Kainate Receptor Editing and Plasticity of AMPARs

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Abstract

Kainate receptors (KARs) are hetero-tetrameric glutamate-gated ion channels. KARs are assemblies of five core subunits (GluK1-GluK5) with the combination GluK2 and GluK5 likely being the most common. KARs can signal via both canonical ionotropic and non-canonical metabotropic pathways to regulate synaptic function, neuronal excitability and maintenance of network activity. Recent evidence has shown that transient activation of KARs can upregulate the surface expression of AMPARs to elicit LTP.

In this thesis I examined whether activation of KARs can induce long-term depression (LTD) of AMPARs. My results suggest that Kainate application increases phosphorylation of GluA1 at S845. Moreover, sustained activation of postsynaptic GluK2 subunit-containing KARs reduces surface AMPARs to induce a novel form of LTD of AMPARs (KAR-LTD$_{AMPAR}$) that is mediated via an ionotropic pathway and is independent of NMDA or mGlu receptors. This KAR-evoked loss of surface AMPA receptors is occluded by inhibiting calcineurin. These data, together with the previously reported KAR-LTP$_{AMPAR}$, demonstrate that KARs bidirectionally regulate synaptic AMPARs and synaptic plasticity.

Finally, I investigated how the trafficking and functions of KARs are regulated by Q/R RNA editing of the GluK2 subunit, by the enzyme adenosine de-aminase 2 (ADAR2). I show that GluK2 editing-deficient mice exhibit higher paired-pulse and frequency facilitation (PPF-FF) along with enhanced KAR/AMPAR ratio. Moreover, the KAR currents in these mice have faster 10-90% rise time and decay kinetics (τ_{decay}) compared to their edited counterparts. Perhaps most interestingly, the GluK2 editing-deficient mice show impaired metabotropic KAR function supported by reduced inhibition of I_{sAHP}. These results demonstrate that GluK2 Q/R editing is crucial in both the biophysical properties and signalling KARs.

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Author's Declaration

Part of the contents of this thesis has been published

1.) As a review paper titled "Kainate receptors and synaptic plasticity" (Nair, J.D., Wilkinson, K.A., Henley, J.M. and Mellor, J.R., 2021. Kainate Receptors and Synaptic Plasticity. Neuropharmacology, p.108540.

2.) In iScience titled "Sustained postsynaptic Kainate receptor activation downregulates AMPA receptor surface expression and induces hippocampal LTD" Nair, J.D., Braksator, E., Yucel, B.P., Fletcher-Jones, A., Seager, R., Mellor, J.R., Bashir, Z.I., Wilkinson, K.A. and Henley, J.M., 2021. Sustained postsynaptic kainate receptor activation downregulates AMPA receptor surface expression and induces hippocampal LTD. *Iscience*, *24*(9), p.103029.

A copy of these publications can be found in Appendix-i

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE: .....

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Abbreviations

A-I	Adenosine to inosine
aa	Amino acid
aCSF	Artificial Cerebro-Spinal Fluid
AC	Adenylate Cyclase
ADARs	Adenosine De-aminase Acting on RNAs
AGE	Agarose Gel Electrophoresis
AHP	After Hyperpolarising Potential
AMPARs	α-Amino-3-Hydroxy-5-Methyl-4-isoxazolepropionic acid receptor
BDNF	Brain Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
C1q	complement component 1q
C1ql	complement component 1q like
CA1	Cornu Amonis 1
CA3	Cornu Amonis 3
cLTP	Chemical Long-Term Potentiation
CNS	Central Nervous System
CP	Calcium Permeable
Cy	Cyanine
DAG	Diacyl Glycerol
DG	Dentate Gyrus
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
dsRNA	Double stranded RNA
EB	Earle's Buffer
ECL	Enhanced Chemiluminescence
ECS	Editing Site Complementary
EDTA	Ethylenediaminetetraacetic Acid
EPSCs	Excitatory Post Synaptic Currents
EPSPs	Excitatory Post Synaptic Potentiation
ER	Endoplasmic Reticulum
FBS	Foetal Bovine Serum

FMRP	Fragile X Mental Retardation Protein
FRET	Fluorescence Resonance Energy Transfer
GABA	γ-Amino Butyric Acid
GFP	Green Fluorescent Protein
GluA1	AMPAR Subunit 1
GluA2	AMPAR Subunit 2
GluA3	AMPAR Subunit 3
GluA4	AMPAR Subunit 4
GluK1	Kainate Subunit 1
GluK2	Kainate Subunit 2
GluK3	Kainate Subunit 3
GluK4	Kainate Subunit 4
GluK5	Kainate Subunit 5
GRIP1 & 2	Glutamate receptor interacting protein 1 & 2
HBSS	Hank's Buffered Salt Solution
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFS	High Frequency Stimulation
I/V	Isoleucine to Valine
iGluRs	Ionotropic Glutamate Receptors
IPSCs	Inhibitory Post Synaptic Currents
IPSPs	Inhibitory Post Synaptic Potentiation
KA	Kainate
KARs	Kainate Receptors
kb	Kilobase
KD	Knock Down
kDa	Kilodalton
KO	Knock Out
LFS	Low Frequency Stimulation
LTD	Long-Term Depression
LTP	Long-Term Potentiation
MAPK	Mitogen Activated Protein Kinases
MAUGK	Membrane Associated Guanylate Kinase

mEPSPs	miniature Excitatory Post Synaptic Potentiation
MF	Mossy Fibre
mGluRs	metabotropic Glutamate Receptors
MW	Molecular Weight
NEM	N-ethylmaleimide
NMDARs	N-Methyl-D-Aspartate Receptor
NSF	N-Ethylmaleimide Sensitive Factor
PAK	P21-Activated Protein Kinase
PICK1	Protein interacting with C-Kinase 1
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	cGMP dependent Protein Kinase
PKMζ	Protein Kinase C isoform
PLC	Phospholipase C
PLL	Poly L-Lysine
PPF-FF	Paired-Pulse Facilitation, Frequency Facilitation
PPR	Paired-Pulse Ratio
PSD 95	Post-Synaptic Density 95
PTM	Post-translational Modifications
PTx	Pertussis Toxin
Q/R	Glutamine to Arginine
R/G	Arginine to Glycine
RT	Room Temperature
sAHP	Slow After Hyperpolarising Potential
SAP97	Synapse Associated Protein 97
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
sEPSPs	spontaneous Excitatory Post Synaptic Potentiation
ShRNA	Short hairpin Ribonucleotide (RNA)
SNAREs	Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors
SNX27	Sorting Nexin 27
Stim	Stimulating electrode
STD	Short-Term Depression

STP	Short-Term Plasticity
SUMO	Small Ubiquitin like Modifiers
SVs	Synaptic Vesicles
TARP	Transmembrane AMPAR Regulatory Protein
TEMED	Tetramethyl ethylenediamine
TM	Transmembrane
TTX	Tetrodotoxin
VGCC	Voltage Gated Calcium Channel
WB	Western Blot
WT	Wild Type
Y/C	Tyrosine to Cystine
YFP	Yellow Fluorescent Protein
αCaMKII	α Calcium/Calmodulin–Dependent Kinase II
β-Me	β-Mercaptoethanol

Chapter 1. General Introduction

1.1 Neuronal communication

1.1.1 Introduction to neuronal communication

The brain and spinal cord constitute the central nervous system (CNS). The CNS is an immensely complex and integrated processing system that simultaneously and constantly processes vast amount of information. The CNS can be considered as one of the most complex structures in the known universe and its function extent from sensory, motor, memory, perception, cognition, emotion, speech, and homeostasis etc. All these sophisticated functions are controlled and coordinated by the inter-connected circuitry formed by morphologically complex and highly excitable post-mitotic cells called the neurons. Glial cells support neurons by providing insulation (oligodendrocytes in CNS and Schwann cells in peripheral nervous system (PNS)), nourishment (astrocytes) and by destroying pathogens (microglia) that invade the CNS. Over and above that it is becoming lucid that glia also participates in the modulation of neuronal signaling (Cornell-Bell et al., 1990, Haydon, 2001). It is estimated that there are equal number of neurons to glia. However, at some regions of the brain, glia outnumbers neurons. (Harvey Lodish, 2016, von Bartheld et al., 2016, Herculano-Houzel, 2009)

The architecture of a typical mature neuron includes a soma (cell body), an axon and dendrites (Ramón y Cajal et al., 2011). The soma contains most of the organelles and cytosol. The soma often serves as a hub for integrating the information received by a neuron. The axon is portrayed as a long tubular projection beginning at axon hillock and extending outward along with varying protein composition compared to the soma. Axons can carry electrical signals over very long distances from the soma, for e.g. axons in the spinal cord extend more than 1m (Nachmansohn, 1950). The electrical signals conveyed through the axon and dendrites is termed as an Action potential.

Generally, dendrites are the shorter branching structures that extend out of soma and functions to convey information to the soma from external stimuli or a neuron (Ramón y Cajal et al., 2011) **[Figure 1-1]**. The size and shape of dendrites vary depending on the type of neurons and hence this is often regarded as a method for neuronal classification (Muller and Nikonenko, 2013) Dendrites encompasses mushroom-like projections called dendritic spines that serves as the ‘antennae’ of neurons. (Nimchinsky et al., 2002) [**Figure 1-1].** Dendritic spines form specialized junctions with

adjacent neurons known as ‘synapse’ the fundamental anatomical structure for information transfer in the CNS (Spruston, 2008, Park et al., 2019).

In the adult brain, there are millions of neurons and trillions of synapses (Harvey Lodish, 2016, Choquet and Triller, 2013).

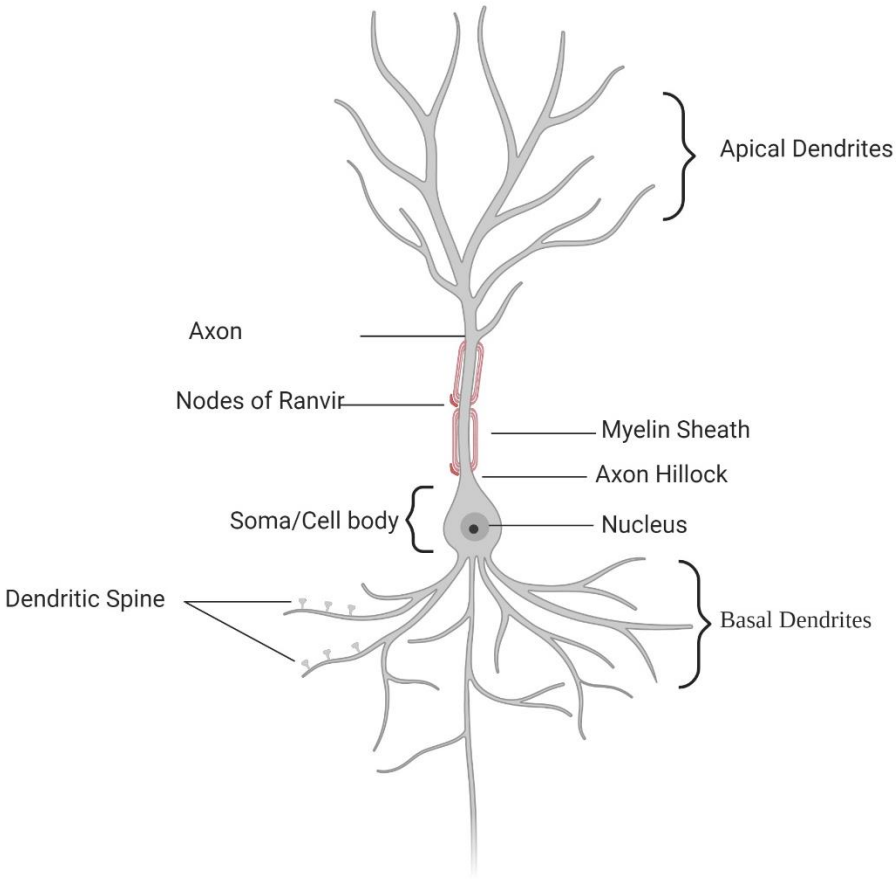

Figure 1-1: Schematic of a hippocampal pyramidal neuron

Neurons are characterized with a spherical structure known as cell body or soma that constitutes organelles and cytosol. Long projections originating from axon hillock are called Axons. Axons are surrounded by insulating sheaths formed by oligodendrocytes (CNS) or myelin (PNS) which accelerate the information transfer. Short projections from the soma are dendrites. Dendrites are classified into apical and distal dendrites depending on their position relative to the cell body.

1.1.2 Synapses and synaptic transmission

Neurons communicate by electrical or chemical signaling. In electrical synapses (Gap Junctions), the neuronal membrane is continuous with another neuron leading to rapid and synchronous activity. However, most neurons in the CNS communicate by

chemical signaling mediated via specialized molecules called the neurotransmitters. Unlike electrical synapses, the neuronal membrane in the chemical synapse is discontinuous (Purves D, 2001). The region formed between the pre, and post synaptic neuron is known as synapse with the space in between termed 'synaptic cleft'. Presynaptic neurons release neurotransmitter to the synaptic cleft where it diffuses and bind to the receptors present at the pre and postsynaptic sites to exert its effect [**Figure 1-2]**.

1.1.2.1 Neurotransmitters

Various neurotransmitters in CNS and PNS include amino acids, amino acid derivatives, small peptides, hormones, etc. (Kandel, 2013). Neurotransmitters are broadly classified into two types depending on their function. The neurotransmitters that can evoke a response by activating their receptors on the postsynaptic cell are known as excitatory neurotransmitters and the ones that inhibit the transmission is termed as an inhibitory neurotransmitter (Collingridge et al., 1983, McCormick, 1989). Glutamate is the major excitatory and GABA is the major inhibitory neurotransmitter in the CNS. However, there are hundreds of other neurotransmitters in the CNS contributing to the diverse signaling of neurons (Purves D, 2001, Kandel, 2013).

1.1.2.2 Mechanism of neurotransmitter release

Activation of receptors on the membrane of an afferent neurons leads to influx of ions generating a receptor potential/generator potential. Depolarization of the membrane due the influx of positively charged ions beyond the threshold trigger the opening of Na^+ channels at the vicinity of axon hillock. This evoke a transient positive charge known as the action potential (Fletcher, 2011). Axon potential travel down the axon at a constant speed which is further enhanced by the insulation. Nodes of Ranvier are the intermittent uninsulated spots on an axon where the axon potential is regenerated **[Figure 1-2]**. Action potential reaching the terminals divides and move further to presynaptic terminals (Tyler and Murthy, 2004). Activation of the voltage gated Ca^{2+} channels (VGCC) at the presynaptic terminals leads to an influx of Ca^{2+}. This transient rise in the Ca^{2+} levels promote their binding with synaptotagmins. Ca^{2+} bound synaptotagmins along with soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) (Ramakrishnan et al., 2012) trigger the fusion and release of docked synaptic vesicles (Svs) containing neurotransmitters into the

synaptic cleft **[Figure 1-2]** (Kandel, 2013, Dolphin and Lee, 2020, Sudhof, 2012, Harvey Lodish, 2016, Heuser et al., 1979).

Binding of neurotransmitter evoke response in the postsynaptic neurons by activating two major types of receptors: ionotropic (ion channels) and/or metabotropic (G-protein coupled) receptors. Depending on the nature of neurotransmitters, activation of the receptor drive opening or closing of the channels. Excess neurotransmitters in the synaptic cleft are cleared by astrocytes, reuptake by neurons or degraded at the synapses (Kaneko, 2000, Colovic et al., 2013) **[Figure 1-2]**. The neurotransmitter in the glia gets converted or degraded to an inactive form (Danbolt, 2001) and is transported back to the presynaptic terminal through the receptors **[Figure 1-2]**. The degradation products are recycled to generate functional neurotransmitters (Mahmoud et al., 2019, Perdan et al., 2009, Pines et al., 1992).

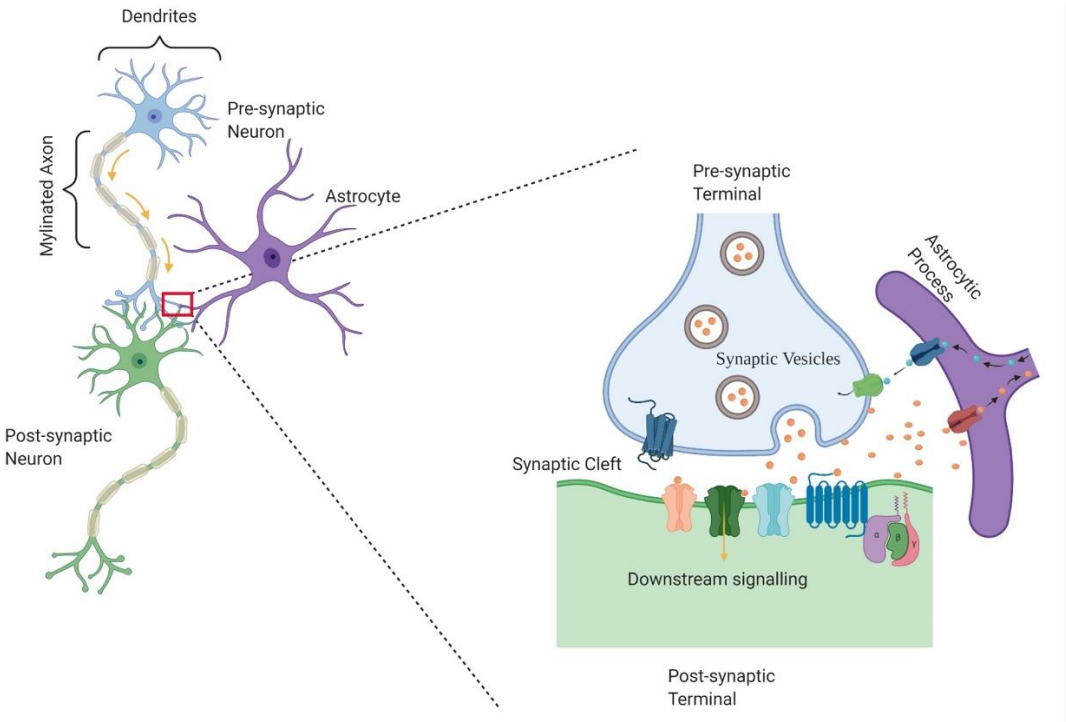

Figure 1-2: Synapse and synaptic transmission

Synapses are the junctions formed by the pre-synaptic membrane of one and the dendritic postsynaptic membrane of another neuron. The neuronal membranes are separated by a gap known as synaptic cleft. Action potential at the terminus of a neuron trigger the release of neurotransmitter from docked synaptic vesicles (Svs) into the synaptic cleft. The released neurotransmitter diffuses and bind to the receptors present at the pre and postsynaptic membrane. This trigger activation of the receptor resulting in influx of ions through the channel (ionotropic receptor) or activation of G-proteins (metabotropic receptor). If the postsynaptic receptor activation reaches values above a critical level action potential is generated in the postsynaptic neuron that further facilitate the information transfer through the network.

1.1.3 Neuronal transmission

1.1.3.1 Inhibitory neurotransmission

The major inhibitory neurotransmitter in the CNS is GABA (γ-aminobutyric acid) (Awapara et al., 1950, Basemore et al., 1957). The postsynaptic GABA receptors are classified into three types $GABA_AR$ $GABA_BR$ and $GABA_CR$. $GABA_A$ and $GABA_C$ are ionotropic receptors whereas $GABA_BR$ relay on metabotropic signaling (Chebib and Johnston, 1999, Kaupmann et al., 1998, Shen et al., 2021, Enz, 2001, Goetz et al., 2007, Mehta and Ticku, 1999, Enna, 2007). Binding of GABA to the postsynaptic $GABA_A$ and $GABA_C$ receptors leads to the opening of the channel and the influx of negatively charged Cl^- ions into the cytosol (Goetz et al., 2007, Enz, 2001, Stephenson, 1995). This hyper-polarizes the postsynaptic membrane and prevent the generation of action potentials.

$GABA_BR$ on the other hand, exerts its action on the postsynaptic membrane by activating K^+ channels and subsequent efflux of ions (Frangaj and Fan, 2018) or alternatively by inhibiting Ca^{2+} channels in presynaptic membrane (Bowery et al., 2002, Hyland and Cryan, 2010). The net potential generated due to the flow of negatively charged ions are known inhibitory postsynaptic potential (IPSPs) or inhibitory post synaptic currents (IPSCs) (Connors et al., 1988, Mehta and Ticku, 1999, Kaupmann et al., 1997).

1.1.3.2 Excitatory neurotransmission

The predominant excitatory transmitter in the mammalian CNS is glutamate (Broman et al., 2000). Release of glutamate from the presynaptic neuron activates pre-and postsynaptic receptors. Like GABA, glutamate can also signal via ionotropic and metabotropic receptors. Activation of ionotropic glutamate receptors induce influx of monovalent and/or divalent cations through the channel (ionotropic). The resulting influx of ions recorded in voltage-clamp electrophysiology (keeping the voltage constant) is observed as excitatory post synaptic currents (EPSCs) and in current clamp (keeping the current constant) is excitatory post synaptic potential (EPSPs) (Binder et al., 2009). Depolarization of the postsynaptic membrane trigger the generation of action potentials thereby facilitating information transfer to adjacent neuron/effector organ (Wisden and Seeburg, 1993b, Pin and Duvoisin, 1995).

1.1.4 Recording synaptic transmission

The vesicular release of neurotransmitters occurs in the form of discrete units called quanta (Fatt and Katz, 1952). The quantal release of neurotransmitter determines the amplitude of resultant EPSP or IPSPs in the postsynaptic neuron, which could potentially generate action potentials.

Electrophysiological techniques can measure the amplitude or frequency of the EPSPs or IPSPs in the postsynaptic neurons which corresponds to the number of synapses, the amount of neurotransmitter released from a presynaptic neuron and the number of receptors present at postsynaptic neuron. EPSPs/EPSCs generated experimentally by stimulating an axon is termed as evoked EPSPs/EPSCs and those without an experimental stimulation due to the spontaneous neurotransmitter release is mEPSPs or sEPSPs (Sutton et al., 2004, Streit and Luscher, 1992). mEPSPs are usually at sub threshold levels and hence fail to evoke action potentials.

1.2 Synaptic plasticity

One of the crucial and intriguing property of mammalian brain is its ability to undergo plasticity. The neural activity generated from various experiences can remodel the neural circuitry and function thereby influencing emotions, feelings, thoughts, and behavior are known as plasticity (Citri and Malenka, 2008, Nelson, 2000). Similarly, the capacity of a pre-existing synapse to undergo changes in their efficacy of synaptic transmission in an activity-dependent manner is termed as synaptic plasticity (Martin et al., 2000, Nair et al., 2021, Malenka, 2003, Takeuchi et al., 2014, Magee and Grienberger, 2020, Bliss et al., 2014, Mellor, 2006, Citri and Malenka, 2008, Rebola et al., 2010). Induction of synaptic plasticity is accompanied by both structural and functional changes of the synapses and is believed to form the basis for learning and memory formation (Takeuchi et al., 2014). Impairments in the synaptic plasticity is implicated in various neurodevelopment, neurodegenerative and neuropsychiatric disorders (Benarroch, 2018, Maggio and Vlachos, 2014). Synaptic plasticity is classified into Hebbian plasticity and homeostatic plasticity. Other forms of plasticity include short term potentiation (STP), early-LTP, late-LTP, short-term depression (Homo/Heterosynaptic), homeostatic scaling, spike time dependent plasticity (STDP) metaplasticity etc. (Lisman, 2017).

1.2.1 Hippocampus and synaptic plasticity

The hippocampus located in temporal lobe of CNS play a critical role in the formation of new memories, consolidation, and spatial navigation (rodents). Much of the studies about the role of hippocampus in memory formation is from case studies of patients with lesion in hippocampus, notably patient H.M (Scoville and Milner, 1957). Patients with trauma in temporal lobe is often associated with deficits in the formation of new memories (Squire, 2009). Studies with pharmacological blockade of hippocampus and controlled lesions hamper the learning or formation of spatial memories in rodents (Tsien et al., 1996, Martin et al., 2005). The putative role of hippocampus in formation and storage of new memories and the simple neural pathways to perform extracellular recordings enabled hippocampus a prime target to study plasticity (Neves et al., 2008).

The hippocampus receives information from entorhinal cortex primarily through dentate gyrus (DG). A tri-synaptic loop comprising DG-CA3-CA1 process the information from visual and associative cortical areas through entorhinal cortex (Urban et al., 2001, Henze et al., 2002). The sensory information form layer II neurons of entorhinal cortex are carried to DG granule cells by the axons forming Perforant pathway (PF). The granule cells, then project their axons to make connections with CA3 pyramidal cells known as mossy fibres (MF). The ipsilateral connections of CA3 pyramidal cells with CA1 is termed as the Schaffer collaterals (SC) and the contralateral connections are commissural fibres [**Figure 1-3]** (Amaral et al., 2007, Neves et al., 2008).

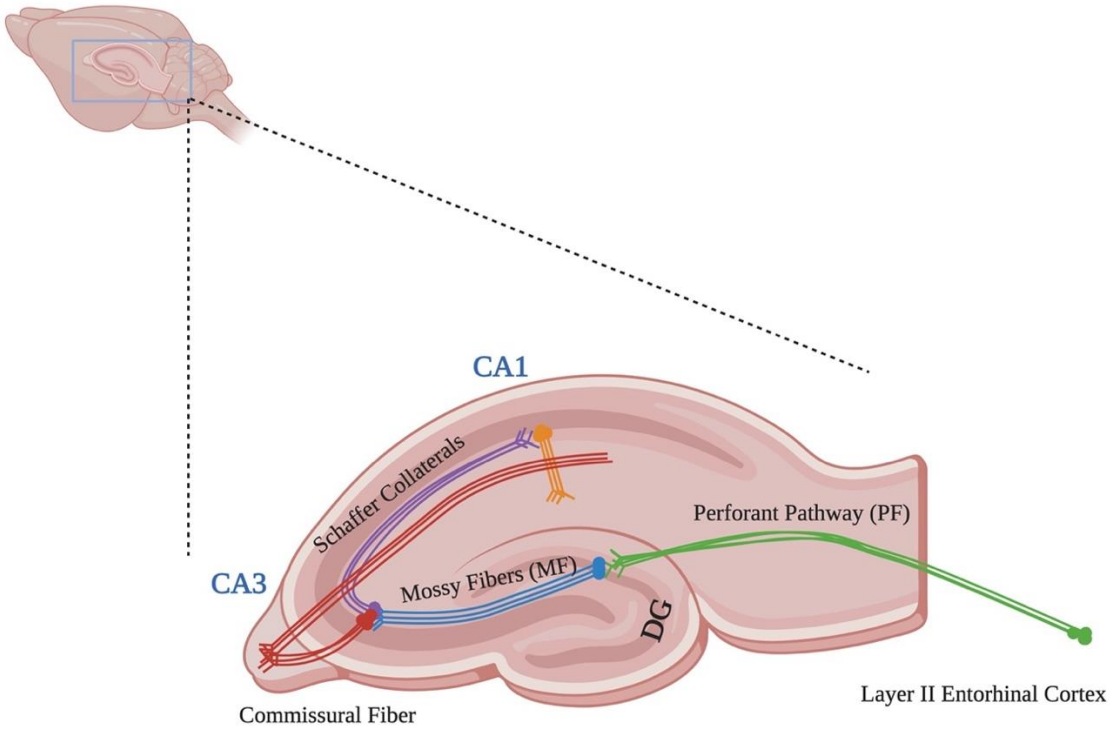

Figure 1-3: Anatomy of hippocampus with tri-synaptic pathway

Information from the layer II entorhinal cortex reach the granules cells of DG via Perforant pathway. The granules cells of DG make contacts with the proximal dendrites of CA3 pyramidal neurons forms the mossy fibre synapses (MF-CA3). The CA3 neurons makes ipsilateral connections to the CA1 pyramidal neurons by Schaffer collateral pathway (SC). The contralateral connections made by CA3 pyramidal neurons are termed commissural Fibres.

1.2.2 Hebbian plasticity

Hebbian plasticity is often considered as the mechanism by which information can be coded and retained in the neurons (Stent, 1973, Hebb, 2005). Activity- dependent long lasting changes leading to an increase - long-term potentiation (LTP) or a decrease - long-term depression (LTD) in the strength of synaptic transmission that is often maintained for minutes to months post induction (Martin et al., 2000, Auclair et al., 2000, Ho et al., 2011, Shepherd and Huganir, 2007, Choquet and Triller, 2013, Luthi et al., 1999, Morris and Frey, 1997, Malenka, 2003, Nicoll and Schmitz, 2005, Magee and Grienberger, 2020, Bliss et al., 2014, Neves et al., 2008, Citri and Malenka, 2008, Nelson, 2000). In a postsynaptic neuron, these long-lasting changes are characterized by an increase or decrease in EPSCs or IPSCs depending on the neuronal subtype.

Hebbian rules of plasticity includes i) Associativity ii) co-operativity and iii) input-specificity. Thus, Hebbian forms of plasticity is thought to underlie the fundamental brain processes such as learning and memory formation, opening of critical window of plasticity during development etc. (Fox and Stryker, 2017). Although synaptic plasticity in physiological conditions are evoked by varying patterns of neuronal activity, in *in vitro*/*ex-vivo* conditions, stimulations of neuronal fibres with specific frequency of stimulus or application of agonist can induce plasticity (Esposito and Pulvirenti, 1992).

1.2.2.1 Long-term potentiation

Persistent and long-lasting enhancement of synaptic efficacy is termed as LTP. *In vitro* induction of LTP in hippocampus and neo-cortex, is achieved by a brief high frequency stimulation (HFS) which usually stable for minutes to months (Bliss and Lømo, 1973). LTP studies were widely conducted in CA1 synapses of hippocampus which require activation of NMDARs and follows the Hebbian rules (Kauer et al., 1988). However, there are mechanistically distinct forms of LTP at different synapses. LTP at MF-CA3 synapses is found to be independent on NMDAR activation whereas Perforant and Schaffer collateral commissural (SCC) pathways requires NMDAR activation (Bliss et al., 2014). Modification of channel properties and rapid insertion of AMPARs into the postsynaptic membrane is often considered as a characteristic feature of LTP (Robert C.Malenka, 2004, Diering et al., 2018, Makino and Malinow, 2009, Malinow and Malenka, 2002, Henley and Wilkinson, 2013, Bredt and Nicoll, 2003, Huganir and Nicoll, 2013, Ho et al., 2011, Shepherd and Huganir, 2007).

1.2.2.2 Long-term depression

LTD is characterized by a persistent and long-lasting decrease in the efficiency of synaptic transmission. In *in vitro* conditions LTD is triggered by agonist dependent or synaptic activation of glutamate receptors by prolonged low frequency stimulations (LFS) (Malinow and Malenka, 2002, Malenka, 2003, Martin et al., 2000, Bliss et al., 2014). Consistent with LTP, LTD is also linked primarily with changes in the surface expression of AMPARs and are dependent or independent on NMDAR activation (Malinow and Malenka, 2002, Lee et al., 2005, Huber et al., 2001, Dore and Malinow, 2021, Kemp et al., 2000).

1.2.2.3 NMDAR dependent LTP

Activation of postsynaptic NMDARs by activity induced depolarization leads to opening of the channel (Lynch et al., 1983, Malenka et al., 1988). This trigger influx of Ca^{2+} through the channel and subsequent activation of calcium sensing protein calcium/calmodulin dependent kinase II (CaMKII) (Giese et al., 1998c, Lledo et al., 1995). Activation of CaMKII involves autophosphorylation of the kinase itself along with the phosphorylation and activation of target proteins such as AMPARs, Rho GTPases (RhoA, Cdc 42), PSD95 etc. CaMK ll dependent phosphorylation of PSD proteins creates slots for accommodation of AMPARs and is critical for remodeling of the synapse (Hell, 2014, Sanhueza and Lisman, 2013). Rapid polymerization of F-actin and an activity-dependent translocation of PSD proteins including CaMKII, cofilin, profilin, actin, Arp 2/3 are required for maintenance and stabilization of structural changes associated with late-LTP (Bosch et al., 2014, Reymann and Frey, 2007).

AMPAR insertion can be via exocytosis of native receptors by recycling endosomes or by lateral diffusion into the PSD (Pickard et al., 2001, Makino and Malinow, 2009). Phosphorylation of C-terminal AMPARs at GluA1 S831 and S845 enhances insertion of AMPARs and increases channel conductance (Diering et al., 2018, Roche et al., 1996, Diering et al., 2016, Lee HK, 2003, Esteban et al., 2003). Phosphorylation and de-phosphorylation of AMPAR interacting protein stargazin also regulate the induction of NMDAR dependent LTP/LTD (Tomita et al., 2005). Experimental conditions to evoke LTP involves stimulation of synapses with HFS or pairing of LFS with a depolarizing voltage (Citri and Malenka, 2008). Alternatively, agonist-induced (chem-LTP) can be achieved by bath application of glycine (Lu et al., 2001).

1.2.2.4 NMDAR dependent LTD

In conventional LTD pathway, activation of NMDARs agonist or electrical stimulation leads to moderate rise in Ca^{2+} concentration causing de-phosphorylation of CaMKII and activation of calcium/calcineurin dependent phosphatase 1 (PP2B) (Lisman and Zhabotinsky, 2001, RM Mulkey, 1993, Neveu and Zucker, 1996, Lisman, 1989, Rebola et al., 2010). Dephosphorylation of GluA1 S831/ S845 and phosphorylation of GluA2 S880 disrupts the interaction with PSD95 proteins such as PICK1, GRIP1 etc. leading to destabilization of the receptors at the synapses (Rocca et al., 2008, Hanley, 2018,

Chung et al., 2000). This together with interaction of calcineurin and dynamin promotes the internalization of de-phosphorylated proteins and receptors from the synapses (Man et al., 2000, Lai et al., 1999, Slepnev et al., 1998). Removal of functional AMPARs from the synapses by endocytosis or lateral diffusion results in decrease in the synaptic efficacy **[Figure 1-4]** (Malinow and Malenka, 2002, Henley and Wilkinson, 2013, Bats et al., 2007).

In *in vitro* conditions de-phosphorylation of GluA1 S845 is crucial for the induction of NMDAR-LTD. Transgenic mice expressing non-phosphorylatable form of GluA1 S845 and GluA2 S880 exhibit deficits in hippocampal LTD (Lee et al., 2010, Diering et al., 2018, Steinberg et al., 2006).

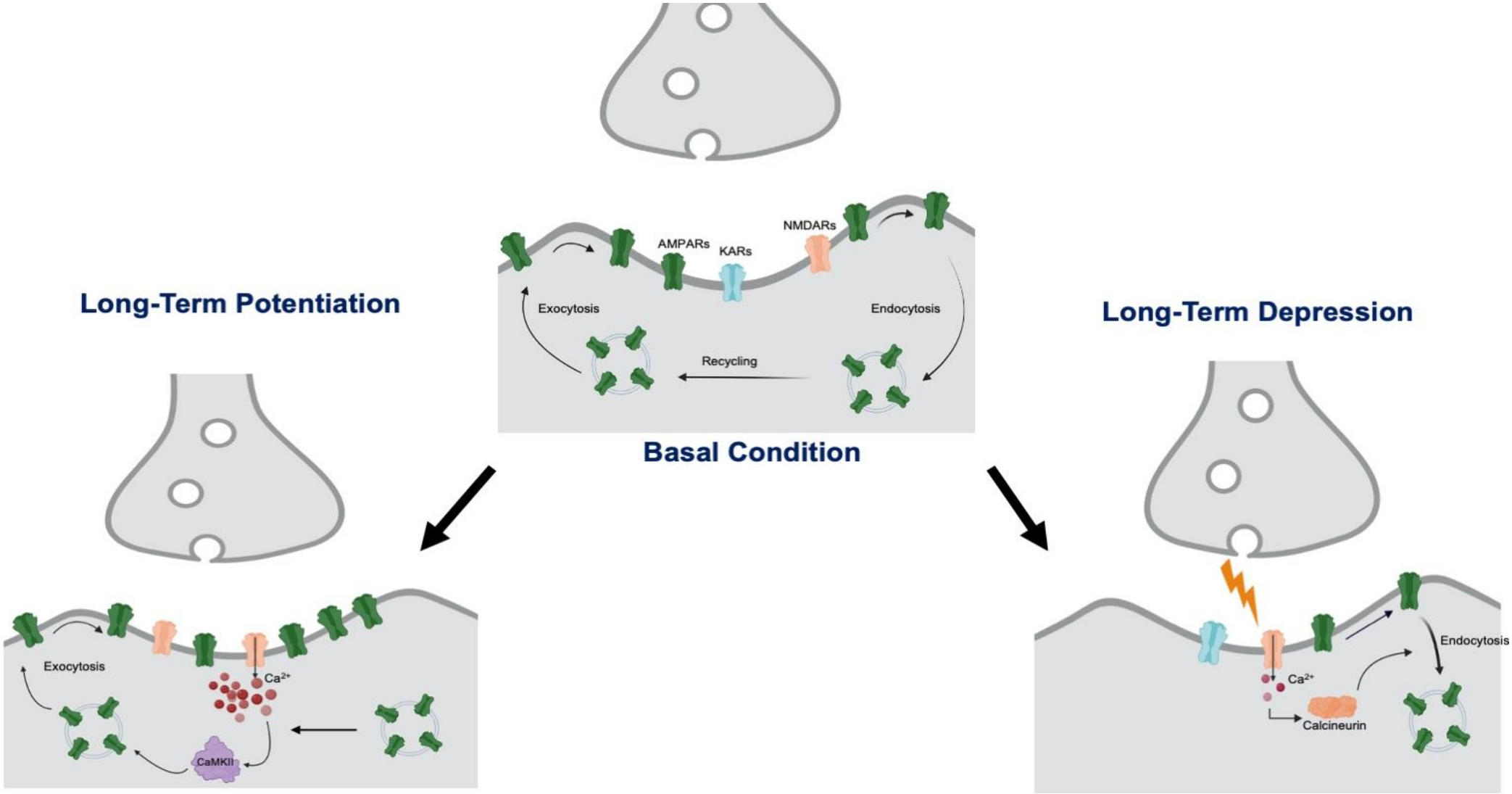

Figure 1-4: Synaptic plasticity- A classical model

A classical NMDAR dependent LTP and LTD. Activation of NMDARs followed by HFS stimulation leads to enhanced Ca^{2+} influx through the channel. This trigger activation of CaMKII and various protein kinases subsequently leading to exocytosis of Internalized AMPARs. LTD is characterized by persistent LFS which leads to activation of NMDARs and downstream activation of calcineurin leading to de-phosphorylation and endocytosis of AMPA.

1.2.3 NMDAR Independent forms of Plasticity

Although the best characterized form of synaptic plasticity in the CNS is dependent on activation of NMDARs, various synapses in the CNS exhibit NMDAR independent forms of pre and postsynaptic plasticity.

1.2.3.1 NMDAR independent LTP

CA1 synapses show NMDAR independent, group I mGluR dependent LTP in CA1 synapses. This form of LTP is dependent on fragile x mental retardation protein (FMRP), Arc signaling and protein synthesis (Fidzinski et al., 2008, Wang et al., 2016). Further, KAR-mediated LTP in CA1 is also independent of NMDAR activation. (Petrovic et al., 2017c).

1.2.3.2 NMDAR independent forms of LTD

Analogous to LTP, NMDAR independent forms of LTD are featured at various synapse. Paired LFS or bath application of group I mGluR agonist DHPG induces mGluR dependent LTD at CA1 synapses and is abolished by application of mGluR specific antagonist MCPG (Massey and Bashir, 2007, Palmer et al., 1997, Bashir et al., 1993). Spike in dendritic Ca^{2+} levels by the activation of VGCC or release from the intracellular stores by PKC also facilitates LTD induction (Anwyl, 2006, Wang et al., 1997). Apart from glutamate, neurotransmitters such as acetylcholine (Massey et al., 2001), nor-adrenaline, serotonin, dopamine (Huang et al., 2004) are associated with LTD induction (Gu, 2002).

Endocannabinoid receptors (CB1R) mediated LTD is prevalent in neostriatum (Ronesi et al., 2004), pre frontal cortex (Auclair et al., 2000), nucleus accumbens (Robbe et al., 2002), visual cortex (Sjostrom et al., 2003) etc. Release of endocannabinoids from postsynaptic sites by elevated intracellular Ca^{2} facilitate their binding with presynaptic CB1R. This depress the presynaptic neurotransmitter release and induce a novel form of LTD (Gerdeman et al., 2002). Furthermore, KAR dependent increase in postsynaptic Ca^{2+} concentration induces $EPSC_{KA}$ LTD in layer II/III of perirhinal cortex (Park et al., 2006, Valbuena and Lerma, 2019, Nair et al., 2021).

1.2.3.3 Presynaptic LTP

Persistent increase in the release of glutamate from the presynaptic terminals can induce presynaptic forms of LTP (Nicoll and Malenka, 1995). Activation of VGCC at presynaptic terminals by HFS leads to influx of Ca^{2+} ions and subsequent release of docked Svs containing neurotransmitters thereby inducing LTP (Zalutsky and Nicoll, 1990).

Alternatively, activation of various protein kinases at the presynaptic terminals can also trigger LTP (Arancio et al., 2001). Studies on knockout mice and pharmacological agents indicated a rise in Ca^{2+} ions in the presynaptic terminals activates PKA through CaMKII dependent-adenylate cyclase (AC) (Zalutsky and Nicoll, 1990). PKA dependent phosphorylation of presynaptic proteins by trigger persistent surge in the neurotransmitter release. (Nicoll and Schmitz, 2005). Furthermore, mice lacking presynaptic vesicle proteins Rab3A (GTPase) or its interacting protein Rim1α are devoid of presynaptic and MF-CA3 LTP (Schoch et al., 2002). In addition, activation of presynaptic KARs also induces various forms of short and long-lasting facilitation of neurotransmitter release in MF-CA3 pathway (Contractor et al., 2001).

1.2.4 Homeostatic plasticity

While Hebbian plasticity is widely considered as the mechanism for coding and storage of information, homeostatic plasticity is the mechanism by which a neuron senses its own activity and returns to its basal state following a perturbation (Turrigiano and Nelson, 2004, Turrigiano, 2012). Counterbalancing this perturbation is crucial in the maintenance and establishment of a functional neuronal circuitry (Fox and Stryker, 2017). Synaptic scaling is one of the mechanisms by which neurons regulate their own firing rate by altering the number of glutamate receptors at the synapses to bring the excitability to a physiological range **[Figure 1-5]** (Davis and Bezprozvanny, 2001, Turrigiano and Nelson, 2004). Moreover, studies on a diverse model organisms have established interconnections between homeostatic plasticity and neurological disorders (Lee and Kirkwood, 2019).

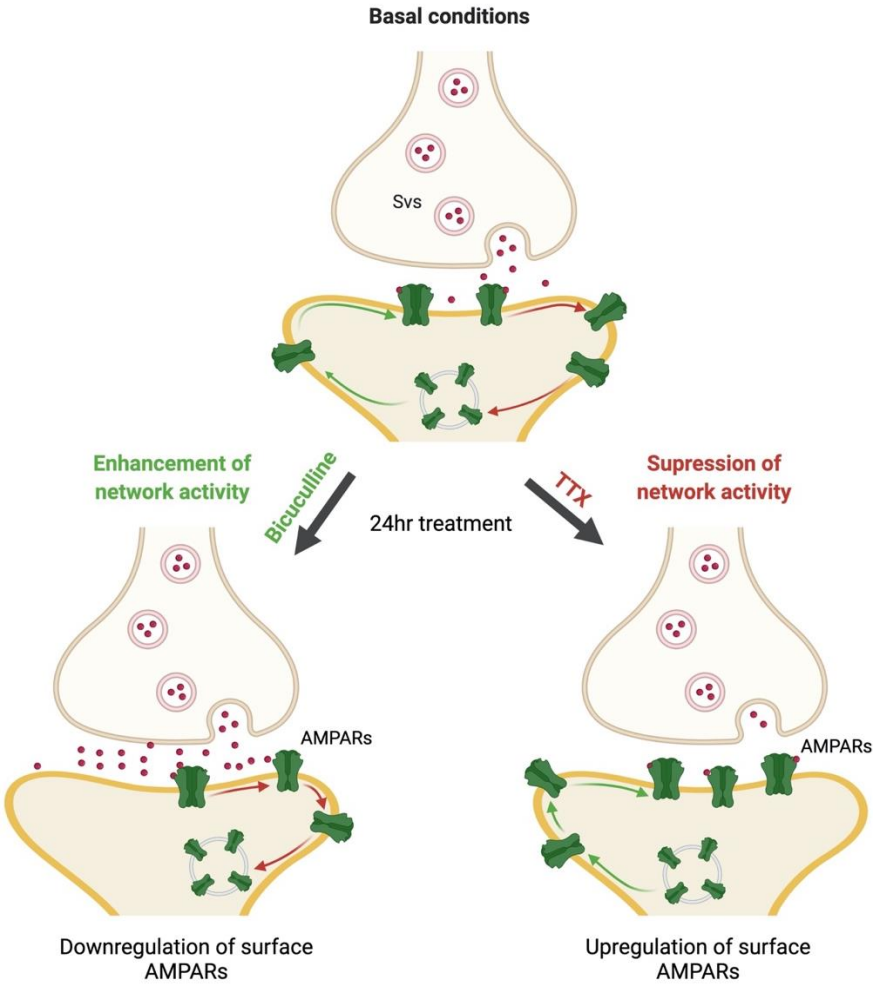

Figure 1-5 :Homeostatic scaling mechanism of glutamate receptors

Chronic bidirectional modulation of the network activity can be induced by bicuculline ($GABA_AR$ blocker) or TTX (Na^+ channel blocker) treatment. An upsurge in network activity by 24hr bicuculline treatment downregulate surface AMPARs. Contra wise, diminution in network activity by 24hr TTX treatment upscale surface AMPARs. This activity dependent modulation in the surface AMPARs counterbalance the perturbations in the network activity.

1.2.4.1 AMPARs

Synaptic scaling was first demonstrated in cortical cultures upon chronic treatment with tetrodotoxin (TTX) that blocks voltage gated sodium channels (O'Brien et al., 1998). Chronic TTX treatment increased the amplitudes of AMPAR mediated mEPSCs (Narahashi, 2008, Soares et al., 2013, O'Brien et al., 1998) whilst bicuculline ($GABA_AR$ blocker) decreased AMPAR mEPSCs (Gina G. Turrigiano, 1998, Wierenga et al., 2005). In *in vitro* conditions, glutamergic neurons respond to disturbances in the network activity by bi-directionally tuning the number of synaptic AMPARs is termed

as synaptic scaling of AMPARs (Moulin et al., 2020, Gina G. Turrigiano, 1998, Huganir and Nicoll, 2013). Consistent with the changes in the number of synaptic AMPARs, the phosphorylation of GluA1 S845 is also enhanced with TTX stimulation and decreased with bicuculline (Diering et al., 2014, Diering et al., 2016, Goel et al., 2011). As summarized above, this regulation helps the neurons to contain the strength of information encoded corresponding to an LTP or LTD induction (Chater and Goda, 2014, Chowdhury and Hell, 2018, Diering et al., 2014).

GluA1 and GluA2 subunits of AMPARs change in a coordinated fashion during scaling (Watt et al., 2000, Thiagarajan et al., 2005, Groth et al., 2011, Soares et al., 2013). Studies on scaling of AMPARs in visual cortex upon TTX treatment revealed that GluA2 containing AMPARs predominate over GluA1 during up-scaling, which is blocked by shRNA induced knockdown of GluA2 (Gainey et al., 2009, Frank et al., 2006, Goel et al., 2006, Goel et al., 2011). Further, TTX mediated scaling up of AMPARs prior to LTP induction enhances subsequent LTP due to the un-silencing of previously silent synapses (Arendt et al., 2013).

