurons, and thus I would predict that reduced KCC2 expression in the GluK1/2<sup>−/−</sup> would not play a prominent role in this effect. The mechanism by which ionotropic KAR activation could
regulate intracellular [Cl\textsuperscript{−}] remains to be determined, but there are several possible explanations for how this could occur and experiments that could give further insight into the mechanism of this effect.

The first possible explanation is the involvement of NKCC1, another Cl\textsuperscript{−} transporter. In addition to KCC2, NKCC1 also plays a role in Cl\textsuperscript{−} homeostasis, and it is upregulated during development and present in mature neurons (Clayton et al., 1998, Wang et al., 2002, Balakrishnan et al., 2003, Hyde et al., 2011, Kaila et al., 2014). In disease states such as neonatal epilepsy (Kahle and Staley, 2008) increases in NKCC1 activity and a subsequent depolarization have been implicated, and this is thought to resemble a phenotype of more immature neurons, in which GABA is excitatory (Ben-Ari, 2002). Conversely, a hyperpolarization of E\textsubscript{GABA} like I observed with ionotropic KAR activation could be related to a decrease in NKCC1-transport.

However, previous studies using similar concentrations of KA to generate epileptiform activity in the hippocampus have found that KA application is associated with increased NKCC1 expression and transporter function (Dzhala et al., 2005, Dzhala et al., 2010, Nogueira et al., 2015), which suggests that decreased NKCC1 activity is unlikely to be the cause of the hyperpolarized E\textsubscript{GABA} observed in my experiments. Despite this, it would be simple to test the possibility of NKCC1’s involvement in KAR-mediated regulation of E\textsubscript{GABA} using bumetanide to block NKCC1 transport. Bumetanide is a potent inhibitor of NKCC1 function, and while it can also inhibit KCC2, it does so at much higher concentrations than are required to inhibit NKCC1 (Flatman, 2002).

A second possible mechanism for ionotropic KAR-mediated regulation of E\textsubscript{GABA} could involve osmotic changes brought on by cation influx through the KAR. Excitotoxic activity brought on by cation influx is associated with a passive influx of other ions and water into the cell (Rungta et al., 2015). If the influx of Na\textsuperscript{+} into the cell via KAR activation was sufficient to
cause water influx as well, E\textsubscript{GABA} could appear to be hyperpolarized. It is important to note however that cell swelling and water influx brought on by Na\textsuperscript{+} in some cases is caused by increased intracellular [Cl\textsuperscript{-}] (Rungta et al., 2015), making it difficult to determine how KAR-mediated influx of Na\textsuperscript{+} could cause a transiently lower intracellular [Cl\textsuperscript{-}].

A third possible explanation for ionotropic KAR-mediated regulation of [Cl\textsuperscript{-}] may involve calcium. KARs can be rendered calcium permeable through RNA editing (Egebjerb and Heinemann, 1993, Burnashev et al., 1995, Burnashev et al., 1996) and activation of KARs has also been associated with increased release of Ca\textsuperscript{2+} from intracellular stores in MF boutons (Scott et al., 2008). Intracellular calcium release can activate calcium-activated chloride channels (CaCCs) (Owen et al., 1984, Hallani et al., 1998, Frings et al., 2000, Hartzell et al., 2005), which would allow Cl\textsuperscript{-} flux across the membrane. However, in my experiments, E\textsubscript{GABA} was hyperpolarized upon KA application, whereas an influx of Cl\textsuperscript{-} down its electrochemical gradient through CaCCs would be expected to depolarize E\textsubscript{GABA} rather than hyperpolarize it. In general, a KCC2-independent hyperpolarization of E\textsubscript{GABA} is difficult to reconcile with changes in chloride channel function, because KCC2 transports Cl\textsuperscript{-} against its electrochemical gradient, whereas Cl\textsuperscript{-} channels such as CaCCs do not actively transport Cl\textsuperscript{-} and can only flux chloride down it’s electrochemical gradient. Despite this, it would be interesting to determine if Ca\textsuperscript{2+} somehow plays a role in ionotropic KAR-mediated regulation of E\textsubscript{GABA}, for example, by preventing calcium signaling by including an intracellular Ca\textsuperscript{2+} chelator such as BAPTA in the pipette internal solution.