1.2.5 Protein kinases and synaptic plasticity

Protein kinases are decisive in induction and maintenance of various forms of plasticity in the mammalian and invertebrate brains. Modulation of the kinase activity by pharmacological and genetic tools have underpinned their role in induction and maintenance of synaptic plasticity (Mayford, 2007, Routtenberg, 1986, Lisman, 1985, Husi et al., 2000, Lee, 2006).

1.2.5.1 Calcium/calmodulin dependent Kinase II (CaMKII)

CaMKII, extensively located at the postsynaptic sites is considered as leadoff molecule to initiate LTP induction (Kennedy et al., 1983, Malenka et al., 1989, Malinow et al., 1989). Ca^{2+} influx through activated NMDARs and subsequent autophosphorylation of CaMKII at T286 preserve CaMKII in persistently active state (Miller and Kennedy, 1986, Barria et al., 1997, Giese et al., 1998a). Although initial activation of CaMKII is dependent on Ca^{2+}, auto phosphorylated state of CaMKII is independent on postsynaptic Ca^{2+} levels for its subsequent activity. This couples the transient Ca^{2+} signal to a long-lasting biochemical signal (Lisman, 1994, Lai et al., 1986). Mutation of CaMKII phosphorylation site at T286 to a non-phosphorylatable

form impaired LTP at CA1 synapses. Moreover, animals expressing non-phosphorylatable form of CaMKII failed to perform in behavioral tasks (Cho et al., 1998, Giese et al., 1998b, Chang et al., 2017). However, interestingly application of CaMKII specific inhibitors post LTP induction failed to block preformed LTP/memory (Chen et al., 2001). This emphasis that CaMKII is critical in induction of early stages of LTP (E-LTP) but not for maintenance (Irvine et al., 2005, Chen et al., 2001).

1.2.5.2 PKMζ

Brain specific form of protein kinase C, (PKMζ) is embroiled in maintenance of L-LTP (late-LTP). Lack of inhibitory domain in PKMζ keeps the kinase in a constitutively active form (Sacktor et al., 1993, Hernandez et al., 2003). Impeding the PKMζ activity by *in vivo* application of cell permeant inhibitor reversed L-LTP and newly formed memories in rodents (Pastalkova et al., 2006, Ling et al., 2002). Activation of CaMKII by NMDARs enhances the synthesis of PKMζ (Boehm et al., 2006). Collectively, studies on CaMKII and PKMζ suggests that CaMKII and PKMζ are important for different phases of memory formation with former in induction (E-LTP) while latter in maintenance of LTP (Mayford, 2007, Hrabetova and Sacktor, 1996).

1.2.5.3 Protein kinase A (PKA)

PKA is significant in the induction of both LTP and LTD. Intrinsically, PKA is activated by intracellular cAMP produced as a resultant of Ca^{2+} /G-protein dependent activation of adenylate cyclase by iGluRs (Eliot et al., 1989). And/or mGluRs (Tang and Gilman, 1991, Lee et al., 2000, Roberson and Sweatt, 1996). G-protein coupled receptor can bidirectionally regulate cAMP thereby emanating or inhibiting the downstream signaling (Tang and Gilman, 1991, Otmakhova et al., 2000). Interaction of R II (type ii regulatory subunit) subunit of PKA with several anchoring proteins such as A kinase anchoring protein (AKAP) facilitate targeting of PKA into the vicinity of glutamate receptors (Hausken et al., 1994, Brandon et al., 1995). Notably, the interaction of AKAP 79/150 with PKA facilitate the phosphorylation of GluA1 S845 (Colledge et al., 2000, Esteban et al., 2003, Diering et al., 2014, Lee et al., 2010). Disrupting PKA-AKAP 79/150 interaction with pharmacological tools decrease the amplitude of AMPAR EPSCs and is reversed by the intracellular application of catalytically active PKA (Rosenmund et al., 1994). Bath application of PKA specific activator forskolin

enhanced glutamate mediated excitatory response in cultured hippocampal neurons and is occluded by PKA specific inhibitors. Furthermore, bath application of PKA inhibitors failed to maintain LTP at Schaffer collateral CA1 pathway (Greengard et al., 1991, Matthies and Reymann, 1993). Overall PKA activity in chorus with PKMζ is key in maintaining late LTP (Matthies and Reymann, 1993, Huang and Kandel, 1994). Interaction of PKA by various vesicle proteins such as synaptic vesicle protein 2 (SV2), Rim1 etc. induce presynaptic forms of LTP by enhancing presynaptic release (Park et al., 2014, Falcon-Moya and Rodriguez-Moreno, 2021).

1.2.6 Synaptic plasticity and neurological disorders

Aberrant synaptic plasticity is often characterized in many forms of neurological disorders such as Alzheimer's Disease (AD), autism spectrum disorders (ASD), mood disorders, cognitive decline etc. Understanding impairments of synaptic plasticity in disease models therefore posits critical towards development of novel therapeutics (Bliss et al., 2014, Klyubin et al., 2014, Henley et al., 2021, Lee et al., 2014, Clayton and George, 1999).

1.2.6.1 Alzheimer's disease

AD is a chronic and incurable form of dementia caused by progressive degeneration of neurons (Masters et al., 2015, Qiu et al., 2009, Du et al., 2021, Aranda et al., 2021). Histopathology of postmortem brains revealed the presence of insoluble amyloid plaques (Aβ oligomer) or neurofibrillary tangles (Tau) along with brain atrophy and synaptic loss (Dickson, 1997, Kinney et al., 2018, Selkoe and Hardy, 2016, Hsiao et al., 1996). This marks the progressive deficits in cognition, memory and language abilities of AD patients (De Strooper and Karran, 2016, Hardy and Selkoe, 2002), Studies on rodent models reveled that infusion of Aβ oligomers into CA3-CA1 synapses block NMDAR dependent LTP as well as VGCC activation (Klyubin et al., 2014, Viana da Silva et al., 2016).

Glycogen synthase kinase (GSK 3) dependent phosphorylation of Tau proteins are required for NMDAR-LTD. Mice lacking Tau proteins failed to evoke NMDAR dependent LTD suggesting the importance of Tau in LTD induction (Jin et al., 2015, Llorens-Martin et al., 2014). Taken together, aberrant Aβ oligomers and

hyperphosphorylation of Tau tangles that impede the basal synaptic function is a hallmark of AD pathology (Santacruz et al., 2005, Hsiao et al., 1996, Citron, 2010).

1.2.6.2 Autism spectrum disorders (ASD)

ASD is a multifactorial neurodevelopment disorder with individuals having deficits in social communication skills, social behaviors, nonverbal interactions, repetitive behaviors, and restricted interests (Lord et al., 2020, Kanner, 1968, Khan et al., 2012, Lord et al., 2018). Impairments in various synapse and synapse associated proteins are identified as prime targets in neuropathology of ASD (Zoghbi and Bear, 2012, Zoghbi, 2003, Piochon et al., 2016).

Shank family of postsynaptic scaffolding proteins act as an intermediary to link NMDARs and mGlu5 receptors to PSD95 (Yoo et al., 2013). Although the exact mechanism remains unclear, mice deficient in Shank 3 show impairments in NMDAR-LTP and is depicted in patients with ASD (Yoo et al., 2013, Uchino et al., 2006, Peter et al., 2016). Another theory on the pathology of ASD implies deregulation of LTD (Hansel, 2019). Impairments in LTD induction and subsequent aberrant synaptic pruning (elimination of dysfunctional synapses) during early stages of brain development correlates with autistic behaviors (Ramiro-Cortes et al., 2014, Park et al., 2016a, Hansel, 2019).

1.2.6.3 Cognitive disabilities

OPHIN1, a Rho-GTPase activating protein (RhoGAP) is associated with cognitive disabilities (Zoghbi and Bear, 2012). Mice deficient in *Ophin1* gene show impaired PKA signaling and PKA dependent presynaptic plasticity in hippocampus and amygdala (Bliss et al., 2014, Myeku et al., 2016). Inhibiting the activity of PKA with ROCK/PKA kinase inhibitor rescued the learning disabilities in mice (Khelfaoui et al., 2013, Buffington et al., 2014, Xu et al., 2021).

1.3 Glutamate receptors

Glutamate released from the presynaptic terminals bind to its receptors present at the pre and postsynaptic sites to trigger the downstream signalling pathways **[Figure 1-6]** (Pinheiro and Mulle, 2008, Kellner et al., 2021, De Blasi et al., 2001). Depending upon the mode of signalling, glutamate receptors are classified into:

- Metabotropic glutamate receptors (mGluRs)
- Ionotropic glutamate receptors (iGluRs)

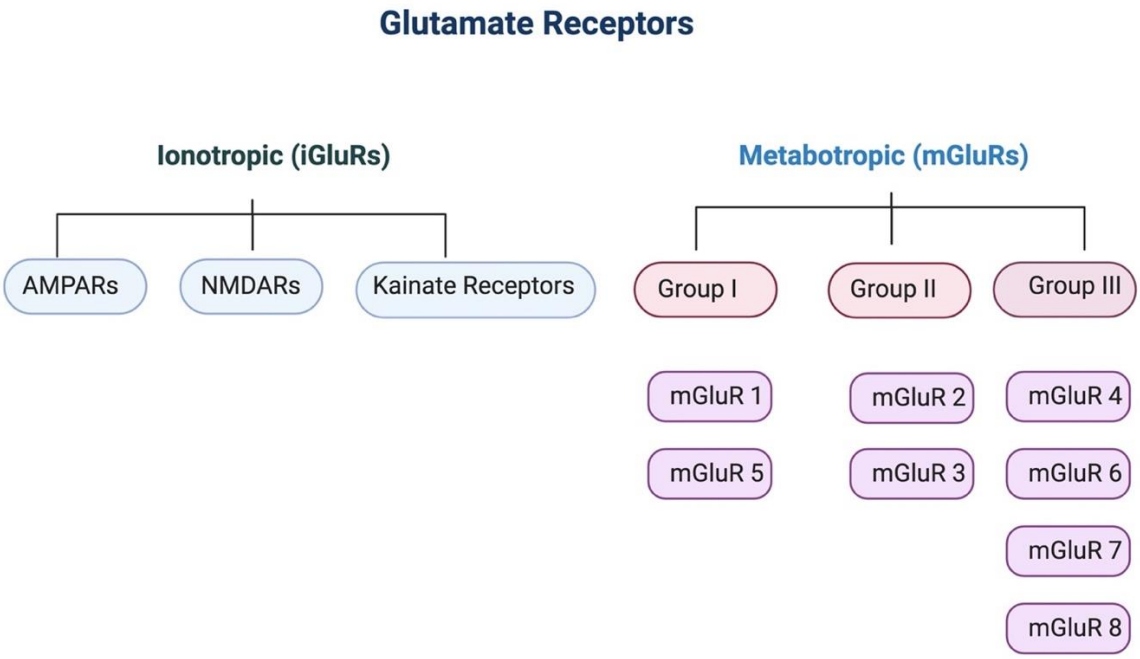

Figure 1-6: Classification of glutamate receptors

Glutamate receptors are classified into iGluRs and mGluRs depending on their mode of signaling. Glutamate binding to iGluRs opens the channel leading to influx of ions whereas mGluRs. This is often associated with depolarization of postsynaptic membrane, activation of various proteins etc. In contrary to this, mGluRs signal via G-protein dependent second messengers to trigger the signaling cascade.

iGluRs are ion channels formed by tetrameric assemblies of various subunits and are activated by the binding of glutamate to the extracellular ligand-binding domain (LBD) **[Figure 1-7 A]**. (Dingledine et al., 1999, Hollmann et al., 1989, Wo and Oswald, 1995, Lodge, 2009). Agonist binding to the channel alter the confirmation of the receptor from closed to open state and subsequent influx of mono and/or divalent cations (Wood et al., 1995). This underpins the reversal of the resting membrane potential and activation of downstream signaling cascade (Traynelis et al., 2010, Tichelaar et al., 2004).

Nevertheless, glutamate binding to mGluRs signal via G-protein dependent mechanism that leads to the activation or inactivation of adenylate cyclase and phospholipase C (PLC) **[Figure 1-7 B]**. (Kew and Kemp, 2005, Vaidya et al., 2013, Sugiyama et al., 1989, Willard and Koochekpour, 2013).Regardless of the signaling

mechanism, mGluRs can also depolarize the membrane and evoke various synaptic response such as LTP, LTD etc. (O'Hara et al., 1993, Paas et al., 1996, Kunishima et al., 2000).

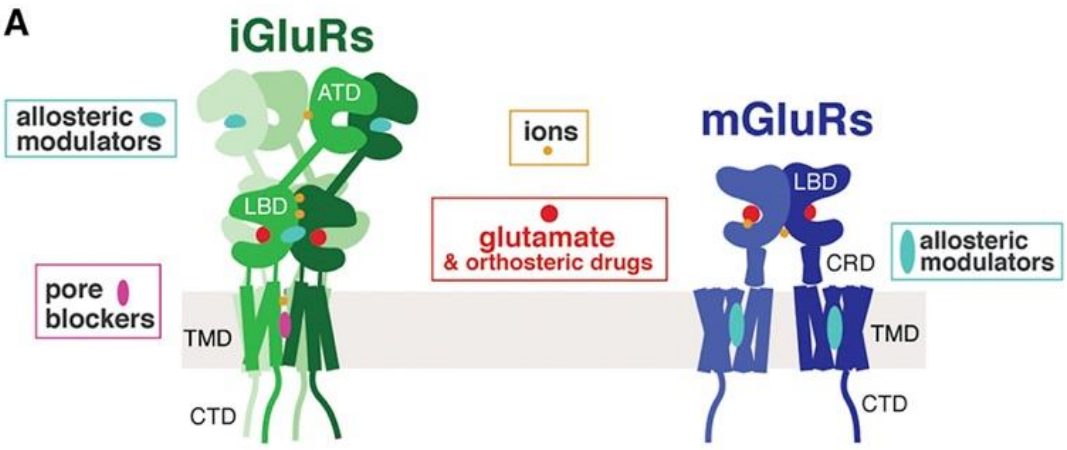

Figure 1-7: Structural topology of glutamate receptors

Structural organisation of iGluRs (green) and mGluRs(blue). Glutamate and other agonists bind to the inter lobe cleft formed in the ligand binding domain (LBD). Adapted from (Reiner and Levitz, 2018).

1.4 Metabotropic glutamate receptors

mGluRs belong to the family of GPCRs (G-protein coupled receptors) and share structural similarities with other GPCR superfamily of proteins (Kunishima et al., 2000, Jingami et al., 2003). The receptor consists of a large N-terminal domain followed by a heptahelical membrane-spanning domain and a C-terminal domain that serves as the prime site for G-protein coupling **[Figure 1-7 B]**. However, unlike other members, mGluRs are constitutive dimers and are key in regulating neuronal excitability and synaptic transmission (Niswender and Conn, 2010, Tsuji et al., 2000). Based on their sequence homology, pharmacology and downstream signaling mechanisms, mGluRs are sub-classified into three groups (Group I, Group II and Group III) encoded by eight genes **[Figure 1-6]** (Spooren et al., 2003, Niswender and Conn, 2010, Nakanishi et al., 1994).

1.4.1 Classification of mGluRs

1.4.1.1 Group I

mGluR1 and mGluR5 belongs classified under group I mGluRs are key players in mediating glutamate induced fast excitatory neuronal transmission, LTP and LTD (Aiba et al., 1994, Lu et al., 1997).

Activated group I mGluRs signal via G_q dependent PLC signaling cascade leading to the production of inositol 1,4,5 triphosphate (IP3) and diacyl glycerol (DAG) (Hermans and Challiss, 2001). This stimulates release of Ca^{2+} from the internal stores and influx from the extracellular sites leading to a rise in intracellular Ca^{2+} levels. In addition to G_q, studies on heterologous systems illustrated the ability of the receptors to activate G_s and $G_{i/o}$ (Aramori and Nakanishi, 1992, Heuss et al., 1999, Upreti et al., 2013). Group I mGluRs, they are present on both pre and postsynaptic sites of glutamatergic, GABAergic neurons as well as in glial cells (Spampinato et al., 2018).

1.4.1.2 Group II

This group is composed of mGluR2 and mGluR3. Unlike other members, group II mGluRs are coupled to G_i proteins that signal by inhibiting AC and subsequent closure of various Ca^{2+} channels (Pin et al., 2003, Niswender and Conn, 2010, Klausnitzer and Manahan-Vaughan, 2008b). Inhibition of group II mGluRs by pharmacological antagonists revealed their role in neuropsychiatric disorders such as anxiety (Helton et al., 1998), schizophrenia (Moghaddam and Adams, 1998), drug addiction (Helton et al., 1997) etc. Group II mGluR specific agonist DCGIV suppress synaptic transmission in MF-CA3 synapses are excellent tool to confirm the activation of MF-CA3 synapses in electrophysiological recordings (Yoshino et al., 1996, Maccaferri et al., 1998, Manahan-Vaughan, 1998, Klausnitzer and Manahan-Vaughan, 2008b).

1.4.1.3 Group III

The subclass contains mGluR4, mGluR6, mGluR7 and mGluR8. This subclass of mGluRs are abundant in both pre and postsynaptic sites of neurons, retinal cells etc. (Pin et al., 2003, Schoepp et al., 1999). Studies on the heterologous system indicated their association with $G_{i/o}$-proteins. In neurons, the primary role of Group II mGluRs is to inhibit Ca^{2+} channels, adenylate cyclase and in activation of K^+ channels. Studies

on cultured cerebellar granule cells revealed the role of mGluR7 in inhibiting P/Q type Ca^{2+} channels and is dependent on metabotropic signaling via $\alpha_{i/o}$, $\beta\gamma$ and PLC (Perroy et al., 2000). Studies on rodents lacking mGluR4$^{-/-}$ and mGluR7$^{-/-}$ affirmed their role in generalized absence epilepsy (Snead et al., 2000) and impairments in motor activity (Pekhletski et al., 1996).

1.4.1.4 Presynaptic mGluRs

Group I mGluRs are usually present on postsynaptic neurons with an exception in lamprey spinal cord where the presynaptic receptors modulate vertebrae locomotion (Takahashi and Alford, 2002). Although the presynaptic activation of mGluRs in physiological conditions remains ambiguous, activated group II and III mGluRs by exogenous glutamate application inhibit presynaptic release (Anwyl, 1999, Schoepp, 2001).

Further, LFS stimulation of MF-CA3 synapses induces a presynaptic form of plasticity that is dependent on mGluR2. Application of group II antagonist or KD mGluR2 inhibit this form of plasticity (Kobayashi et al., 1996, Yokoi et al., 1996). Presynaptic mGuR7 acts as a bidirectional switch in inducing presynaptic forms of LTP and LTD in MF-striatum lucidum CA3 interneurons (Pelkey et al., 2005, Pelkey et al., 2006). This bidirectional modulation of signal is dependent on PKC. Interestingly, another theory prevalent on mGluR7 in inducing presynaptic LTD involves activation of calcium permeable AMPARs (CP-AMPARs) (Laezza et al., 1999). Retrograde signaling by postsynaptic CP-AMPARs, trigger the activation of presynaptic mGluR7 and regulate the neurotransmitter release by a feedback mechanism (Laezza et al., 1999, Pelkey et al., 2005). Thus, mGluR7 bidirectionally control neurotransmitter release by both feedforward and feedback mechanisms.

In summary, mGluRs regulate various synaptic functions that are critical in regulating neuronal network activity and their impairments are implicated in various neurological and neurodegenerative disorders (Pilc et al., 2008, Swanson et al., 2005, Conn et al., 2009, Lee et al., 2004).

1.5 Ionotropic glutamate receptors

iGluRs are ligand gated ion channels that mediate vast majority of fast excitatory neurotransmission in the CNS (Twomey and Sobolevsky, 2018, Lodge, 2009, Wo and

Oswald, 1995, Wood et al., 1995). Activated postsynaptic iGluRs can depolarize the membrane by influx of cations and/or trigger downstream signaling cascade for modulating various functions (Traynelis et al., 2010, Henley et al., 2021, Henley and Wilkinson, 2013, Ashby et al., 2008).

iGluRs at pre and postsynaptic sites perform distinct functions. Presynaptic iGluRs are central in determining synaptic functioning and network activity (Valbuena and Lerma, 2016, Nakanishi et al., 1994, Hollmann et al., 1989, Pinheiro and Mulle, 2008). iGluRs are tetrameric subunit receptors with each subunit containing an N-terminal domain, three membrane spanning transmembrane domains (TM I – TM III) a small re-entrant loop and a long C-terminal domain (Wo and Oswald, 1995, Sobolevsky, 2015, Wollmuth and Sobolevsky, 2004) **[Figure 1-8].**

Different subunits of iGluRs combine to form a tetrameric receptor constituting a functional ion channel with distinct properties (Kew and Kemp, 2005, Chen and Wyllie, 2006).

The major subtypes of iGluRs are.

- α-Amino-3-Hydroxy-5-Methyl-4-isoxazolepropionic acid receptor (AMPARs)
- N-Methyl-D-Aspartate Receptor (NMDARs)
- Kainate Receptors (KARs)

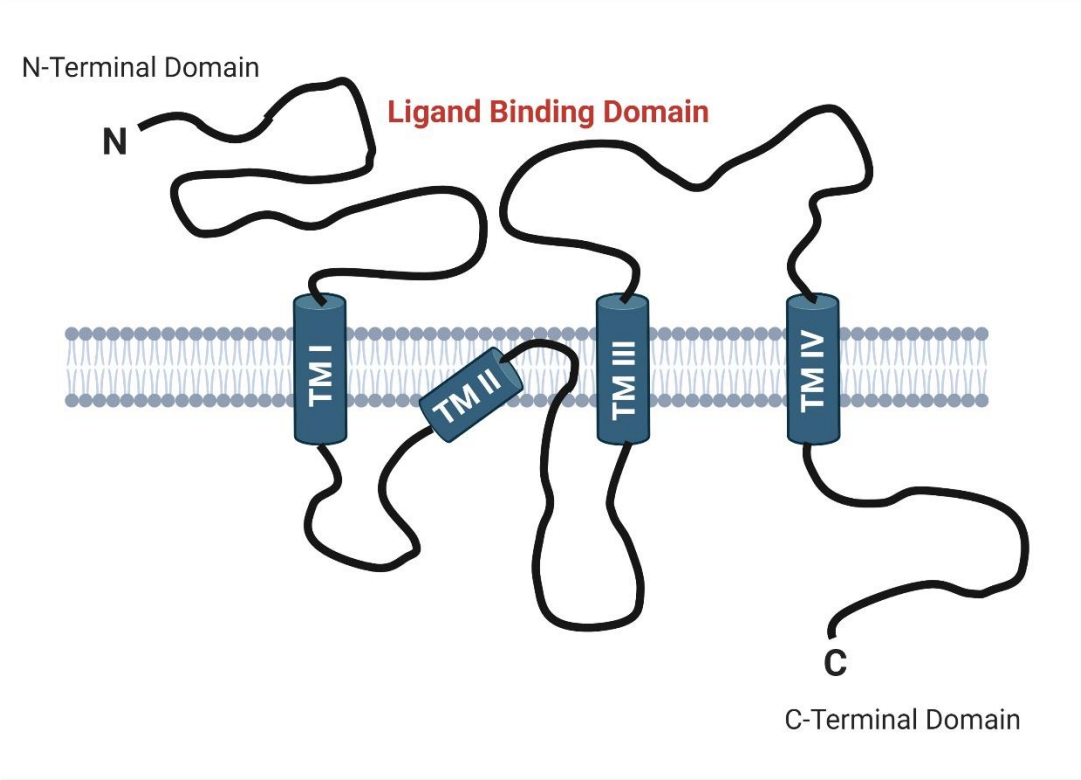

Figure 1-8: General structure and topology of ionotropic glutamate receptor subunits

Ionotropic glutamate receptors share similar membrane topology with an extracellular N-terminal region followed by three transmembrane region (TMI, TMIII and TMIV) a re-entrant loop (TMII) and a long C-terminal tail facing the cytosol which is key in various protein-protein interactions and post-translational modifications.

1.6 Presynaptic iGluRs

Biochemical studies on brain slices and isolated synaptosomes revealed the presence of functional presynaptic iGluRs including AMPARs, NMDARs and KARs subtypes (Valbuena and Lerma, 2016, Pinheiro and Mulle, 2008). The key function of presynaptic iGluRs is vested in the modulation of synaptic transmission and maintenance of network activity (Engelman and MacDermott, 2004, Chittajallu et al., 1999, Frerking and Nicoll, 2000, Huettner, 2003, Berretta and Jones, 1996).

Studies on different brain regions indicated that presynaptic GluN2B containing NMDARs activated by endogenous glutamate can increase the frequency of spontaneous EPSCs (Berretta and Jones, 1996, Li and Han, 2007, Corlew et al., 2007, Woodhall et al., 2001, Yang et al., 2006). Further, retrograde activation of presynaptic NMDARs enhances GABA release from interneurons to Purkinje cells. This depolarization-induced potentiation of inhibitory neurons alters the synaptic efficacy

(Duguid and Smart, 2004). Although activation of presynaptic NMDARs is still a matter of debate due to the persistence of voltage-dependent Mg^{2+} block, it is believed that GluN2C and GluN2D subunit-containing receptors confer lower sensitivity to the blockade of the channel by Mg^{2+} ions and hence facilitating activation (Pinheiro and Mulle, 2008, Berretta and Jones, 1996, Li and Han, 2007). In visual cortex, activation of presynaptic NMDARs induces LTD and is dependent on activation of presynaptic endocannabinoid receptor (Banerjee et al., 2016). Blockade of NMDARs with specific antagonist D-APV significantly reduce the release of glutamate from presynaptic terminals (Sjostrom et al., 2003).

Studies on rat auditory cortex revealed the presence of presynaptic AMPARs (Takago et al., 2005a). Presynaptic AMPARs via metabotropic pathway inhibit glutamate release by hindering the activation of VGCC (Takago et al., 2005b). Furthermore, in cerebellar interneurons, activation of presynaptic AMPARs impede GABA release from presynaptic terminals (Satake et al., 2000, Rusakov et al., 2005, Pinheiro and Mulle, 2008).

Presynaptic role of KARs is mentioned in section 1.7.3.3

1.7 Postsynaptic iGluRs

1.7.1 AMPARs

AMPARs are ion channels formed by tetrameric assemblies of four subunits GluA1-GluA4 (previous named GluR1-4) in varying combinations encoded by the genes *GRIA1-A4.* Functional receptors are formed by tetramerization of homo/hetero dimers (Wenthold et al., 1996, Herguedas et al., 2016, Hollmann M, 1994, Sobolevsky et al., 2009, Clayton et al., 2009, Tichelaar et al., 2004).

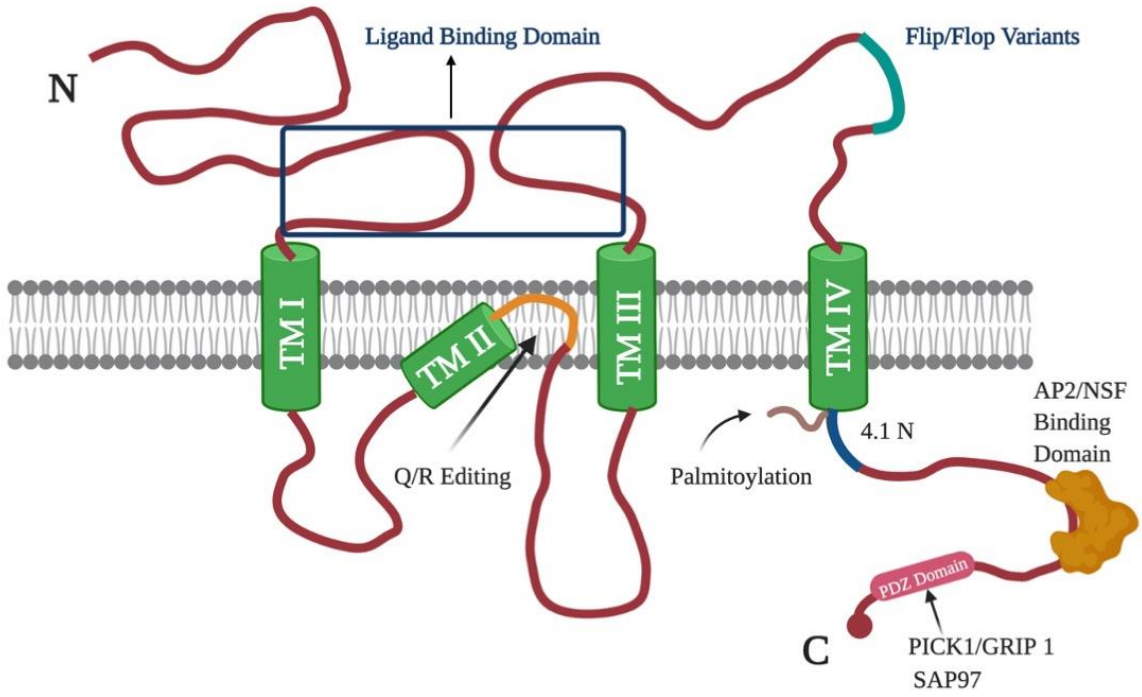

Figure 1-9: General structural topology and PTM of AMPAR subunits

AMPARs share structural similarities with other iGluRs. Glutamate binding site is formed in between the N terminal and transmembrane domains (TM) III and IV. Q/R editing of AMPAR subunits occurs at the TM II domain, alternative splicing at regions corresponding to TM IV and C-terminal attributes to the variation within the AMPAR subunits. A palmitoylation site adjacent to TM IV is key for anchoring of the receptors to the neuronal membrane. C-terminal tail serves as a hub for the binding of proteins including PSD 95, PICK1, GRIP1 that regulates the synaptic localization and stability of the receptor.

1.7.1.1 AMPAR Subunits

AMPARs subunits share conserved sequence homology in LBD and transmembrane domains (TM) (Sobolevsky et al., 2009, Midgett and Madden, 2008, Tichelaar et al., 2004). The difference arises in the C and N-terminal domains. Further, post-translational modifications (PTMs), RNA editing, alternate splicing adds to repertoire of AMPA receptor diversity (Shepherd and Huganir, 2007, Hollmann et al., 1994) **[Figure 1-9]**.

1.7.1.2 Classification of AMPAR subunits

Depending upon the length of the C-terminal tail and rate of insertion into the plasma membrane, AMPAR subunits are classified into two groups. Long-tailed (GluA1 and GluA4) and a short-tailed AMPARs (GluA2 and GluA3) **[Figure 1-10]** (Henley and

Wilkinson, 2013, Diering et al., 2018). Long-tailed AMPARs show an enhanced trafficking and hence the homo/heterodimers composed of long-tailed subunits predominate the short-tailed ones (Henley and Wilkinson, 2013). Despite this, GluA2 alternative splice form GluA2L also have a long C-terminal tail like GluA1 and A4. Conversely GluA4c, the alterative splice form of GluA4 is short-tailed (Gallo et al., 1992).

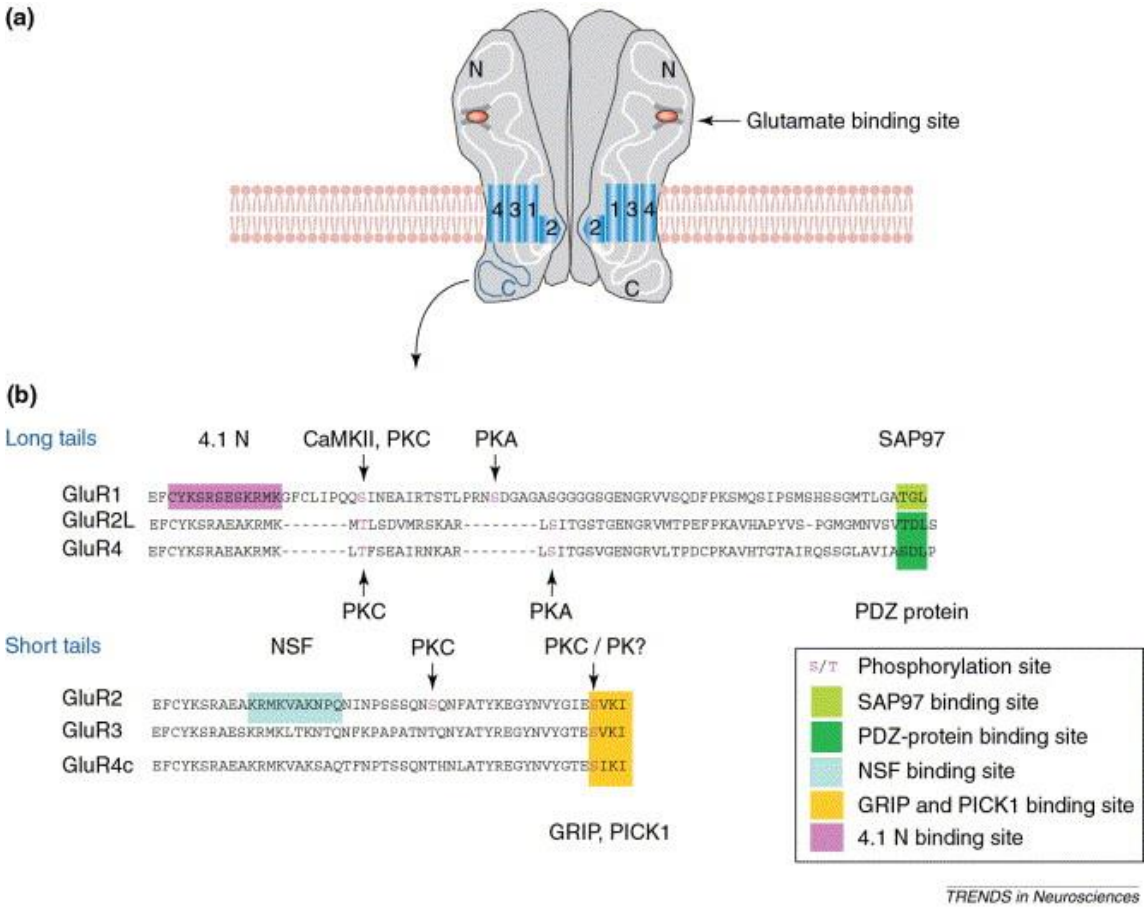

Figure 1-10: Classification of AMPAR subunits

AMPARs subunits are classified into long and short tailed AMPARs based on the length of their C-terminal tails. GluA1, GluA4 and splice variant GluA2L have long C-terminal tails whereas GluA2, GluA3 and splice variant GluA4c are short tailed. Various protein-protein interactions with the C-terminal domain regulate the surface expression and channel properties of the receptor. Adapted from (Song and Huganir, 2002)

1.7.1.3 Alternative splicing of AMPARs

Flip/flop form of alternative splicing produce two more variants for each subunit by altering the segment preceding TM IV (Sommer et al., 1990, Penn et al., 2012) **[Figure 1-19]**. The resultant splice variants show altered channel kinetics. Flop variants tend to desensitize more rapidly upon agonist binding compared to flip variants. Further, the flop variant is less sensitive to desensitization by pharmacological antagonists (Pei et al., 2009, Dingledine et al., 1999, Salussolia and Wollmuth, 2012). The above-mentioned alternative splicing of AMPARs can therefore regulate the channel kinetics and functions in response to perturbations in physiological conditions (Pei et al., 2009, Sommer et al., 1990, Lomeli et al., 1994, Mosbacher et al., 1994).

1.7.1.4 Post-Translational modifications of AMPARs

AMPARs subunits undergo various post-translational modification that regulate the channel kinetics, trafficking and surface expression of the receptor (Diering et al., 2018, Mah et al., 2005, Roche et al., 1996). The major post-translational modifications of AMPARs are listed below.

Table 1-1: Showing post-translational modifications of AMPA receptors and auxiliary subunits

Subunit	Type of PTM	Protein	Site of PTM	Significance	Cross reference
GluA1	Phosphorylation	PKA	S845	• Increases open channel probability/conductance. • Promotes LTP	(Diering et al., 2016, Diering et al., 2014, Lee et al., 2010, Roche et al., 1996)
GluA1	Phosphorylation	PKC/CaMKII	S831	• Increases single channel conductance. • Enhances synaptic retention	(Diering et al., 2016, Diering et al., 2014, Lee et al., 2010, Roche et al., 1996)
GluA1	Phosphorylation	PKC	S818	• LTP expression • Synaptic incorporation	(Boehm et al., 2006)
GluA1	Phosphorylation	PKC/p70S6	T840	• Regulate LTD induction post NMDAR activation	(Lee et al., 2007, Delgado et al., 2007)

GluA1	Phosphorylation	PAK3	S863	• Regulates GluA1 trafficking	(Hussain et al., 2015)
GluA1	Phosphorylation	CaMKII	567	• Trafficking • Synaptic incorporation	(Lu et al., 2010)
GluA1	Phosphorylation	Casein Kinase	579	• Surface expression	(Lussier et al., 2014)
GluA2	Phosphorylation	PKC	S863, S880	• Regulates clustering of AMPARs at excitatory synapses. • Promotes internalisation	(Matsuda et al., 1999, Chung et al., 2000)
GluA2	Phosphorylation	Src family of Tyrosine kinase	Y876	• Homeostatic strengthening of synapse • Enhances GRIP1 interaction	(Yong et al., 2020)
GluA4	Phosphorylation	PKA/PKC	S842	• Receptor trafficking	(Diering et al., 2018)
GluA1	Palmitoylation	Palmitoyl acyl transferase GODZ	C811	• Receptor trafficking and synaptic transmission	(Diering et al., 2018, Lin et al., 2009, Hayashi et al., 2005)
GluA2			C836		
GluA3			C841		
GluA4			C817		
GluA1	Ubiquitination	E3 ubiquitin ligase Nedd 4-1	K868	• Endocytosis and targeting to lysosomes	(Schwarz et al., 2010)

GluA2		E3 ubiquitin ligases	K870, K882	• Post-endocytic sorting and degradation of receptors	(Widagdo et al., 2015, Widagdo et al., 2017, Lussier et al., 2015)
GluA1	S-nitrosylation	Nitric oxide	C875	• Enhances GluA1 S831 Phosphorylation • Increases single channel conductance	(Selvakumar et al., 2013)
Auxiliary AMPAR subunits					
TARP γ-8 S227	Phosphorylation	CaMKII	S227, S281	• Enhances AMPAR mediated transmission. • Required for LTP induction	(Park et al., 2016b)
Stargazin	S-nitrosylation	Nitric oxide	C302	• Enhances binding to GluA1. • Increased surface expression of AMPARs	(Selvakumar et al., 2009, Selvakumar et al., 2014)

1.7.1.5 Protein-Protein interactions with AMPARs

Studies on heterologous expression systems alongside interaction studies on brain samples revealed multiple protein-protein interactions with C-terminal tail of AMPAR subunits (Henley, 2003, Mah et al., 2005, Hayashi et al., 2005). A wide range of proteins interact to regulate trafficking and functional properties of the receptor (Henley, 2003, Hayashi et al., 2005, Widagdo et al., 2017, Hussain et al., 2015, Jiang et al., 2006, Henley and Wilkinson, 2013). Proteins interacting with AMPAR subunits can be broadly classified into two categories: PDZ and non-PDZ domain mediated interactors.

1.7.1.6 Interaction of PDZ proteins with AMPARs

PDZ domains are scaffolding motifs that facilitate protein-protein interactions in multiprotein complexes (Bezprozvanny and Maximov, 2001, Long et al., 2003, Hung and Sheng, 2002). The PDZ domain is an 80-110 amino acid stretch present in varying numbers (Songyang et al., 1997, Pollard et al., 2017). In general, most of the PDZ domain mediated interactions require recognition of a short specific sequence motifs (PDZ ligands) at the C-terminal region of targeted protein that facilitates the binding of target protein with PDZ domain containing interacting protein. However, this is not a prerequisite as there can be other modes of interactions (Sheng and Sala, 2001, Ponting et al., 1997).

The membrane associated guanylate kinase (MAGUK), family of PDZ ligands include the archetypal postsynaptic protein PSD 95 (Colledge et al., 2000, McGee et al., 2001, Tavares et al., 2001). Interaction of PSD 95 with C-terminal tail of AMPARs is crucial for synapse specific trafficking of AMPARs and their retention (Song and Huganir, 2002, Christopherson et al., 2003, Long et al., 2003).

Chronic suppression of network activity enhances palmitoylation of PSD 95 and clustering of AMPARs at the synapses (Noritake et al., 2009) . Conversely elevation of network activity is linked with de-palmitoylation and removal of AMPARs (Chowdhury et al., 2018, Noritake et al., 2009). The binding of another MAGUK, synapse associated protein 97 (SAP97) with GluA1 C-terminus is necessary for insertion of GluA1-containing AMPARs specifically to the synapses during LTP (Leonard et al., 1998, Song and Huganir, 2002, Jiang et al., 2006) **[Figure 1-11]**.

GluA1 specific interaction with 4.1N and 4.1G, a homologous cytoskeletal membrane protein in erythrocyte promotes the interaction with actin filaments and stabilize the receptors at the synapse. In heterologous system, hampering the interaction of GluA1 with 4.1N reduces surface expression of GluA1 containing AMPARs (Shen et al., 2000) **[Figure 1-9]**.

Other PDZ domain interacting proteins of AMPARs include protein interacting with c-kinase 1 (PICK1) **[Figure 1-11]** (Hanley, 2018, Hanley and Henley, 2005) and glutamate receptor interacting protein 1 & 2 (GRIP1 & 2 aka AMPA receptor binding protein (ABP)) (Dong et al., 1997, Dong et al., 1999). The dynamic interaction of PICK1 and GRIP complexes regulate the synapse specific targeting, surface retention and activity of AMPARs. *For detailed reviews see (Hanley, 2018, Henley and Wilkinson, 2013, Huganir and Nicoll, 2013, Henley et al., 2020).*

Further, PICK1 and GRIP1 modulate trafficking of AMPARs to the surface (Hanley, 2018, Rocca et al., 2008). Scaling up of AMPARs following prolonged inhibition of network activity increase interaction of GRIP1 and GluA2 subunit of AMPAR (Hashimoto et al., 1999, Letts et al., 1998, Bats et al., 2007). Conversely, chronic inhibition of network activity reduces endogenous PICK1 protein and increases GluA2 containing surface AMPARs (Anggono et al., 2011).

1.7.1.7 Non-PDZ interactions with AMPARs

1.7.1.7.1 N-ethylmaleimide-sensitive factor

N-ethylmaleimide-sensitive factor (NSF) is a key component of SNARE dependent fusion complex. NSF interacts with GluA2 subunits by binding to their C-terminal tail (Henley et al., 1997, Nishimune et al., 1998). Inhibiting NSF-GluA2 interaction using synthetic peptides partially occlude synaptic transmission and LTD of AMPARs, [**Figure 1-11]**. This emphasis the role of NSF in removal of AMPAR from the synapses and for constitutive recycling (Nishimune et al., 1998, Luthi et al., 1999).

1.7.1.7.2 Retromer

Retromer is heteromeric endosomal coat protein complex originally identified to be crucial for retrograde trafficking to trans-Golgi network (Seaman et al., 1998, Gallon and Cullen, 2015, Burd and Cullen, 2014). Retromer complex is formed by the

combination of 5 proteins including vacuolar sorting proteins (VPS) that forms the cargo complex, and the sorting nexins (SNXs), that attaches the cargo proteins to the vesicles (Horazdovsky et al., 1997, Seaman et al., 1997, Seaman et al., 1998, van Weering et al., 2012, Carlton et al., 2005). Interestingly, recent studies indicate retromers are pivotal in membrane trafficking and recycling of various cargo proteins (Burd and Cullen, 2014). Interaction of AMPARs with retromers regulate the basal and activity-dependent trafficking of AMPARs (Hussain et al., 2014, Choy et al., 2014, Moretto and Passafaro, 2018, Jiang et al., 2006, Temkin et al., 2017). SNX27, a PDZ domain containing protein in retromer complex is decisive in AMPAR recycling and surface expression (McMillan et al., 2021). Genetic ablation of SNX27 reduce surface levels of AMPARs and NMDARs (Wang et al., 2013, Hussain et al., 2014, McMillan et al., 2021, McMillan et al., 2017).

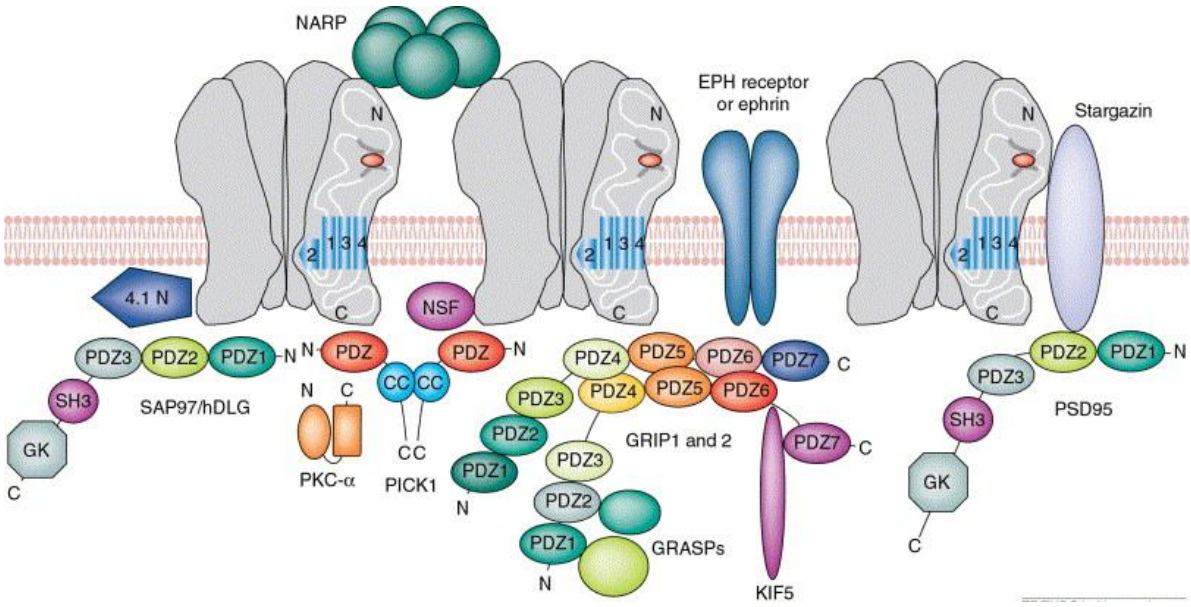

Figure 1-11: Proteins interacting with AMPARs and regulation of trafficking.

AMPARs interact with myriad of proteins that regulate trafficking, synaptic localization, and functions of the receptor. Interactions of PDZ domain proteins such as PSD95, GRIP1, PICK1 SNX27 surface trafficking and synaptic localization. Binding of auxiliary subunits such as TARPs modulate channel kinetics. Adapted from (Song and Huganir, 2002).

1.7.1.7.1 Stargazin

Stargazin (also known as γ2 and CACNG2) belongs to the family of γ-1 calcium channels that are known to interact with AMPARs (Kim et al., 2005, Hashimoto et al., 1999) **[Figure 1-11]**. The indirect PDZ interaction of stargazin with AMPARs, via

PSD95, is vital for the trafficking and stabilization of AMPARs at the synapse (Hashimoto et al., 1999, Letts et al., 1998, Bats et al., 2007, Louros et al., 2018). The PDZ domain in the C-terminal tail of stargazin facilitate their interaction with PSD95 proteins. Genetic ablation of the PDZ domain of stargazin results in a decline in the number of synaptic AMPARs (Chen et al., 2000b).

Expression of phospho-dead forms of stargazin in cultured neurons prevent scaling up of AMPARs during suppression of network activity. On the other hand, over expression of phosphomimetic mutants increased the levels of AMPARs in basal conditions and prevent scaling up (Louros et al., 2014, Chowdhury and Hell, 2018, Louros et al., 2018).

1.7.1.8 AMPARs in diseases

Erroneous expression, trafficking or functioning of AMPARs culminates in impaired brain functions (Henley and Wilkinson, 2013, Salpietro et al., 2019, Bleakman et al., 2007, Chappell et al., 2007, Zarate and Manji, 2008). A decrease in functional synaptic AMPARs and subsequent impairments in synaptic plasticity is a prominent clinical feature in early onset of Alzheimer's disease (AD) (Shankar et al., 2008, Li et al., 2009), Inhibition of AMPAR trafficking by amyloid β (Aβ) lead to impairments in synaptic function and loss of functional synapses (Walsh and Selkoe, 2007). Moreover, intracellular application of oligomerized Aβ increase AMPAR mediated EPSCs and ablation of GluA1 subunits or expression of phosphor null mutant of GluA1 845 reversed the effects. This suggest that the preliminary response of neurons to Aβ oligomers is by enhancing the number of CP-AMPARs (Whitcomb et al., 2015). Together, enhancement in CP-AMPARs can be an early sign of AD pathology and can emerge as a therapeutic target in early onset of AD.

Amyotrophic lateral sclerosis (ALS) is fatal neurodegenerative disorder characterized by rapid and progressive degeneration of motor neurons in the brain and spinal cord (Rowland and Shneider, 2001, Zarei et al., 2015, Renton et al., 2011). Whilst several genes have linked to pathology of ALS, one of the prominent hypotheses is glutamate receptor excitotoxicity through CP-AMPARs (Cleveland and Rothstein, 2001, Gregory et al., 2020). Ca^{2+} permeability of AMPARs is tightly regulated by curbing the subunit expression and RNA editing of GluA2 subunits (Pachernegg et al., 2015, Henley et al., 2021, Greger et al., 2003, Salpietro et al., 2019).

Patients with ALS exhibit spike in synaptic CP-AMPARs and a decline in the expression of ADAR2 enzymes (Yamashita and Kwak, 2014, Henley and Wilkinson, 2016). Over activation of CP-AMPARs and associated excitotoxicity is prevalent in ischemic brain injury as well as in some forms of epilepsies (Chang et al., 2012, Williams et al., 1997, Gregory et al., 2020).

Taken together, the aforesaid neurological and neurodegenerative disorders emphasise the importance of AMPARs in various brain functions.

1.7.2 Kainate receptors

KARs belong to the family of iGluRs. functional KARs comprised of hetero-tetrameric assemblies of combinations of 5 core subunits (GluK1-5) (previously named GluR5, GluR6, GluR7, KA1 and KA2) encoded by genes *GRIK 1-5*, respectively (Lerma, 2003, Chittajallu et al., 1999, Pinheiro and Mulle, 2006, Graham L.Collingridge, 2009, Henley et al., 2021). Interestingly, KARs can signal through both canonical ionotropic signalling and a non-canonical metabotropic signalling **[Figure 1-12]** (Frerking et al., 2001, Lerma and Marques, 2013, Rozas et al., 2003, Fernandes et al., 2009, Vignes and Collingridge, 1997). Functional KARs are located at pre-, post- and/or extra-synaptic sites where they perform distinct functions. This includes modulation of neurotransmitter release, depolarisation of postsynaptic membrane, regulation neuronal excitability etc. (Pinheiro and Mulle, 2006, Castillo et al., 1997, Vignes and Collingridge, 1997, Melyan et al., 2002, Chittajallu et al., 1996, Frerking and Nicoll, 2000, Frankle et al., 2003, Collingridge et al., 2004, Nair et al., 2021, Henley et al., 2021).

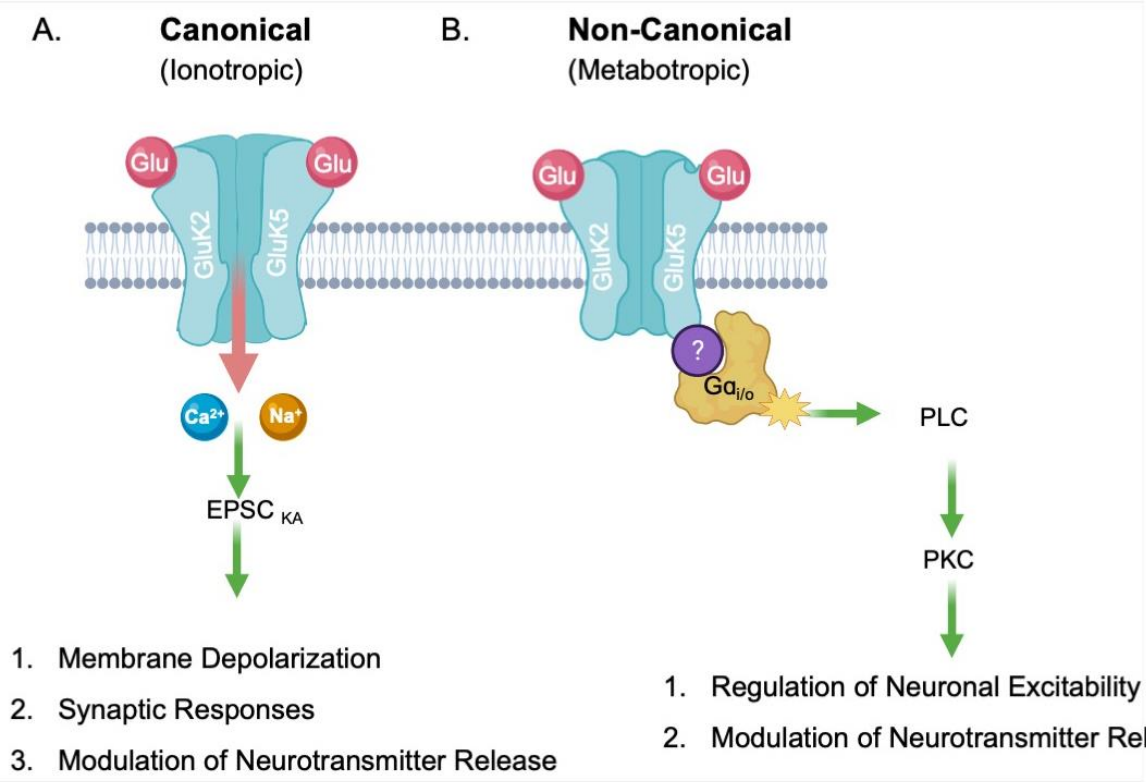

Figure 1-12: Signalling of KARs

KARs can signal through canonical ionotropic signalling and a non-canonical metabotropic signalling pathway. A.) Activation of KARs by endogenous glutamate opens the channel pore by inducing conformational changes in the subunits leading to influx of mono and divalent cations. Canonical signalling of KARs have been attributed in depolarisation of postsynaptic membrane, modulating receptor trafficking of KARs and AMPARs and facilitation of neurotransmitter release at some synapses. B.) Non-canonical signalling of KARs activates PLC and PKC via G-protein coupled second messenger. Metabotropic signalling of KARs regulate the excitability of neurons by inhibiting I_{sAHP}, modulate glutamate and GABA release at some synapses, regulates AMPAR trafficking, and maturation of neuronal circuitry during development. Adapted from (Nair et al., 2021).

1.7.2.1 KAR subunits

KAR subunits are categorized into low-affinity KARs (GluK1-GluK3), and high-affinity subunits (GluK4, GluK5) based on their sensitivity for agonist-KA (Chittajallu et al., 1999, Carta et al., 2014, Lerma and Marques, 2013, Pinheiro and Mulle, 2006). Although all low-affinity KARs can form homomeric channels, high-affinity subunits can only form functional receptor when combined with low-affinity subunits (Ayalon and Stern-Bach, 2001, Chittajallu et al., 1999, Lerma and Marques, 2013, Nair et al.,

2021). Furthermore, genetic ablation of high-affinity subunits revealed that one or more high-affinity subunits are required for the ionotropic functions of KARs **[Figure 1-13]**.(Fernandes et al., 2009).

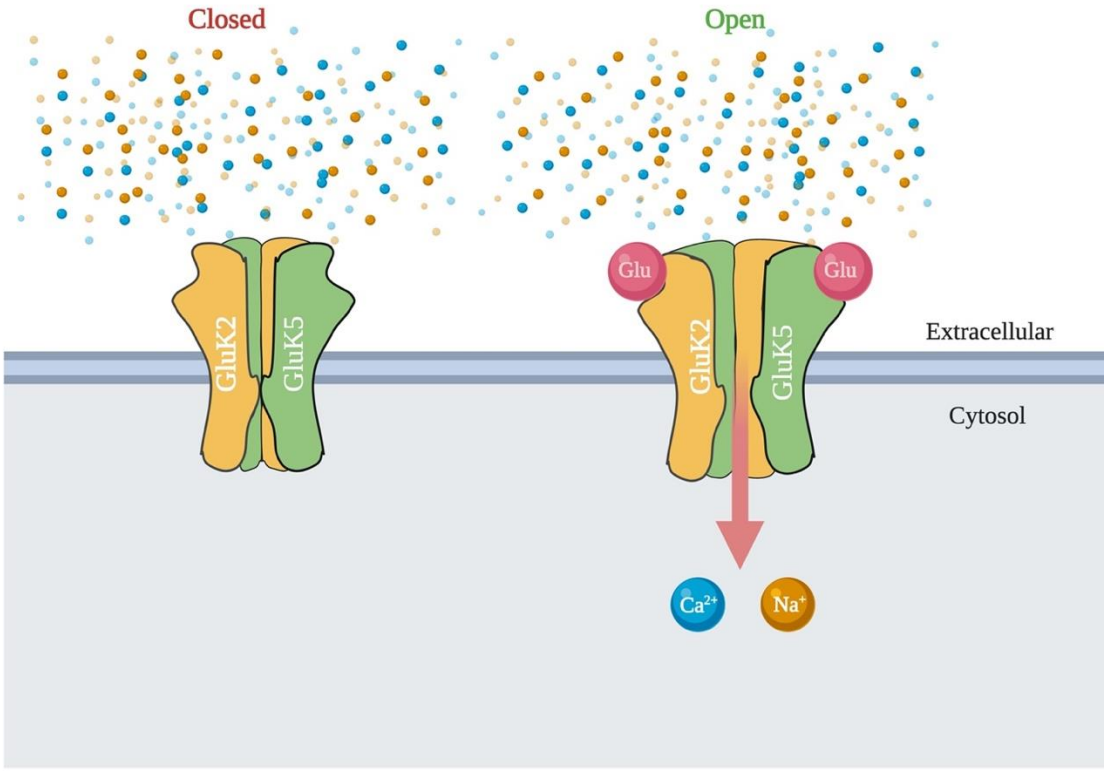

Figure 1-13: General structure of a tetrameric KAR

Functional KARs are mainly hetero-tetramers composed of various subunit composition ranging from GluK1-GluK5. Most abundantly expressed form of KARs are composed of GluK2 and GluK5 subunits. Activity-dependent release and binding of glutamate to the receptor induces conformational change allowing the influx of ions through the channel.

1.7.2.1.1 Alternative splicing of KARs

Beyond subunit composition, diversity in KARs arises due to pre- and post-translational modifications inclusive of alternative splicing and editing of the pre-mRNAs encoding specific subunits (Lerma, 2003, Kortenbruck et al., 2001, Hirbec et al., 2003, Wilding et al., 2005, Bernard et al., 1999, Pinheiro and Mulle, 2006, Evans et al., 2017c). Splicing of GluK1 generates two N-terminal variants, GluK1$_{1-2}$, and four C-terminal variants, GluK1$_{a-d}$ (Sommer et al., 1992, Gregor et al., 1993, Jaskolski et al., 2004). GluK2 and GluK3 undergoes alternative splicing at their C-terminal tail to produces GluK2$_{a-c}$ and

GluK3$_{a-b}$ variants (Schiffer et al., 1997) **[Figure 1-14]**. Further, alternative splicing of KARs promote release of the receptors from the ER enhancing their surface expression (Coussen et al., 2005). The splice variants were characterized with different physiological properties and distinct protein-protein interactions with the C-terminal tails (Ren et al., 2003, Evans et al., 2017a) .

1.7.2.1.2 Editing of KARs

Additionally, GluK1 and GluK2 mRNA undergo Q/R editing in the channel pore as well as I/V and Y/C editing at the re-entrant TM domain (Pahl et al., 2014, Köhler et al., 1993, Bernard et al., 1999, Egebjerg and Heinemann, 1993) **[Figure 1-14]**. Edited subunits show varying channel kinetics and alter surface expression. Q/R editing of GluK2 subunit hinders their ability to interact with other subunits in the ER and thereby hampering the surface expression (Ball et al., 2010a, Contractor et al., 2011).

ADAR dependent editing of KARs is further discussed in **Chapter 5**

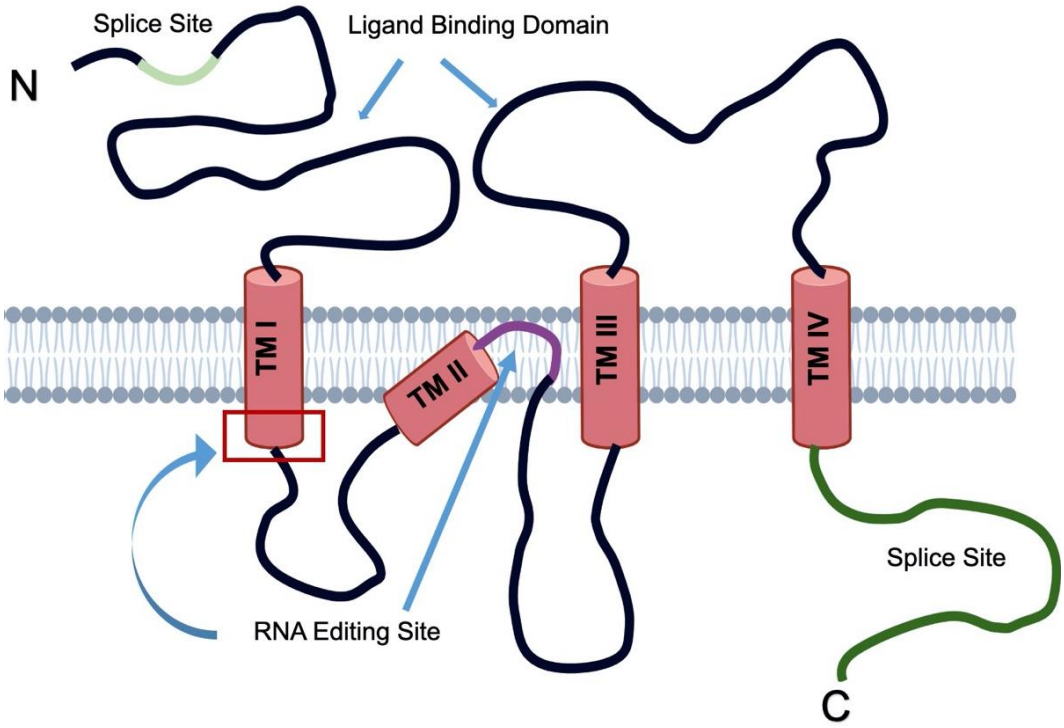

Figure 1-14: General structural topology and PTM of KAR subunits

KARs share structural similarities with the other members of iGluRs family. The extracellular N-terminal region in combination with TMIII and TMIV forms the glutamate binding domain. Alternative splicing and RNA editing adds to the repertoire of the receptor variants along with regulation of channel kinetics and trafficking. The C-terminal serves as a hub for various protein-protein interaction with the receptor.

1.7.2.2 Regional expression of KARs

KARs are expressed widely in the brain and spinal cord with the mRNA expression levels peaking early in postnatal development (Wisden and Seeburg, 1993a, Bureau et al., 1999). The mRNA of GluK1 subunit is widely expressed in the layers of sensory cortex, subiculum, pontine nuclei, interneurons of CA1 hippocampus, purkinje cells etc. (Bettler et al., 1990, Wisden and Seeburg, 1993a). GluK2, GluK4 and GluK5 express predominantly in CA1, CA3 and dentate gyrus (DG) layers of the hippocampus (Bahn et al., 1994, Carta et al., 2014). Studies using antipeptide antibodies against KAR subunits revealed highest amount of GluK2/GluK5 in hippocampus (Petralia et al., 1994, Wenthold et al., 1994, Mulle et al., 2000, Bureau et al., 1999).

High affinity GluK4 and GluK5 containing KARs are expressed widely in brain regions with GluK5 predominating in MF-CA3 neurons whilst GluK4 in CA3-CA1, nuclei of layer II – IV of neocortex, septal nucleus etc. (Wisden and Seeburg, 1993a). However, the lack of specific antibodies to probe individual KAR subunits have limited immunohistochemical studies and hence unraveling the definite expression patterns of KARs in the CNS.

1.7.2.3 Subcellular localization of KARs

Localization of KARs within the neurons is decisive of their function. KARs are expressed in pre-/postsynaptic sites in addition to cell body, axonal terminals, dendritic spines etc. (Jaskolski et al., 2005, Kieval et al., 2001) GluK2/GluK5 containing KARs postsynaptically expressed at the MF-CA3 synapses mediate majority of KAR-induced excitatory transmission (Kieval et al., 2001, Wenthold et al., 1994). Functional mapping of KARs using focal glutamate uncaging revealed that KARs are restricted to postsynaptic sites in MF-CA3 synapses (Fièvre et al., 2016).

Immunoreactivity studies on monkey striatum indicate 70% of the GluK2/GluK5 containing KARs are expressed in intracellular regions such as ER and Golgi whereas 30% of the heteromeric assemblies are found in synaptic and/or extra-synaptic sites (Kieval et al., 2001).

GluK3 subunit is a key component of presynaptic KARs (Sakha et al., 2016, Pinheiro et al., 2007, Perrais et al., 2009). Genetic ablation of GluK3$^{-/-}$ subunits in mice failed to exhibit presynaptic forms of long-term and short-term KAR plasticity at MF-CA3 synapses (Pinheiro et al., 2007, Schiffer et al., 1997, Contractor et al., 2001). Nonetheless, GluK2/GluK5 subunits exhibit low sensitivity to glutamate (Pinheiro et al., 2007). However, presynaptic KAR activation at MF-CA3 synapses requires higher glutamate concentration suggesting that presynaptic receptors are devoid of GluK2/GluK5 receptors (Pinheiro et al., 2007). Furthermore, presynaptic KAR functions are severely impaired in GluK3$^{-/-}$ mice emphasis their presynaptic localization (Pinheiro et al., 2007, Lauri et al., 2003). Moreover, the subcellular localization is also dependent on the subunit composition of KARs. Disruption of GluK1 containing KARs failed to exhibit loss of KA-induced inhibition of GABAergic transmission onto CA1 pyramidal cells. However, in mice lacking both GluK1$^{-/-}$ and GluK2$^{-/-}$ subunits this function of KARs is severely affected (Mulle et al., 2000). In addition, heteromerization of GluK1 with GluK2 and or GluK5 is decisive in the expression in distal dendrites (Pahl et al., 2014). Interaction of heteromeric KARs containing GluK1/GluK2/GluK5 with microtubule motor protein KIF17 promotes the expression of the receptor in distal dendrites (Kayadjanian et al., 2007, Lauri and Taira, 2011) **[Figure 1-15]**.

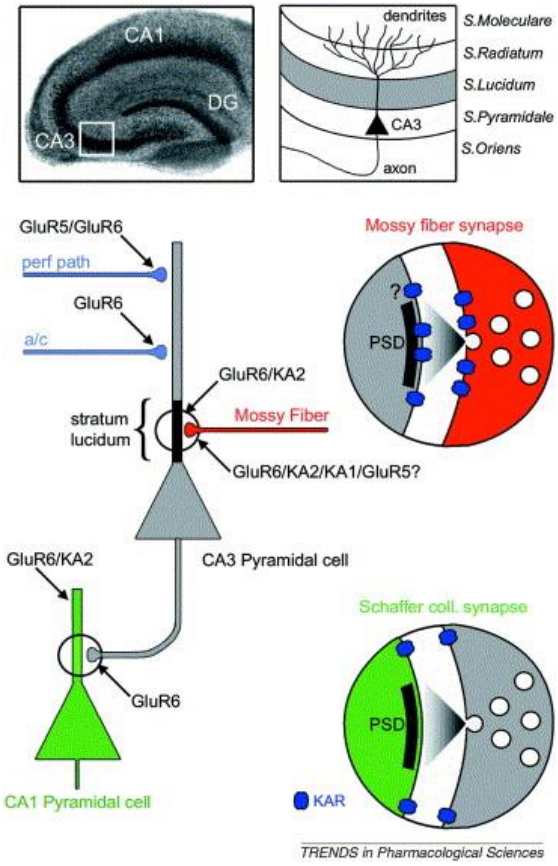

Figure 1-15: Subcellular localization of KARs in hippocampal pyramidal neurons

KARs distributed in neuronal compartments have varied functions. GluK2/GluK5 containing KARs present on the postsynaptic sites mediates glutamate mediated excitatory transmission. KARs on the presynaptic sites regulate the neurotransmitter release by functioning as auto receptors. In striatum lucidum, CA3 neurons express KARs in dendritic region. In CA1 neurons, extra synaptic KARs express without a notable KAR-mediated EPSCs. (Adapted from (Jaskolski et al., 2005).

1.7.2.4 Post-translational modifications of KARs

KAR subunits undergo various post-translational modifications that regulate the trafficking, receptor clustering at postsynaptic sites and channel kinetics (Evans et al., 2017a, González-González et al., 2012, Copits and Swanson, 2013, Henley et al., 2020, Nair et al., 2021, Henley et al., 2021). Various PTM of KARs are listed:

Table 1-2: Showing post-translational modifications of KARs

Subunit	Type of PTM	Protein	Target site	Significance	Cross reference
GluK2	Phosphorylation	PKC	S846	• Surface expression during basal and activity induction. • Trafficking of the receptors • Internalisation	(Konopacki et al., 2011, Evans et al., 2017c) Refer **[Figure 1-16]**
GluK2	Phosphorylation	Src Kinase	Y590	• Opening of KAR channel and proapoptosis after brain ischemia	(Zhu et al., 2014)
GluK2	Phosphorylation	PKC	S868	• Increased KAR surface expression	(Wilkinson et al., 2012, Evans et al., 2017a, Evans et al., 2017c, Nasu-

					Nishimura et al., 2010) Refer **[Figure 1-16]**
GluK2b	Phosphorylation	PKC	S880, S886	• Stabilisation of KARs at synapse • KAR-mediated synaptic responses	(Hirbec et al., 2003)
GluK5	Phosphorylation	CaMKII	S859, S892, T976	• Promotes lateral movement of the receptor • Increased extra synaptic expression	(Carta et al., 2013)
GluK2a	Phosphorylation	PKA	S825, S837	• Potentiates whole-cell currents in GluK2 expressing HEK cells	(Kornreich et al., 2007)
GluK2	SUMOylation	SUMO conjugating enzymes Ubc9 and SUMO ligase PIAS3	K886	• Agonist induced internalisation	(Martin et al., 2007) Refer **[Figure 1-16]**
GluK2	SUMOylation and Phosphorylation	SUMO conjugating	K886/S868	• KAR-LTD at MF-CA3 synapses	(Wilkinson et al., 2012, Chamberlain et al., 2012,

		enzymes Ubc9 and SUMO ligase PIAS3/PKC			Evans et al., 2017a, Henley et al., 2020)
GluK2	De-SUMOylation	SENP-1	?	• Enhances KAR EPSCs	(Chamberlain et al., 2012, Martin et al., 2007)
GluK2	Palmitoylation	Palmitoyl transferase	C858, C871	• Surface expression • Promotes association with 4.1N	(Pickering et al., 1995, Copits and Swanson, 2013) Refer **[Figure 1-16]**
GluK2	Ubiquitination	E3 Ubiquitin ligase - Parkin	Unknown	• Increased surface expression of GluK2 KARs • Enhances susceptibility to KAR-induced excitotoxicity	(Maraschi et al., 2014) Refer **[Figure 1-16]**
GluK2	Ubiquitination	Cullin3 (Cul3)	Unknown	• KAR degradation	(Salinas et al., 2006) Refer **[Figure 1-16]**
GluK3	Glycosylation	?	N402, N247	• Desensitisation of the receptor	(Kumari et al., 2019)

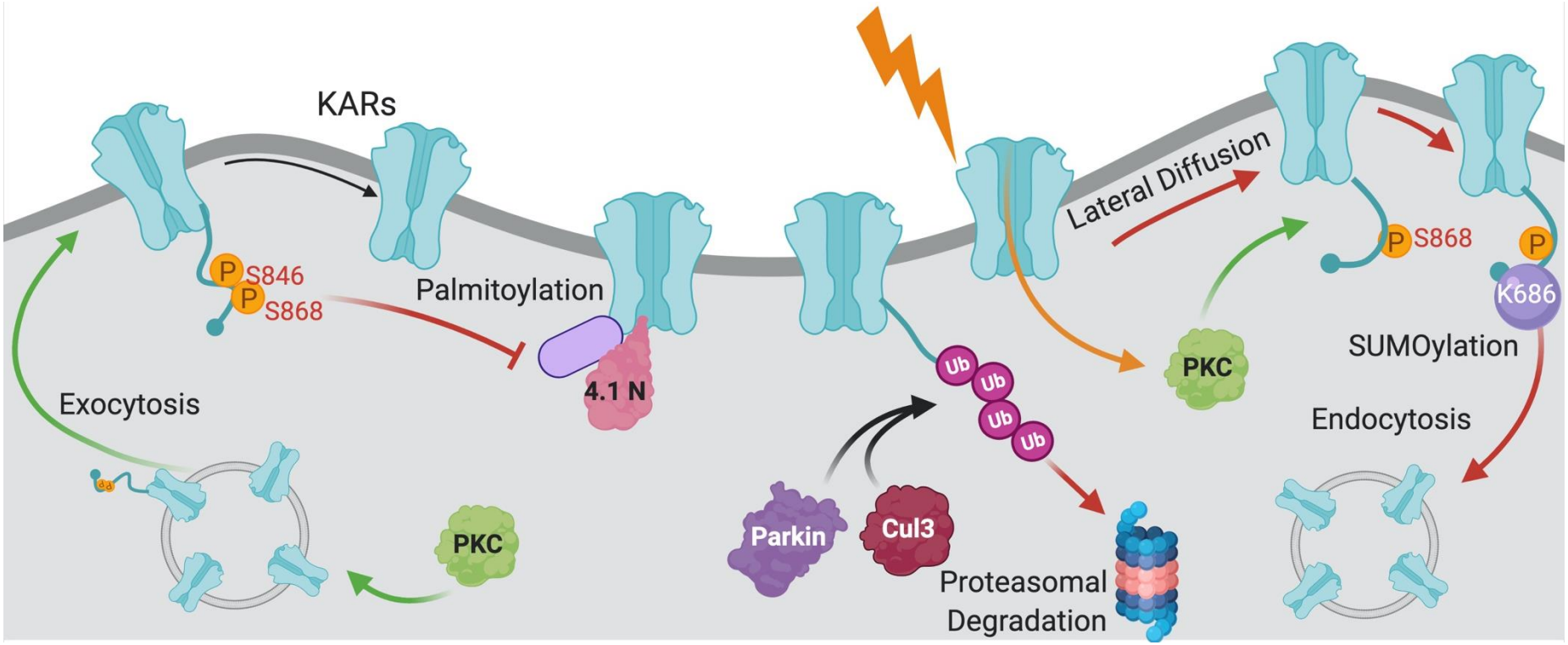

Figure 1-16: Regulation of KAR surface expression by PTM

KAR activation enhances PKC activity and subsequent phosphorylation of GluK2 S846 and S868. This leads to an increase in the surface expression of KARs. Phosphorylation of GluK2 S868 alongside SUMOylation at K886 trigger endocytosis of the receptor. Increased palmitoylation and subsequent interaction with 4.1 N protein increases the stability and surface expression of KARs which is inhibited by PKC dependent phosphorylation. Ubiquitination of KARs by E3 ubiquitin ligases Parkin and Cul3 enhances the receptor endocytosis and degradation by the proteasomal complex.

1.7.2.5 Protein-protein Interactions of KARs

Various proteins interact with C-terminal tail of KAR subunits to regulate the receptor trafficking, stabilization at PSD and channel kinetics (Fièvre et al., 2016). The major proteins interacting with KARs are listed below:

Table 1-3: Showing protein-protein interactions of KARs and auxiliary subunits

Subunit	Interacting protein	Interacting region	Significance	Cross reference
Proteins interacting with PDZ domain				
GluK1/2/5	PSD-95	C-terminal PDZ ligand	• Receptor clustering • Channel kinetics • Desensitisation • LTD at MF-CA3 synapses	(Hirbec et al., 2003, Bowie et al., 2003, Suzuki and Kamiya, 2016, Carta et al., 2013). Refer **[Figure 1-17]**
GluK2/GluK5	SAP90	GluK2- C-terminal amino acid sequence ETMA GluK5- C-terminus of GluK5 with SH3 and GK domains of SAP97	• Clustering of receptors at postsynaptic sites • Reduced desensitization	(Garcia et al., 1998)

GluK2	SAP97	C-terminal amino acid sequence ETMA	• Clustering of receptors at postsynaptic sites	(Garcia et al., 1998)
GluK2/GluK5	SAP102	Unknown	• Clustering of receptors at postsynaptic sites	(Garcia et al., 1998)
GluK1/2	PICK1	-EVTA PDZ domain	• Stabilisation of receptor at synapse	(Hirbec et al., 2003) Refer **[Figure 1-17]**
GluK1/2	GRIP	-EVTA PDZ domain	• Stabilisation of receptor at synapse	(Hirbec et al., 2003) Refer **[Figure 1-17]**
GluK1/GluK2	Syntenin	-EVTA PDZ domain	• Unknown	(Hirbec et al., 2003)
Non-PDZ interactions				
GluK2	4.1N	C-terminal domain	• Receptor trafficking • Regulation of endocytosis	(Copits and Swanson, 2013) Refer **[Figure 1-17**
GluK2	Actinfillin	C-terminal domain	• Degradation by Cul3 mediated ubiquitination	(Salinas et al., 2006)
GluK2	Seizure protein-6 (SEZ6)	Unknown	• Trafficking • Glycosylation	(Pigoni et al., 2020)

			• Surface expression	
GluK2	β-catenin/Cadherin complex	Indirectly with last 14 aa of C-terminus	• Stabilisation of receptor during synapse formation	(Coussen et al., 2002)
GluK2	Calcineurin	C-terminal domain	• Trafficking • Ca2+ regulation	(Traynelis and Wahl, 1997, Pahl et al., 2014, González-González et al., 2012)
GluK2	Calmodulin	C-terminal domain	• Unknown	(Coussen et al., 2005)
GluK2	Contactin	C-terminal domain	• Unknown	(Coussen et al., 2005)
GluK2	Dynamin	C-terminal domain	• Unknown	(Coussen et al., 2005)
GluK2	Dynamitin	C-terminal domain	• Unknown	(Coussen et al., 2005)
GluK2	VLIP1	C-terminal domain	• Unknown	(Coussen et al., 2005)
GluK1/GluK2/GluK5	14-3-3	Unknown	• Trafficking of KARs	(Coussen et al., 2005, Vivithanaporn et al., 2006)
GluK5	COPI	Arginine rich retention/retrieval determinant	• Trafficking of receptor	(Vivithanaporn et al., 2006)
GluK2b	Profilin IIa	C-terminal domain	• Modulate KAR exocytosis	(Coussen et al., 2005, Mondin et al., 2010,

				González-González et al., 2012) Refer **[Figure 1-17]**
GluK2a	Spectrin	C-terminal domain	• Unknown	(Coussen et al., 2005)
GluK2b	NSF	C-terminal domain	• Unknown	(Coussen et al., 2005)
GluK2a	Ubc9, PIAS3	C-terminal domain	• Receptor endocytosis and degradation	(Martin et al., 2007)
GluK5	SNAP25	C-terminal domain	• Synaptic removal of KARs • Receptor trafficking	(Selak et al., 2009)
GluK2/GluK5	KIF17	Unknown	• Localisation of subunits to distal dendrites	(Kayadjanian et al., 2007)
GluK2/GluK4	C1ql2/3	N-terminal domain	• Recruits functional KARs to postsynaptic sites of CA3 neurons • Modulation KAR function	(Matsuda et al., 2016) Refer **[Figure 1-17]**
	Neurexin3	Binds to C1ql2/3 via splice site and subsequent interaction with KARs	• Clustering of KARs at post synapses • Receptor function	(Matsuda et al., 2016) Refer **[Figure 1-17]**

Auxiliary subunits of KARs				
GluK1-5	Neto1	Neto CUB domain interacts with N-terminal Domain of the receptor.	• Trafficking • Channel kinetics • Promotes recovery from desensitization. • Rectification • Enhances GluK1 desensitization	(Laezza et al., 2007, Zhang et al., 2009, Li et al., 2019, Fisher and Mott, 2012, Sheng et al., 2015a) Refer **[Figure 1-17]**
GluK1-5	Neto2	Neto CUB domain interacts with N-terminal Domain of the receptor.	• Trafficking • Channel kinetics • Promotes recovery from desensitization. • Rectification • Slows GluK1 desensitization	(Laezza et al., 2007, Zhang et al., 2009, Li et al., 2019, Fisher and Mott, 2012, Tang et al., 2012, Sheng et al., 2015a) Refer **[Figure 1-17]**
GluK2a	KRIP6	C-terminal residues 842-909	• Alteration of channel kinetics	(Laezza et al., 2007)

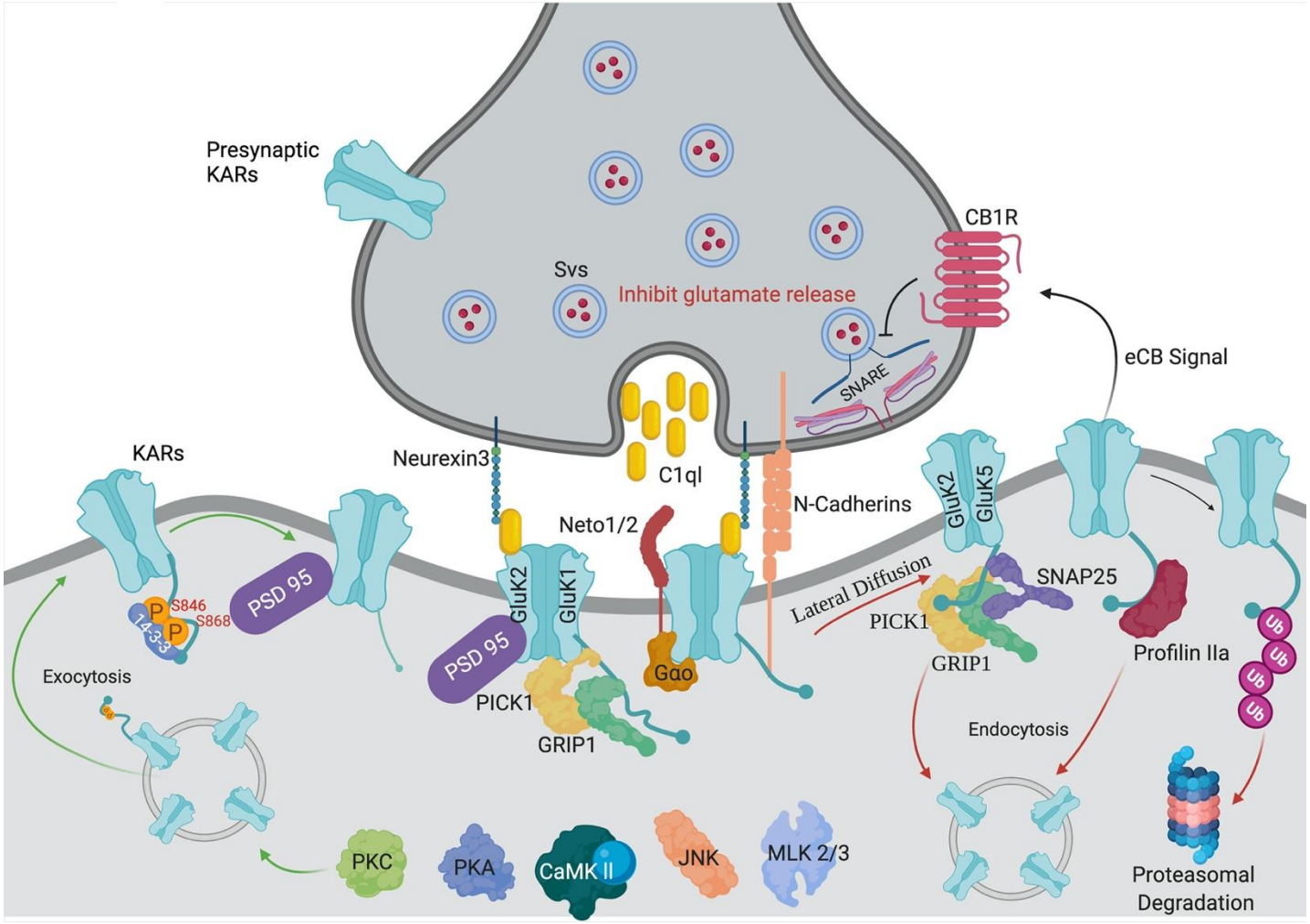

Figure 1-17: KAR interactions and trafficking

KAR C-terminal tail have multiple interacting partners which are key in synaptic retention, localization, channel functions etc. Binding of 14-3-3ζ on C-terminal tail of KARs post phosphorylation on S846 and S868 stabilizes the receptor at the surface. Release of soluble C1ql protein from the presynaptic terminals and their interaction through neurexin 3 along with GluK2 subunits are required for synaptic clustering of KARs at MF-CA3 synapses. PSD95 interacting with KARs are crucial for the synaptic localization. C-terminal interacting proteins such as PICK1, GRIP1, Profilin IIa, SNAP25 regulate the stability and surface expression of the receptors. Single transmembrane auxiliary proteins such as Neto1 and Neto2 interacting with KARs alter the synaptic properties of the channel. Further, polyubiquitination of the receptor trigger proteasomal degradation. Retrograde signaling through KARs activate presynaptic CB1 receptors and inhibit the release of glutamate from the presynaptic.

1.7.2.0 Metabotropic signaling of KARs

KARs can signal through a non-canonical G-protein dependent pathway (Rodrigues and Lerma, 2012, Contractor et al., 2011, Petrovic et al., 2017b, Nair et al., 2021, González-González et al., 2012, Henley et al., 2021). The pertussis toxin (PTx) ($G_{i/o}$-protein inhibitor) sensitive metabotropic functions of KARs is known to regulate a myriad of synaptic functions in both pre- and post-synapses (Gonzalez-Gonzalez and Henley, 2013, Ruiz et al., 2005). Although the precise mechanism of how KARs activate G-proteins remains elusive and remain controversial, GluK1, GluK2 and GluK5 subunits of KARs are attributed in this pathway. Whilst GluK2$^{-/-}$ mice highlighted the prerequisite for metabotropic signalling, proteomic analysis listed various G-proteins interacting with GluK1 subunits (Rutkowska-Wlodarczyk et al., 2015, Rodrigues and Lerma, 2012, Pinheiro et al., 2013) **[Figure 1-18]**.

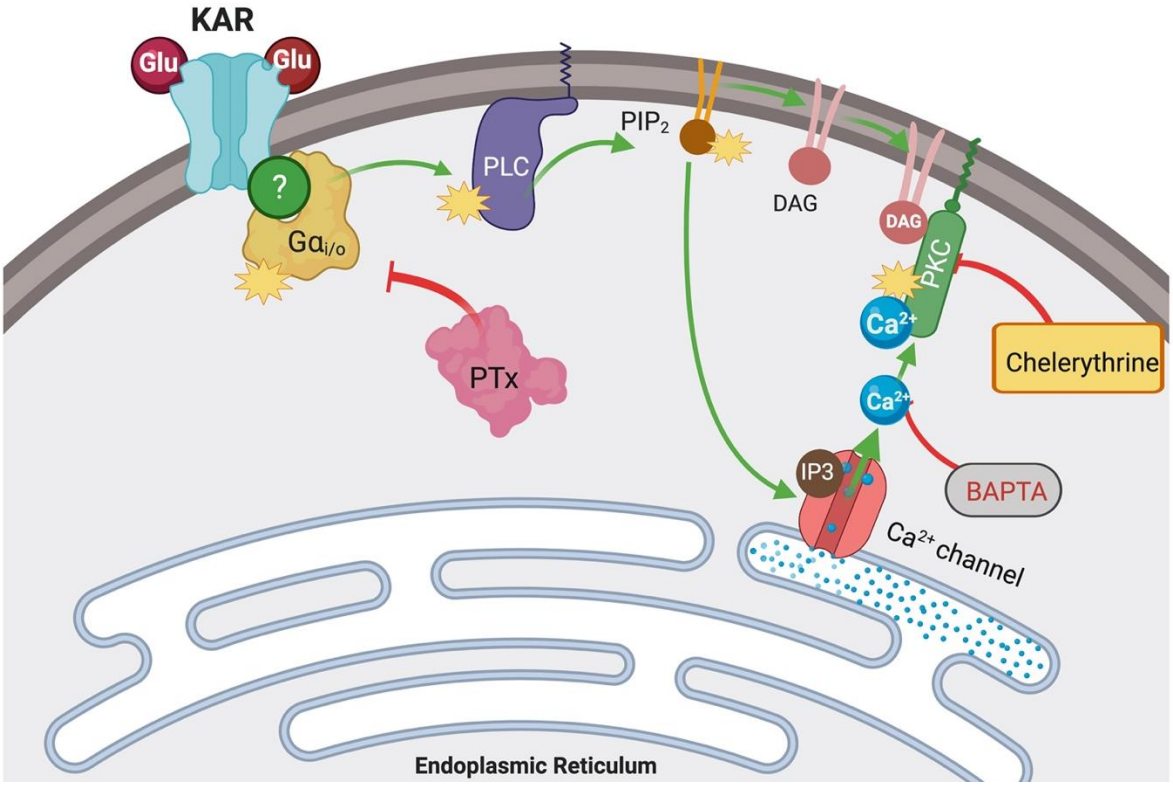

Figure 1-18: Metabotropic signalling of KARs

KAR subunits GluK1, GluK2 and GluK5 are implicated in their ability to signal via metabotropic action. KAR activation by glutamate by a yet unknown mechanism trigger $G\alpha_{i/o}$ protein mediated downstream signaling cascade. The pathway involves activation of PLC and PKC. pertussis toxin (PTx) inhibits the metabotropic action of KARs.

Metabotropic signaling of KAR is crucial for various functions, including neuronal transmission, neuronal development, neuronal network formation, regulation of neuronal excitability etc. (Rodriguez-Moreno et al., 1997, Valbuena and Lerma, 2016, Fisahn et al., 2005, Melyan et al., 2004, Vignes et al., 1998, Vignes and Collingridge, 1997, Carta et al., 2014).

Studies to understand the direct or indirect interaction of KAR subunits with G-proteins availed unsuccessful. Biochemical studies revealed the interaction of GluK5 subunits of KARs with $G_{\alpha q}$ (PTx insensitive G- protein) (Ruiz et al., 2005, Lerma and Marques, 2013). However, PTx sensitive blockade of metabotropic KAR functions failed to explain the mechanism of $G_{\alpha q}$ as a second messenger. Proteomic analysis indicated the interaction of GluK1 with $G_{\alpha i/o}$ protein which was further validated in GluK1$^{-/-}$ mice (Rutkowska-Wlodarczyk et al., 2015).

KARs via metabotropic signaling can regulate the excitability of neurons by inhibiting outward potassium channels that mediate medium and slow after hyperpolarization (mAHP and I_{sAHP} respectively) (Melyan et al., 2004, Chamberlain et al., 2013). (Ruiz et al., 2005) revealed that this metabotropic signaling of KARs is dependent on GluK5 subunits of KARs. The mice deficient in GluK5$^{-/-}$ subunits failed to inhibit I_{sAHP}. However, another group refuted this study by showing that GluK5$^{-/-}$ mice can inhibit I_{sAHP} but not GluK2$^{-/-}$ mice (Fernandes et al., 2009, Fisahn et al., 2005). KA-induced upregulation of postsynaptic KAR is via the metabotropic function of KARs (Gonzalez-Gonzalez and Henley, 2013). More recently, KA-induced LTP of AMPARs is dependent on metabotropic action of GluK2 containing KARs. GluK2$^{-/-}$ mice failed to exhibit this form of LTP suggesting a possible interaction between GluK2 subunits and G-proteins activation (Petrovic et al., 2017c).

Taken together, despite the existing controversies in deciphering the subunit and interaction of G-protein in metabotropic signaling of KARs, it is well understood that metabotropic functions of KARs can regulate various synaptic functions that are crucial for brain function and neuronal development.

1.7.3 Kainate receptors and neuronal transmission

KAR mediated regulation of network activity and neuronal transmission is dependent on the synaptic positioning of the receptors (González-González et al., 2012, Pahl et al., 2014, Vignes and Collingridge, 1997, Lauri et al., 2001, Lerma et al., 2001, Chittajallu et al., 1999, Carta et al., 2014, Nair et al., 2021, Pinheiro and Mulle, 2006, Henley et al., 2021).

1.7.3.1 Biophysical properties of KARs

AMPARs mediate majority of all the fast EPSCs in glutamatergic synapses (Henley and Wilkinson, 2013, Henley et al., 2011, Anggono and Huganir, 2012). Unlike AMPARs, KARs at various synapses is characterized with a slow activation and deactivation kinetics (Cossart et al., 1998, Frerking et al., 1998, Bureau et al., 2000). At MF-CA3 synapses GluK2/GluK5 containing KAR activation by contributes to a slow $EPSC_{KA}$ with delayed activation and deactivation kinetics in comparison with AMPARs (Castillo et al., 1997, Lerma et al., 1997, Carta et al., 2014, Barberis et al., 2008). Further, $EPSC_{KA}$ show altered kinetics in the slice recordings compared to the recombinantly expressed channels in heterologous system. This is likely due to their interactions with auxiliary subunits such as Neto1 and Neto2 (Contractor et al., 2003b, Zhang et al., 2009). Co-expression of Neto1 with homomeric GluK2 subunits decreased desensitization and increased recovery from desensitization (Fisher and Mott, 2013). In agreement with the studies on heterologous system, KARs show a linear current-voltage (I-V) relationship in hippocampal MF-CA3 neurons whereas a rectification in thalamo-cortical neurons (Li and Rogawski, 1998, Frerking et al., 1998, Castillo et al., 1997). The variation is due to difference in the post-translational modifications, RNA editing with thalamo-cortical neurons being more permeable to Ca^{2+} but not in hippocampal neurons and their sensitivity for intracellular polyamines (Li and Rogawski, 1998, Copits and Swanson, 2012, Zhang et al., 2009, Contractor et al., 2011).