One final possibility that could contribute to a relatively lower [Cl\textsuperscript{-}] upon KA application could involve other members of the solute carrier (SLC) family, such as KCC3 or members of SLC4 family of bicarbonate exchangers. KCC3, like KCC2, cotransports Cl\textsuperscript{-} and K\textsuperscript{+} across the neuronal membrane, and KCC3 is expressed abundantly in the CNS. However, while KCC2 can
transport under isotonic conditions, KCC3 cannot transport under isotonic conditions and is instead activated by changes in cell volume (Mount et al., 1999, Pearson et al., 2001, Williams and Payne, 2004, Blaesse et al., 2009). If ionotropic KAR activity were to cause sufficient changes in cell volume to activate KCC3, it may be possible that this could lower intracellular [Cl-]. In addition to KCC2 and KCC3, some members of the SLC4 family of bicarbonate exchangers can also extrude Cl- from the neuron. Sodium-driven chloride bicarbonate exchangers such as NDAE (also referred to as NDCBE) and NCBE use the transmembrane sodium gradient to cotransport Na+ and HCO3- into the neuron while transporting Cl- outside of the neuron in a 1:2:1 ratio (Hübner and Holthoff, 2013). NDAE and NCBE are widely expressed throughout the brain but their contribution to the neuronal Cl- gradient in mature neurons remains largely unclear (Hübner, C. A. et al., 2004, Hübner, Christian A. and Holthoff, 2013). It is possible that KCC2-independent KAR-mediated hyperpolarization of $E_{\text{GABA}}$ could occur by activating other transporters that extrude chloride, such as KCC3, NDAE, or NCBE. This would be a difficult hypothesis to test using pharmacological agents, because the antagonist usually used to block bicarbonate exchangers, 4,4'-Diisothiocyanato-2,2'-stilbenedisulfonic acid disodium salt (DIDS), also nonspecifically blocks other chloride transporters, including NKCC1, KCC2, and other Cl- channels (Delpire et al., 2009).

In summary, while the mechanism by which ionotropic KAR signaling could regulate chloride homeostasis remains unclear, I have shown that ionotropic KAR activation modulates IPSCs and hyperpolarizes $E_{\text{GABA}}$. The resulting change in the driving force for Cl- would ensure robust inhibition upon activation of the GABA$_A$ receptor, which could represent a mechanism for the cell to ensure strong inhibition during periods of strong excitatory activity.
6.3 Metabotropic KAR signaling regulates $E_{\text{GABA}}$ by increasing KCC2 transport.

In addition to KCC2-independent KAR-mediated regulation of $E_{\text{GABA}}$, I have also shown that activation of KARs can regulate $E_{\text{GABA}}$ in a KCC2-dependent fashion. Metabotropic KAR activation using a nanomolar concentration of KA (0.1μM) produced a hyperpolarization of $E_{\text{GABA}}$ with a delayed onset, 10 minutes after KA washout. This effect was shown to be dependent on KCC2 because blockade of KCC2 transport function with VU prevented the effect. This effect was also shown to be mediated by metabotropic KAR signaling, as it was abolished when G-protein blockers NEM or GDP-β-S were included in the perfusion or in the pipette solution. I also showed that this effect depended on GluK1/GluK2 containing KARs, as it was not present in GluK1/2−/− CA3 pyramidal cells. These results represent a novel mechanism for KCC2 regulation that is mediated by metabotropic KAR signaling.

While I have shown that metabotropic signaling can regulate $E_{\text{GABA}}$ via KCC2, the mechanism by which metabotropic KAR signaling regulates KCC2 function remains to be determined. I hypothesized that metabotropic KAR signaling would be able to regulate KCC2 because metabotropic KAR signaling has been shown to cause increased PKC activity. PKC is known to be able to phosphorylate KCC2 at Ser940, which increases KCC2 transport function as well as surface expression (Lee et al., 2007). This leads to several important questions that would need to be addressed to determine the mechanism of metabotropic KAR-mediated regulation of KCC2. Does metabotropic KAR activation lead to changes in KCC2 surface expression, phosphorylation status, or both? Is the activation of PKC by metabotropic KAR signaling required to change KCC2 surface expression or phosphorylation status?
Figure 6.1 Schematic of the effects of 0.1μM KA Application on KCC2 function.