1.7.3.2 KARs in post-synaptic excitatory transmission

Understanding KAR functions in synaptic transmission has been challenging until the discovery of GYKI53655 which specifically antagonise AMPARs but not KARs (Paternain et al., 1995, Wilding and Huettner, 1995). $EPSC_{KA}$ is impetrative in integrating synaptic inputs and maintenance of functional synaptic networks (Pinheiro et al., 2013, Frerking and

Ohliger-Frerking, 2002, Mulle et al., 2000). KAR-mediated responses are well characterized at the MF-CA3 synapses (Castillo et al., 1997, Vignes and Collingridge, 1997). Further, KAR-mediated responses are also detected at various synapses including Schaffer collaterals, CA1 interneurons (Cossart et al., 1998, Frerking et al., 1998) synapse of parallel fibres and Golgi in cerebellum (Bureau et al., 2000) amygdala (Ryazantseva et al., 2020, Li and Rogawski, 1998) thalamocortical synapses (Isaac, 1999) dorsal horns of spinal cord etc (Li et al., 1999). Studies employing quantal release of glutamate successfully identified the activation of postsynaptic KARs in both MF and CA1 interneurons (Cossart et al., 2002). Alongside postsynaptic KARs, facilitation of KAR EPSCs by repetitive MF stimulation is an indicative of KARs at extra-synaptic locations (Castillo et al., 1997, Vignes and Collingridge, 1997, Wyeth et al., 2014, Sheng et al., 2015b, Li et al., 2019, Orav et al., 2019, Zhang et al., 2009, Fièvre et al., 2016).

Electrophysiological studies have identified that the single channel conductance of postsynaptic KARs can range from ~10pS (like AMPARs) to ~1pS. This variation in the channel conductance correlate with the post-translational modification of the subunits and also their interactions with auxiliary subunits (Swanson et al., 1996). Furthermore, due to the prolonged decay of kinetics of $EPSC_{KA}$ and their ability to generate postsynaptic spiking, suggest the substantial role of KARs in depolarising postsynaptic membrane and in integrating synaptic inputs over a large period (Frerking et al., 1998, Li and Rogawski, 1998, Frerking and Ohliger-Frerking, 2002).

1.7.3.3 Pre-synaptic KARs

KARs are expressed on presynaptic terminals of both glutamatergic and GABAergic synapses throughout the brain (Chittajallu et al., 1996, Anwyl, 1999, Lerma, 2003). These presynaptic KARs can lead to increases or decreases in the probability of neurotransmitter release, principally by altering the presynaptic Ca^{2+} concentration (Falcon-Moya and Rodriguez-Moreno, 2021). A number of mechanisms by which KARs may regulate presynaptic Ca^{2+} have been reported, including: (i) direct depolarization of presynaptic axons and terminals altering voltage-gated Ca^{2+} channel (VGCC) activity (Semyanov and Kullmann, 2001, Frerking et al., 2001, Schmitz et al., 2000, Kidd et al., 2002), (ii) Ca^{2+} permeable KARs (Lauri et al., 2003, Griffiths et al., 2008), and (iii) metabotropic signalling, which can regulate VGCCs and vesicle release (Rodríguez-Moreno and Lerma, 1998, Rodriguez-Moreno and Sihra, 2004) **[Figure 1-19]**. Where KARs are located

presynaptically at glutamatergic synapses they can act as auto-receptors and consequently contribute to short-term plasticity dynamics (Rodriguez-Moreno and Sihra, 2004, Scott et al., 2008). In addition, presynaptic KARs can also contribute to long-term plasticity at glutamatergic synapses, such as hippocampal mossy fibre synapses, where LTP and LTD are expressed predominantly by presynaptic mechanisms (Contractor et al., 2001, Schmitz et al., 2003, Pinheiro et al., 2013, Negrete-Diaz et al., 2018).

At developing thalamocortical synapses in the somatosensory cortex, pharmacological activation of presynaptic Kainate auto-receptors induces depression of synaptic transmission on a short-term and rapid timescale (Kidd et al., 2002). This regulation of presynaptic release is not seen after the first postnatal week and may be crucial in the maturation of the sensory network and high frequency information transfer to the cortex by thalamocortical neurons (Kidd et al., 2002).

At mossy fibre synapses between dentate gyrus granule cells and CA3 pyramidal cells in the hippocampus KARs are located presynaptically as well as postsynaptically. In contrast to their function at thalamocortical synapses, Kainate auto-receptors at mossy fibre synapses (and cerebellar parallel fibre synapses) can both depress and facilitate glutamate release depending on the concentration of agonist, with lower concentrations resulting in facilitation and higher concentrations resulting in depression (Delaney and Jahr, 2002, Contractor et al., 2001, Schmitz et al., 2001, Schmitz et al., 2000). Synaptic glutamate release generally results in lower KAR activation and therefore synaptic facilitation. This decrease in KAR opening is a major component of the observed facilitation of synaptic transmission in response to repetitive synaptic stimulation, for stimulation frequencies ranging from ~1 Hz – 100 Hz. However, longer high frequency bursts of synaptic activity can release sufficient glutamate to depress subsequent glutamate release (Schmitz et al., 2001).

The receptor subunit complement of the presynaptic KARs at mossy fibre synapses was initially thought to comprise predominantly GluK1 (Lauri et al., 2001, Bortolotto et al., 1999), but subsequent reports supported the inclusion of GluK2, GluK3 and GluK5 (Schmitz et al., 2003, Contractor et al., 2003a, Pinheiro et al., 2007). These presynaptic KARs can regulate vesicle release by several reported mechanisms. The depolarization of presynaptic terminals and axons causes a broadening of the action potential, leading to enhanced Ca^{2+} influx through VGCCs and increased likelihood of

vesicle release (Schmitz et al., 2001), although this has not been measured (Scott et al., 2008). This mechanism relies on ionotropic KAR function and can therefore act rapidly, on the millisecond timescale found with high frequency synaptic stimulation (Kidd et al., 2002, Schmitz et al., 2001). On an equally fast timescale, increases in presynaptic Ca^{2+} could result from Ca^{2+} influx through Ca^{2+} permeable KARs that in turn can promote release from Ca^{2+} stores (Scott et al., 2008, Lauri et al., 2003). However, there are also reports of metabotropic signaling by presynaptic KARs to regulate neurotransmitter release (Frerking et al., 2001, Rodríguez-Moreno and Lerma, 1998). These would necessarily act on a slower timescale where G-protein coupled signaling might regulate presynaptic VGCCs or directly interact with vesicle exocytosis.

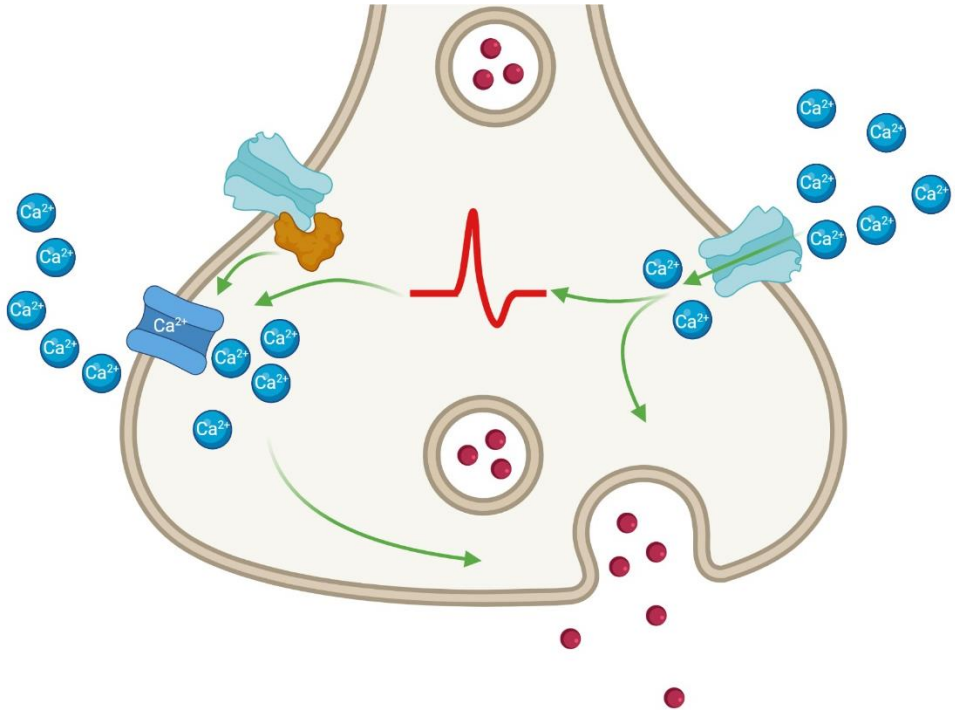

Figure 1-19: Regulation of neurotransmitter release by presynaptic KARs

Presynaptic KARs bidirectionally regulate the release of glutamate. Proposed mechanisms include regulation of presynaptic Ca^{2+} by depolarization, Ca^{2+}-permeable KARs and metabotropic signaling. Adapted from (Nair et al., 2021).

Hippocampal mossy fibre synapses also exhibit a presynaptically expressed form of long-term plasticity that is dependent on presynaptic Ca^{2+} (Nicoll and Schmitz, 2005). Since presynaptic KARs regulate short-term plasticity principally via the influx of Ca^{2+} they can therefore also regulate the induction of this form of long-term plasticity. Initially, KARs were thought to be required for mossy fibre long-term potentiation (mfLTP) (Bortolotto et al., 1999), but subsequently they were shown to alter the threshold for mfLTP induction in line with their role in short-term plasticity (Schmitz et al., 2003). In addition, the receptor subunits important for mfLTP (including GluK2, GluK3 and GluK5) mirror those underlying short-term plasticity (Contractor et al., 2001, Schmitz et al., 2003, Contractor et al., 2003a, Pinheiro et al., 2007).

1.7.3.4 Kainate receptors in Inhibitory neurons

KARs are acclaimed for regulating inhibitions in hippocampal synapses by altering the GABA release from presynaptic terminals (Evans et al., 2017a, Sloviter and Damiano, 1981, Westbrook and Lothman, 1983, Kehl and McLennan, 1985). Dual signaling of KARs are employed in modulating GABA release from the presynaptic terminals. KA induced depression of GABA release is dependent on metabotropic signaling of KARs and requires $G_{i/o,}$ Phospholipase C (PLC) and PKC. This is insensitive to blockade by a $GABA_BR$ specific antagonist (Rodriguez-Moreno et al., 2000, Rodríguez-Moreno and Lerma, 1998, Cunha et al., 2000, Lourenço et al., 2010). Further, KA-induced depression of GABA release is absent in $GluK1^{-/-}$ or $GluK2^{-/-}$ mice. However, mice deficient in both KAR subunits (GluK1 and GluK2 double KO) failed to depress presynaptic GABA release, suggesting the requirement of either of the two subunits (Mulle et al., 2000). Furthermore, facilitation of GABA release is attributed to the ion channel activity of KARs in various synapses including hippocampus, neocortex, and hypothalamus (Mathew et al., 2008, Liu et al., 1999, Jiang et al., 2001). Antidromic action potentials are the ones travelling from axon to soma (Langdon et al., 1993, Henze et al., 1997). Interestingly, activation of ionotropic KARs in the axons and soma reduces the threshold for action potential firing by generating antidromic action potential. This can further enhance the likelihood of action potential generation by extra-synaptic KARs (Semyanov and Kullmann, 2001, Ishizuka et al., 1990, Li et al., 1994, Scharfman, 1994).

1.7.3.4.1 KARs and KCC2

A fine interplay between the excitatory and inhibitory balance is crucial for the maintenance and refinement of neuronal networks. Impairments in GABAergic system during development underlie in various neurological and psychiatric disorders (Roberts, 2006, Jahangir et al., 2021, Steiner et al., 2016, Blum and Mann, 2002). In adults, neurons maintain a lower level of intracellular Cl^- concentration by expressing neuron specific K^+/Cl^- co-transporter type 2 (KCC2). The co-transporter couples the energy to import K^+ ions from outward directed gradient to eliminate intracellular Cl^- ions. This confers $GABA_AR$ with hyperpolarizing properties (Rivera et al., 1999, Li et al., 2002, Moore et al., 2019).

KARs dependent modulation of GABAergic transmission is further influenced by KCC2 (Pressey et al., 2017, Acton et al., 2012). Direct interaction of GluK2 with KCC2 and Neto2 is key in oligomerization and basal surface expression of KCC2 (Mahadevan et al., 2017, Mahadevan et al., 2014). Activation of metabotropic and ionotropic KARs differentially regulate the reversal potential for GABA (E_{GABA}) via KCC2 (Garand et al., 2019). In addition, the ionotropic KARs can hyperpolarize E_{GABA} independent of KCC2 (Pressey et al., 2017). KAR-induced inhibition of E_{GABA} is absent in $GluK1^{-/-}$ and $GluK2^{-/-}$ mice emphasizing the involvement of GluK1 and GluK2 subunits of KARs in regulating inhibitory transmission (Garand et al., 2019, Pressey and Woodin, 2020). Overall, this above mechanisms of KARs in regulation of GABA further advocates the role of KARs in maintaining functional neuronal network (Courtney and Christian, 2019).

1.7.3.5 Developmental role of KARs

KARs play major roles in the development of CNS, growth cone dynamics, synapse formation and stabilization, filopodial motility regulation etc. (Lerma and Marques, 2013, Vesikansa et al., 2007, Henley et al., 2021, Pinheiro and Mulle, 2006, Carta et al., 2014, Vissel et al., 2001a).

Regulation of filopodial movement is crucial in the formation of functional synapses during development. Synaptic KARs activation regulate filopodial movement by enhancing the movement in immature and inhibiting in mature slices. This bidirectional regulation is proposed to establish synaptic contacts (Tashiro et al., 2003). During first week of postnatal development, KARs activated by ambient glutamate release is

critical in the formation of synaptic connections and network activity (Lauri et al., 2005, Lauri et al., 2006, Segerstråle et al., 2010). In thalamo-cortical and in some silent synapses, KARs expressed during early stages of development contribute to neuronal transmission which is later replaced by AMPARs via NMDAR mediated signaling pathway (Isaac, 1999).

KARs expressed during early postnatal days regulate the development of glutamatergic synapses. GluK1, GluK2 and GluK5 subunits of KARs are highly expressed in amygdala during first week of postnatal development (Ryazantseva et al., 2020, Li et al., 2001, Shin et al., 2010). Early expression of KARs regulate the glutamate release via G-protein dependent signaling mechanism (Ryazantseva et al., 2020). Genetic manipulation of KAR expression in newborn rodents perturbed the formation of glutamatergic inputs to central amygdala (Ryazantseva et al., 2020). In addition, GluK2 containing KARs are decisive in the formation of functional MF-CA3 synapses. Mice deficient in GluK2 subunits exhibit delay in functional and morphological maturation of MF-CA3 synapses (Lanore et al., 2012). Further, formation of functional synaptic networks require regulated firing of neurons. (Yang et al., 2007). In the CA3- striatum lucidum synapses of neonatal brain, the spontaneous hyper firing of neurons decreases markedly towards early postnatal days due to changes in the properties of GluK1 KARs (McBain and Fisahn, 2001, Segerstråle et al., 2010). Together, increasing evidence suggest the importance of KARs in modulation of network activity for the proper synaptic circuit formation during development.

1.8 KARs in diseases

1.8.1.1 Epilepsy

Dysfunction of KARs is strongly associated with temporal lobe epilepsies (TLE). In animal models and epileptic patients, formation of aberrant MF to granule cell synapses is characterized by recruitment of KARs (Epsztein et al., 2005, Artinian et al., 2011, Henley et al., 2021). Furthermore, upregulation of GluK1 is evident in the hippocampus of patients with drug-resistant epilepsies (Li et al., 2010, Khalilov et al., 2002). In addition, GluK2 Q/R editing is higher in samples of patients with drug-resistant form of epilepsies (Kortenbruck et al., 2001). Moreover, in neonatal mice

models of epilepsy blockade of ionotropic KARs using specific antagonists or mice lacking GluK2 are less susceptible to hypoxia induce seizures during reoxygenation period suggesting the role of aberrant KARs in diseases (Grosenbaugh et al., 2018, Lerma and Marques, 2013, Henley et al., 2021).

1.8.1.0 Nociception

KARs are implicated in pain sensation (nociception). Modulation of excitatory signals between sensory and spinal neurons by KARs are pivotal in pain perception (Bhangoo and Swanson, 2013). Dorsal root ganglion (DRG) neurons express high amount of GluK1 and GluK5 KARs which are necessary to depolarize afferent fibres. Inhibition of GluK1 containing KARs by pharmacological antagonists or genetic ablation of genes reduces the pain response in rats (Simmons et al., 1998, Agrawal and Evans, 1986, Sommer et al., 1992).

1.8.1.0 Schizophrenia

A prominent theory on the pathophysiology of schizophrenia is the expression of aberrant glutamate receptors in prefrontal cortex (Meador-Woodruff et al., 2001). *In-situ* hybridization studies alongside receptor autoradiography indicated an increased expression of GluK3 and decreased expression of GluK5 mRNA in multiple regions of prefrontal cortex (Meador-Woodruff et al., 2001). Further, a reduction in the mRNA levels of GuK1 in hippocampus, para hippocampus and GluK3 in pre-frontal cortex of patients with schizophrenia raise the possible role of KARs in pathophysiology of the disease (Benes et al., 2001, Scarr et al., 2005, Wilson et al., 2006). SNPs for *GRIK4* gene encoding GluK4 is also predominant in patients with schizophrenia (Paddock et al., 2007). Moreover, exon sequencing studies on patients with schizophrenia exposed a loss of function and/or missense mutations in genes encoding GluK2 and GluK4 subunits of KARs (Koromina et al., 2019).

1.8.1.1 Huntington's Diseases

A major contributor of Huntington's diseases (HD) is the trinucleotide repeat of glutamine (CAG) (Snell et al., 1993). GluK2 is identified as one of the genes associated with age-onset excitotoxity in HD patients (MacDonald et al., 1999) however, there is currently a lack of evidence to establish a link between GluK2 to HD pathology (Rubinsztein et al., 1997, Diguet et al., 2004).

Overall, KAR dysfunction implicated in various neurological disorder open a new avenue for investigation.

1.9 Adenosine de-aminase acting on RNAs

Adenosine de-aminases acting on RNAs (ADARs) are enzymes that hydrolytically deaminate adenosine (A) on C6 position of the double stranded RNA (ds RNA) to Inosine (I) **[Figure 1-20]**. (L.BassHaroldWeintraub, 1988, Wagner et al., 1989), the inosine is then read as guanosine (G) by the transcriptional machinery enabling the incorporation of amino acid arginine (R).

Even though, ADAR mediated editing is predominant in introns and un-translated regions (UTRs) (Picardi et al., 2015, Ramaswami et al., 2013), a minor portion of protein coding regions, precursors of certain miRNAs known as pre-miRNAs and various other non-coding RNAs (ncRNAs) are also edited (Pinto et al., 2014). This adds to the repertoire of transcripts with novel properties (Nishikura, 2016, Aquino-Jarquin, 2020).

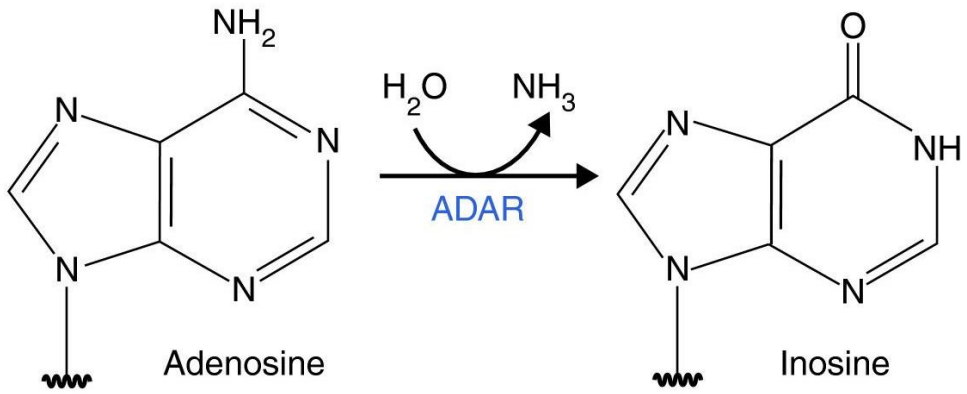

Figure 1-20: ADAR mediated hydrolytic de-amination of Adenosine (A) to Inosine (I)

Hydrolytic de-amination of Adenosine to Inosine by ADAR. The converted Inosine in the RNA is read as Guanosine by the translational machinery. Figure taken from (Slotkin and Nishikura, 2013).

1.9.1 ADAR Family of Enzymes

Mammals express two catalytically active ADAR1 and ADAR2 and a catalytically inactive ADAR3 enzymes encoded by the genes *ADAR1-3* respectively (Chen et al., 2000a, Kim et al., 1994a, Kim et al., 1994b).

ADAR1 express a shorter ADAR1-p110 form under a constitutively active promoter and a longer ADAR1-p150 under Interferon (INF) promoter (Patterson and Samuel, 1995). ADAR1-p110$^{-/-}$ and ADAR1-p150$^{-/-}$ mice die in-utero due to the failure in erythropoiesis alongside fetal liver disintegration (Hartner et al., 2004, Pestal et al., 2015). Furthermore, ADAR2$^{-/-}$ animals die within 3 weeks after birth (Brusa et al., 1995), however this can be rescued by expressing edited *GRIA2* transcript that translate into GluA2 (R) subunit of AMPAR (Higuchi et al., 2000).

1.9.2 Structure of ADARs

ADAR enzymes have a common structural and functional domain **[Figure 1-21]**. A 65 amino acid long highly conserved α-β-β-α configuration forms the basis of dsRNA binding domain (dsRBD) (Ryter and Schultz, 1998). ADAR1 have three dsRBD while ADAR2 and ADAR3 is characterized with two dsRBD that aids in interaction with the targeted dsRNA substrates (Valente and Nishikura, 2007).

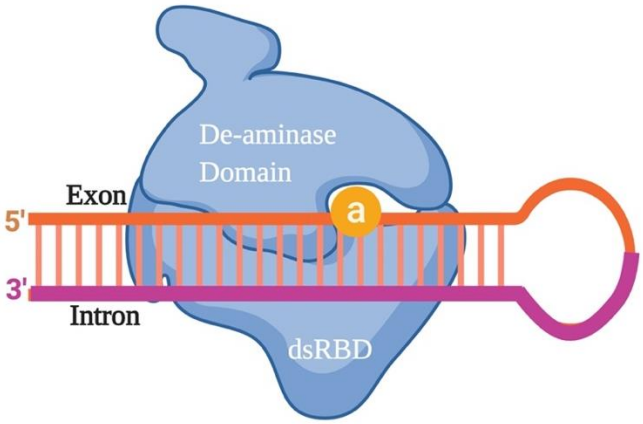

Figure 1-21: Interaction of ADAR with dsRNA

The dsRNA is formed by the base pairing of the intronic editing complimentary sequence to the exonic Q/R editing site. The substrate dsRNA is recognized by the dsRBD of the enzyme that aids in recognizing and editing.

Difference in the number of dsRBD confers specificity to their substrate over others edited by a different family of ADARs (Stefl et al., 2006). In addition, ADAR1 consist of two Z DNA binding domains Zα and Zβ with an exception of p150 isoform which is restricted to have a single Zα domain (Herbert et al., 1997). Zα domain of p150 is vital for rendering negative regulation of interferon induced dsRNA response. Contra wise,

functional significance of Zβ domain remains unresolved [**Figure 1-22]** (Athanasiadis et al., 2005, Herbert, 2019, Herbert et al., 1997).

The catalytic domain of ADAR lies in its C-terminal region and is conserved over several cytidine de-aminases with a Zn^{2+} in the catalytic centre (Valente and Nishikura, 2007, Slotkin and Nishikura, 2013, Kim et al., 1994a, Nishikura et al., 1991). Unravelling the crystal structure of human ADAR2 protein revealed the presence of Inositol hexakiphosphate (IP6) in the protein core which aids in folding as well as for the enzymatic activity by stabilizing the amino acid residues in the catalytic centre (Macbeth et al., 2005, Tomaselli et al., 2013).

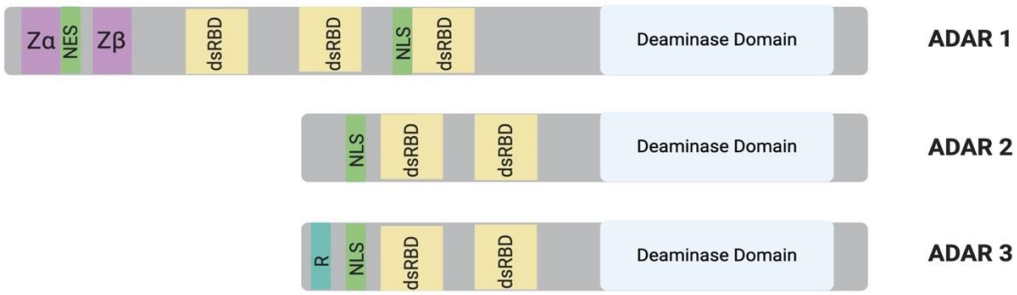

Figure 1-22: Structure of ADAR enzymes

All three family of ADAR enzymes share a conserved deaminase domain along with varying number of dsRBD and a nuclear localisation signal (NLS). In addition, ADAR1 contain Z DNA binding domains Zα and Zβ along with a nuclear export signal (NES). ADAR3 possess an arginine rich, single stranded RNA binding domain (R Domain).

1.9.3 ADAR mediated editing of glutamate receptors

Three among four AMPAR subunits and two of the five KAR subunits undergo ADAR mediated editing as listed below.

Table 1-4: List of known glutamate receptor subunits edited by ADARs and their functional implication in the channel properties.

Gene	Protein	Editing	Functional implications	Type of ADARs
GRIK1	GluK1 (KAR)	Q to R	Modulation of Ca^{2+} permeability of the channel	ADAR2 ADAR1
GRIK2	GluK2 (KAR)	Q to R I to V Y to C	Modulation of Ca^{2+} permeability of the channel, ER retention	ADAR2 ADAR1 ADAR2
GRIA2	GluA2 (AMPAR)	Q to R R to G	Modulation of Ca^{2+} permeability of the channel and receptor desensitization kinetics, ER retention	ADAR2 ADAR1 **[Figure 1-23]**
GRIA3	GluA3 (AMPAR)	R to G	Modulation of receptor desensitization kinetics	ADAR1 ADAR2
GRIA4	GluA4 (AMPAR)	R to G	Modulation of receptor desensitization kinetics	ADAR1 ADAR2

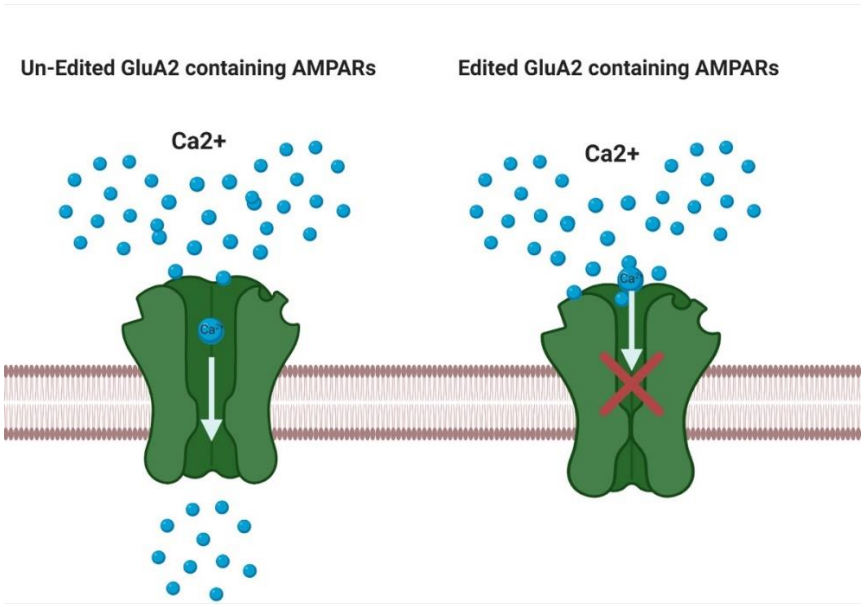

Figure 1-23: Schematics showing differences in the Ca^{2+} permeability of edited vs un-edited GluA2 containing AMPARs.

Q/R editing of GluA2 containing AMPARs, leads to changes genetically encoded negatively charged Glutamine (Q) to positively charged Arginine (R). This conversion in the channel pore restricts the Ca^{2+} influx through the channel.

1.9.4 Q/R editing in GluK2 subunits of KARs.

Editing of GluK1 and GluK2 KARs at Q/R site is a developmentally regulated process with very low levels of edited subunits in embryonic brain and subsequently increasing towards the adulthood.ADAR2 dependent editing converts genetically encoded glutamine (Q) in the pre-mRNA to arginine (R) (Peng et al., 2006, Evans et al., 2017c, Köhler et al., 1993, Herb et al., 1996, Kortenbruck et al., 2001). In adult brain 40% of GluK1 and 80% GluK2 pre-mRNA is edited (Belcher SM, 1997, Paschen et al., 1997, Bernard et al., 1999). The short dsRNA structure formed in the pre-mRNA by the interaction of exonic sequences in the editing site and the downstream intronic editing site complementary sequence (ECS) in the nearby intron is a prerequisite for ADAR2 mediated editing of iGluRs. ECS can be located near or far from the editing site (Herb et al., 1996). For GluK1 and GluK2, the ECS is located ~1900 bp downstream of editing site whereas for GluA2, the ECS site is situated ~310 bp downstream (Bernard et al., 1999).

In line with GluA2 (R), GluK2 (R) show a reduced Ca^{2+}permeability and a subsequent alteration in the I-V relationship (Evans et al., 2017c, Egebjerg and Heinemann, 1993, Kwak and Kawahara, 2005, Herb et al., 1996). The I-V relationship of a receptor is due to cytoplasmic polyamine preventing the outflow of intracellular cations ions through the channel, interestingly the edited receptors renders insensitivity to the polyamine block are less trafficked to the surface due to the exposed ER retention sequence (Bernard et al., 1999, Evans et al., 2017c, Wilding et al., 2005).

1.9.5 Glutamate receptor editing in neurological disorders

Pre- and post-translational modifications including editing is a key feature in regulating the maneuvering and function of AMPARs and KARs at the synapses (Diering et al., 2018, Mah et al., 2005, Roche et al., 1996, Coussen et al., 2005). Dysregulation of editing has been implicated in various neurological disorders. Studies on rat forebrain ischemia models displayed a reduction in GluA2 Q/R editing as a resultant of reduced ADAR2 enzyme and altered CREB protein levels. Restoration of ADAR2 and GluA2 Q/R editing protect neurons from post-ischemic insult (Peng et al., 2006). Interestingly, downregulation of Q/R editing is attributed to sporadic amyotrophic lateral sclerosis (ALS). Progressive loss of cholinergic motor neurons is characterized

in the absence of ADAR2 enzyme linking their role in pathophysiology of ALS (Yamashita and Kwak, 2014, Kwak and Kawahara, 2005).

Increased expression of surface GluK2 (Q) enhances influx of Ca^{2+}through the channel contributing to neuronal excitotoxicity (Kwak and Kawahara, 2005, Hideyama et al., 2012, Hideyama et al., 2010). Further, GluK2 Q/R editing deficient mice exhibit increased vulnerability to Kainate-induced seizures implicating the role of ADAR2 mediated editing in seizure induction (Vissel et al., 2001a). However, contrary to this, studies on patients with drug-resistant temporal lobe epilepsies display increase in editing of GluK1 and GluK2 subunits (Kortenbruck et al., 2001). This can be an adaptive response to regulate excessive Ca^{2} influx and prevent the glutamate induced excitotoxic insult (Kortenbruck et al., 2001).

Chronic treatment of anti-depressants drugs such as fluoxetine and desipramine regulate the expression and editing of GluA2 and GluK2 subunits of AMPARs and KARs respectively (Barbon et al., 2006). Fear conditioning and behavioral studies on mice models indicate an increase in the levels of unedited GluK1 in amygdala of fear conditioning trained animals (Brande-Eilat et al., 2015).

Mass sequencing approach to study the RNA editing in Alzheimer's patients revealed a downregulation in the editing of GluA2, GluA3 and GluA4 linking editing of glutamate receptors with AD pathology (Khermesh et al., 2016). Further, downregulation of ADAR2 activity and a resultant decrease in GluA2 Q/R editing is a prominent clinical feature in patients with Astrocytoma, a form of glioblastoma multiforme (GBM). Over expression of ADAR2 in cell lines indicate a correlation between ADAR2 dependent editing and cancer progression (Maas et al., 2001, Cenci et al., 2008).

Taken together, alteration in ADAR2 mediated editing of glutamate receptors in various diseases emphasize the role of editing in maintaining homeostasis and physiological functions.

1.10 Aims and Objectives

KARs participate in forms of Hebbian and homeostatic plasticity of AMPARs and can themselves undergo plasticity (Park et al., 2006, Petrovic et al., 2017c).

More specifically, recent studies form the lab revealed that KARs are capable of inducing LTP of AMPARs (KAR-LTP_{AMPAR}) by the metabotropic signaling of the receptors, independent of NMDAR and AMPAR activation (Petrovic et al., 2017c). Building on these findings, I wanted to further investigate whether KARs can induce LTD of AMPARs and study the mechanism underlying this novel form of KAR-LTD_{AMPAR}.

Additionally, Q/R editing of GluK2 KARs affects the trafficking and channel properties in both physiological and pathological conditions (Ball et al., 2010a, Evans et al., 2017c, Swanson et al., 1996). We have established a breeding colony of GluK2 Q/R editing-deficient mice to interrogate, in *ex vivo* hippocampal slice preparations, how the complete loss of Q/R editing of GluK2 alters synaptic transmission and plasticity.

Briefly, the overall Aims of my PhD were to address the following broad questions:

- What are the signalling molecules underlying KAR-mediated plasticity of AMPARs?
- Can Kainate stimulation induce AMPAR LTD?
- What are the synaptic and network consequences of the loss of GluK2 Q/R editing?

Each of these questions are subdivided into specific objectives in the corresponding results chapters.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

All the chemicals and reagents used were from Merck (formerly Sigma) unless stated.

2.1.2 Molecular biology reagents

2.1.2.1 Agarose gel

4% Agarose gel was prepared for gel electrophoresis from Ultra-pure agarose purchased from Thermo scientific. Ethidium bromide was used to visualize the DNA under UV- illuminator. 1Kb DNA ladder was used as a standard marker.

2.1.3 Protein biochemistry reagents

20X stock of protein inhibitor cocktail is made by dissolving Complete™ protease inhibitor tablets purchased from Roche in 2ml distilled water and was stored at 4ºC.

AcrylaGel (30% w/v Acrylamide stock solution) obtained from Geneflow limited was used to make SDS-PAGE gels.

Immobilon-P PVDF membrane (Polyvinylidene difluoride) with 0.45µM pore size, 3MM Chr cellulose chromatography grade Whatman™ papers from GE healthcare life sciences, PageRuler Protein ladder was purchased from ThermoScientific. Membrane blocking solutions included BSA, and non-fat milk powder from Co-op. For the visualization of protein bands, the following HRP substrates were used.

- Super Signal® West Pico Enhanced Chemiluminescence (ECL) - ThermoScientific
- Luminata™ Crescendo
- Luminata™ Forte
- West Femto ECL-ThermoScientific

For X ray detection, CL-Xposure™ Xray film was purchased from ThermoScientific, and the cassettes were obtained from Amersham Biosciences. Fixer and developer solutions were obtained from Fixaplus.

For biotinylation, the EZ-Link® Sulfo-NHS-SS-Biotin and Streptavidin-Agarose from *Streptomyces Avidinii* beads were from ThermoScientific and Merck (Sigma) respectively. Chromotek provided GFP Trap beads.

2.1.4 Mammalian cell culture reagents

Stocks of Human Embryonic Kidney Cells (HEK) 293 T obtained from The European Collection of Cell Cultures (ECACC) is maintained in 1% DMSO and stored at -196 ^{o}C. The stocks are revived and passaged depending on the requirements.

The cell culture medium was prepared by supplementing Dulbecco's modified eagle's medium (DMEM) with 10% heat-inactivated foetal bovine serum (FBS), 1% glutamine (Gibco, Invitrogen) and 1000 units penicillin and 0.1mg streptomycin (Gibco). Poly-L-Lysine (PLL) was purchased from Merck. Transfection reagent Lipofectamine® 2000 was obtained from ThermoFisher.

2.1.5 Electronic instruments

Laminar Airflow for cell culture were installed by Holten LaminAir. The cell culture incubators and shaking incubators for bacterial culture were obtained from LTE Scientific and New Brunswick Scientific, respectively. Biofuge and Eppendorf supplied benchtop microcentrifuges and the high-speed ultra-centrifuges were from Beckman-Coutler or Jouan. Low speed centrifuges were obtained from Jouan and Hettick. The powerpack and the buffer tanks for SDS-PAGE gel electrophoresis were from Bio-Rad laboratories. Electrophoretic tank for agarose gel was obtained from Takara. Eppendorf provided heating blocks and water bath was from Grant. UV-Transilluminator for DNA visualisation was obtained from BioDoc-It^{TM} imaging system. The thermal cycler PCR instrument was obtained from MJ Research PTC-2000. Konica (SRX-101A automatic medical X-ray film developer) and Odyssey Fc-detection system from LI-COR were used for X-ray film developing and chemiluminescence detections respectively. Dissection microscopes for primary culture was obtained from Leica.

2.1.6 Materials for electrophysiology

Micromanipulators for electrophysiology rig is obtained from Scientifica, amplifier was from Axon Instruments, Data Acquisition unit (Digitizer) is from Cambridge electronic

design (CED) limited. Stim electrodes were obtained from Life science and glass capillaries from Harvard Apparatus.

2.1.7 Laboratory glassware and plasticwares

Nonsterile pipette tips of volume 10µl to 1000µl and PCR tubes (0.5ml and 0.2ml) were from StarLabs and the gel loading tips were supplied by Fischer. Sterile serological pipettes and cell culture plastic wells (6 well to 96 well) were obtained from Cell star. Glass coverslips and glass slides for fixed cell imagining were from VWR international. Falcon supplied tubes of volume 15ml and 50ml. syringes (1ml to 10ml) were from Therumo and needles from BD Microlance™3.

2.1.8 Reagents for fixed cell imaging

Pierce™ 16% Formaldehyde and Fluoromount-G mounting™ media with DAPI were obtained from ThermoScientific.

2.1.9 Antibodies

2.1.9.1 Primary Antibodies

Immunocytochemistry (ICC) and Western Blotting (WB) were performed using the following primary antibodies. The antibodies for WB were diluted in 5% milk or BSA in PBS-T and for ICC were diluted in 3% BSA in PBS.

Table 2-1: List of primary antibodies used

Antibody Used	Species	Supplier	Catalogue number	Dilution	Type
GluA1 (WB)	Rabbit	Millipore	AB1504	1:1000	Polyclonal
GluA1 (ICC)	Mouse	Millipore	MAB2263	1:100	Monoclonal
GluA2 (WB)	Mouse	BD Pharmingen	556341	1:1000	Monoclonal

GluA2 (ICC)	Mouse	Millipore	MAB397	1:70	Monoclonal
GluK2 (WB)	Rabbit	Millipore	04-921	1:1000	Monoclonal
pGluA1 S845 (WB)	Rabbit	Millipore	AB5849	1:1000	Polyclonal
pGluA1 S831 (WB)	Rabbit	Millipore	AB5847	1:1000	Polyclonal
EGFR (WB)	Rabbit	Abcam	Ab52894	1:3000	Monoclonal
GAPDH (WB)	Mouse	Abcam	Ab8245	1:10000	Monoclonal
SUMO 1 (WB)	Rabbit	CST	4930S	1:1000	Polyclonal
APP (WB)	Rabbit	Abcam	Ab241592	1:4000	Polyclonal
GFP (WB)	Rat	Chromotek	3h9-100	1:3000	Monoclonal
Phospho-Ser PKC Substrate (WB)	Rabbit	CST	2261	1:1000	Polyclonal

2.1.9.2 Secondary Antibodies

The HRP-Conjugated secondary antibodies form Merck. Anti-rabbit (raised in goat), anti-rat (raised in rabbit) and anti-mouse (raised in goat). The antibodies were used in 1:10,000 dilution in 5% milk or 5% BSA in PBS-T.

Secondary antibodies for immunocytochemistry Cy2, Cy3 and Cy5 were raised in donkey. The antibodies were used at 1:400 diluted in 3% BSA in PBS and were purchased from Jackson ImmunoResearch.

2.1.10 Plasmid constructs

The following plasmid prepared by Dr. K. A. Wilkinson were used.

Table 2-2: List of plasmid constructs used

Protein	Species	Backbone	Tag
shGluK2	Rat	pXLG3-100bp stuffer	GFP
GluK2a	Rat	pcDNA3.1	N-ter YFP and Myc
GluK2 K886R	Rat	pcDNA3.1	N-ter YFP and Myc
GluK2 S846A, S868A	Rat	pcDNA3.1	N-ter YFP and Myc
GluK2 C858A	Rat	pcDNA3.1	N-ter YFP and Myc

2.1.11 Drugs

The drugs listed below were used for various experiments.

Table 2-3: List of various drugs used

Drugs	Stock Concentration	Final Concentration	Diluted in	Supplier
Chelerythrine	5mM	5µM	DMSO	Sigma
CNQX	10mM	10µM & 50µM	DMSO	Sigma
CGP55845 Hydrochloride	10mM	1µM	DMSO	Hellobio

DCGIV	10mM	2µM	ddH2O	Tocris
D-APV	10mM	50µM	ddH2O	Tocris
FK506	50mM	50µM	DMSO	Hellobio
Forskolin	20mM	20µM	ddH_2O	Tocris
Glycine	20mM	20µM	ddH_2O	Sigma
GYKI53655	10mM	40µM	ddH_2O	Abcam
H89	5mM	10µM	ddH_2O	Tocris
Kainate (KA)	10mM	10µM	ddH_2O	Tocris
KT5720	1mM	1µM	ddH_2O	Tocris
MPEP	10mM	10µM	DMSO	Tocris
NMDA	50mM	20µM	ddH_2O	Tocris
Okadaic acid	1mM	1µM	DMSO	Hellobio
Picrotoxin	1mM	50µM	ddH20	Sigma
PTx	0.1mg/ml	1µg/ml	ddH_2O	Tocris
Rp-cAMP	25mM	200µM	ddH_2O	Tocris
TTX	1mM	1µM	ddH_2O	Tocris
UBP310	10mM	20µM	DMSO	Tocris
YM29818	1mM	1µM	ddH20	Tocris

2.2 Molecular biology methods

2.2.1 Agarose gel electrophoresis

4% agarose gels were prepared by dissolving the required amount of agarose in 0.5X TAE buffer containing 40mM Tris acetate, 1mM EDTA, heated to dissolve the agarose. The mixture was then cooled (but not solidified) and ethidium bromide (0.5µl/ml) was added, mixed thoroughly, and poured (without bubbles) to the mould with setting combs attached beforehand. The gels were then left for solidifying.

The agarose gels were submerged in a Mupid®-eXu gel tank containing 0.5X TAE buffer. Samples with loading dye were added to the wells and 5µl of 1kb Hyperladder (Bioline) as a standard to identify DNA size. The electrophoresis was run for 20 minutes at 135 volts (V) to resolve the DNA. Gels were visualised in a UV-transilluminator.

2.2.2 Genotyping and PCR

Ear notches of animals were obtained by request from the animal sciences unit (ASU). The genomic DNA isolation was performed by using KAPA mouse genotyping kit purchased from Merck. The DNA extraction was performed in 100µl volume as follows.

Table 2-4: List of components required for genomic DNA isolation (Genotyping)

Component	Volume for 100µl	Final Concentration
PCR Grade water	88µl	-
10X KAPA Extract Buffer	10µl	1X
1U/µl KAPA Express Extract Enzyme	2µl	2 U /reaction
Ear notch	-	2 mm section

The mixture was pipetted up and down to thoroughly mix the solution and heated at 75°C for 10 minutes in a heating block for lysis followed by enzyme inactivation at 95°C for 5 minutes. The samples were centrifuged briefly to pellet the debris. The DNA extract was diluted 10-fold with 10mM Tris-HCl (pH 8.0-8.5). The samples were stored at -4°C or -20°C for future use.

PCR reaction was set up in individual PCR tubes by mixing the following components.

Table 2-5: PCR reaction mixture for genotyping

Component	Volume (µl)	Stock Conc.	Final Conc.
PCR-grade water	7.75	N/A	N/A
2xKAPA2G Fast (HotStart) Genotyping Mix with dye	12.5	2x	1x
Forward Primer	1.25	10µM	0.5µM
Reverse Primer	1.25	10µM	0.5µM
Reverse Primer 2	1.25	10µM	0.5µM
Genomic DNA	1µl	-	-
	Total=25µl		

The reaction was setup in a PCR thermocycler with following settings.

Table 2-6: PCR reaction setup for genotyping

Polymerase activation	95°C for 3 minutes
Denature DNA	95°C for 15 seconds
Annealing primers	60°C for 15 seconds
Polymerase extension	72°C for 30 seconds
Repeat Cycles	Repeat steps 2-4, 30 times
Final extensions	72°C for 2 minutes
Cooling	10°C for 10 minutes

Following PCR reaction, the samples were run on a 4% Agarose gel and the bands were visualized using a UV-Transilluminator.

2.2.3 Lentivirus preparation

Lentivirus preparation was performed in HEK293T cells grown in DMEM (with 1% L-glutamine, and 10% FBS). The same media was used for transfections.

2.2.3.1 Plating Cells

10^6 cells were plated in a 10cm cell culture dish containing 7ml of complete DMEM and left in the incubator overnight to achieve required confluency (70-80%).

2.2.3.2 Transfection

For transfection, the following day adequate amount of DMEM was filter sterilized using a 0.2µM syringe filter. A sterile bijou containing 2.5ml of plain DMEM was mixed with 20µl of XLG viral vector consisting of shRNA expressed under H1 promoter (pXLG3-100bp stuffer), 5µg pDMD2. G packaging vector (Addgene) and 15µg of p8.91 helper vector (Addgene). 2.5ml of plain DMEM media was mixed with 4.8% 1mg/ml polyethylenimide (PEI) in a sterile 15ml falcon tube. The mixture was mixed thoroughly by inverting the falcon tube several times and was filter sterilized into a fresh 15ml falcon tube with a 0.2µM syringe filter. This was left at RT for 2-3 mins with occasional mixing. The transfection media was prepared by mixing PEI-DMEM into the bijou

containing DNA mixture. The media was mixed by inverting several times and was left at RT for 30 mins.

The culture plates containing HEK293T cells were washed twice with 6ml of plating media carefully without dislodging the cells. 5ml of transfection media was slowly added after aspirating the media from the second wash and left for 4 hours at 37^{o}C incubator. After 7 hours of incubation, the transfection media was aspirated and supplied with 7ml of pre-warmed neuronal feeding media or DMEM. The cells were placed back in the incubator for 2-3 days.

2.2.3.3 Harvesting lentivirus

After 60-72 hours of transfection, the media containing the virus was transferred into a fresh 15ml falcon tube and centrifuged at to 3000g for 10 minutes at 4^{o}C to pellet cellular debris. A syringe filter of 0.45µM was wetted with plain DMEM containing FBS or neurobasal medium and the virus containing supernatant was filtered into a fresh 15ml falcon. The filter sterilized virus was aliquoted and stored at -80^{o}C until further use.

2.3 Cell culture methods

Cell culture works were performed under sterile conditions using laminar airflow. The cells were incubated in cell culture incubator maintained at 37^{o}C with 5% CO_2 and 95% O_2.

2.3.1 HEK293T culture

Stocks of HEK293T cells were stored with 10% DMSO in DMEM at -196^{o}C (liquid nitrogen). The stock was thawed in 37^{o}C water bath, transferred to a sterile 15ml falcon tube, and resuspended in 10ml of complete DMEM containing 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. The cells were centrifuged for 2 mins RT at 1500g. 5ml of complete DMEM was added to a sterile T25 flask. The supernatant was discarded, and the pellet was resuspended in 1ml complete DMEM before transferring to a T25 flask containing 5ml of medium. The flask was placed at 37^{o}C incubator overnight and passaged to a T75 flask containing pre-warmed 15ml of DMEM. Cells were passaged regularly the subsequent days until 20 passages.

2.3.2 Passaging HEK293T cells

HEK293T cells were passaged upon reaching a confluency of 70-90%. Sterile 1X PBS (10X PBS (Gibco) and sterile cell culture grade water (Hyclone™ HyQPAK)) were prepared and prewarmed prior to passaging. The cells were washed gently with 10ml of 1X PBS prior to adding 1ml of 0.05% trypsin-EDTA and were incubated at 37°C for 5 mins. This activates the trypsin to dislodge the attached cells. After 5 mins, the activity of trypsin is inhibited by adding 10ml of prewarmed complete DMEM. The cells were collected to a sterile 15ml falcon tube and were pelleted down at 1500g for 2 mins at RT. The supernatant was discarded, and the pellet was resuspended in 10ml of complete DMEM and triturated with a 10ml serological pipette. 1ml of the cell suspension was added to a fresh T5 flask containing prewarmed complete DMEM and placed back in the incubator.

2.3.3 Plating HEK293T cells

To facilitate the adhesion of cells, the culture dishes (6 wells or 3cm) were coated with sufficient volume of 0.1mg/ml PLL to cover bottom of the well/dish. The plate/dish was placed back in incubator at least for an hour. Post-incubation, the plate/dish was washed thrice with sterile cell culture grade water and 2ml of transfection media (Plain DMEM with 1% L-glutamine, and 10% FBS). After passaging the cells in T75 flask, the. Cells were counted using a haemocytometer. 200,000 or 1million cells were added to a 6 well dish or a 3 cm dish, respectively. The plates were left in incubator overnight to for the cells to adhere.

2.3.4 Counting HEK293T cells using a hemocytometer

The resuspended cells were mixed with 0.4% trypan blue (Gibco) in 1:10 dilution. The cells with dye were mixed by pipetting up and down several times and was added to the hemocytometer to determine the concentration of cells expressed as million per ml.

2.3.5 Transfection of HEK293T cells

Required amount of DNA and 1.5µl of Lipofectamine® 2000 (per µg of DNA) were added to two 1.5ml Eppendorf containing 100µl of plain DMEM media respectively. The Eppendorf's were gently vortexed and left at RT for 5 mins. Following the

incubations, the Eppendorf containing lipofectamine was added to the tube containing DNA. The solution was gently vortexed and was left to incubate at RT for 25 mins. After incubation, the transfection mixture was again vortexed before adding in a drop-wise manner to the wells/dish. The plates were placed back in the incubator. For overexpression constructs, the mixture was left for 48 hours and 72 hours for knockdown constructs.

2.4 Primary neuronal cell culture

2.4.1 Preparation of borate buffer

The buffer was prepared by adding required amount of borax and boric acid powder to a 50ml falcon to achieve the final concentration of 10mM Borax and 50mM Boric acid in a final volume of 500ml in sterile cell culture grade distilled water. 50ml of sterile cell culture grade distilled water was added to 50ml falcon containing borax and boric acid under laminar airflow chamber and left to dissolve on a rotating wheel at RT. The dissolved 50ml solution was filter sterilized using vacuum filtration method with 0.2μM membrane and the final volume was made up to 500ml with sterile cell culture grade distilled water. The filtered borate buffer is left at RT for future use.

2.4.2 Coating plastic cell culture plates with Poly-L-Lysine (PLL)

To ensure adhesion of non-adherent cells such as neurons, 6-well containing tissue culture dishes were coated with 0.5mg/ml of PLL diluted in borate buffer under laminar airflow chamber and left overnight in a 37°C,5% CO_2 incubator. The plates were washed three times with sterile cell culture grade water and replaced it with 1.5ml of plating media (Neurobasal® medium supplemented with 5% horse serum, 2% B27 and 1% glutamax) and left at 37°C/5% CO_2.

2.4.3 Embryonic rat dissection

Dissection area and surgical tools were sterilized with 70% ethanol before use. Pregnant E18 (Embryonic Day 18) Han Wistar rats were anesthetised with isoflurane and humanely sacrificed using schedule 1 method (cervical dislocation) after checking for reflexes. The abdominal region was sterilized with 70% ethanol and a vertical incision was made through linea-alba to expose the abdominal cavity. Uterus containing embryos were removed

leaving behind both the ovaries marking the termination of uterus. The embryos were kept in Hank's buffered salt solution at RT throughout the dissection. Embryos were removed from the uterine sac and decapitated. Brains of embryos were removed from severed heads using sharp forceps under a dissection microscope. Left and right hemispheres were separated, and meninges were removed carefully without damaging the cortical and inner anatomical structures. Hippocampus from both the hemispheres were excised for preparing hippocampal cultures and the rest of the cortex was used for preparation of cortical cultures.

2.4.4 Dissociation of cortical and hippocampal tissues, plating and feeding of neurons

Dissected hippocampi and cortices were transferred to 15ml and 50ml falcon tubes respectively under a laminar airflow chamber. Cortices were made further into small sections using a sterile scalpel blade. Tissues were washed three times with 10ml HBSS for hippocampi and 30ml for cortices. Cortices were incubated for 15 mins in 30ml HBSS containing 0.005% trypsin/EDTA and hippocampi for 9 mins in 10ml HBSS containing 0.005% trypsin/EDTA at 37ºC with frequent inverting. The trypsinised tissues were washed three times with HBSS (same volume as mentioned above) and one wash with 1ml plating media for hippocampi and 5ml plating media for cortices. The cell suspensions were triturated by pipetting up and down several times with 1ml pipette for hippocampus and 5ml serological pipette for cortical tissues. Hippocampal and cortical cell suspension were diluted up to 4ml and 20ml respectively with plating media.

2.4.5 Counting of neurons using hemocytometer

The cells were counted in a haemocytometer. For biochemistry experiments, 500,000 cells per well were plated in pre-treated 6 well tissue culture dish containing plating media and incubated in a 37°C incubator. 2 hrs after seeding the cells, the plating media was replaced with 2ml of feeding media (Neurobasal® medium supplemented with 2% B27 and 1% glutamax) and replaced in a 37°C incubator. 7 days later the neurones were fed with a further 1ml of feeding media and stored until use.

2.4.6 Viral transduction of neurons

For viral transduction of neurons, the aliquots of lenti or sindbis viruses stored at -80 were thawed. The optimized volume of virus was added to the hippocampal or cortical neuronal cultures. Sindbis virus infected cells were left for 24 hrs prior to experiments. The sindbis virus were used to over express GFP, WT-GluK2 and GluK2-K886R and were prepared by Dr. Enaam Al Momany or Dr. K. A Wilkinson. For knockdown experiments, lentivirus was added to neurons at DIV 10, and the infected cells were left for 7 days at 37°C incubator before treatment with KA.

2.5 Biochemical methods

2.5.1 Cell lysate preparation

Phospho proteins and total protein samples were studied by lysing the cells directly by adding 250µl of 1X sample buffer (2% SDS, 5% glycerol, 5% β-mercaptoethanol (β-ME), 0.002% bromophenol blue, 0.0625M Tris-HCl pH6.4) to the wells containing neurons after aspirating the media. The cells were scraped using a cell scraper and transferred to an Eppendorf. The lysate was prepared by heating the samples at 95°C for 10 mins. The samples were stored at -20°C until use.

2.5.2 Neuronal surface biotinylation

Live hippocampal neurones were cooled down to 4°C post treatment to prevent further trafficking of receptors. The wells were washed twice with 2ml of cold Earle's buffer (EBS) (140mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgCl_2$, 25mM HEPES and 0.9g/L D-glucose). The surface proteins were tagged with 1.5ml of Sulfo NHS-SS-Biotin (0.3mg/ml diluted in 1X EBS) for 10 mins with gentle movement every 2 mins and washed three times with 2ml of EBS. 2ml of 100mM NH_4Cl was added to scavenge the free biotin for 1 min. The cells were washed with 2ml of EBS and were lysed in 200µl lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1% triton X-100, 0.1% SDS, 1X protease inhibitor in ddH_2O), scraped and transferred to a 1.5ml Eppendorf. The cells were sonicated briefly with 3 pulses and were incubated on ice for 30 mins. After incubation cells were centrifuged at 20,000g at 4ºC for 30 mins to get rid of cell debris.

The surface proteins were pulled down with 150µl Pierce™ Streptavidin UltraLink™ resin beads. The beads were washed twice with lysis buffer devoid of protease inhibitor cocktail by centrifuging at 1500g at 4ºC for 2 mins followed by aspirating the buffer containing supernatant. 80µl of lysate was added to the beads and mixed on a rotating wheel at 4°C for 1 hr. Following incubation, the beads were washed three times in lysis buffer and the supernatant was aspirated. The beads were re-suspended in 2X sample buffer and were heated at 95°C for 10 mins on a heating block before Western blotting.

2.5.3 SDS-PAGE and Western blotting

Sodium dodecyl sulphate- poly acrylamide gel electrophoresis (SDS PAGE) was used to separate proteins based on their molecular weight. 10% of acrylamide resolving gel (375mM Tris-HCL pH 8.8, 10% acrylamide, 0.1% SDS, 0.1% APS and 0.01% TEMED) was filled into a 1.5mM glass plate and layered with 100% ethanol to ensure even polymerisation. The ethanol was washed off with double distilled water (ddH_2O) and stacked with 5% stacking gel (125mM Tris-HCL pH 6.8, 5% acrylamide, 0.1% SDS, 0.1% APS, 0.01% TEMED) and a 1.5mM comb was inserted.

The gels were assembled into an electrode after polymerisation and the assembly was inserted into an appropriate electrophoresis tank. Reservoirs were filled with 1 X SDS PAGE running buffer (25mM Tris, 250mM glycine and 0.1% SDS in ddH_2O). The wells were loaded using gel loading tips and 3µl of PageRuler™ Prestained protein ladder was used as a maker. The electrophoresis was run at 100V initially and was increased to 150V once the samples entered the resolving gel.

The proteins in acrylamide gel were then transferred to a PVDF membrane. The membrane was activated by 100% methanol for a few seconds and was left in the transfer buffer for 10 mins (50mM Tris, 40mM glycine and 20% methanol in ddH_2O). Cellulose chromatographic papers and sponges were soaked in the transfer buffer before assembling into a wet transfer cassette. The resolving gel was then sandwiched between sponges and chromatographic papers and PVDF membrane and the cassette was placed in a tank with gel facing cathode and membrane facing anode. Buffer was filled in the tank; ice packs were used, and the wet transfer was carried out at a constant Amperes of 400mA for 70 mins with constant stirring.

2.5.4 Immunoblotting

The membrane was removed after completion of transfer and was blocked in 5% skimmed milk in PBS-T or 5% BSA in PBS-T (PBS (recipe: 0.137M NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM K_2HPO_4, pH to 7.4 with HCl) + 0.001% Tween-20) for 1 hr at RT. The PVDF membrane was then incubated overnight with primary antibody diluted to appropriate concentration in 5% milk/BSA in PBS-T. After incubation, the membrane was washed three times with PBS-T to get rid of unbound antibody. Secondary antibody was diluted 1:10,000 in 5% milk/BSA in PBS-T and the membrane was incubated for an hour at RT. The membrane was washed three times with PBS-T for 5 mins on a shaker before adding the chemiluminescence substrate.

2.5.5 Chemiluminescence detection

The protein was visualized by incubating the membrane for 1 min at RT with the any of the following substrates in the increasing order of their sensitivity.

- SuperSignal® West Pico Enhanced Chemiluminescence (ECL)
- Luminata™ Crescendo
- Luminata™ Forte
- West Femto ECL

The excess substrate was removed, and the membrane was placed in Odyssey Fc detection machine for detection of signal. The signal was recorded by exposing the membrane in chemiluminescence channel for 2-5 mins and the protein ladder was detected using 700-flurophore channel for 1-2 mins.

2.5.6 Stripping and re-probing of the membrane

Previously used membrane was washed three times with PBS-T and incubated with 5ml of Restore™ Stripping buffer (Thermo Scientific) for 15-20 mins at 65°C. The stripping buffer was discarded, and the membrane was washed four times with PBS-T on a shaker at RT. The membrane was then blocked for 30 mins and was re-probed with another primary antibody.

2.5.7 Quantification and analysis of blots

The data acquired from the Odyssey Fc detection system was quantified using ImageStudio Lite (LI-COR) version 5.2 as pixel signal intensity per protein bands. The signal intensity for the selected area was calculated by subtracting the product of background and area from total signal. The values obtained were copied to Microsoft excel and the intensity of the bands were normalised with an appropriate internal control.

2.6 Fixed cell imaging

2.6.1 Immunocytochemistry

Hippocampal neurons for imaging were plated at a cell density of 70,000 per well in a 25mm glass coverslip. After stimulation with KA, the cells were taken out of the incubator and leave to cool at RT for 5-10 mins to prevent receptor trafficking. The cells were incubated for 20 mins in the primary antibody. To do this, appropriate concentration N-terminal antibody was added **(Section 2.1.9.1**) and mixed in a 1.5ml Eppendorf containing 100µl of conditioned media (per coverslip). 90µl of the antibody containing media pipetted onto a parafilm and the coverslips were gently placed with cells facing down on to the primary antibody. After incubation, the coverslips were placed back into a 6 well plate containing 2ml of DPBS (cells side facing up). The coverslips were washed 3-5 times in DPBS and fixed with 1ml of pre-warmed 4% formaldehyde + 5% sucrose for 12 mins. The cells were again washed 3 times in DPBS followed by a wash with 1ml of 100mM glycine dissolved in DPBS to quench residual formaldehyde. To remove glycine, the coverslips were again washed 3 times with DPBS. The cells were permeabilised and blocked using 3% BSA in DPBS containing 0.1% Triton-X 100 for 20 minutes with gentle shaking at RT. Secondary antibodies were diluted 1:400 in 3% BSA in 1XDPBS and were incubated for 1 hr (same as primary antibody incubation). After the incubation, the coverslips were placed back into the wells and were washed 3 times with DPBS. 40µl of mounting media (Fluoromount-GTM with DAPI (Thermo Fisher)) was pipetted on to slides and the coverslips were mounted (cells facing down) after gently dipping into ddH2O (to prevent salt crystal formation). The slides were left overnight to dry before imaging or storage at 4^{o}C

2.6.2 Image acquisition

The fixed neurons were imaged using an inverted epifluorescence Leica DMI 6000 microscope attached to a Leica laser scanning confocal microscope SP5-II. Image acquisition was performed with a resolution of 1024X1024 pixels, 1X optical zoom using a 63x HCxPL APO CS oil-immersion objective lens. Z stacking of 6-8 planes was taken with a frame average of 2 and frame in an interval of 0.5µM. 405nm diode laser of 50mW, 561nm solid-state laser of 20mW were used to excite DAPI and Cy3, respectively. All the parameters including the gain power were kept constant throughout the complete set of experiments. The number of neurons imaged, quantified and the number of dissections used to obtain the neurons is mentioned in the figure legends.

2.6.3 Image analysis

Z-stacks were taken at 0.5 µm intervals, covering the whole depth of the cell. Using ImageJ (Fiji) software, images were compressed using maximum intensity projection. For optimization of background subtraction, rolling ball radius was adjusted to 30 pixels. Random but clearly selectable and isolated dendrites were selected from initial dendrite (starting after cell body to first branch point) and first branch (from initial dendrite point to first branch point) of dendrites **[Figure 2-1]**. For both categories, at least 4 dendrites were selected, and the branches were traced using the Simple Neurite Tracer tool, and then were filled out automatically **[Figure 2-1]**. to the thickness of the neurite to create masks of the dendritic structure (Region of Interest (ROI)). The mean values of the surface GluA2 intensity was measured from the masks by Fiji software. The intensity values were averaged for 2 categories (first and second branches) for each neuron and analysed in Graph Prism 9.2.

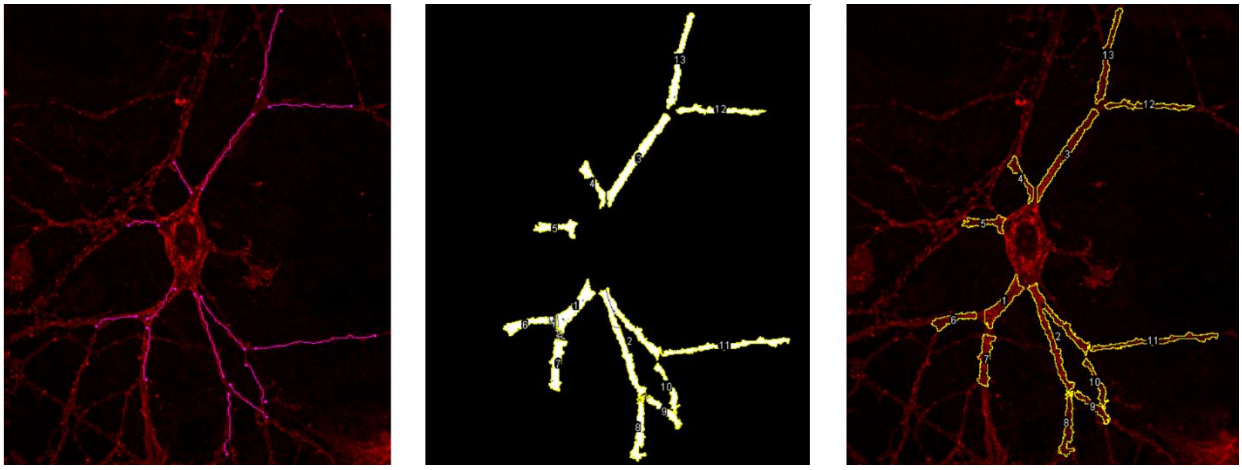

Figure 2-1: Confocal image analysis

The initial and first branch dendrites were tracked by Simple Neurite Tracker tool and then the masks were created and labelled. 1,2,3,4 and 5 are the initial dendrites and 6,7,8,9,10,11,12 and 13 are first branch dendrites. This approach applied for all neurons to see differences near and further away from the cell body. Initial and first branch of dendrites were measured and analysed as one neuron.

2.7 Pharmacological stimulation

2.7.1 Solution

- Earle's buffer

Table 2-7: Components in Earle's buffer

Components	Concentration (mM)
NaCl	140
KCl	5
CaCl2	1.8
MgCl2	0.8
HEPES	25
D-Glucose	0.9g/L

pH of the solution was adjusted to 7.4 with 1M Tris and the osmolarity was adjusted to the osmolarity of the media containing neurons. The buffer was warmed to 37°C in water bath before simulation.

2.7.2 Sustained KA stimulation

Stimulation protocol for transient and sustained KA were adapted from (Petrovic et al., 2017c, Henley, 2004, Martin et al., 2008, Gonzalez-Gonzalez and Henley, 2013). Cortical neurons were incubated in 2ml of prewarmed EBS containing 1μM TTX and other drugs for at least 30 mins at 37°C incubator. After 30 mins, the cells were taken out, the media containing drugs were aspirated and supplied with 2ml of fresh EBS containing drugs along with 10μM KA for 20 mins. Controls were treated the same but with vehicle instead of KA. All stimulations were performed with 10μM KA for 20 mins along with preincubation for 30 mins with 1μM TTX unless stated specifically.

2.8 Ethical approval

All the animal experiments and procedures were performed in compliance with the UK Animal Scientific Procedures act (1986) and were guided by the Home Office Licensing Team at the University of Bristol. All animal procedures relating to this study were approved by the Animal Welfare and Ethics Review Board at the University of Bristol.

2.9 Statistical analysis

For each experiment, the signal for each condition was divided by the mean overall signal from that experiment. This analysis was performed for each replicate experiment, and for presentation purposes, the mean of the control condition set to 100%.

All the graphs and statistical tests were performed using GraphPad Prism version 9.2. The details of the statistical test performed on each experiment are explained in the figure legend along with p-values and error bars. The n (number of cells) and N (number of independent dissections/ Number of animals) were also mentioned.

2.10 Figures

All the images and cartoons were created with BioRender.com. Images adapted from previously published works are acknowledged and mentioned with the source.

Chapter 3. KAR dependent phosphorylation of GluA1 S845

3.1 Introduction

3.1.1 Phosphorylation of GluA1 S845 as a marker of synaptic plasticity

The trafficking and functional properties of AMPAR subunits are regulated by post translational modifications, including phosphorylation, ubiquitination, nitrosylation, SUMOylaton and palmitoylation (Michaelis, 1998, Diering et al., 2018, Huganir and Nicoll, 2013, Diering et al., 2016, José A. Esteban, 2003, Henley and Wilkinson, 2016). Phosphorylation of C-terminal AMPAR subunits by various protein kinases are critical regulator of receptor properties and synaptic plasticity at least in *in vitro* conditions **[Figure 3-1]** (Ho et al., 2011, José A. Esteban, 2003, Chater and Goda, 2014, Baudry et al., 2015). However, studies on animals models indicated that trafficking of the subunits and synaptic plasticity is independent on C-terminal tail of AMPARs (Díaz-Alonso et al., 2020).

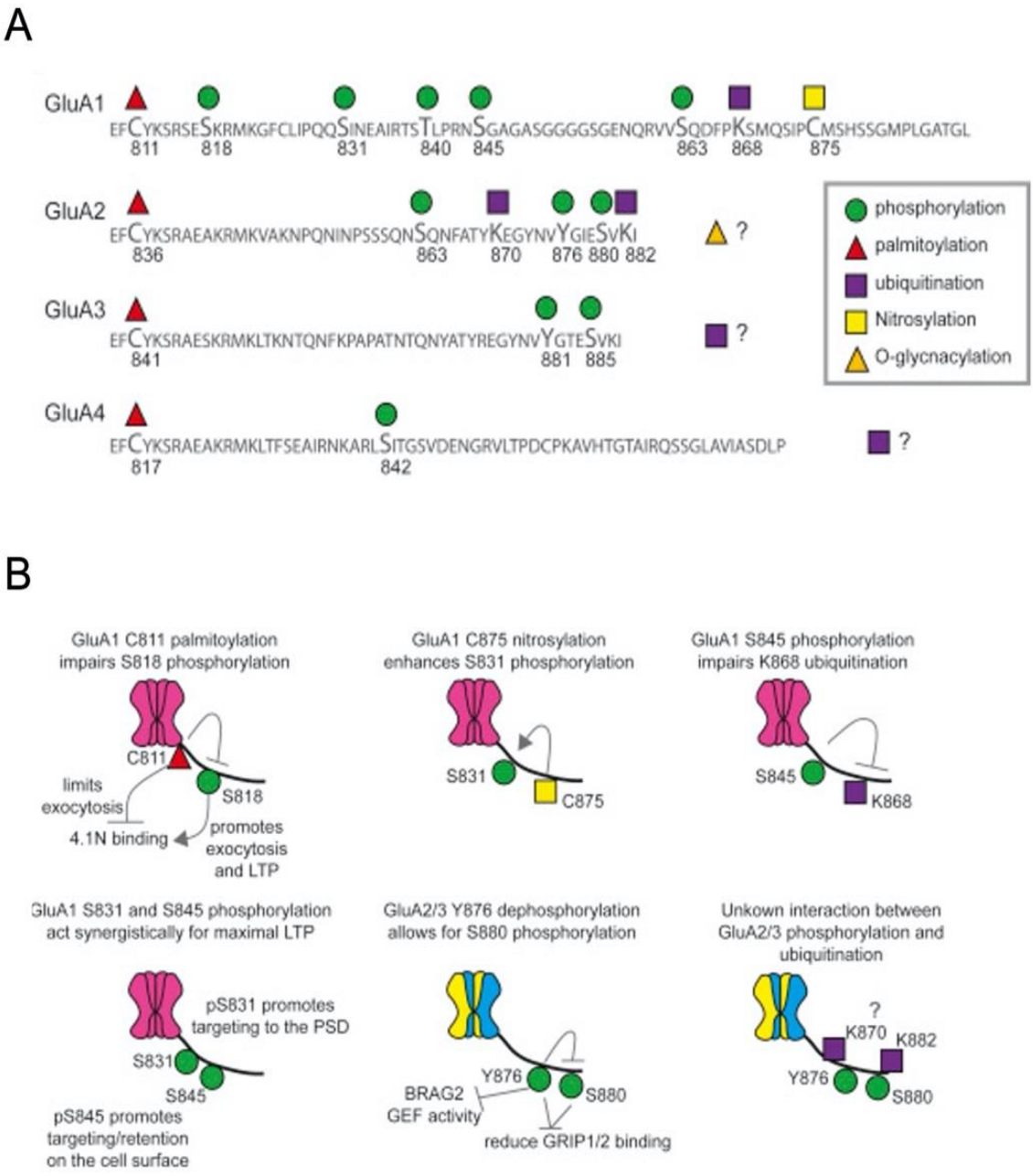

Figure 3-1: post-translational modifications of AMPARs

(A) Amino acid sequence of AMPAR subunits with sites of various post-translational modifications. (B) Cartoon showing various post-translational modifications of AMPARs. Adapted from (Diering et al., 2018).

Activity-dependent synaptic plasticity is characterized by an increase or decrease in the synaptic efficacy along with structural modulation of synapses (Bliss et al., 2014, Malinow and Malenka, 2002, Henley and Wilkinson, 2013, Díaz-Alonso et al., 2020, Majewska et al., 2006). These events are often associated with the endo/exocytosis

of the AMPARs from the recycling pool (Ho et al., 2011). Post-translational modifications and protein-protein interactions with the C-terminal tail of tetrameric AMPARs regulate their trafficking during plasticity at least in *in vitro* conditions (Henley and Wilkinson, 2016). Indeed, a well characterized marker of the activity dependent AMPAR trafficking in plasticity is the phosphorylation of C-terminal tail of GluA1 S845 (José A. Esteban, 2003).

Under basal conditions, the GluA2/GluA3 predominates over GluA1/GluA2 containing AMPARs. Due to the short-tail, GluA2/GluA3 subunits constantly recycle in an out of synapses (Henley and Wilkinson, 2013, Wenthold et al., 1996). GluA1 S845 is basally phosphorylated, at relatively low levels, under normal conditions. This regulation in the phosphorylation is achieved by maintaining low level activity of PKA and PKC. However, synaptic potentiation enhances PKA and PKC leading to the phosphorylation of subunits **[Figure 3-2]** (Jurado et al., 2010, Sanderson et al., 2012).

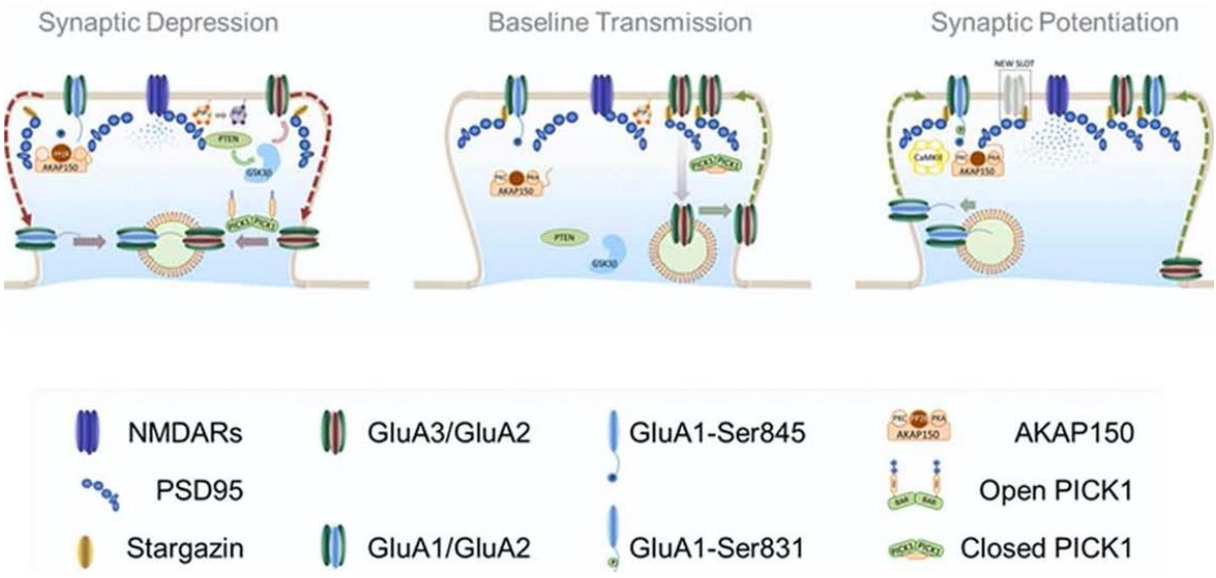

Figure 3-2: PKA-dependent phosphorylation of GluA1 S845

Fewer AMPARs are phosphorylated under basal conditions. Enhanced synaptic activity (right) increases PKA and PKC activity. Phosphorylation of GluA1 S845 increases synaptic incorporation of AMPARs from recycling pool. During depression of synaptic transmission, activation of various phosphatases such as calcineurin trigger dephosphorylation and endocytosis of the receptors. Adapted from (Jurado, 2017).

Conversely, during synaptic depression activation of the phosphatases PP1, PP2A and PP2B (calcineurin) dephosphorylates GluA1S845 and S831 triggering

endocytosis of AMPARs **[Figure 3-3]** (He et al., 2009, Jurado, 2017). The localization of protein kinases and phosphatases to the vicinity of AMPARs is carried out by the isoform of A-kinase anchoring protein (AKAP-150) (Guercio et al., 2018, Sanderson et al., 2018). Transgenic mice expressing mutant form of AKAP150 with defective calcineurin binding site shows enhanced phosphorylation of S845 and surface expression of GluA1 containing AMPARs at the synapses. However, the transgenic mice failed to exhibit LTD at CA3-CA1 synapses but not LTP (Sanderson et al., 2012).

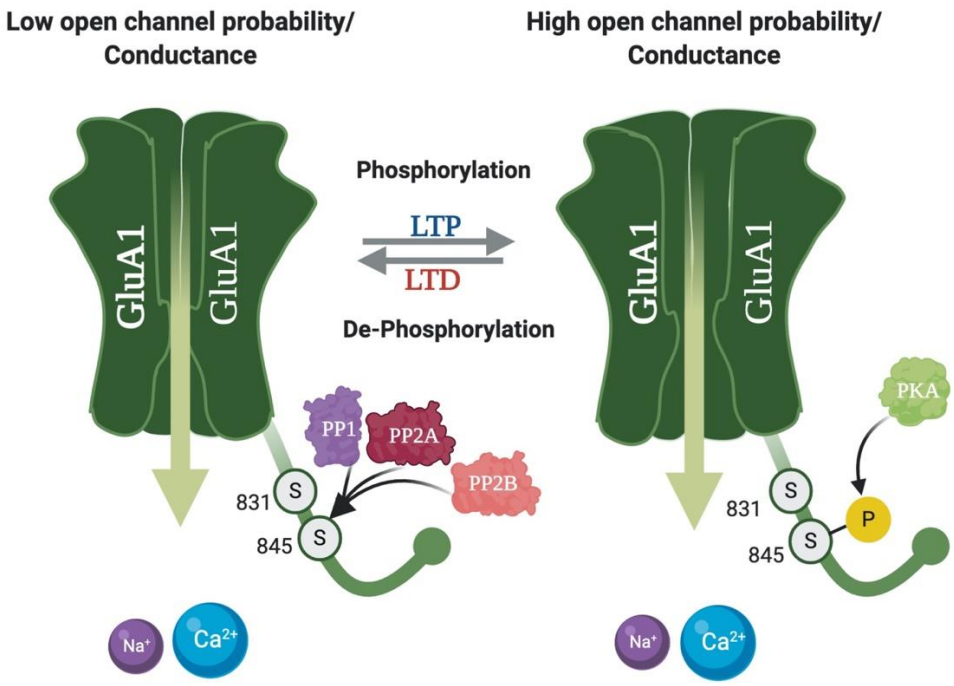

Figure 3-3: Regulation of AMPAR channel function by phosphorylation of GluA1 S845 and GluA1 S835

The channel properties of AMPARs are regulated by phosphorylation of GluA1 S845. PKA mediated phosphorylation increases open channel probability of the receptor along with enhanced channel conductance to divalent and monovalent cations. Phosphorylation of GluA1 S845 and S831 during LTP induction promotes channel function whereas dephosphorylation during LTD abate. LTD induction trigger dephosphorylation by phosphatases such as PP1, PP2A and PP2B(calcineurin).

Activation of PKA by Ca^{2+}-sensitive adenylate cyclase or via G-protein dependent pathways leads to phosphorylation of GluA1 S845 (Joiner et al., 2010). PKA dependent phosphorylation of GluA1 S845 enhances surface expression GluA1

containing AMPARs during induction of LTP by promoting surface retention and targeting to the cell surface (Man et al., 2007b, Diering et al., 2018). This also enhances the single channel open probability of the receptor further augmenting the channel activity alongside lowering the threshold for LTP **[Figure 3-3]** (Banke et al., 2000) (Esteban et al., 2003, Oh et al., 2006). Conversely, dephosphorylation of GluA1 S845 leads to internalization of GluA1 AMPARs corresponding to LTD induction (MD, 2000).

Transgenic mice expressing phospho-null or phospho-mimetic mutants of GluA1 revealed that phosphorylation of GluA1 is a prime inducer of synaptic plasticity (Lee et al., 2010). Mice expressing non-phosphorylatable form of GluA1 S845A/S831A displayed impairments in synaptic plasticity and deficits in spatial navigation (Lee HK, 2003, Ho et al., 2011). Single mutations on phosphorylation of GluA1 S845 hampered LTD in mice however, LTP of Schaffer collateral-CA1 synapses remained intact. These data suggest that for LTP phosphorylation of GluA1 S845 and S831 have synergistic effects (Lee et al., 2010).

Only 15-20% of GluA1 are phosphorylated at S845 or S831 under basal conditions with minimal phosphorylation at dual sites (Diering et al., 2016). However, exposure of mice to an enriched environment or stimulation of PKA/PKC increases the dual phosphorylation of GluA1 to 50%. Subcellular localization studies of phosphorylation status of GluA1 reported an increase in expression of phosphorylated GluA1 S831 at the PSD but not GluA1 S845 **[Figure 3-4]** (Diering et al., 2016).

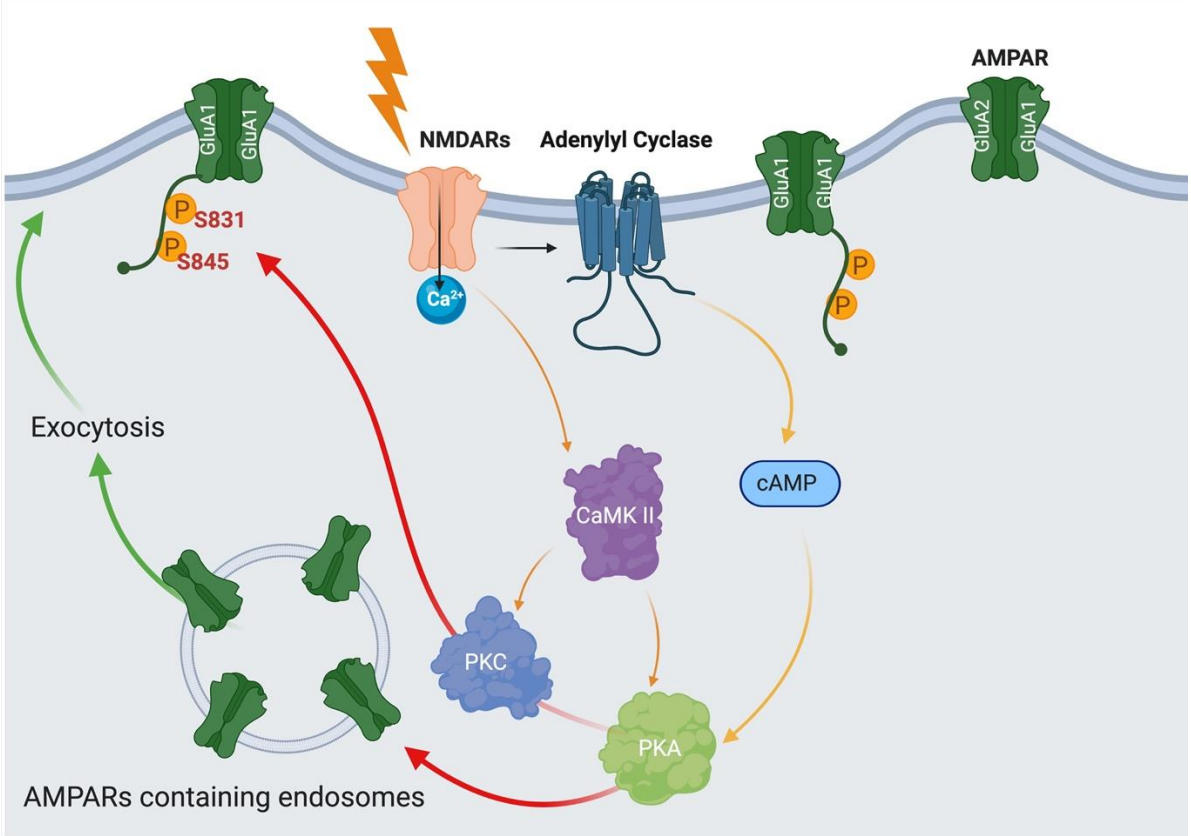

Figure 3-4: Phosphorylation of GluA1 C-terminus of AMPARs

AMPARs undergo PKA and PKC dependent phosphorylation at GluA1 S845 and S831 respectively. Activation of NMDARs and subsequent kinase activity leads to phosphorylation at GluA1 C-terminal S845 and GluA1 S831 sites. Phosphorylation of these sites leads to enhancement in exocytosis of AMPARs from the recycling endosomes and promote surface expression and retention of GluA1 containing AMPARs at the synapses.

3.1.2 Mechanisms of PKA activation and AMPAR phosphorylation

Dynamic regulation of post synaptic AMPARs are crucial for the network activity and neuronal signaling. The number of AMPARs at synapse are regulated by trafficking as well as anchorage of the receptors to the postsynaptic sites including the postsynaptic densities (PSD) (Henley and Wilkinson, 2013, Buonarati et al., 2019). Many proteins regulate the trafficking and expression of AMPARs during basal and induction of synaptic plasticity (Buonarati et al., 2019).

PKA has been shown to regulate synaptic AMPARs by inhibiting endocytosis as well as by promoting insertion of newly synthesized and recycling AMPARs in an activity

dependent manner (MD, 2000, Sun et al., 2005). Under basal conditions, only 25-30% of AMPARs are expressed at the surface with constant recycling. Among these, only 15% of GluA1 subunits are phosphorylated at S845 (Oh et al., 2006, Babiec et al., 2016). During activity, PKA dependent phosphorylation of GluA1 S845 increases to 60% promoting the delivery of AMPARs to extra-synaptic sites and priming their movement to PSD by lateral diffusion in an activity dependent manner **[Figure 3-5]** (Oh et al., 2006, Tardin et al., 2003). Moreover, increase in synaptic activity leads to activation of NMDARs and subsequent rise in the intracellular Ca^{2+} concentration that trigger the activation of CaMKII. This promotes the lateral diffusion of AMPARs from extra synaptic sites to PSD triggering the functional changes associated with LTP (Esteban et al., 2003). Conversely during LTD, phosphorylation of GluA1 S845 decreases to 10%. (Oh et al., 2006).

Recent studies revealed that β-adregenic receptors induced activation of Ras GTPase coupled with cAMP dependent PKA activity enhances the channel conductance of GluA2/GluA3 containing AMPARs and induces potentiation of the synapses (Renner et al., 2017) .

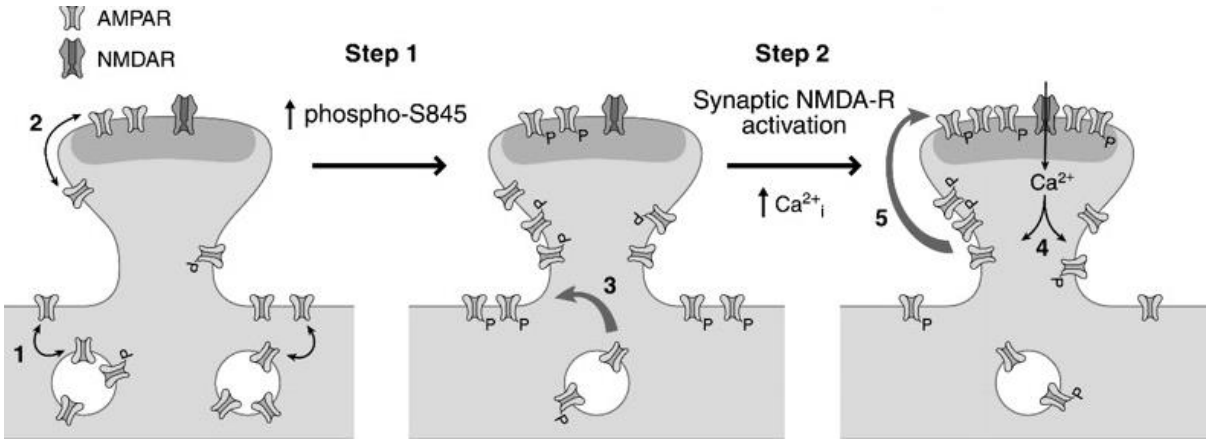

Figure 3-5: Model of AMPAR trafficking by phosphorylation of GluA1 S845

Under basal conditions, AMPARs move in and out of synapses and along with exo- and endocytosis from recycling pool of receptors (1). During activity or cLTP, PKA dependent phosphorylation of GluA1S845 in internal pool of AMPARs promotes their delivery to extra synaptic location (2) Phosphorylated AMPARs move to PSD, wherein the activity triggers the activation of NMDARs (3) Activated NMDARs promotes Ca^{2+} influx through the channel and subsequently trigger the activation of CaMKII (4). This promotes the lateral movement and synaptic incorporation of AMPARS (5). Adapted from (Oh et al., 2006).

3.1.3 KAR dependent activation of PKA

KARs are crucial modulators of network activity (Evans et al., 2017a). Activation of various kinases regulate KAR-mediated signaling pathways (Henley, 2004, Petrovic et al., 2017c, Chamberlain et al., 2013). However, the majority of KAR-mediated pathways are dependent of PKC activation such as inhibition of I_{sAHP} (Chamberlain et al., 2013, Melyan and Wheal, 2011) and regulation of trafficking of AMPARs and KARs (Petrovic et al., 2017c, Henley, 2004).

PKA activation by presynaptic KARs regulate excitatory neuronal transmission at various synapses (Lauri et al., 2003, Rodriguez-Moreno and Sihra, 2004, Ji and Staubli, 2002). At MF-CA3 synapses, higher concentration of KA inhibits presynaptic release via metabotropic action of KARs by dampening the PKA activity (Negrete-Diaz et al., 2006). Contrary to this, low concentration of KA or endogenous glutamate enhances presynaptic release and is correlated with enhanced PPF (Schmitz et al., 2001). It is proposed that this facilitation of glutamate release by presynaptic KARs containing GluK2 and GluK5 subunits require Ca^{2+} influx through the channel (Contractor et al., 2000). KAR-mediated Ca^{2+} influx enhances intracellular Ca^{2+} concentration by Ca^{2+}-activated Ca^{2+}-release from intracellular stores (Lauri et al., 2003, Scott et al., 2008). This rise in Ca^{2+} activates PKA via Ca^{2+}-sensitive adenylate cyclase and cAMP and thereby enhancing the glutamate release **[Figure 3-6]** (Rodriguez-Moreno and Sihra, 2004, Negrete-Diaz et al., 2006).

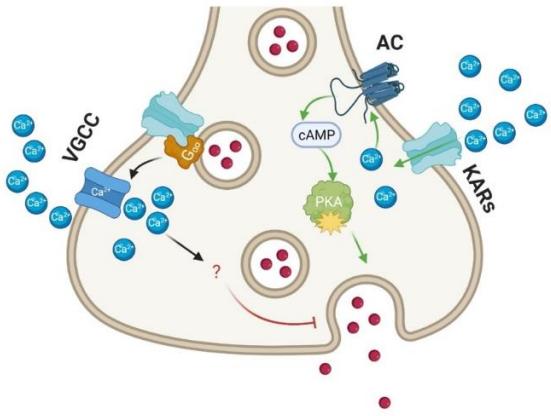

Figure 3-6: Presynaptic KAR-mediated enhancement of glutamate release requires PKA

Activation of calcium permeable KARs. Increase in cytosolic Ca^{2+} concentration activates Ca^{2+}-calmodulin and downstream activation of AC and PKA.

3.2 Objectives

Phosphorylation of GluA1 S845 is a well-established marker of NMDAR-dependent synaptic plasticity. Increased phosphorylation of GluA1 S845 triggers the forward trafficking of AMPAR subunits and their incorporation to postsynaptic sites (Henley and Wilkinson, 2013, Henley et al., 2021, Diering et al., 2018). Thus, phosphorylation contributes to the functional and structural changes associated with synaptic plasticity (Diering et al., 2018).

As a first step towards understanding the role of KAR activation in AMPAR trafficking, I decided to study the changes in phosphorylation of the AMPAR subunit GluA1 at S845. All the studies in this chapter are restricted to GluA1 S845, as in initial experiments I detected no significant changes in the phosphorylation status at other sites. The specific objectives were to address the following questions.

1. Can sustained stimulation of KAR induce changes in phosphorylation of AMPARs?
2. Do KARs employ ionotropic or metabotropic signaling to alter the phosphorylation of AMPAR subunits?
3. What are the signaling mechanism underlying KAR-induced phosphorylation of AMPAR subunits?

3.3 Results

3.3.1 Sustained KA stimulation increases phosphorylation of GluA1 S845

To study the role of KARs in synaptic plasticity, I set off my study by investigating whether KA stimulation altered the phosphorylation of C-terminus S845 of the AMPAR subunit GluA1 since the changes in the phosphorylation of GluA1 S845 is correlated with the induction of synaptic plasticity (Lee HK, 2003, Diering et al., 2016, Diering et al., 2018, Heng-Ye Man, 2007, José A. Esteban, 2003).