(A) Under resting conditions, KCC2 extrudes Cl\(^-\) from the cell and maintains a low intracellular Cl\(^-\) level. KARs have not been activated.

(B) Upon 0.1μM KA application, metabotropic KAR signaling is activated, KCC2 function is increased, which results in a lower [Cl\(^-\)] and hyperpolarized E\(_{\text{GABA}}\). The mechanism of this effect has not been tested, but KARs are known to be able to activate PKC, and PKC is a known phosphorylator of KCC2, so this signaling pathway represents a likely mechanism for KAR-mediated hyperpolarization of E\(_{\text{GABA}}\).

(C) Co-application of KA and zinc chelator ZX1 establish that mZnR activation, which has been previously shown to increase KCC2 function, is not required for KAR-mediated hyperpolarization of E\(_{\text{GABA}}\).

(D) Blockade of G-proteins prevents KAR-mediated hyperpolarization of E\(_{\text{GABA}}\), supporting a metabotropic, G-protein mediated mechanism for KAR-mediated hyperpolarization of E\(_{\text{GABA}}\).
There are several experiments that could be performed to further determine if PKC-mediated phosphorylation of KCC2 is involved in the mechanism of metabotropic KAR-mediated regulation of KCC2. Changes in KCC2 phosphorylation status can be assessed using a phospho-specific KCC2 antibody (Lee et al., 2011, Conway et al., 2017). This antibody could be used to compare levels of phosphorylated KCC2 in neurons treated with KA to levels of phosphorylated KCC2 in untreated neurons. Additionally, KCC2 surface expression could be assessed using a surface biotinylation assay that compares surface expression of KCC2 in slices treated with 0.1μM KA to untreated slices. Finally, to determine if PKC contributes to any changes in KCC2 phosphorylation or surface expression mediated by KAR activation, there are several inhibitors of PKC available, such as calphostin. It is important to note that PKC inhibitors such as calphostin have been shown to decrease basal levels of KCC2 phosphorylation (Lee et al., 2007), but it would be interesting to determine if co-application of a PKC inhibitor and KA acid would produce a difference in KCC2 phosphorylation levels compared to cell treated only with PKC or only with KA, to further establish to potential role of PKC in KAR-mediated regulation of KCC2.

It is also important to consider whether metabotropic KAR-mediated regulation of KCC2 is relevant in a more physiological setting. In addition to this, due to the subcellular localization pattern of post-synaptic KARs in the hippocampus, it would also be interesting to determine if metabotropic KAR regulation of KCC2 function is relevant to specific interneuronal inputs in the CA3. CA3 pyramidal cell activity is regulated by many different types of interneurons, and different subpopulations of interneurons innervate different subcellular domains of CA3 pyramidal cells and are important for different functions in the hippocampus. In particular, PV+ interneurons usually innervate the somatodendritic area of PCs, while SOM+ interneurons innervate the dendrites (Higley, 2014). Postsynaptic GluK2-containing receptors kainite
receptors are found primarily in the striatum lucidum, where they mediate a postsynaptic KAR current (Castillo et al., 1997, Jaskolski et al., 2005). This area is also where the pyramidal cell is innervated by both mossy fibres terminals and PV+ interneurons. It is therefore important to examine if Gluk2-KAR regulation of KCC2 occurs at inhibitory synapses made with specific interneuron types. PV+ and CCK+ interneurons innervate all subcellular domains of hippocampal pyramidal cells, particularly the perisomatic area, but play different roles in regulating PC firing (Klausberger et al., 2005, Klausberger and Somogyi, 2008, Somogyi et al., 2014). In contrast, SOM+ interneurons typically innervate only the dendrites. Because of differences in subcellular localization of KARs and in innervation by interneurons, it is possible that KAR-mediated regulation of E_{GABA} would be more relevant to PV+ interneuronal input than for example, SOM+ interneuronal inputs. To determine if different GABAergic inputs are affected by GluK2-KAR regulation of KCC2 function in a more physiological setting, rather than using a stimulating electrode to trigger GABA release from interneurons, an optogenetic strategy could be used to selectively activate different subpopulations, such as PV+ and SOM+. Combined with laser uncaging of glutamate targeted to CA3 pyramidal cells, this approach could be used to determine if KAR-mediated regulation of E_{GABA} occurs with levels of glutamate release that more closely mimic physiological conditions than bath application of KA. Additionally, it could be used to determine if KAR-mediated regulation of E_{GABA} is relevant to inputs from specific interneuron subtypes.