Cortical neurons at DIV 18 were pre-treated with 1μM TTX for 30 mins to inhibit activity induced release of glutamate. Phosphorylation of GluA1 S845 was analysed following application of 10μM KA for 20 mins in parallel to 3 mins of 20μM NMDA and 20μM glycine, (a well-established NMDAR-mediated chem-LTD protocol) (Anwar et al., 1998, Ashby

et al., 2004) was performed. A significant increase in the phosphorylation was detected following 20 mins of KA stimulation. NMDA treatment was used as a control wherein no increase in the phosphorylation was expected. **[Figure 3-7].**

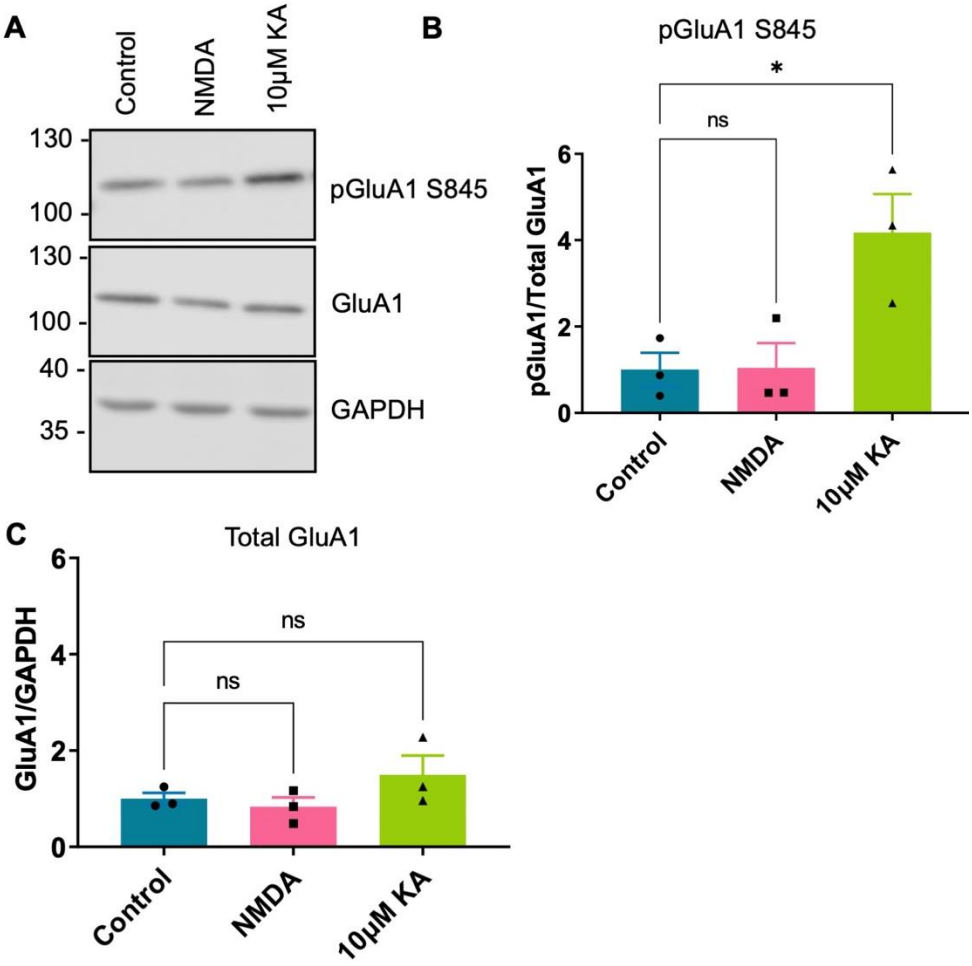

Figure 3-7: 10μM KA stimulation increases phosphorylation of GluA1 S845 in cortical neurons

Phosphoproteins were analysed by western blotting following 30 min of pre-treatment with 1μM TTX prior to stimulation with 10μM KA for 20 mins. 20μM NMDA 20μM glycine were used in parallel, wherein the NMDA treatment did not increase the phosphorylation.

Representative western blots of pGluA1 S845, Total GluA1. GAPDH was used as a loading control to ensure equal amount of protein was added in each well. 10μM (A).

Quantification of pGluA1 to Total GluA1 (B), Total GluA1 to GAPDH (C), N=3 independent dissections, ns $p>0.05$, *$p<0.05$; One-way ANOVA with Dunnett's multiple comparisons test, error bars = SEM.

3.3.2 KA stimulation increases phosphorylation of GluA1 S845 in hippocampal neurons

Several neuronal cell types are present in the cortical cultures. In contrast, primary cultures made from hippocampus consist mostly of CA1 pyramidal neurons (Kaech and Banker, 2006). Further, induction and maintenance of synaptic plasticity is diverse in different regions of brain. *In vitro* and *in vivo* studies have shown that the hippocampus is crucial for learning and formation of spatial memories (Tsien et al., 1996, Martin et al., 2005). To investigate whether there is region specific difference in KA induced phosphorylation of GluA1 S845, I next repeated the sustained KA stimulation experiments in hippocampal cultures. Like cortical cultures, 20 mins of KA stimulation increased the phosphorylation of GluA1 S845 **[Figure 3-8]**. Again, no change was observed in NMDA treated samples.

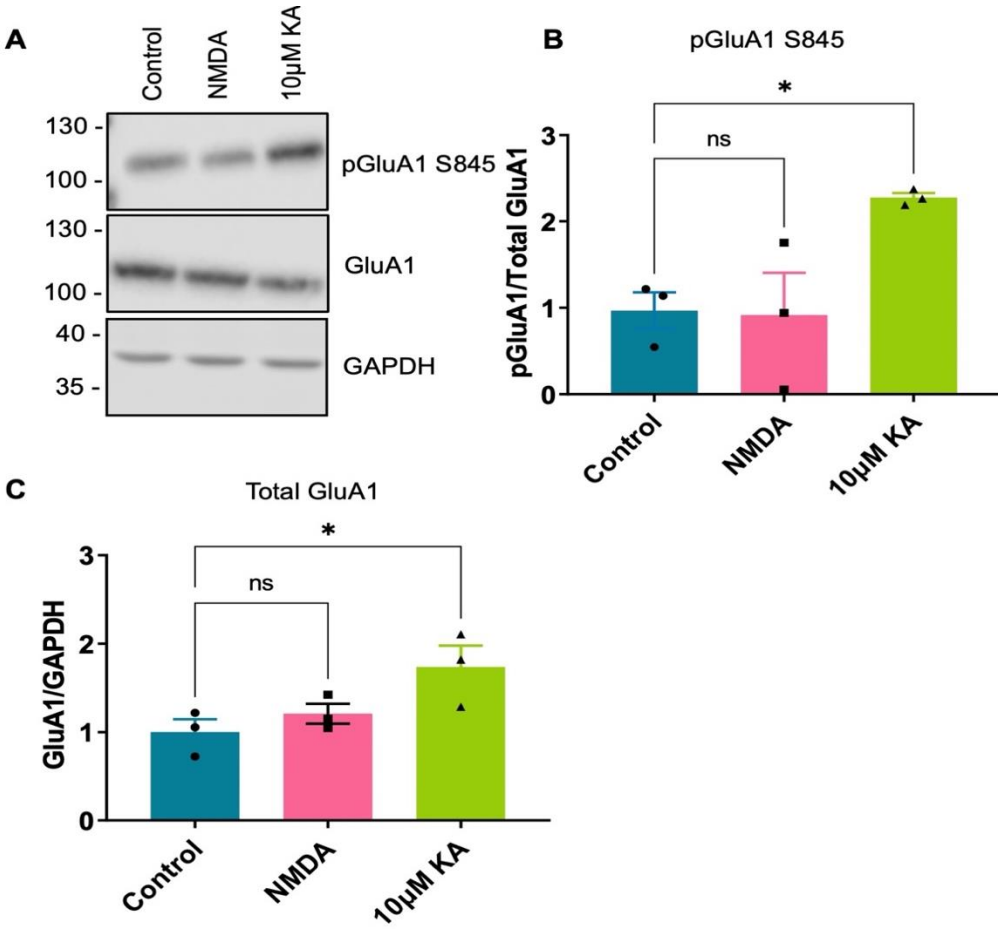

Figure 3-8: 10μM KA stimulation increases phosphorylation of GluA1 S845 in hippocampal neurons

Phosphoproteins were analysed by western blotting following 30 min of pre-treatment with 1μM TTX prior to stimulation with 10μM KA for 20 mins. 20μM NMDA 20μM glycine were used as a control, wherein the NMDA treatment did not increase the phosphorylation.

Representative western blots of pGluA1 S845, Total GluA1. GAPDH was used as a loading control to ensure equal amount of protein was added in each well. 10μM (A).

Quantification of pGluA1 to Total GluA1 (B), Total GluA1 to GAPDH (C), N=3 independent dissections, ns $p>0.05$, *$p<0.05$; One-way ANOVA with Dunnett's multiple comparisons test, error bars = SEM.

3.3.3 Increase in the phosphorylation of GluA1 S845 is independent of AMPAR activation

Previous studies indicated that 10μM KA stimulation can non-specifically activate AMPARs (Bureau et al., 1999, Petrovic et al., 2017b). To eliminate the possible activation of AMPARs, I used a lower concentration of KA that is subthreshold to activate AMPARs (Levchenko-Lambert et al., 2011, Ruiz et al., 2005). Consistent with previous results,

3μM KA stimulation significantly increased the phosphorylation of GluA1 S845 **[Figure 3-9]**. These observations suggest the phosphorylation of GluA1 S845 is dependent on activation of KARs.

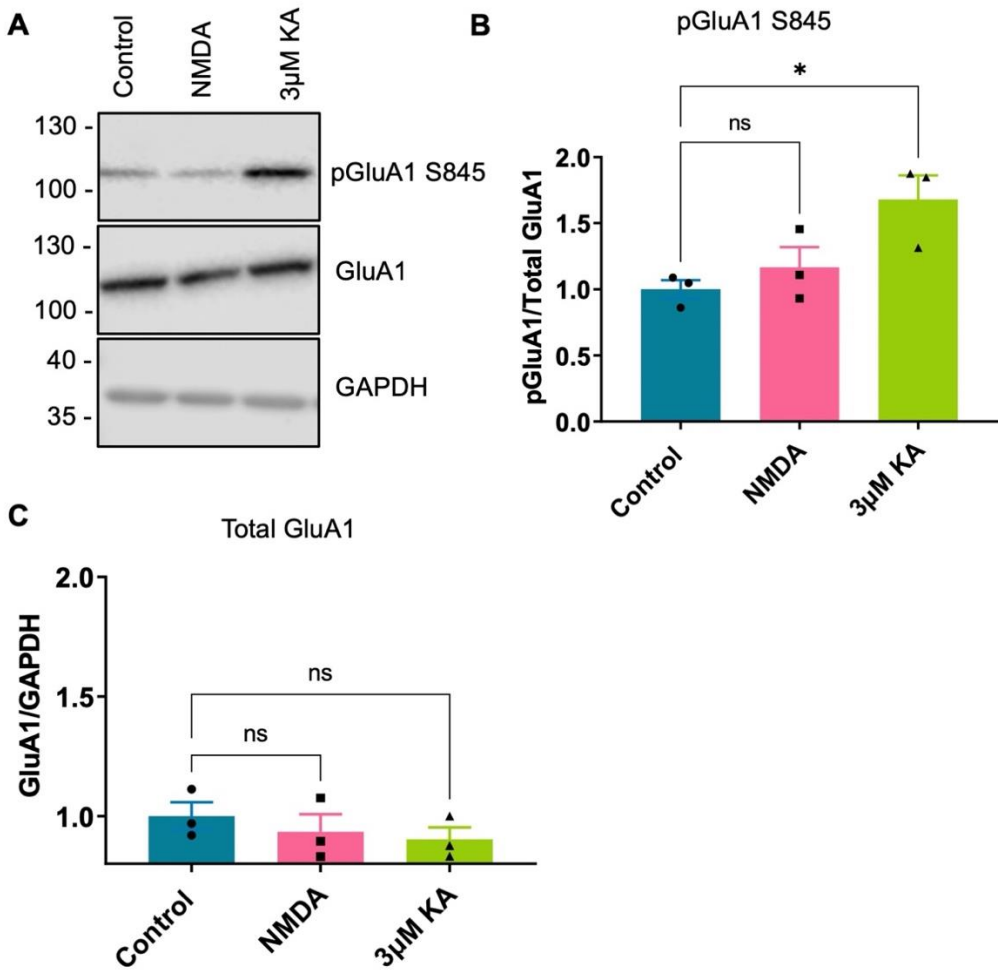

Figure 3-9: 3μM KA stimulation increases phosphorylation of GluA1 S845

Phosphoproteins were analysed by western blotting following 30 min of pre-treatment with 1μM TTX prior to stimulation with 3μM KA for 20 mins. 20μM NMDA 20μM glycine were used as a control, wherein the NMDA treatment did not increase the phosphorylation.

Representative western blots of pGluA1 S845, Total GluA1. GAPDH was used as a loading control to ensure equal amount of protein was added in each well (A).

Quantification of pGluA1 to Total GluA1 (B), Total GluA1 to GAPDH (C), N=3 independent dissections, ns $p>0.05$, *$p<0.05$; One-way ANOVA with Dunnett's multiple comparisons test, error bars = SEM.

3.3.4 KA induced increase in phosphorylation of GluA1 S845 is independent of metabotropic signaling

Although a significant increase in phosphorylation was evident with 3μM KA, to completely exclude the possibility of non-specific AMPAR activation, neurons were pre-treated with 40μM GYKI53655 (Partin and Mayer, 1996) alongside 1μM TTX for 30 mins prior to 10μM KA treatment. Post 20 mins stimulation, a significant increase in phosphorylation of GluA1 S845 was detected, confirming the KA induced increase in phosphorylation of GluA1 S845 is independent of AMPAR activation. However, interestingly, a partial blockade was evident in samples treated with GYKI53655 **[Figure 3-10 B]**.

KARs employs both G-protein mediated metabotropic and ionotropic signalling to modulate neuronal functions (Lerma and Marques, 2013, Petrovic et al., 2017c, Evans et al., 2017a). To decipher the signalling mechanism underlying phosphorylation of GluA1 S845, I used the G- protein inhibitor pertussis toxin (PTx). Cortical neurons were incubated with 1μg/ml PTx for 1 hr and 1μM TTX for 30 mins prior to 20 mins of KA stimulation. PTx failed to block the increase in phosphorylation of GluA1 S845 suggesting the pathway is independent of metabotropic KAR signalling **[Figure 3-10]**.

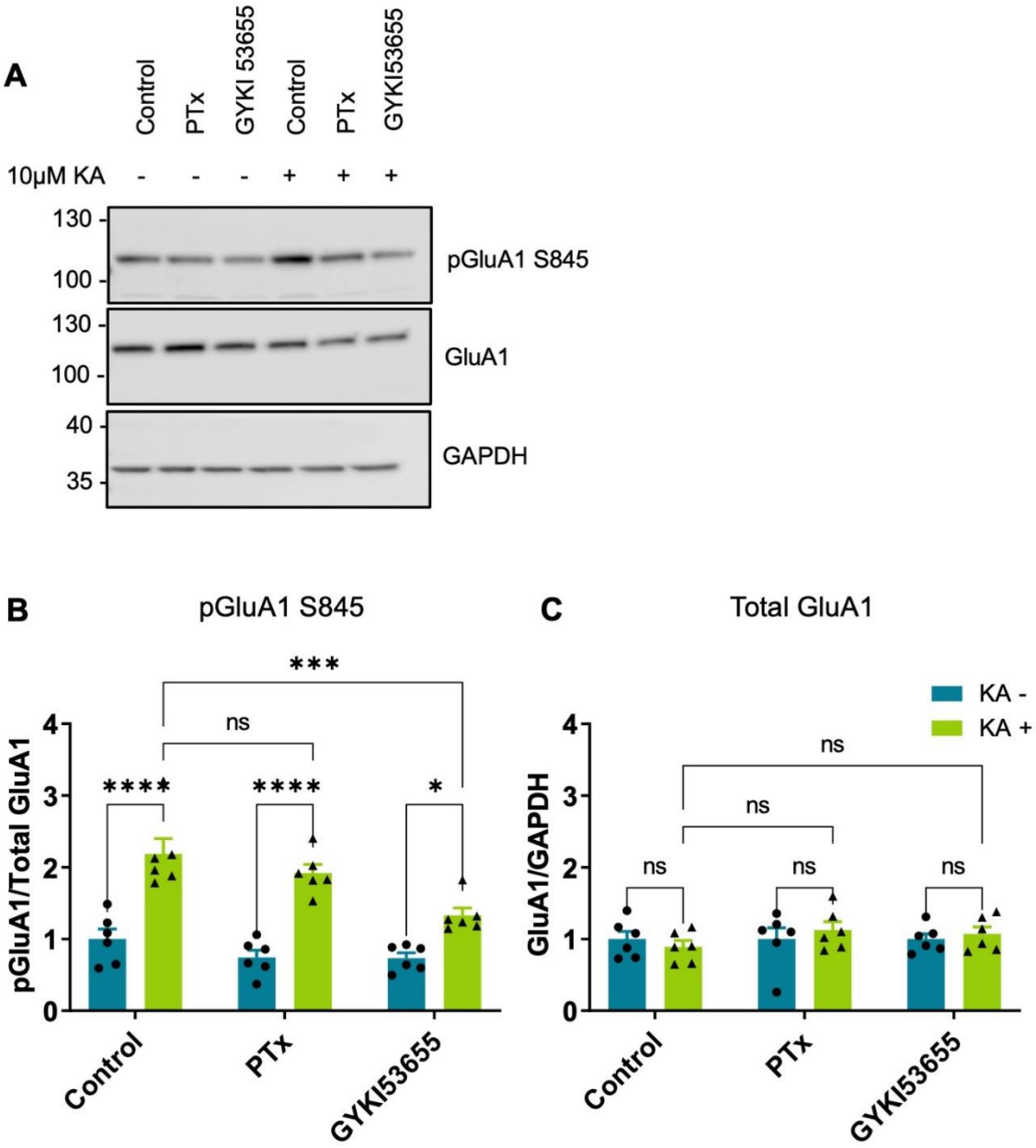

Figure 3-10: Phosphorylation of GluA1 S845 is independent of AMPARs or metabotropic signalling of KARs

Phosphoproteins were analysed by western blotting following 1hr of pre-treatment 0.1μg/ml PTx and 30 mins with 1μM TTX prior to 20 min of 10μM KA treatment on DIV 18 cortical neurons.

Representative images of western blots of pGluA1 S845, Total GluA1.GAPDH was used as a loading control to ensure equal amount of protein was added in each well (A).

Quantification of pGluA1 S845 to Total GluA1 (B), Quantification of GluA1 to GAPDH (C), N=5 independent dissections, ns $p>0.05$, *$p<0.05$, ****$p<0.0001$; Two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

3.3.5 KA-induced phosphorylation of GluA1 S845 is blocked by KAR/AMPAR antagonist CNQX.

I next investigated whether KA induced increase in the phosphorylation of GluA1 S845 require ionotropic signaling of KARs. UBP310, is a synthetic compound initially developed as an antagonist of GluK1 and GluK3 KARs (Pinheiro et al., 2013, Perrais et al., 2010, Dolman et al., 2007) but was later shown to inhibit ionotropic function of KAR containing GluK2/GluK5 subunits (Pinheiro et al., 2013, Petrovic et al., 2017c) without affecting AMPARs and NMDARs. Neurons were pre-treated with 10μM UBP310 and 1μM TTX for 30 mins prior to 20 min stimulation with 10μM KA. An increase in the phosphorylation was still evident in the presence of UBP310 **[Figure 3-11].**

KAR and AMPAR activation is blocked by KAR/AMPAR specific antagonist CNQX (Petrovic et al., 2017c). To confirm the KAR-mediated signalling in phosphorylation of GluA1 S845. I used KAR/AMPAR antagonist CNQX. A complete blockade in the phosphorylation of GluA1 S845 was evident in CNQX treated samples suggesting KAR or AMPAR activation in phosphorylation of GluA1 S845 **[Figure 3-11]**.

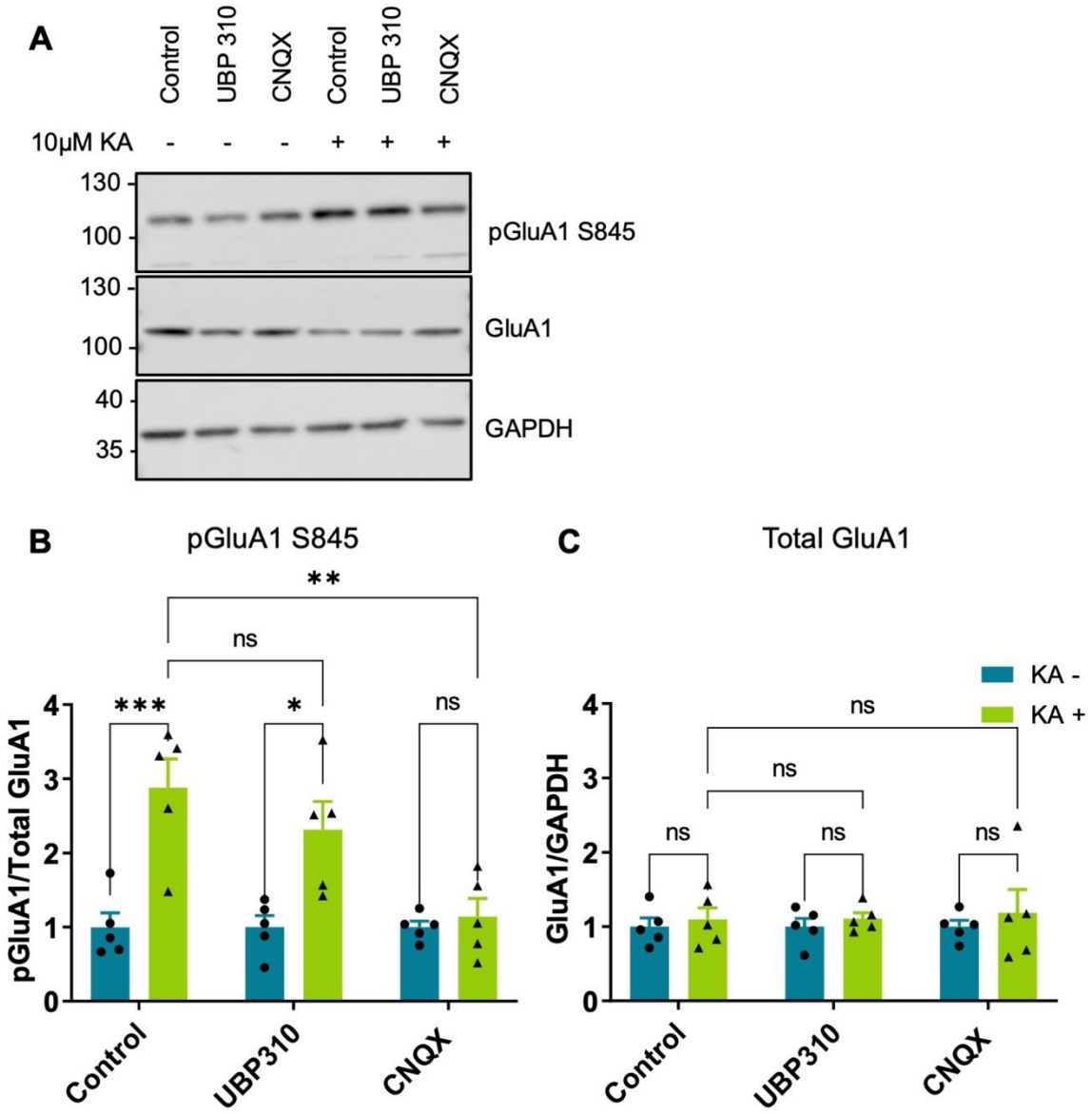

Figure 3-11: Phosphorylation of GluA1 S845 is blocked by CNQX

Phosphoproteins were analysed by western blotting following 30 mins of pre-treatment with 10μM CNQX or 10μM UBP310 along with 1μM TTX prior to 20 min of 10μM KA treatment on DIV 18 cortical neurons.

Representative images of western blots of pGluA1 S845, Total GluA1. GAPDH was used as a loading control to ensure equal amount of protein was added in each well (A).

Quantification of pGluA1 S845 to Total GluA1 (B), Quantification of GluA1 to GAPDH (C), N=5 independent dissections, ns $p>0.05$, ** $p<0.01$, *** $p<0.001$; Two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

3.3.6 KA-induced phosphorylation of GluA1 S845 is independent of NMDAR activation

KARs are present at both pre and postsynaptic sites regulate the neurotransmitter release in both excitatory and inhibitory neurons (Sihra and Rodriguez-Moreno, 2013). Activation of presynaptic KARs can therefore inhibit or enhance the glutamate release (Contractor et al., 2000, Vignes et al., 1998, Kamiya and Ozawa, 2000, Schmitz et al., 2000, Falcon-Moya and Rodriguez-Moreno, 2021). Considering this, one possibility for KA-induced phosphorylation of GluA1 S845 is enhanced glutamate release. Glutamate released from presynaptic terminals can activate postsynaptic receptors and trigger downstream signalling cascade resulting in the phosphorylation of GluA1. To investigate the potential role of NMDARs in GluA1 S845 directly or indirectly by KA application, the neurons were pre-treated with 50μM D-APV (NMDAR antagonist) to block NMDARs activation (Davis et al., 1992, Petrovic et al., 2017c). As expected, D-APV did not block KA induced phosphorylation of GluA1 S845 **[Figure 3-12]**.

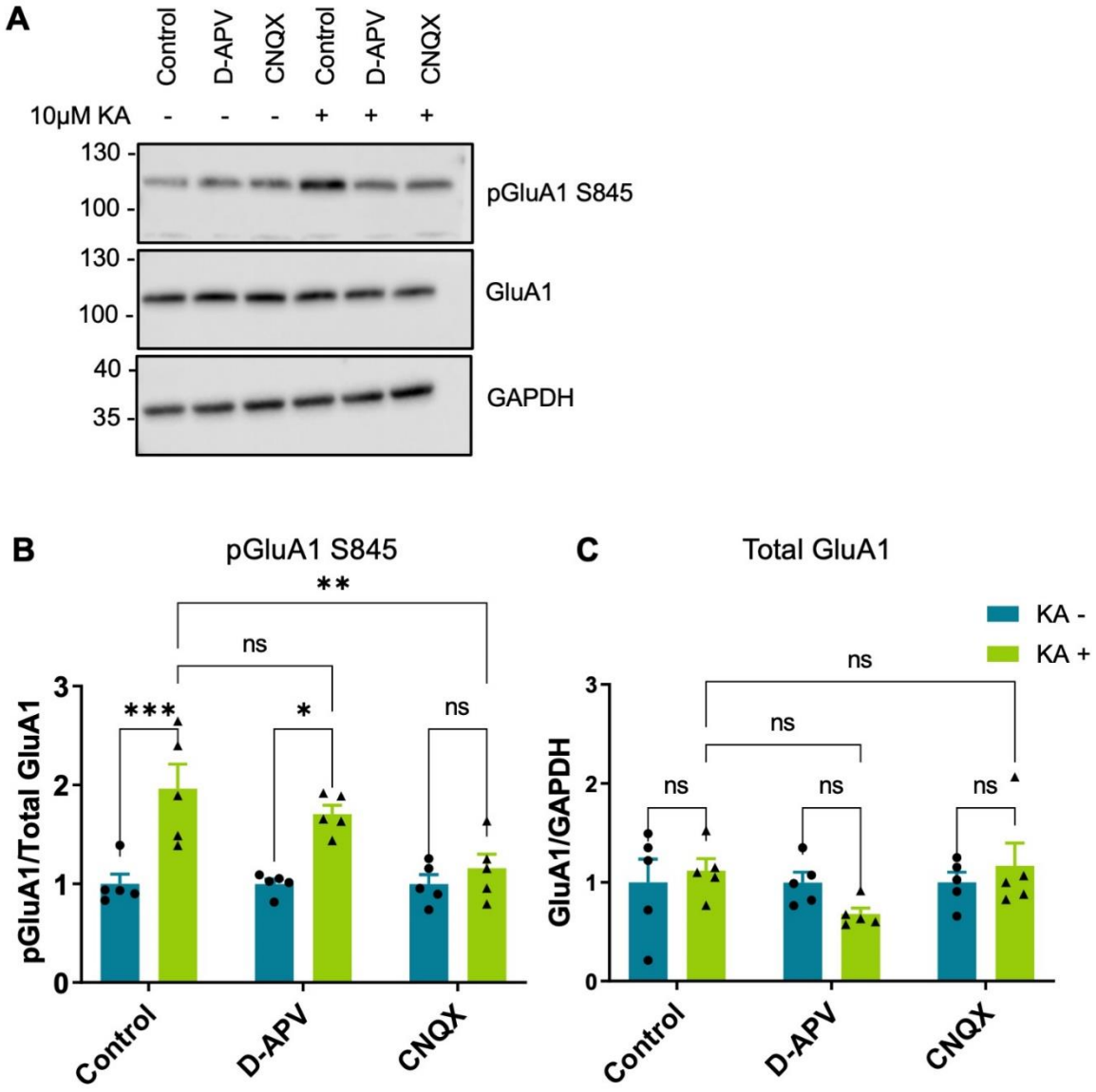

Figure 3-12: KA-induced increase in phosphorylation is independent on NMDAR activation

Phosphoproteins were analysed by western blotting following 30 mins of pre-treatment with 10μM CNQX or 50μM D-APV along with 1μM TTX prior to 20 min of 10μM KA treatment on DIV 18 cortical neurons.

Representative images of western blots of pGluA1 S845, Total GluA1. GAPDH was used as a loading control to ensure equal amount of protein was added in each well (A).

Quantification of pGluA1 S845 to Total GluA1 (B), Quantification of GluA1 to GAPDH (C), N=5 independent dissections, ns $p>0.05$, ** $p<0.01$, *** $p<0.001$; Two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

3.3.7 KA-induced phosphorylation of GluA1 S845 is dependent on PKA activation

I was keen to understand the downstream signalling mechanism of GluA1 S845 phosphorylation following KA stimulation. Previous studies have shown that postsynaptic KAR-mediated signalling pathways activate PKC but not PKA (Petrovic et al., 2017c, Henley, 2004) whereas presynaptic KAR-mediated facilitation of glutamate release require activation of cyclic-AMP dependent protein kinase A (PKA) (Sihra and Rodriguez-Moreno, 2013). Further, phosphorylation of GluA1 S845 is also dependent on PKA (Lee HK, 2003, Diering et al., 2016, Diering et al., 2018, Heng-Ye Man, 2007, José A. Esteban, 2003).

To confirm the activation of PKA, neurons were pre-treated with 10μM H89 (PKA inhibitor) (Henley, 2004) and 5μM chelerythrine (PKC inhibitor) (Konopacki et al., 2011) along with 1μM TTX for 30 mins prior to 20 mins of KA stimulation **[Figure 3-13]**. Neurons treated with PKA inhibitor completely abolished phosphorylation of GluA1 S845 after KA treatment, confirming the requirement for PKA activation. Interestingly, increases in the total levels of GluA1 was detected in chelerythrine treated samples post KA stimulation **[Figure 3-13 C]**.

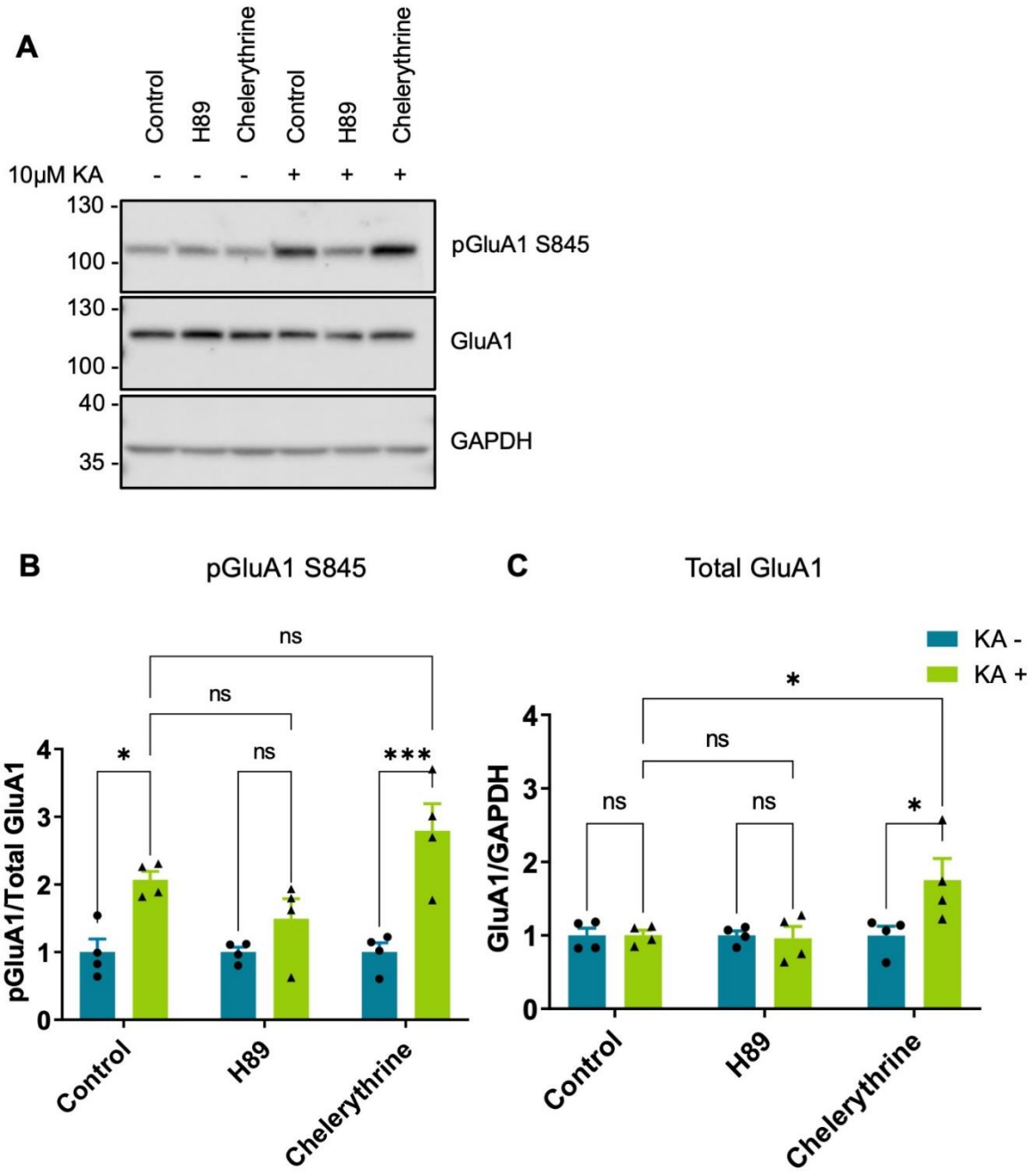

Figure 3-13:KA-induced increase in phosphorylation of GluA1 S845 is mediated by PKA

Phosphoproteins were analysed by western blotting following 30 mins of pre-treatment with 10μM H89 or 5μM chelerythrine along with 1μM TTX prior to 20 min of 10μM KA treatment on DIV 18 cortical neurons.

Representative images of western blots of pGluA1 S845, Total GluA1. GAPDH was used as a loading control to ensure equal amount of protein was added in each well (A).

Quantification of pGluA1 S845 to Total GluA1 (B), Quantification of GluA1 to GAPDH (C), N=4 independent dissections, ns $p>0.05$, *$p<0.05$, **$p<0.01$; Two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

3.4 Discussion

3.4.1 Sustained KA stimulation increases PKA-dependent phosphorylation at S845 of GluA1

Previous studies indicated that NMDAR activation leads to an increase in the phosphorylation of GluA1 at S845 (Diering et al., 2018). This facilitates LTP induction by promoting surface trafficking of AMPARs (Henley and Wilkinson, 2013, Ashby et al., 2004). Here I investigated whether KAR activation could also enhance phosphorylation of GluA1 S845.

Sustained stimulation of neurons with 10μM KA significantly increased phosphorylation of GluA1 S845. However, no significant change was detected in NMDA treated neurons **[Figure 3-7]** and **[Figure 3-8].**

To eliminate the possibility of 10μM KA activating AMPARs, I repeated the experiments with 3μM KA, a concentration that is reported to be selective for KARs (Bureau et al., 1999). Consistent with previous results an increase in GluA1 phosphorylation was detected even with low concentration of KA **[Figure 3-9]**. To further confirm the effect of KA was independent of activation of AMPARs, the experiments were performed with 10μM KA alongside the AMPAR-specific blocker GYKI53655 (Partin and Mayer, 1996). Surprisingly, a partial block in phosphorylation was detected in the presence of GYKI53655 **[Figure 3-10].** This indicates that the increase in phosphorylation of GluA1 might be dependent on direct or indirect activation of AMPARs by KA. Moreover, the KAR/AMPAR-specific blocker CNQX abolished the KA-induced phosphorylation of GluA1 S845, reinforcing the role of KAR or AMPAR activation **[Figure 3-12]**.

To determine the kinase(s) involved in GluA1 S845 phosphorylation, I used previously reported inhibitors of PKA and PKC, H89 and chelerythrine, respectively (Henley, 2004, Konopacki et al., 2011). In agreement with previous findings (Ho et al., 2011, José A. Esteban, 2003, Chater and Goda, 2014, Baudry et al., 2015), the PKA inhibitor H89 effectively abolished the KA-induced phosphorylation of GluA1 S845 **[Figure 3-13]**.

Together, my results indicate that KA application increases phosphorylation of GluA1 S845 via PKA is largely dependent on AMPAR or KAR activation.

3.4.2 The KAR-dependent increase in GluA1 phosphorylation might be dependent on presynaptic KARs

The phosphorylation of GluA1 S845 by KA is reduced in the presence of an AMPAR-specific blocker GYKI53655 **[Figure 3-10]**. Activation of presynaptic KARs can enhance glutamate release, therefore activating postsynaptic receptors and downstream signaling cascades wherein activation of PKA and phosphorylation of GluA1 S845 may be affected. This could go some way to explaining the partial blockade in GluA1 phosphorylation by AMPAR inhibitors **[Figure 3-10]**.

3.5 Conclusion

Overall, the data in this chapter demonstrate that the increase in phosphorylation of GluA1 S845 is primarily mediated by KARs. Potentially, one of the mechanisms involved may be activation of presynaptic KARs leading to a PKA-dependent increase in phosphorylation of GluA1 S845. This may have a completely novel function that is different from the previously reported NMDAR-dependent increase in surface expression of AMPARs. However, investigating this contrasting direction of change in phosphorylation at the same site (GluA1 S845) with chem-LTD protocol for NMDAR versus sustained KA treatment poses significant challenges. This may suggest that the NMDAR and KAR activation might be targeting different receptor pools. One way, a partial blockade in the extent of phosphorylation by GYKI53655 may be mediated by activation of presynaptic KARs and a subsequent increase in glutamate release, or by KA application non-specifically activating AMPARs **[Figure 3-14]**.

Although GluA1 S845 phosphorylation is a well-established marker of LTP (Diering et al., 2018), measuring the levels of surface receptors using surface biotinylation post-KAR stimulation is crucial to translate the biochemical changes in phosphorylation to functional effects on trafficking and on KAR-evoked plasticity of AMPARs (discussed in chapter 4).

Thus, to my knowledge, the KA-dependent increase in the phosphorylation of GluA1 S845 described here is completely novel. However, a lack of specific pharmacological

inhibitors to further investigate the precise mechanisms and signalling pathways involved (other than non-specific Ca^{2+} channel blockers) led me to discontinue investigations of the upstream mechanisms of KAR-mediated AMPAR phosphorylation. Rather, I next focused on how KARs impact on AMPAR trafficking, function, and plasticity.

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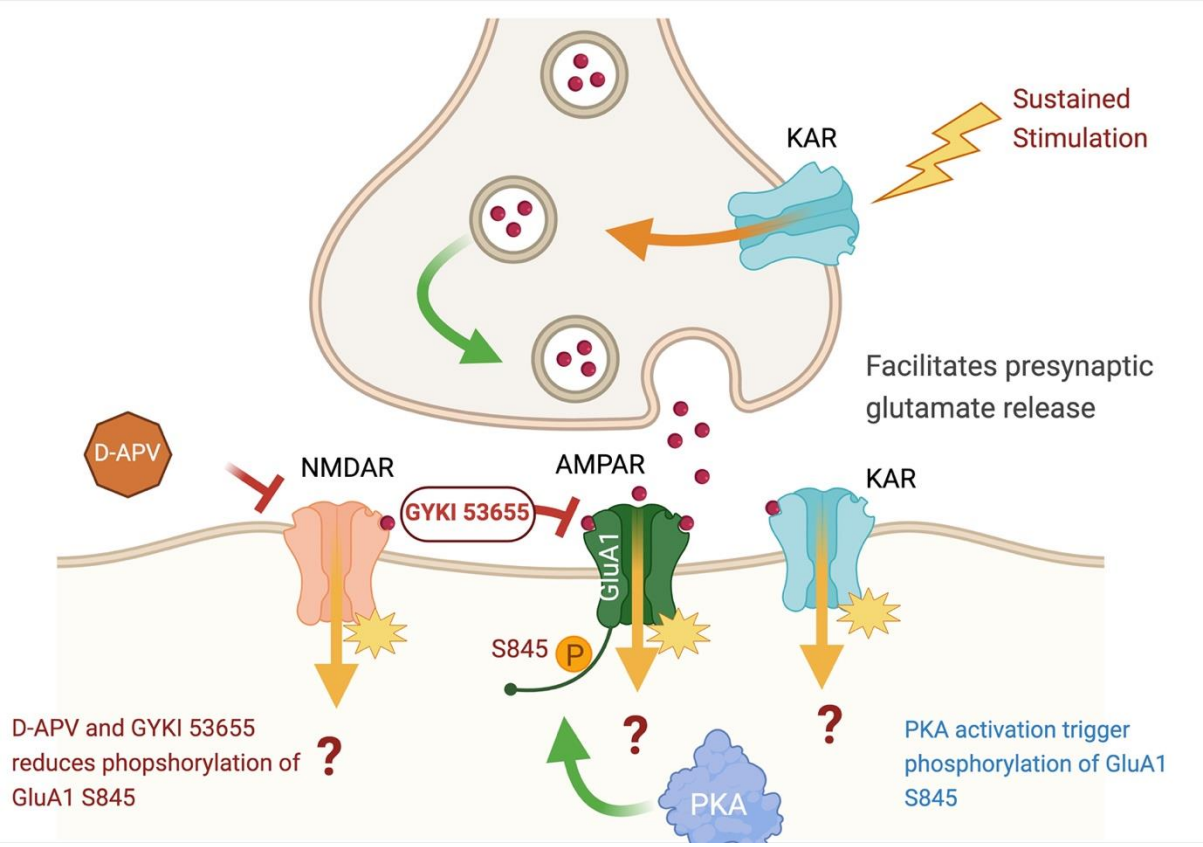


Figure 3-14: Proposed mechanism for the presynaptic KAR-dependent increase in the phosphorylation of GluA1 S845

Activation of presynaptic KARs facilitates the release of glutamate from presynaptic terminals via an unknown mechanism. The released glutamate activates postsynaptic glutamate receptors leading to the activation of PKA and subsequent increase in phosphorylation of GluA1 S845. Application of the AMPAR blocker GYKI53655 reduces the phosphorylation of GluA1 S845.

Chapter 4. Sustained stimulation of postsynaptic Kainate receptors induces LTD of AMPARs

4.1 Introduction

4.1.1 Plasticity of KARs

Synaptic plasticity at glutamatergic synapses is underpinned by alterations in the surface expression of postsynaptic glutamate receptors. This is achieved through protein-protein interactions and post-translational modifications that stabilise or destabilise receptor complexes at the postsynaptic density (Henley et al., 2021)(Nair et al., 2021). Each glutamate receptor subtype has its own complement of interacting proteins that enable synapses to selectively control the surface expression of individual glutamate receptors. KAR subunits have both PDZ and non-PDZ ligand mediated protein-protein interactions, including with the Neto proteins, C1ql 2/3, PICK1 and GRIP1 (Hirbec et al., 2003, Straub et al., 2016, Matsuda et al., 2016, Zhang et al., 2009, Straub et al., 2011b), and post-translational modifications including phosphorylation and SUMOylation that regulate their activity-dependent trafficking and surface expression (Konopacki et al., 2011, Martin et al., 2007, Jaskolski et al., 2005, Chamberlain et al., 2012, Gurung, 2018, Gurung et al., 2018, Copits and Swanson, 2013, Lerma and Marques, 2013).

Activation of KARs bidirectionally regulates their own surface expression, as well as that of other receptors **[Figure 4-1]**. Transient KAR stimulation (10μM KA for 3 mins) increases the surface expression of GluK2 containing heteromeric KAR complexes in the postsynaptic membrane **[Figure 4-1B]** (Martin et al., 2008). This KA-induced increase in surface expressed KARs is sensitive to the G-protein inhibitor pertussis toxin, indicating mediation by metabotropic KAR signaling. It also requires Ca^{2+}, PKC and PLC, and results in recycling of KARs and recruitment into spines via Rab 11-dependent endosomal pathways, promoting the upregulation of postsynaptic KARs (Gonzalez-Gonzalez and Henley, 2013). These findings strongly predict that KARs will undergo LTP (KAR-LTP) in response to brief bursts of activity at glutamatergic synapses, but this has yet to be demonstrated especially since majority of KARs are not expressed at synaptic sites.

Conversely, sustained KAR stimulation (10μM KA for 10 mins) decreases the surface expression of postsynaptic KARs by enhancing GluK2 SUMOylation, leading to endocytosis and targeting to lysosomes for degradation **[Figure 4-1C]** (Konopacki et al., 2011, Martin et al., 2007, Henley, 2004, Martin et al., 2008). This pathway is dependent on phosphorylation of GluK2 by PKC, but not PKA. Activation of NMDARs

can also trigger internalization of KARs via a Ca^{2+}, PKA and PKC dependent pathway. However, this pathway is independent of SUMOylation (Henley, 2004, Martin et al., 2008).

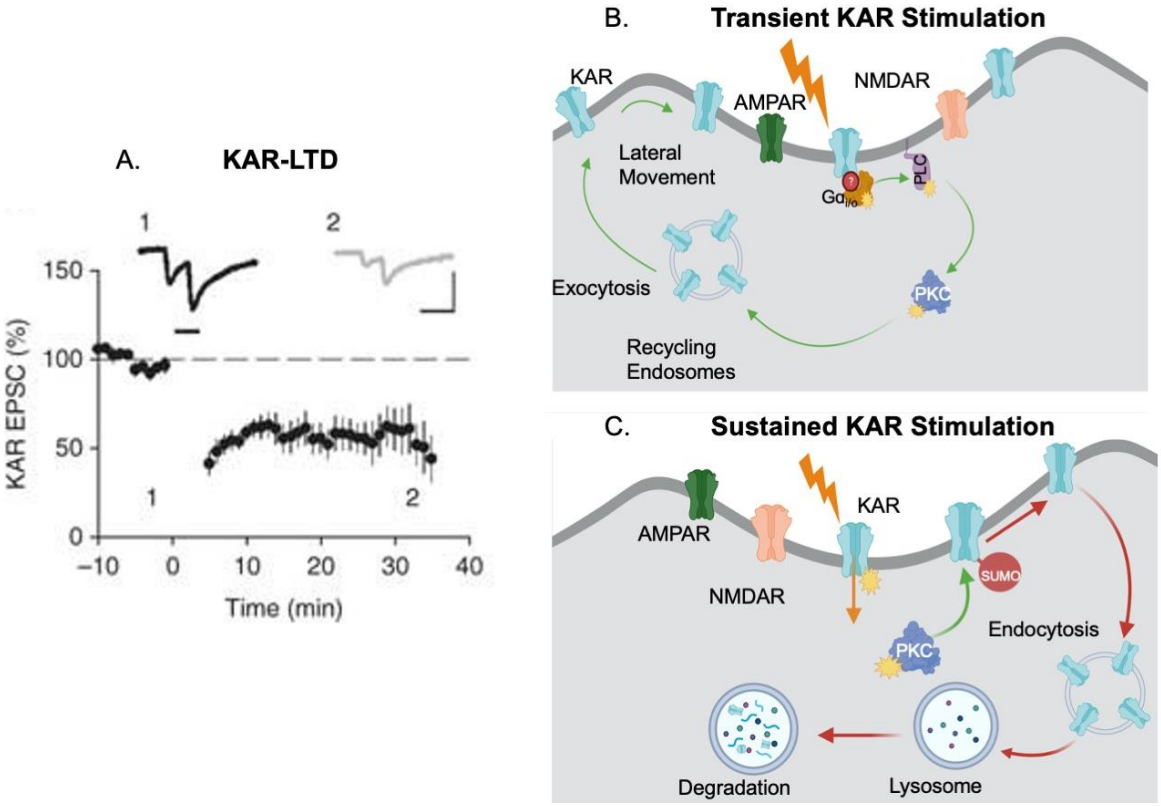


Figure 4-1: Bidirectional regulation of KAR surface expression

A) Sustained low frequency (black bar, 1 Hz for 5 min) stimulation of hippocampal mossy fibre synapses induces KAR-LTD. Numbers represent timepoints for example Kainate responses (Adapted from (Chamberlain et al., 2012)). B) Transient stimulation of KARs leads to enhanced surface expression of KARs via activation of metabotropic KARs, PLC, PKC and via recruitment of Rab 11-positive endosomes. C) Sustained stimulation of KARs leads to activation of PKC and increased SUMOylation of GluK2. Downregulation of surface KARs occurs via endocytosis and lysosomal degradation. Adapted from(Nair et al., 2021).

SUMOylation of GluK2 at K886, promoted by prior phosphorylation at S868, triggers internalisation of the receptor during the induction of KAR-LTD at mossy fibre synapses in the hippocampus, a phenomenon resulting from sustained low frequency stimulation of synapses releasing glutamate to activate postsynaptic KARs (Chamberlain et al., 2012). KAR-LTD also requires GluK5 participation in a complex with the PDZ proteins PICK1 and GRIP1, and the tSNARE complex protein SNAP25, which has been reported to recruit KARs into vesicles for endocytosis (Selak et al., 2009). In CA3 neurons, blockade of

SNAP25 increased KAR EPSCs, while cells over expressing SNAP25 exhibited increased intracellular accumulation of GluK5 containing KARs, suggesting a role for SNAP25 in destabilising GluK5 containing KARs at the surface and thereby promoting their internalisation (Selak et al., 2009).

KARs can themselves undergo plasticity. KAR-LTD can be induced in MF-CA3 pathways by brief high frequency stimulation that mimics natural spike patterns in dentate gyrus granule cells, illustrating the physiological relevance of KAR plasticity. KAR-LTD induced by high frequency stimulation requires coactivation of adenosine A2A receptors, in addition to activation of mGluR5, which is also required for sustained low frequency induced KAR-LTD (Chamberlain et al., 2012, Chamberlain et al., 2013, Carta et al., 2014). Surprisingly, the major consequences of KAR-LTD do not result from the reduced synaptic current, even though these small but prolonged currents can display significant influence on cellular spiking (Sachidhanandam et al., 2009), but instead arise from the reduced ability to inhibit channels mediating slow afterhyperpolarizations (I_{sAHP}), leading to a reduction in cell excitability (Melyan et al., 2002, Chamberlain et al., 2013). This in turn offsets the excitatory effect of NMDAR-LTP at mossy fibre synapses, which is also induced by brief high frequency stimulation, potentially protecting CA3 neurons form excitotoxic insults (Rebola et al., 2008, Kwon and Castillo, 2008, Chamberlain et al., 2013).

KAR-LTD also exists at synapses onto layer II/III neurons in the perirhinal cortex, where 5 Hz synaptic stimulation induces LTD of KAR-mediated transmission, and at developing thalamocortical synapses in somatosensory cortex (Park et al., 2006). KAR-LTD in perirhinal cortex has many mechanistic similarities to mossy fibre KAR-LTD, relying on postsynaptic Ca^{2+} levels, mGluR5, PKC activation and PICK1 PDZ domain interactions (Park et al., 2006). In layer IV cells of somatosensory cortex, postsynaptic KARs undergo activity-dependent LTD and are replaced by AMPARs during the first postnatal week (p3-p7), consistent with an important developmental role for KAR-LTD (Isaac, 1999).

4.1.2 KARs induce LTP of AMPARs

LTP and LTD occur at different synapses throughout the CNS and are crucial for processes including network formation during development, synaptic stabilisation, higher levels of cognitive functioning and in modulating multiple learning and memory (Robert C.Malenka, 2004). Although the best characterized forms of LTP and LTD are

evoked by NMDARs activation, (Bliss et al., 2014) there are several forms of NMDAR independent plasticity (Gladding et al., 2009, Anwyl, 2009).

Recently, studies from the lab reported that transient activation of KARs induces a novel form of LTP of AMPARs in hippocampal CA1 neurons (Petrovic et al., 2017a). Transient activation of GluK2 containing KARs induces LTP of AMPARs in hippocampal CA1 neurons (Petrovic et al., 2017a) **[Figure 4-2]**. This KAR-dependent AMPAR plasticity (AMPAR-LTP$_{KA}$) requires metabotropic signalling and PKC activation that cause increased surface expression of AMPARs via exocytosis from recycling endosomes (Petrovic et al., 2017a). When combined with KAR-LTD, AMPAR-LTP$_{KA}$ may provide a mechanism for the observed developmental switch in glutamate receptor expression at several synapses where KARs are replaced by AMPARs during specific developmental windows (Lauri et al., 2006, Isaac, 1999, Lanore et al., 2012).

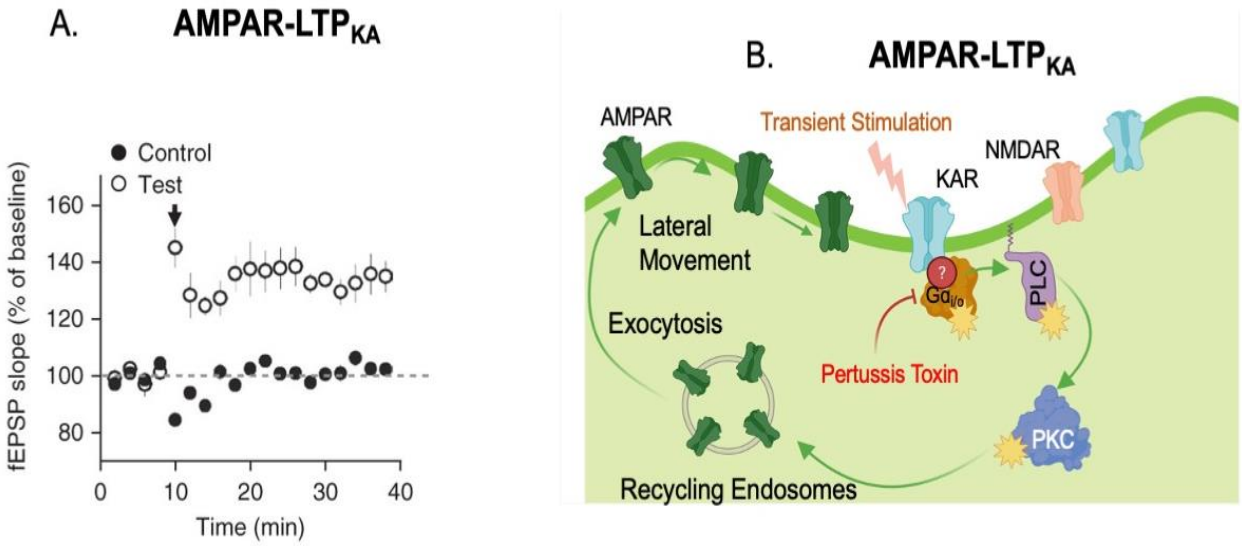


Figure 4-2: Induction of AMPA receptor LTP by KARs

A) High frequency stimulation (4 bursts of 100 stimuli at 200 Hz) of Schaffer collateral synapses induces AMPAR-LTP$_{KA}$ (adapted from (Petrovic et al., 2017a)). B) Transient stimulation of KARs induces increased surface expression of AMPARs. The increase in surface AMPAR expression requires metabotropic signaling of GluK2 containing KARs and activation of PKC. Adapted from (Nair et al., 2021)

4.2 Objectives

Building on the discovery of KAR-LTP$_{AMPAR}$ and the experiments in the previous chapter demonstrating that KAR activation can regulate AMPAR GluA1 phosphorylation, I investigated if KARs can also induce LTD of AMPARs.

I have focussed on GluA1 and GluA2 subunits of AMPARs, as the major heteromeric AMPARs expressed in hippocampal neurons are composed of GluA1/GluA2 and GluA2/GluA3 (Henley and Wilkinson, 2013, Wenthold et al., 1996). However, during synaptic plasticity, it is predominantly surface expression of the GluA1 and GluA2 subunit that are subject to altered trafficking and surface expression (Plant et al., 2006). The majority of hippocampal KARs are likely to be composed of GluK2/GluK5 (Mulle et al., 1998, Contractor et al., 2003b, Wenthold et al., 1994). Due to the availability of high-quality antibodies and importance of GluK2 subunits in regulating the majority of KAR-mediated signalling, including KAR-LTP$_{AMPAR}$, I focussed on the GluK2 subunit of KARs.

Specifically, my objectives were to address the following questions:

1. Can sustained KA stimulation cause a reduction in surface AMPARs and induce KAR-LTD$_{AMPAR}$?
2. Which signalling mode of KARs regulates this form of plasticity?
3. Is this form of plasticity dependent on postsynaptic KARs?
4. What are the signalling mechanisms underlying this form of plasticity?

4.3 Materials and Methods

4.3.1 Sustained KA and NMDA stimulation

Stimulation protocol for transient and sustained KA treatment were adapted from (Petrovic et al., 2017c, Henley, 2004, Martin et al., 2008, Gonzalez-Gonzalez and Henley, 2013). Hippocampal neurons were incubated in 2ml of prewarmed Earle's buffer (EBS) containing 1μM TTX with or without other drugs for at least 30 mins in a 37°C incubator. After 30 mins, the cells were taken out, the media containing drugs were aspirated and supplied with 2ml of fresh pre-warmed EBS containing drugs along with KA and placed in the incubator for 20 mins **[Figure 4-3]**. Controls were treated

the same way but with vehicle instead of KA. For NMDA stimulation, 20μM NMDA and 20μM glycine were added to the cells and incubated for 3 mins (Ashby et al., 2004). This stimulation protocol was followed for all the biochemical as well as imaging experiments.

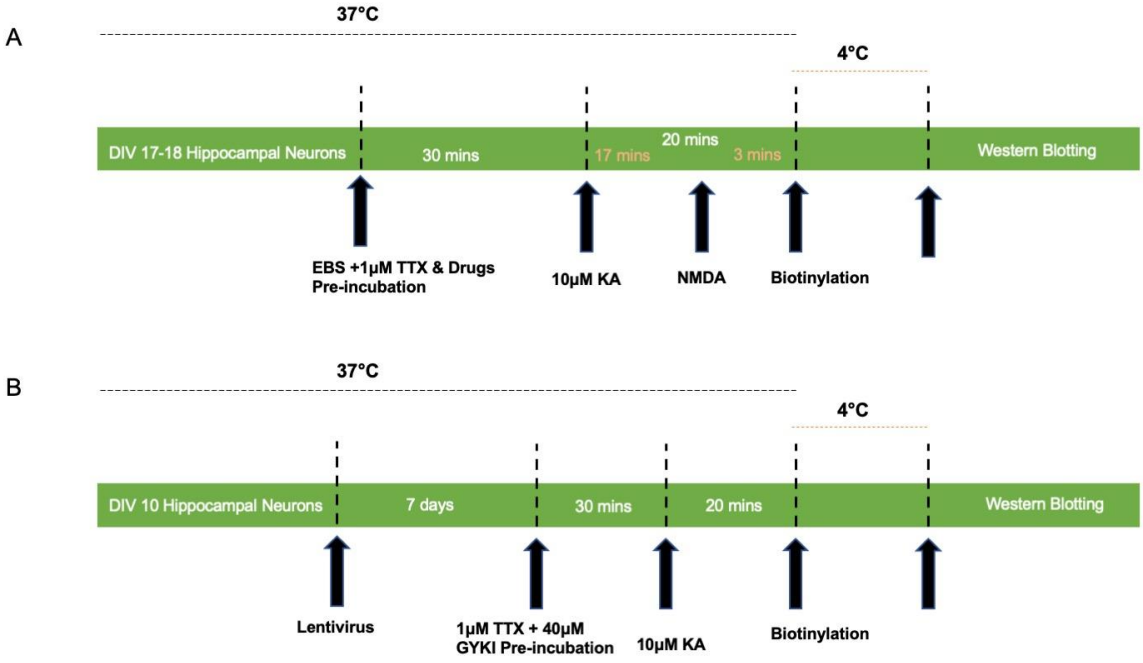


Figure 4-3: Schematics showing timeline of sustained KA stimulation

A.) Pre-treatment of neurons were performed at 37°C for 30 mins after adding 2ml of prewarmed EBS containing 1μM TTX and drugs prior to 20 mins of sustained stimulation with KA or 3 mins with NMDA and glycine. The cells were removed from incubator and left it at 4°C to inhibit receptor trafficking. Surface receptors were isolated by biotinylation followed by pull down with streptavidin beads and Western blotting.

B.) Lentivirus (ShRNA and GFP) were added to hippocampal neurons at DIV10 and left it for 7 days to knock down GluK2 and/or express GFP. At DIV17, Neurons were pre-treated with 2 ml of prewarmed EBS containing 1μM TTX and 40μM GYKI53655 for 30 mins prior to 20 mins of sustained stimulation with KA. The cells were removed from incubator and left it at 4°C to prevent receptor trafficking. Surface receptors were isolated by biotinylation followed by pull down with streptavidin beads and Western blotting.

4.3.2 Live surface staining

Hippocampal neurons for imaging were plated at a cell density of 70,000 per well in a 25mm glass coverslip. The protocol for sustained KA stimulation is the same as that of biotinylation experiments **[Figure 4-4]**. After stimulation with KA, the cells were

taken out of the incubator and leave to cool at RT for 10 mins to prevent receptor trafficking. The cells were incubated for 20 mins in the primary antibody. Appropriate concentration N-terminal antibody was added (Section:2.1.9.1) and mixed in a 1.5ml Eppendorf containing 100µl of conditioned media (per coverslip). 90µl of the antibody containing media pipetted onto parafilm and the coverslips were gently placed with cells facing down on to the primary antibody. After incubation, the coverslips were placed back into a 6 well plate containing 2ml of DPBS (cells side facing up). The coverslips were washed 3-5 times in DPBS and fixed with 1ml of pre-warmed (37ºC) 4% formaldehyde + 5% sucrose for 12 mins. The cells were again washed 3 times in DPBS followed by a wash with 1ml of 100mM glycine dissolved in DPBS to quench residual formaldehyde. To remove glycine, the coverslips were again washed 3 times with DPBS. The cells were permeabilised and blocked using 3% BSA in DPBS containing 0.1% Triton-X 100 for 20 mins with gentle shaking at RT. Secondary antibodies were diluted 1:400 in 3% BSA in 1XDPBS and were incubated for 1 hr (same as primary antibody incubation). After the incubation, the coverslips were placed back into the wells and were washed 3 times with DPBS. 40µL of mounting media (Fluoromount-GTM with DAPI (Thermo Fisher)) was pipetted on to slides and the coverslips were mounted (cells facing down) after gently dipping into ddH2O (to prevent salt crystal formation). The slides were left overnight to dry before imaging or storage at 4ºC.

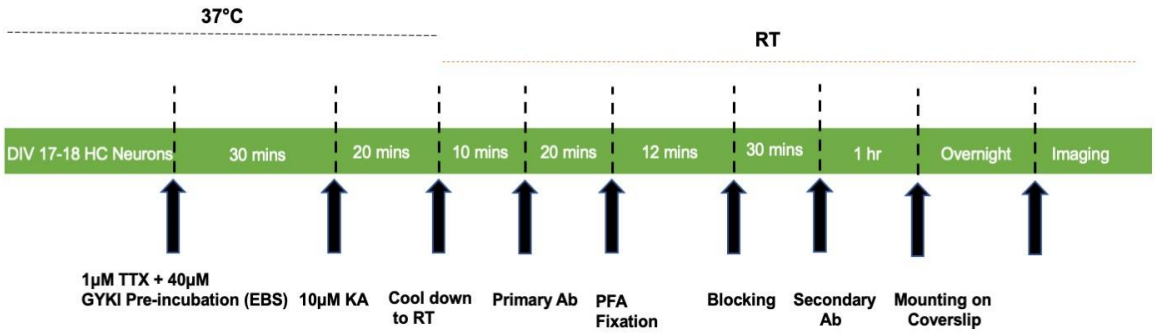


Figure 4-4: Timeline of Live surface staining post-KA stimulation

Neurons were pre-treated with 2 ml of prewarmed EBS containing 1µM TTX for 30 mins along with drugs prior to 20 mins of sustained stimulation with KA. The cells were removed from incubator and left it at RT to prevent receptor trafficking. Neurons containing coverslips were incubated with primary Ab of appropriate concentration for at least 20 mins. The cells were fixed, incubated with secondary Ab and mounted on a glass slide before imaging.

4.3.3 Electrophysiology

4.3.3.1 Artificial cerebrospinal fluid (aCSF)

The below listed reagents were used to prepared aCSF.

Table 4-1: List of components in aCSF

Reagent	Concentration (mM)
NaCl	124
KCl	3
$NaHCO_3$	26
NaH_2PO_4	1
D-Glucose	10
$CaCl_2$	2
$MgSO_4$	1

aCSF for the experiments were prepared by adding required amounts of D-APV and picrotoxin and was constantly bubbled with 95% O_2 and 5% CO_2 at 35ºC.

4.3.3.2 Sucrose slicing solution

The below listed reagents were used to prepare sucrose slicing solution.

Table 4-2: List of components in sucrose cutting solution

Reagent	Concentration (mM)
Sucrose	189
KCl	3
$NaHCO_3$	26
NaH_2PO_4	1.25
D-Glucose	10
$CaCl_2$	1
$MgSO_4$	5

1X solution was prepared from 10X stock solution of sucrose cutting solution and was kept in freezer to obtain ice-cold cutting solution prior to slicing. The solution was constantly bubbled with 95% O_2 and 5% CO_2.

4.3.3.3 Internal solution

The following reagents were used to prepare internal solution.

Table 4-3: List of components in patch solution (internal solution)

Reagents	Concentration (mM)
NaCl	8
$CsMeSo_4$	130
HEPES	10
EGTA	0.5
MgATP	4
NaGTP	0.3
QX314.Cl	5

pH and osmolarity of the solution were adjusted to 7.4 and 290 mOsm respectively. The solution was aliquoted into 1.5ml Eppendorf and stored at -20°C until further use.

4.3.3.4 Acute hippocampal slice preparation

Male and female Han Wistar rat pups of age postnatal day 13-15(P13-15) were anesthetized using 4% isoflurane and decapitated. Brain of the animal was removed and placed in ice-cold sucrose slicing solution constantly bubbled with 95% O_2 and 5% CO_2. Hippocampus were carefully removed and parasagittal sections of 400µm thickness were obtained using a vibratome (7000smz-2, Campden Instruments). Slices were kept for recovery in slice holder containing aCSF constantly bubbled with 95% O_2 and 5% CO_2 at 37°C for 20 mins and later transferred to room temperature and left it for 30 mins before performing experiments.

4.3.3.5 Electrophysiology analysis

Hippocampal slices were placed in a submerged holding chamber continuously perfused with oxygenated aCSF at 32-35^{o}C at a flow rate of 2ml^{-1}. Hippocampal CA1 pyramidal cells were patch-clamped in whole cell configuration using a Harvard borosilicate glass capillary of resistance 2-5M MΩ filled with WCS. The cells were held at -70mV and evoked AMPAR EPSCs were obtained by stimulating Schaffer collateral pathway. CA3 region of hippocampi was carefully removed to eliminate epileptiform activity. 50µm D-APV and 50µm picrotoxin were applied in bath to isolate AMPAR EPSCs. Cells with series resistance above 20 MΩ or deviated by 20% were discarded.

A stable baseline was achieved after 20 mins of stimulation, 1µm KA was bath applied for 10 mins followed by 20 mins of washout period.

At 2kHz signals were low-pass filtered and digitalized at 10kHZ using Axonpatch 200B amplifier (Molecular Devices) and WInLTP v1.11acquisition software (Anderson and Collingridge, 2007).

4.1 Statistical analysis

For each experiment, the signal for each condition was divided by the mean overall signal from that experiment. This analysis was performed for each replicate experiment, and for presentation purposes, the mean of the control condition set to 100%.

All graphs were generated, and statistical tests performed, using GraphPad Prism version 9.2. The sample sizes correspond to previous published results and no statistical tests were performed to predetermine the sample size (Gonzalez-Gonzalez and Henley, 2013, Petrovic et al., 2017a). The details of the statistical tests performed on each experiment are explained in the figure legend along with p-values and error bars. Number of cells = n and number of independent dissections/number of animals = N.

4.2 Results

4.2.1 Sustained KAR stimulation on cortical cultures reduces GluA1 but not GluA2 AMPARs and GluK2 KARs

To study the role of sustained KA stimulation on the levels of surface expression of AMPARs and KARs, I performed surface biotinylation on cortical cultures following 20 mins of 10μM KA stimulation. A significant decrease in the surface GluA1 receptors were detected **[Figure 4-5]**. In contrast, the surface expression of GluA2 and GluK2 subunits remain unchanged **[Figure 4-5]**. Although the precise reasons remain ambiguous, lack of reproducibility, together with failure to recapitulate previously reported decrease in the surface expression of GluK2 KARs and GluA2 AMPARs (Evans et al., 2017a, Gurung, 2018, Henley, 2004) led me to discontinue further experiments on cortical cultures.

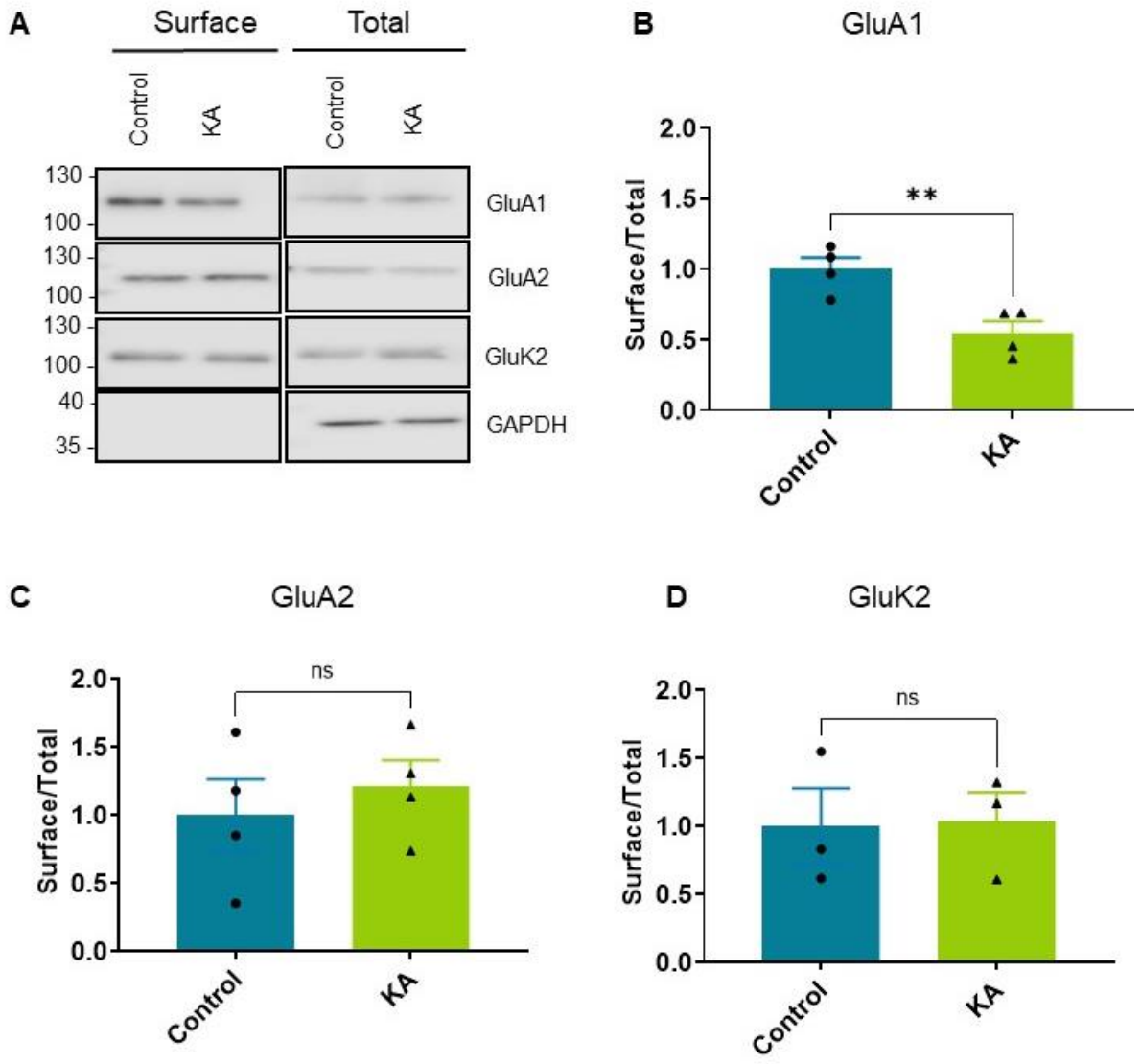


Figure 4-5: Sustained KA stimulation reduces surface expression of GluA1 but not GluA2 or GluK2, in cortical neurons

Surface biotinylation was carried out on DIV 18 cortical neurones following 20 mins sustained stimulation with 10μM KA. The neurons were pre-treated with 1μM TTX to inhibit ambient glutamate release.

Representative western blots of surface and total levels of GluA1, GluK2, GluA2 and GAPDH (A). GAPDH was used as a control to ensure no internal proteins were biotinylated.

Quantification of surface to total ratio of GluA1 (B), GluA2 (C), GluK2 (D), N=4 independent dissections, ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$; Un paired t test with Welch's correction, error bars = SEM.

4.2.2 KA stimulation on hippocampal neurons reduces surface GluA1 and GluK2 receptors in the absence of GYKI53655

Because of the lack of consistency using cortical neurons, I switched to using hippocampal neuronal cultures. Interestingly, KA treatment significantly reduced surface levels of both GluA1 AMPARs and GluK2 KARs **[Figure 4-6]**. This effect was consistent with previous findings from the lab (Henley, 2004, Gurung, 2018).

NMDAR dependent chem-LTD was used in parallel as a control to ensure neuronal health and activity **[Figure 4-6]**. In agreement with previous reports application of NMDA significantly decreased the surface expression of GluK2 containing KARs (Henley, 2004), along with GluA1 and GluA2 AMPARs (Luthi et al., 1999, Beattie et al., 2000). EGFR was used as a non-glutamate receptor control to ensure the specificity of KA stimulation **[Figure 4-6 E]**. Together, these results suggest that KA treatment can reduce the number of GluA1 containing AMPARs expressed on the surface of both cortical and hippocampal neurons.

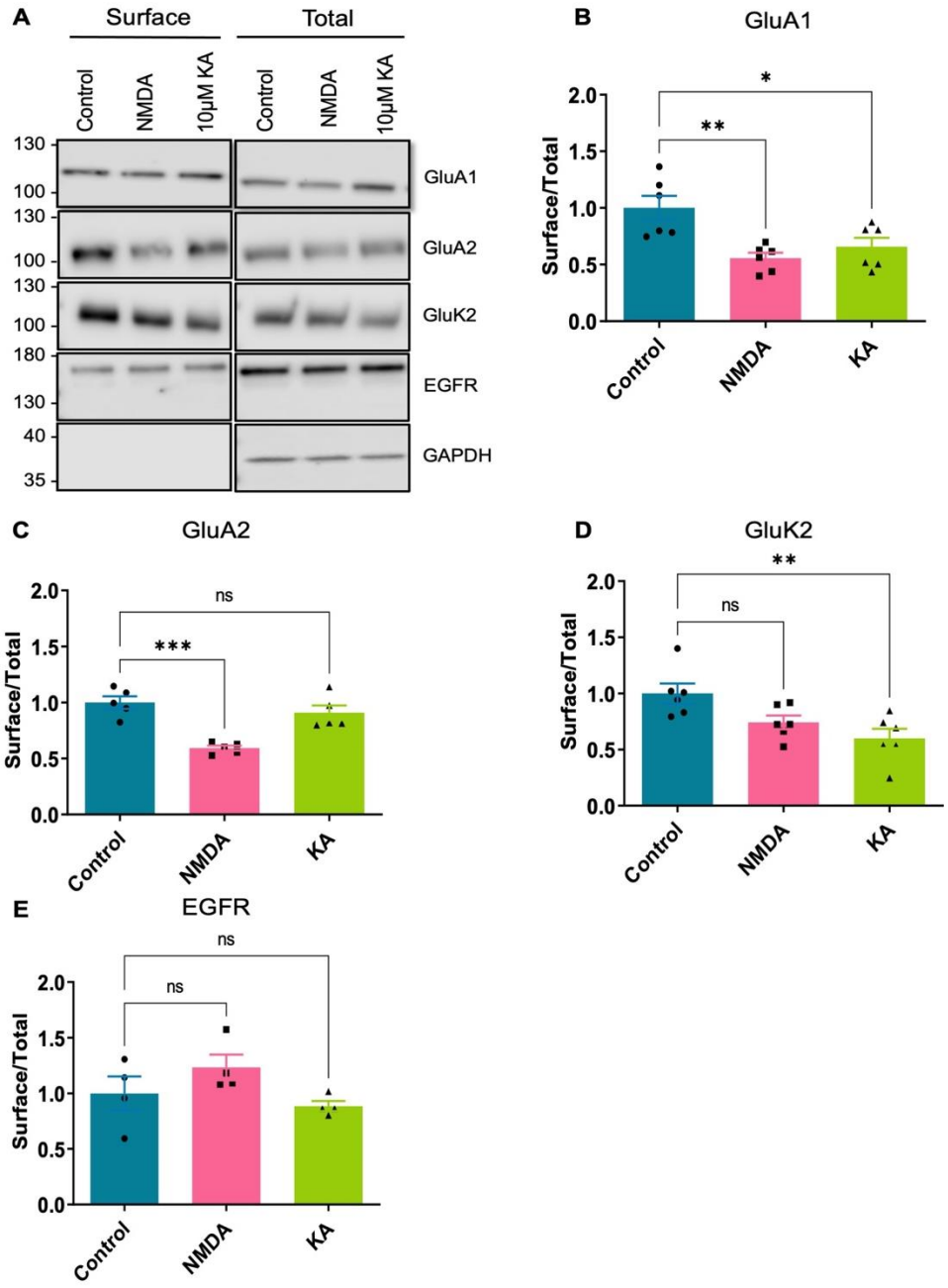


Figure 4-6:Sustained KA stimulation reduces surface AMPARs and KARs

Surface biotinylation was carried out on DIV 18 hippocampal neurons following 20 mins sustained stimulation with 10μM KA. The neurons were pre-treated with 1μM TTX to inhibit ambient glutamate release

Representative western blots of surface and total levels of GluA1, GluK2, GluA2, EGFR and GAPDH (A). EGFR was used as a non-glutamate receptor expressed on surface which ideally should not respond to KA stimulation. GAPDH was used as a control to ensure no internal proteins were biotinylated.

Quantification of surface to total ratio of GluA1 (B), GluA2 (C), GluK2 (D), EGFR (E); N=6 independent dissections, ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$; One-way ANOVA with Dunnett's multiple comparisons test, error bars = SEM.

4.2.3 KA decreases surface levels of AMPARs and KARs independent of AMPAR activation

I next investigated the effects of sustained KA stimulation in the presence of AMPAR specific antagonist GYKI53655 (Partin and Mayer, 1996, Paternain et al., 1995). The cells were incubated for 30 mins with 1µM tetrodotoxin (TTX), to inhibit spontaneous glutamate release and 40µM GYKI53655 to exclude the possibility of direct activation of AMPARs by KA. In parallel, NMDAR-mediated chem-LTD protocol was induced on neurons from the same dissections by applying 20µM NMDA and 20µM glycine for 3 minutes (Anwar et al., 1998, Ashby et al., 2004) to ensure that neuronal health and activity was not compromised.

Surface expression of GluA1, GluA2 and GluK2 were significantly reduced to similar levels by KA or NMDA stimulation **[Figure 4-7].** To confirm that these effects are selective, EGFR levels was measured in neurons treated with KA or NMDA stimulation. As expected, no change was detected in the levels of EGFR indicating the effects are specific to iGluRs.

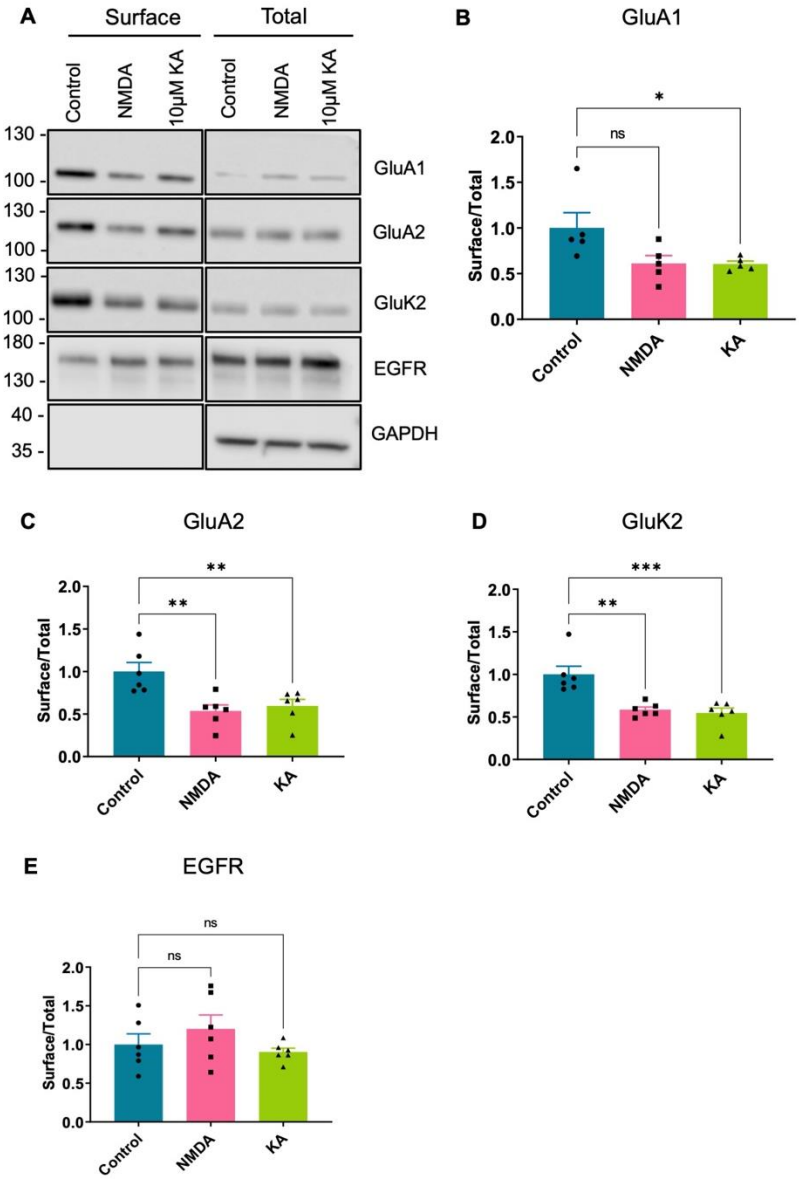


Figure 4-7: KAR activation reduces surface expression of AMPARs and KARs independent of AMPAR activation

The effects of 10μM KA on surface expression of GluA1, GluA2 AMPARs and GluK2 KARs for 20 mins following 30 min pre-treatment with 1μM TTX, 40μM GYKI53655 on DIV 18 hippocampal neurons was investigated by surface biotinylation. For NMDA treatment includes pre-treatment with 1μM TTX for 30 mins followed by 3 mins of treatment with 20μM NMDA and 20μM glycine. Control samples were treated same as KA but with vehicle for 20 mins.

Representative western blots of surface and total levels of GluA1, GluK2, GluA2, EGFR and GAPDH (A). EGFR was used as a non-glutamate receptor expressed on surface that do not respond to KA stimulation. GAPDH was used as a control to ensure no internal proteins were biotinylated.

Quantification of surface to total ratio of GluA1 (B), GluA2 (C), GluK2 (D), EGFR (E). N=6 experiments from independent dissections, *p<0.05, **p<0.01, ***p<0.001; One-way ANOVA with Dunnett's multiple comparisons test, error bars = SEM.

At concentrations above 3µM, KA is a partial, weakly desensitising agonist of AMPARs (Levchenko-Lambert et al., 2011, Ruiz et al., 2005). Therefore, to further confirm KAR specific activation rather than KA induced agonism of AMPARs, I also investigated 1µM KA, a concentration below the threshold for AMPAR activation without inclusion of GYKI53655 (Massey et al., 2001) on reducing surface AMPARs. In this experiment, I measured the change in the surface levels of GluA2, a prominent and extensively used reporter for AMPAR endocytosis (Nishimune et al., 1998, Lee et al., 2010). Again, as expected, surface levels of GluA2 were significantly reduced by 1µM KA, further confirming KAR activation in loss of surface AMPARs **[Figure 4-8]**.

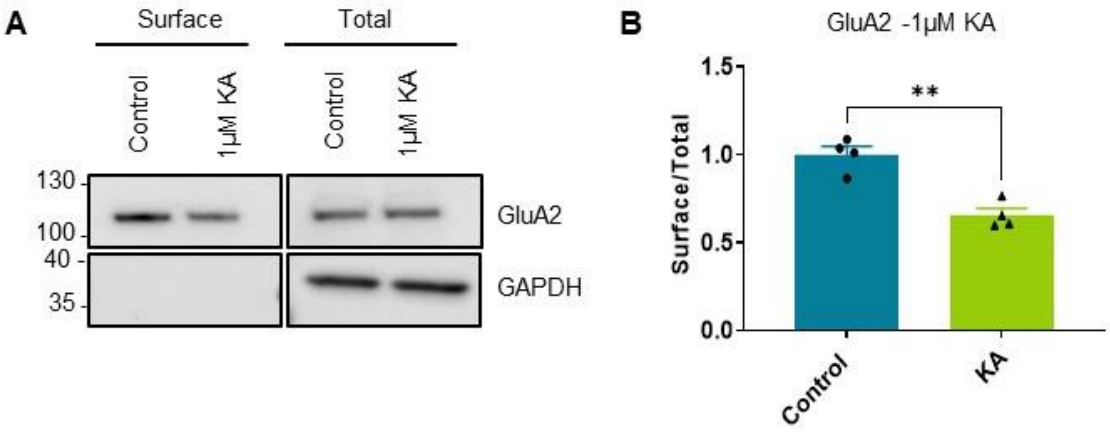


Figure 4-8: 1µM KA reduces surface expression of GluA2-containing AMPARs

The effects of 1µM KA for 20 mins following 30 min pre-treatment with 1µM TTX, on DIV 18 hippocampal neurons was investigated by surface biotinylation. Control samples were treated same as KA but with vehicle for 20 mins.

Representative western blots of surface and total levels of GluA2 and GAPDH (A). GAPDH was used as a control to ensure no internal proteins were biotinylated.

Quantification of surface to total ratio of GluA2 (B), N=4 experiments from independent dissections **p<0.01, Unpaired t test with Welch's correction., error bar = SEM.

4.2.4 KA application decreases surface GluA2 in dendrites

To study the compartment specific changes in KA evoked reduction in surface AMPARs, live surface staining and imaging of the GluA2 subunit was performed in neurons. Hippocampal neurons were pretreated with 40µM GYKI53655 and 1µM TTX for 30 mins prior to KA stimulation. Non-permeabilised cell surface staining and

confocal imaging was performed to monitor the alteration in GluA2 surface expression with KA treatment in the region of interest (ROI). Consistent with the biotinylation data, the imaging results showed a significant reduction in the surface levels of GluA2 in both proximal and branched dendrites [**Figure 4-9].** Due to the unavailability of reliable N-terminus GluK2 antibody, I restricted my imaging study to GluA2 AMPARs. The confocal images were taken and analysed by Ms. Busra Perihan Yucel.

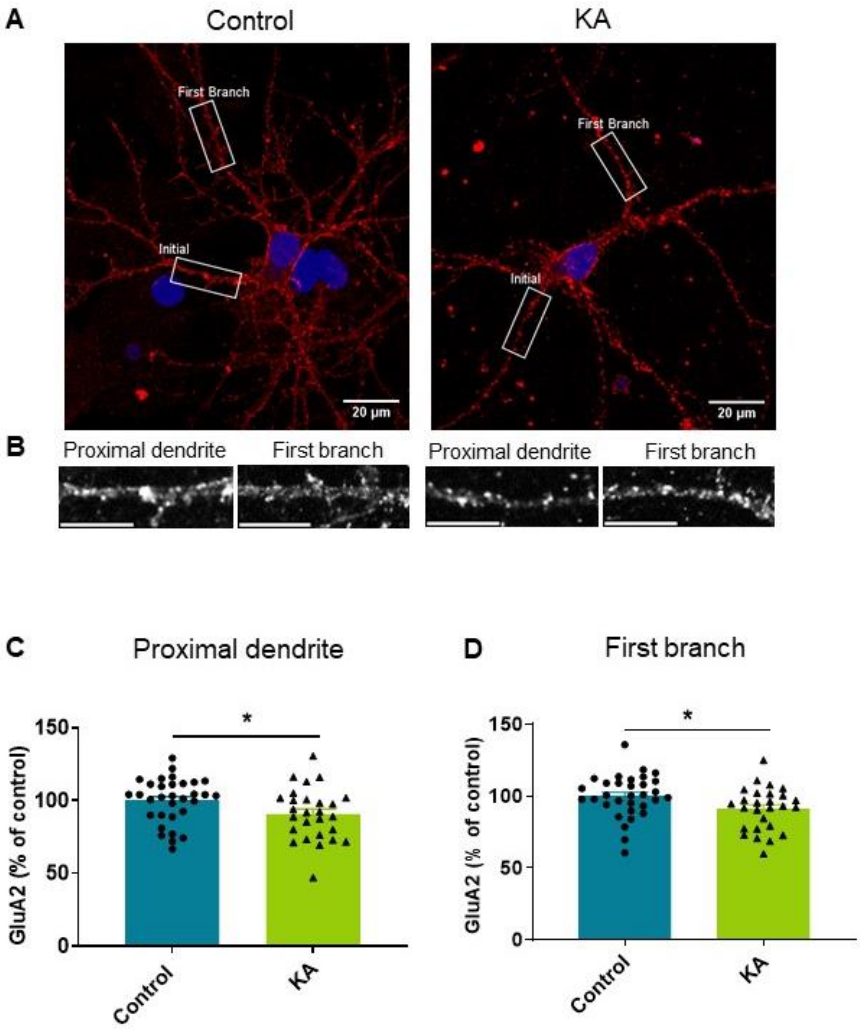


Figure 4-9: Live surface staining of GluA2 shows a significant reduction post-KA treatment

Representative image of neurons (A) with ROI (B) for GluA2 Control and KA treatment Quantification of intensity of GluA2 in proximal dendrites (C). Quantification of intensity of GluA2 after the first dendritic branch (D). n=32 cells, N=3 independent dissections, *p<0.05; Un-paired t test with Welch's correction, Error bars = SEM.

4.2.5 KA decreases synaptic surface expressed GluA2

To determine if the decrease in GluA2-containing AMPARs occurred at synapses, we repeated live surface staining of GluA2 subunits post 20 mins of KA stimulation along with a postsynaptic marker Homer. Hippocampal neurons were pretreated with 40µM GYKI53655 and 1µM TTX for 30 mins prior to KA stimulation. Following fixation and permeabilisation, the neurons were labelled with synaptic marker Homer. Confocal imaging was performed to monitor the changes in the surface expression of GluA2 at the puncta postive for Homer. Consistent with the biotinylation and imaging data. KA treatment showed a significant decrease in the surface expression of GluA2 at Homer-positive puncta consistent with a loss of surface expressed AMPARs at the synapses **[Figure 4-10].** No change in the fluorescence of Homer was detected indicating the KA treatment did not alter the number of synapses. The confocal images were captured and analysed by Dr. Alexandra Fletcher-Jones.

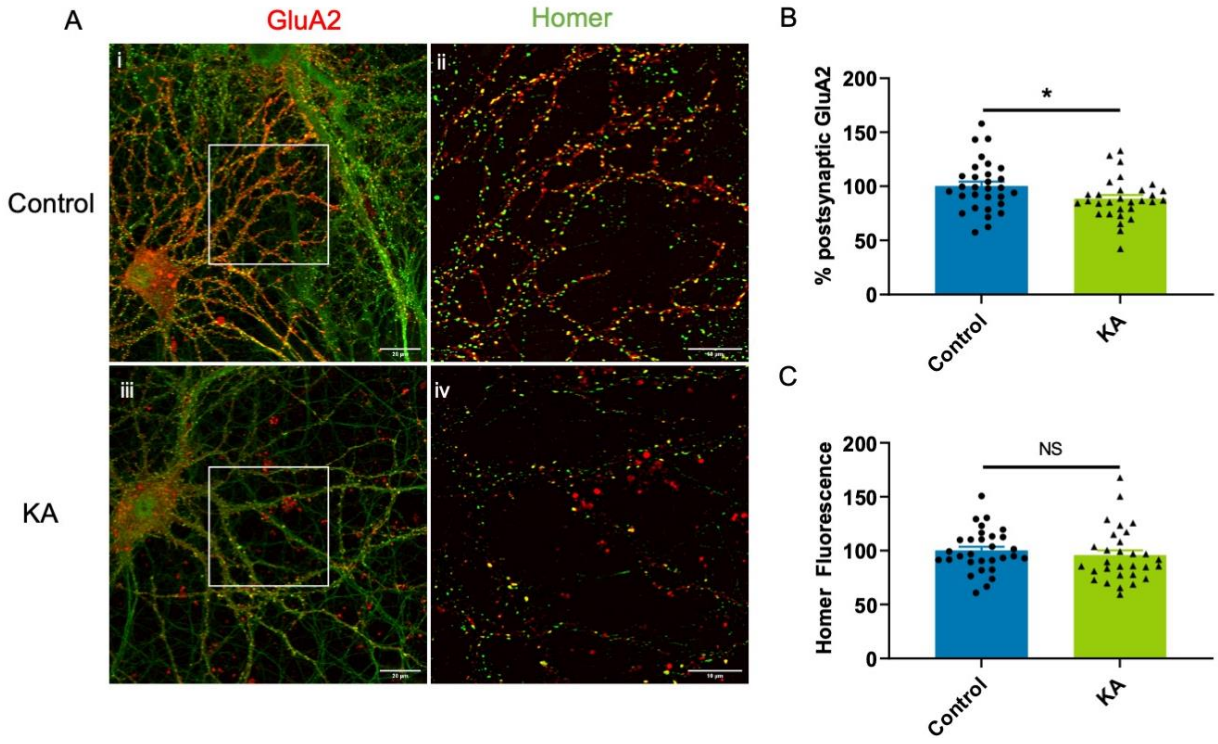


Figure 4-10 : KA induced reduction of GluA2 AMPAR is synaptic

Fixed confocal images of DIV18 hippocampal neurons show a significant reduction in postsynaptic GluA2 surface expression after 10µM KA treatment for 20 mins.

Representative images of control and KA-treated neurons (Ai and Aiii). Scale bar = 20µm. Representative ROIs of control and KA-treated neurons (Aii and Aiv). Scale bar = 10µm.

Quantification of mean fluorescent intensity of postsynaptic surface GluA2 staining (B) and fluorescent intensity of Homer (C). In all cases n=10 ROIs, N=3 independent dissections, *p<0.05, Un-paired t test, error bars = SEM.