Although the mechanism of KAR-mediated regulation of E_{GABA} still remains to be elucidated, my findings represent a novel mechanism for regulating KCC2 function and demonstrate a novel functional interaction between GluK1/2 containing KARs and KCC2. I have shown that a G-protein mediated form of KAR signaling leads to a hyperpolarization of E_{GABA} that depends on KCC2 transport. This finding is in line with several recent discoveries that have
shown that KCC2 can be regulated by other metabotropic, G-protein signaling proteins and adds KARs to a growing list of proteins that signal via G-proteins and can regulate KCC2, which includes mGluRs (Banke and Gegelashvili, 2008), mZnRs (Chorin et al., 2011, Gilad et al., 2015). In addition to this, serotonin and adenosine receptors, both of which are also GPCRs, have been shown to regulate KCC2 function in models of spinal cord injury (Bos et al., 2013, Ford et al., 2015). It has been proposed that GPCR signaling may be a conserved mechanism for chloride homeostasis (Mahadevan and Woodin, 2016), and my finding that metabotropic KAR signaling regulates $E_{\text{GABA}}$ through a KCC2-dependent mechanism further supports this hypothesis.

6.4 Conclusion

In conclusion, the major findings of my research have established a novel functional role for GluK1/GluK2 containing KAR activation in CA3 pyramidal cells. I have demonstrated that both ionotropic and metabotropic KAR signalling is able to regulate $\text{Cl}^-$ homeostasis by hyperpolarizing $E_{\text{GABA}}$ and increasing the driving force for $\text{Cl}^-$. While metabotropic KAR signaling regulates $E_{\text{GABA}}$ through a KCC2-dependent mechanism, ionotropic KAR signalling can regulate $E_{\text{GABA}}$ independent of KCC2 function through yet undetermined mechanism. These results suggest that the two signaling modes of KARs may serve different functional roles for chloride homeostasis, allowing the cell to regulate the strength of GABAergic inhibition in response to excitatory activity in both an ionotropic, KCC2-independant manner and a metabotropic, KCC2-dependent manner. Metabotropic KAR-mediated regulation of KCC2 function places KARs on a growing list of proteins that are able to regulate KCC2 via G-Protein signalling (Banke and Gegelashvili, 2008, Chorin et al., 2011, Mahadevan and Woodin, 2016),
which supports the hypothesis that G-protein signaling may be a conserved mechanism for regulating chloride homeostasis.

In addition to establishing a novel role for KAR signaling in the hippocampus, my findings have also established a novel functional interaction between KARs and KCC2 and reveal a novel mechanism for regulation of KCC2 function. Postsynaptic KARs that exist as part of a larger macromolecular complex that includes KCC2 (Mahadevan et al., 2014) would be well positioned to influence inhibitory activity via KCC2 during periods of high glutamate activity, which may be one mechanism by which neurons are able to maintain excitatory : inhibitory balance. It is well known that disruptions in the Cl⁻ gradient are implicated in many neurological diseases and disorders that are characterized by excessive glutamatergic excitation, including epilepsy and neuropathic pain (Kahle et al., 2014, Coull et al., 2003). My findings place KARs, and in particular metabotropic regulation of KCC2 via KARs, as a potential target for the treatment of these diseases. These results represent a novel mechanism for regulation of excitatory : inhibitory balance in the mature nervous system and could have important implications for both normal physiological function of neurons as well as disease states.
References