4.2.6 KA-induced decrease in surface AMPARs is independent of NMDAR or mGluR activation

KAR-LTD induced by high or sustained low frequency requires activation of mGluR5 (Chamberlain et al., 2012, Chamberlain et al., 2013). To exclude any indirect effect mediated by either NMDARs or mGluRs, the neurons were pre-treated with the NMDAR antagonist D-APV (50µM) (Morris, 1989) or a combination of the mGluR5 antagonist (MPEP) (Lea and Faden, 2006) and the mGluR1 antagonist YM298198 (Kohara et al., 2005) (10µM and 1µM, respectively) along with 40µM GYKI53655 and 1µM TTX for 30 mins prior to 20 mins of sustained stimulation with KA. As expected, both NMDAR and mGluR antagonists failed to block KAR-induced reduction in the

surface expression of GluA2 **[Figure 4-11]**. 10μM KA still caused a significant decrease in GluA2 surface expression in the presence of these drugs, indicating the KA-induced decrease in surface GluA2 occurs in the absence of NMDAR and mGluR activity **[Figure 4-11]**.

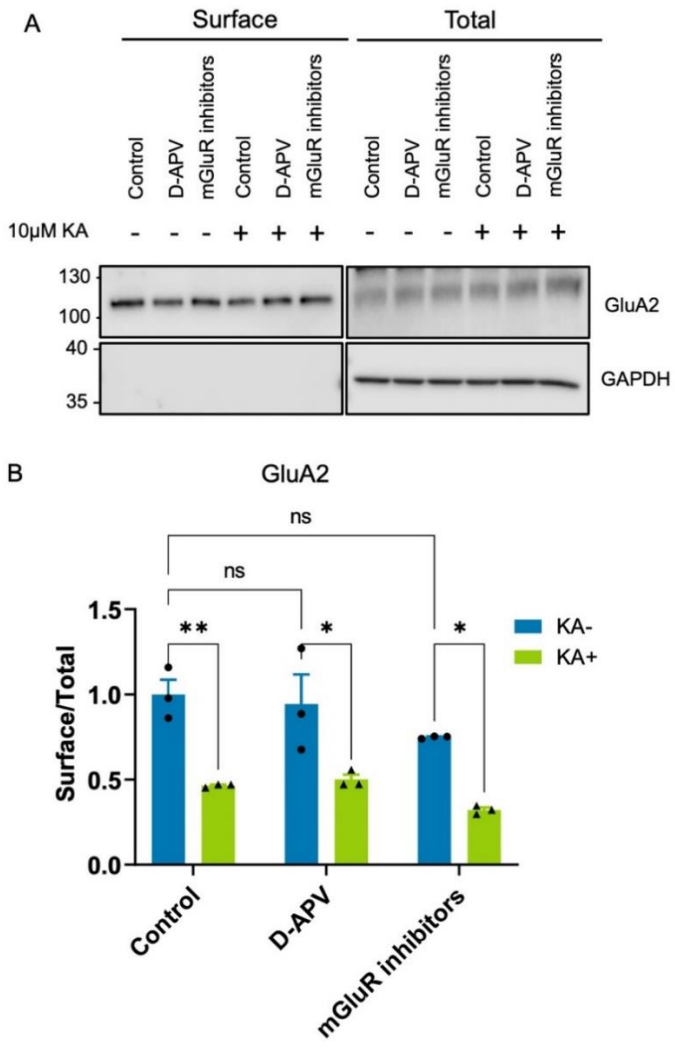


Figure 4-11: KA-induced decrease in surface AMPARs is independent of NMDAR or mGluR activation

The effects of 10μM KA on surface expression of GluA1, GluA2 AMPARs for 20 mins following 30 mins with 50μM D-APV or 10μM MPEP and 1μM YM298198 ,40μM GYKI53655 and 1μM TTX were investigated by surface biotinylation on DIV 18 Hippocampal neurons. Control samples were treated same as KA but with vehicle for 20 mins.

Representative western blots of surface and total levels of GluA2 and GAPDH (A). GAPDH was used as a control to ensure no internal proteins were biotinylated.

Quantification of surface to total ratio of GluA2 (B). N=3 independent dissections, *p<0.05, **p<0.01, ****p<0.0001; Two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

4.2.7 CNQX occludes the KA-induced reduction in surface AMPARs

To further validate the involvement of KARs in reducing surface AMPARs, I used KAR/AMPAR-specific antagonist CNQX (Petrovic et al., 2017c). Hippocampal neurons were pre-treated with 10µM CNQX along with 40µM GYKI53655 and 1µM TTX for 30 mins, prior to 20 mins of sustained stimulation with KA. CNQX reduced basal surface levels of GluA1 and GluA2 AMPARs indicating that KAR-mediated signalling pathways regulate the surface expression of AMPARs **[Figure 4-12]**.

However, interestingly, an increase in the GluA1 levels compared to no-drug treated control were detected in samples treated with CNQX and KA **[Figure 4-12 A]**.

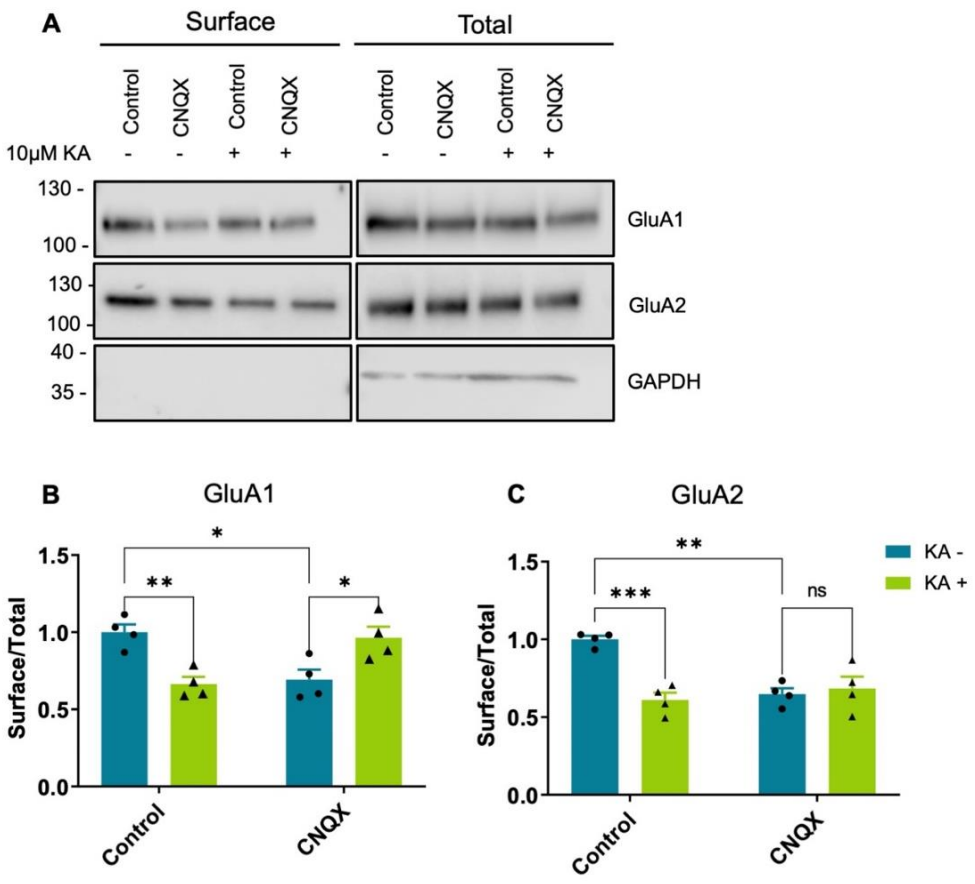


Figure 4-12: KA-induced reduction in surface AMPARs is blocked by CNQX

The effects of 10μM KA on surface expression of GluA1, GluA2 AMPARs for 20 mins following 30 mins of pre-treatment with 10μM CNQX and 1μM TTX were investigated by surface biotinylation on DIV 18 Hippocampal neurons. Control samples were treated same as KA but with vehicle.

Representative western blots of surface and total levels of GluA1,GluA2 and GAPDH (A). GAPDH was used as a control to ensure no internal proteins were biotinylated.

Quantification of surface to total ratio of GluA1 (B), GluA2 (C), N=4 independent dissections, *p<0.05, **p<0.01, ***p<0.001; Two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

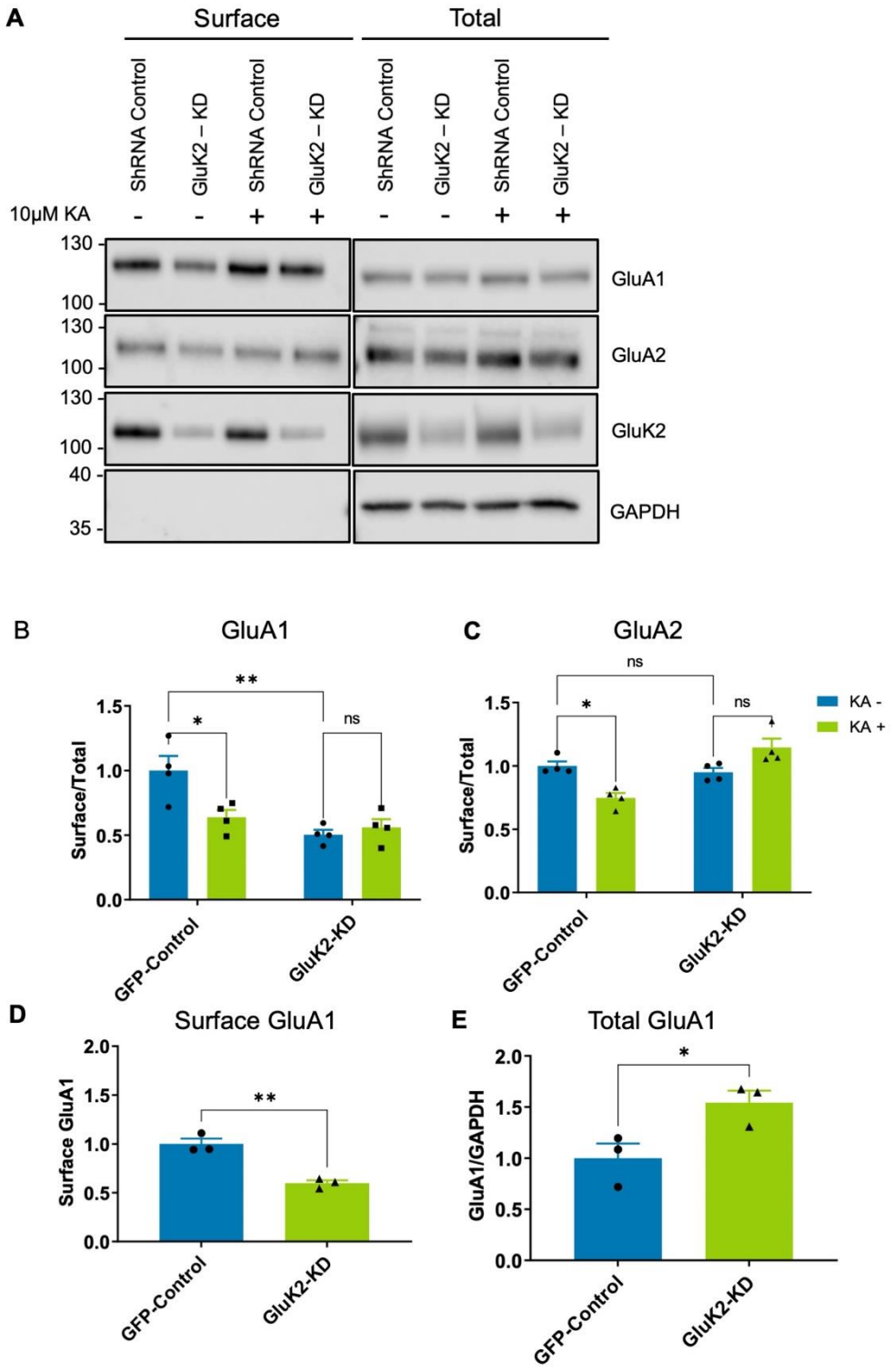
4.2.8 The KAR subunit GluK2 is required for the KA-evoked decrease in AMPAR surface expression

Previous work from the lab has shown that the KAR GluK2 subunit is crucial for induction of KAR-LTP$_{AMPAR}$ (Petrovic et al., 2017c). To understand the dependence of GluK2 containing KARs in reduction of surface expressed AMPAR, I knocked down GluK2 subunits in neurons by using lentiviral constructs to express shRNA targeted against GluK2 or a control virus expressing GFP (Gurung, 2018). Consistent with the

previous reports (Gurung et al., 2018), the GluK2 shRNA reduced total GluK2 levels by ~70% [**Figure 4-13 H**].

7 days after transduction, neurons were pre-treated for 30 mins with 40µM GYKI53655 and 1µM TTX prior to KA application for 20 mins. As expected, the shRNA treated neurons failed to exhibit KAR-induced reduction in surface AMPARs suggesting the importance of GluK2 subunits in regulating KAR-induced reduction in surface AMPARs **[Figure 4-13 A-C].**

Furthermore, a significant increase in the total and a decrease in surface GluA1 levels were detected in GluK2 knockdown cells compared to GFP virus infected control **[Figure 4-13 D, E].** However, no statistically significant difference was observed for GluA2 in GluK2 knockdown cells **[Figure 4-13 F, G].**


A
Surface
Total
ShRNA Control
GluK2 – KD
ShRNA Control
GluK2 – KD
ShRNA Control
GluK2 – KD
ShRNA Control
GluK2 – KD
10μM KA
- - + + - - + +
130
100
GluA1
GluA2
GluK2
40
35
GAPDH
B
GluA1
Surface/Total
GFP-Control
GluK2-KD
C
GluA2
KA -
KA +
D
Surface GluA1
Surface GluA1
E
Total GluA1
GluA1/GAPDH

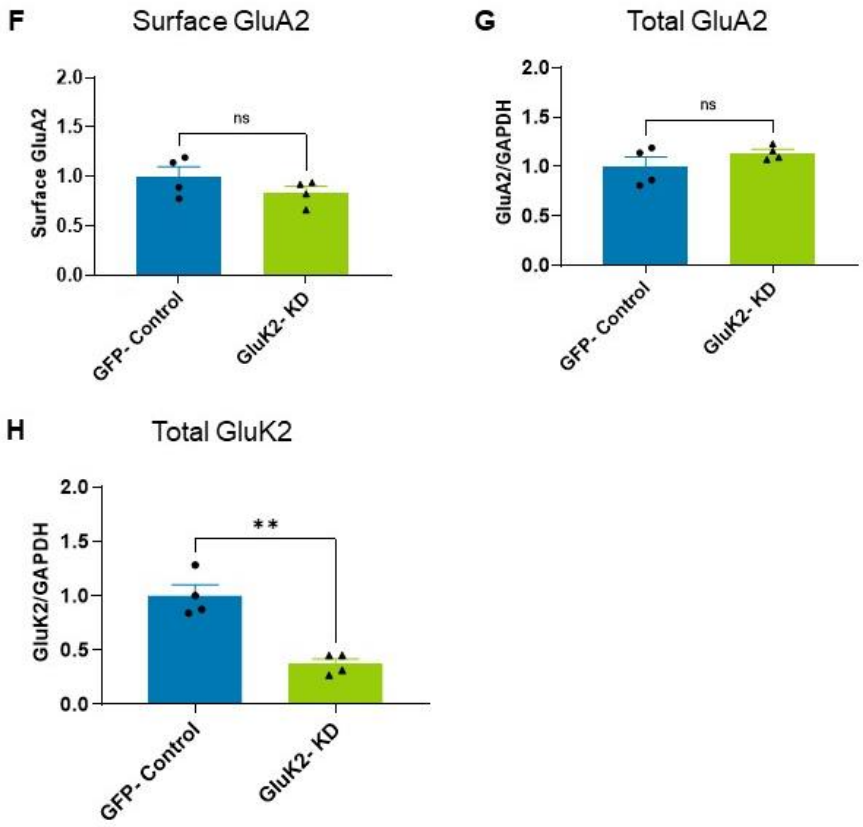


Figure 4-13: KA-induced reduction in the surface AMPARs requires GluK2-containing KARs

500μl of Lentivirus (Scr and Sh1) was added to the neurons on DIV 10 and left in the incubator for 7 days following which surface Biotinylation was carried out on DIV 17 hippocampal neurons following 30 mins of pre-treatment with 1μM TTX and 40μM GYKI53655, and 20 mins of 10μM KA treatment.

Representative western blots of surface and total levels of GluA1, GluK2, GluA2 and GAPDH (A). GAPDH was used as a control to ensure no internal proteins were biotinylated.

Quantification of surface to total ratio of GluA1 (B), GluA2 (C), Surface GluA1(D) Total GluA1 (E), Surface GluA2 (F) Total GluA2 (G), Total GluK2 (H). N=4 independent dissections, ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$; Two-way ANOVA with Tukey's multiple comparisons test (B, C); Un-paired t test with Welch's correction (D-H), error bars = SEM.

4.2.9 KA regulation of AMPAR surface expression requires ionotropic KAR signaling

KARs employ both canonical ionotropic and non-canonical $G\alpha_{i/o}$ protein dependent metabotropic signalling mechanisms to regulate neuronal functions (Evans et al., 2017a). To investigate the KAR-mediated signalling pathway underlying the reduction in surface AMPAR expression, I used the ionotropic KAR blocker UBP310 (Pinheiro et al., 2013, Petrovic et al., 2017a, Grosenbaugh et al., 2018) and $G\alpha_{i/o}$-protein inhibitor pertussis toxin (PTx) (Petrovic et al., 2017c). In contrast to KAR-LTP_{AMPAR}, blocking KAR metabotropic signalling for 1hr with 1µg/ml PTx prior to KA stimulation did not prevent the KA-induced reduction in GluA2 surface expression **[Figure 4-14 C]**. However, 10µM UBP310, an antagonist of postsynaptic ionotropic signalling through GluK2/GluK5-containing KARs, did block the KA-evoked reduction in surface GluA2 **[Figure 4-14 C].** Interestingly, both PTx and UBP310 treatment inhibited reduction of GluA1 **[Figure 4-14 B]**. Moreover, that UBP310 caused a significant reduction in surface GluA2 levels in the absence of KA stimulation, suggesting KAR activity may be required to maintain surface AMPAR expression. Consequently, the lack of KA-induced loss of surface GluA2 in the presence of UBP310 may represent an occlusion effect.

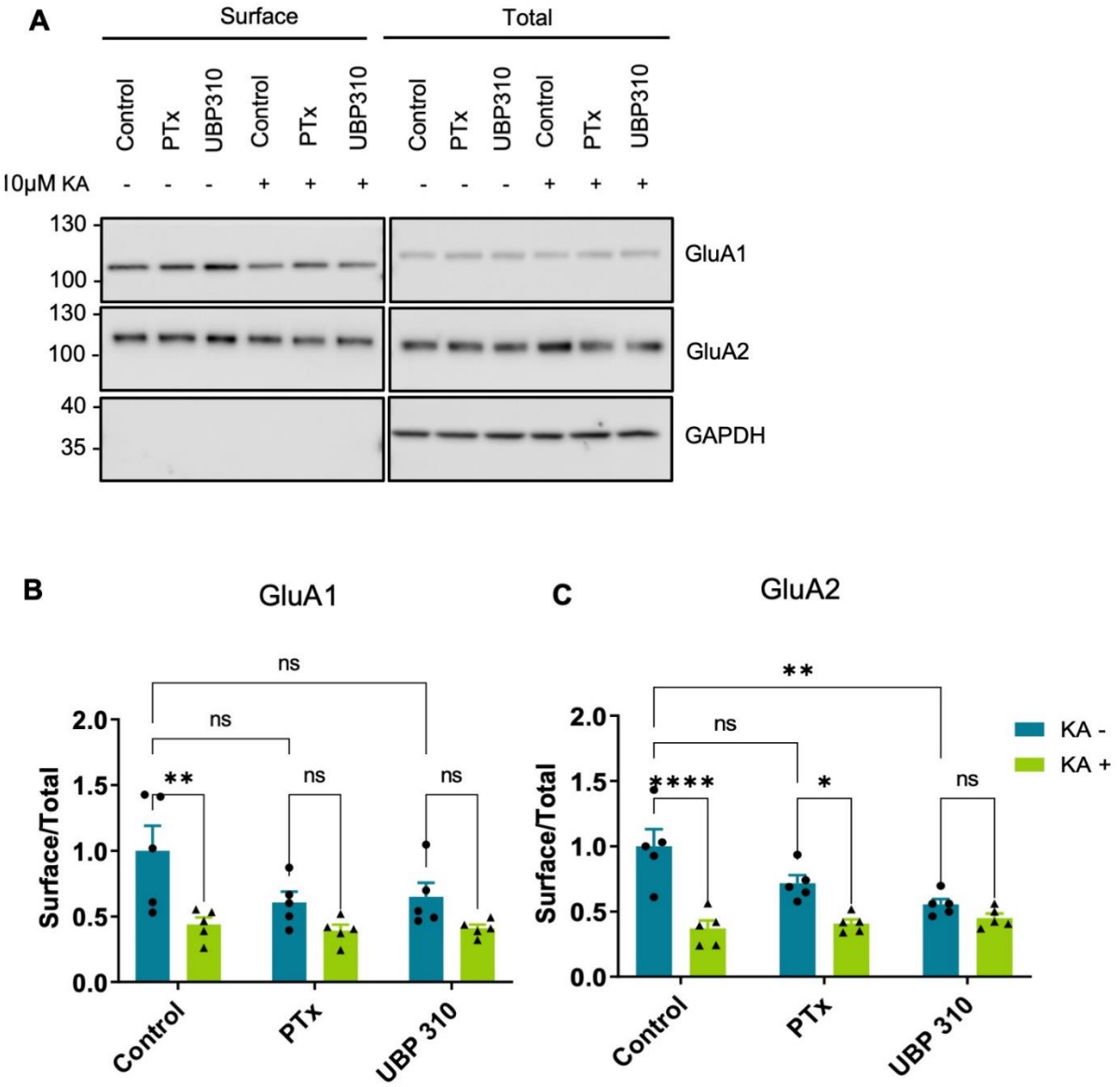


Figure 4-14: KA-induced regulation of AMPAR surface expression requires ionotropic KAR signalling

The effects of 10μM KA on surface expression of GluA1, GluA2 AMPARs for 20 mins following 1hr of pre-treatment with 1μg/ml PTx and 30 mins with40μM GYKI53655, 10μM UBP310 and 1μM TTX were investigated by surface biotinylation on DIV 18 Hippocampal neurons. Control samples were treated same as KA but with vehicle for 20 mins.

Representative western blots of surface and total levels of GluA1, GluA2 and GAPDH (A). GAPDH was used as a control to ensure no internal proteins were biotinylated.

Quantification of surface to total ratio of GluA1 (B), GluA2 (C). N=5 independent dissections, *p<0.05, **p<0.01, ****p<0.0001; Two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

4.2.10 Effects of PKA and PKC on KA-dependent regulation of surface AMPARs

AMPAR subunits are phosphorylated by multiple protein kinases (Diering et al., 2018). Alteration in the phosphorylation of subunits play key roles in receptor trafficking, induction and maintenance of LTP and LTD (Lu et al., 2012). KAR-mediated ionotropic and metabotropic signalling pathways activates protein kinases as an integral component of downstream signalling pathways to regulate network activity (Petrovic et al., 2017c, Martin et al., 2008, Henley, 2004). KAR-mediated induction of LTP of AMPARs require activation of PKC (Petrovic et al., 2017c). Therefore, I next decided to explore the role of protein kinase(s) PKA and PKC in regulating the surface expression of AMPARs. Hippocampal neurons were pre-treated with 10μM H89, PKA specific inhibitor or 5μM chelerythrine specific inhibitor for 30 mins along with TTX (1μM) prior to 20 mins of sustained KA stimulation. Both kinase inhibitors caused a trend towards a decrease in surface expression of GluA1 and GluA2 AMPARs indicating that PKA/PKC signalling pathways support the surface expression of GluA1 and GluA2-containing AMPARs **[Figure 4-15].** I hypothesise that H89 and chelerythrine, like UBP310, may lead to the loss of 'removable' AMPARs from the neuronal surface to a minimal level so that KA-stimulation cannot decrease them any further. Thus, blocking PKA or PKC occludes the KA-induced reduction in GluA2 surface expression **[Figure 4-15].**

These data suggest that both PKA and PKC are required for maintaining tone of AMPARs.

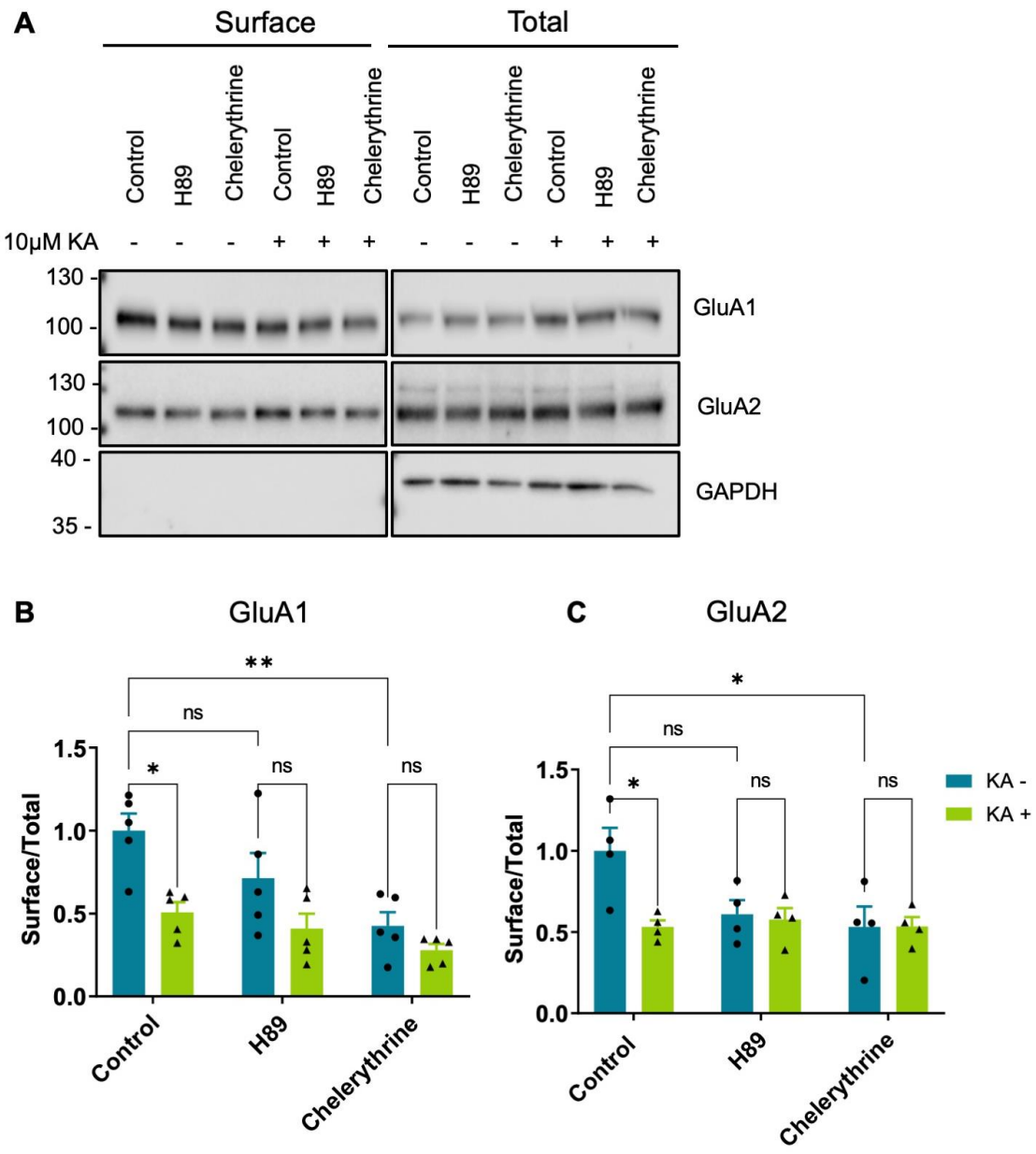


Figure 4-15: Protein kinases activity on KA dependent surface expression of AMPARs

The effects of 10μM KA on surface expression of GluA1, GluA2 AMPARs for 20 mins following 30 mins of pre-treatment with inhibitors 40μM GYKI53655, 10μM H89, 5μM Chelerythrine and 1μM TTX were investigated by surface biotinylation on DIV 18 Hippocampal neurons. Control samples were treated same as KA but with vehicle.

Representative western blots of surface and total levels of GluA1, GluA2 and GAPDH (A). GAPDH was used as a control to ensure no internal proteins were biotinylated.

Quantification of surface to total ratio of GluA1 (B), GluA2 (C). N=5 independent dissections, **p<0.01; Two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

4.2.11 The KAR-mediated reduction in AMPARs requires activation of PP2B (Calcineurin)

In addition to kinases, the protein phosphatases PP1, PP2A, and PP2B (calcineurin) have been reported to be involved in forms of LTD (Belmeguenai and Hansel, 2005, Groth et al., 2003). I therefore investigated the effects of pre-treatment with inhibitors of these phosphatases **[Figure 4-16]**.I investigated the effects of 1µM okadaic acid to inhibit PP1 and PP2A (Schnabel et al., 2001), or 50µM FK506 to inhibit calcineurin/PP2B (Beattie et al., 2000), on the KA-evoked decrease in GluA2 surface expression. Okadaic acid reduced surface levels of GluA2 in non-stimulated conditions, indicating roles for the protein phosphatases PP1/PP2A in regulating basal surface expression of AMPARs. These data suggest that okadaic acid occludes, whereas FK506 blocks, the KA-evoked decrease in GluA2 surface expression **[Figure 4-16].**

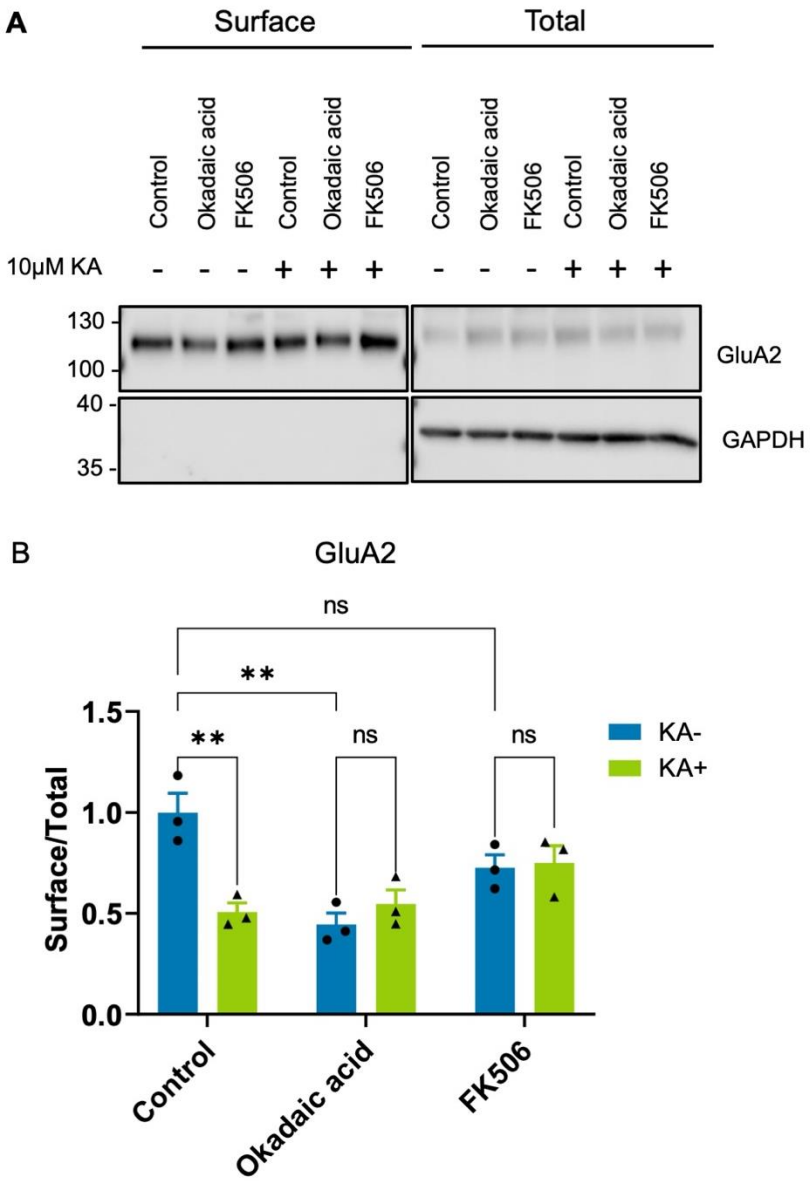


Figure 4-16: KAR-mediated reduction of AMPARs require activation of PP2B (calcineurin)

The effects of 10μM KA on surface expression of GluA2 AMPARs for 20 mins following 30 mins with 1μM Okadaic acid or 50μM FK506 and40μM GYKI53655 and 1μM TTX were investigated by surface biotinylation on DIV 18 Hippocampal neurons. Control samples were treated same as KA but with vehicle for 20 mins.

Representative western blots of surface and total levels of GluA2 and GAPDH (A). GAPDH was used as a control to ensure no internal proteins were biotinylated.

Quantification of surface to total ratio of GluA2 (B). N=3 independent dissections, ns $p>0.05$, *$p<0.05$; Two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

4.2.12 KAR activation induces long-term synaptic depression

Biochemical and imaging studies with sustained KAR stimulation have shown that activation of KARs leads to decrease in the surface expression of AMPARs. We next investigated the effect of KAR stimulation on synaptic function by measuring AMPAR mediated excitatory post synaptic currents (EPSCs) and induction of LTD in CA1 region of acute rat hippocampal slices. The electrophysiology recordings in this chapter were performed and analysed by Dr. Ellen Braksator.

Bath application of 1μM KA for 10 min in the presence of 50μM picrotoxin and 50μM D-APV significantly reduced AMPAR mediated EPSCs 20 mins post KA washout **[Figure 4-17]**.

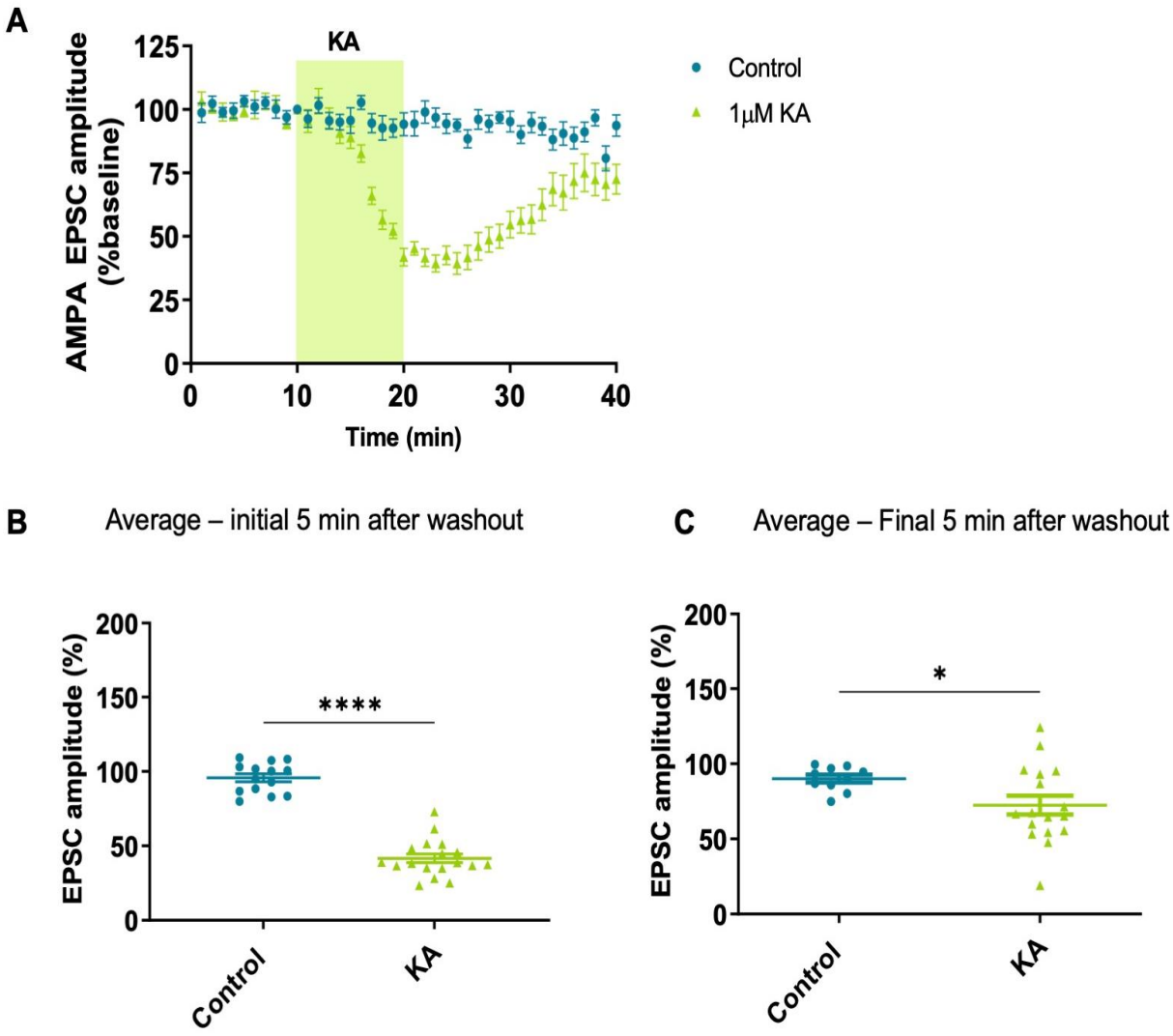


Figure 4-17: 10 mins of KA application induces depression of $EPSC_{AMPA}$

Graph showing the effects of 1μM KA on AMPAR EPSCs in CA1 region of acute hippocampal slices (A). EPSCs were normalised to baseline corresponding to initial 10 mins prior to KA application. The traces were recorded in the presence of 50μM D-APV and 50μM picrotoxin. N=9-14, cells from different animals.

Quantification of average EPSC amplitudes initial 5 mins after washout (B) average EPSC amplitudes 35-40 mins after washout (C). N=17 slices, ns $p>0.05$, *$p<0.05$, ****$p<0.0001$ Unpaired t test with Welch's correction. Error bar= SEM.

4.2.13 KAR activation induces a presynaptic form of short-term plasticity

KARs present at both pre-, and postsynaptic sites regulate network activity and neuronal transmission (Henley et al., 2021, Nair et al., 2021). To assess the role of presynaptic KARs in short- and long-lasting changes in the depression of AMPAR mediated EPSCs, we measured the paired pulse ratio (PPR). A ratio of the amplitude of second peak to the first peak was measured after application of two stimuli which are 50 ms apart. PPR measures the probability of glutamate release from presynaptic terminals and the ratio is inversely proportional to release probability (Manabe et al., 1993, Debanne et al., 1996). A higher ratio is associated with presynaptic receptor mediated modulation of release (Zucker and Regehr, 2002, Xu-Friedman and Regehr, 2004).

Immediately 10 mins post KA application, we observed an increase in PPR indicating a reduction in the probability of release. This along with an initial decrease in AMPAR EPSCs are characteristics of presynaptic KAR-mediated short-term plasticity (STP) **[Figure 4-18]** (Lauri et al., 2003). Interestingly, however, the PPR returned to baseline levels within 10-20 mins post KA washout while AMPAR EPSCs remained depressed **[Figure 4-18]**. Together, these data indicate that bath application of 1µM KA for 10 min activates both pre- and postsynaptic KARs or activate postsynaptic KARs and triggers a retrograde signalling inducing a dual form of plasticity (Lourenco et al., 2011). However, the long-term KAR-LTD$_{AMPAR}$ is mediated by postsynaptic KARs.

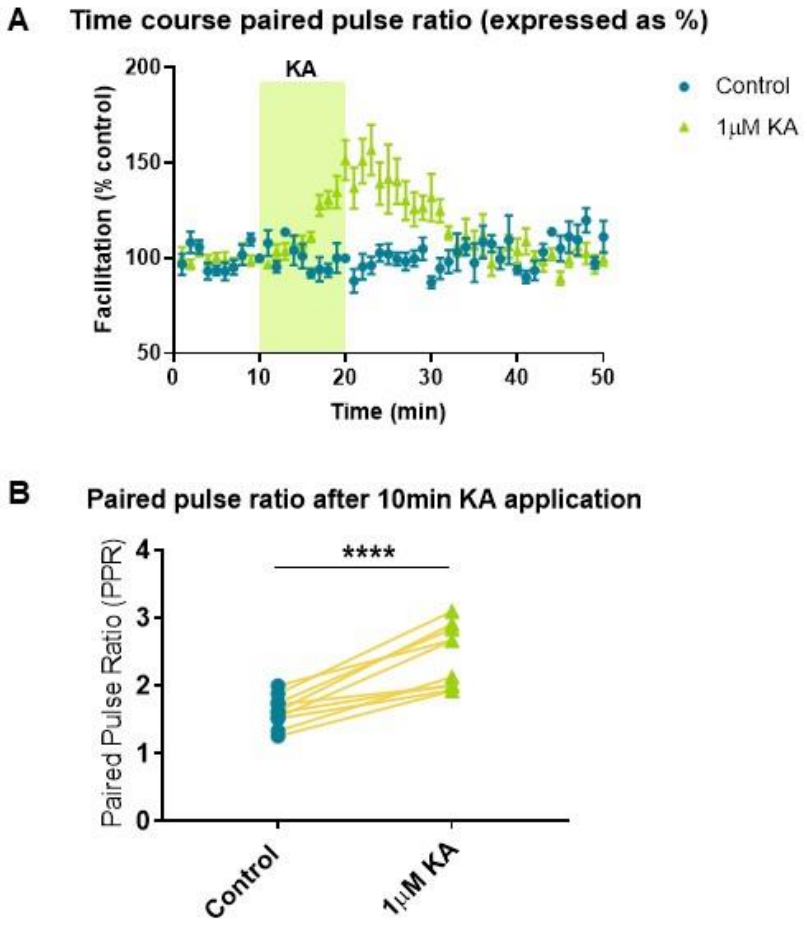


Figure 4-18: Sustained stimulation of KARs induces an early presynaptic form of plasticity

Graph showing the traces of PPR upon 40 mins post 10 mins of sustained stimulation with 1μM KA in CA1 region of acute hippocampal slices (A). N=5-9, cells from different animals. Error bars =SEM.

Quantification of PPR after 10 mins of stimulation with 1μM KA (B). N=10 cells, ns p>0.05, *p<0.05, **p<0.01, ***p<0.001; ****p<0.0001; paired t test.

4.3 Discussion

4.3.1 Sustained KARs stimulation in cortical cultures lacked consistency

I initially set out to define the mechanism of KAR-LTD$_{AMPAR}$ in cortical neurons by performing sustained KA stimulation followed by surface biotinylation and pull down with streptavidin. Although I was able to detect a significant reduction in the level of GluA1 AMPARs, my results lacked reproducibility **[Figure 4-5]**. Unaltered levels of GluK2 were another enigma in my study with cortical cultures. This was because 10µM KA, for 20 mins did not lead to internalization of surface KARs **[Figure 4-5 D]**. Due to lack of reproducibility even after changing variables I discontinued my further experiments with cortical cultures.

I reasoned the lack of unaltered levels of surface AMPARs and KARs might be due to the heterogeneity in the cortical neuronal cultures which contain multiple neuronal cell types. Thus, changes occurring in a relatively small proportion of cell types might go undetected due to the vast number of neuronal populations.

4.3.2 KAR stimulation in the absence of GYKI might be specifically targeting GluA1 AMPARs

My results with surface biotinylation without GYKI53655 following sustained KA stimulation showed significantly reduced GluA1 but not GluA2-containing AMPARs **[Figure4-6]**. This begs the question as to whether the KA-dependent loss of surface AMPARs is specific to GluA1 but not GluA2-containing AMPARs. A possible explanation is that without GYKI53655, KA is both activating KARs and AMPARs with the net effect to cause a decrease in GluA1, but not GluA2. However, using GYKI5655, to allow KA to act solely on KARs, results in decrease of both GluA1 and GluA2-contaning AMPARs **[Figure4-7]**.

4.3.3 The KAR-induced reduction in surface AMPARs is dependent on ionotropic KAR signaling and requires GluK2 and calcineurin

KARs employ both ionotropic and metabotropic signalling pathways to regulate excitatory and inhibitory neurotransmission (Evans et al., 2017a). Previous studies from the lab have shown that both transient pharmacological and synaptic activation of KARs induces an increase in surface expression of AMPARs and evoked a novel KAR-dependent, NMDAR-independent form of hippocampal LTP (KAR-LTP$_{AMPAR}$) (Petrovic et al., 2017a). This form of plasticity requires metabotropic signalling of KARs, GluK2 and activation of PLC and PKC (Petrovic et al., 2017a).

This raised the question as to whether KAR activation can downregulate the surface expression of AMPARs. In agreement with this hypothesis, I report that sustained activation of GluK2-containing KARs can downregulate AMPAR surface expression and evoke a KAR-dependent, NMDAR- and mGluR-independent form of LTD (KAR-LTD$_{AMPAR}$) **[Figure 4-7]** to **[Figure 4-11]**. Moreover, the KA-mediated reduction in surface AMPARs did not occur in GluK2 knock down neurons, indicating the requirement of GluK2-containing KARs in this form of LTD **[Figure 4-13]**. In contrast to KAR-LTP$_{AMPAR}$, inhibition of metabotropic signalling did not prevent KAR-LTD$_{AMPAR}$, whereas UBP310, an antagonist of postsynaptic ionotropic signalling through GluK2/GluK5-containing KARs did **[Figure 4-14 C]** (Pinheiro et al., 2013, Petrovic et al., 2017a, Grosenbaugh et al., 2018). However, both PTx and UBP310 failed to inhibit the KA-induced reduction in surface GluA1 levels **[Figure 4-14 B]**. The simplest explanation for this change might be due to the differences in the trafficking rate of GluA1 and GluA2 AMPARs or indicate that G-protein dependent signalling is a prerequisite for GluA1 trafficking.

The basal surface expression of GluA1 and GluA2 appear to be reduced in the presence of metabotropic and/or ionotropic inhibitors, although this was not significant for PTx, and it should be noted that PTx inhibits the signalling of multiple G-protein-coupled receptors in addition to the metabotropic actions of KARs. The significant decrease in basal GluA2 surface levels in the presence of UBP310 suggests KARs may also be required for maintaining tonic activity of AMPARs through a yet unidentified pathway and may indicate that UBP310 occludes LTD by resulting in a

loss of ‘removable’ AMPARs from the cell surface such that they can no longer be removed by KA **[Figure 4-14 C]**.

Phosphorylation and dephosphorylation of AMPAR subunits has been an area of intense studies for many years (Diering et al., 2018). Both GluA1 and GluA2 contain multiple phosphorylation sites that are targeted by myriad of protein kinases (Lee HK, 2003, José A. Esteban, 2003, Diering et al., 2014, Yong et al., 2020). The functional implications have been questioned by one study which reported almost no phosphorylated GluA1 *in vivo* (Hosokawa et al., 2015). However, a subsequent study has directly refuted that finding, showing that 12%-50% of the total population of GluA1 is phosphorylated under basal and stimulated conditions *in vitro* and *in vivo* (Diering et al., 2016). Indeed, it has been reported previously that activation of PKA with forskolin promotes surface trafficking of GluA1-containing AMPARs without affecting rates of endocytosis, thereby resulting in increased AMPAR surface expression (Man et al., 2007a, Diering et al., 2016). My experiments using the PKA inhibitor H89 resulted in a decrease in AMPARs surface expression consistent with reduced surface trafficking but unaffected endocytosis. Thus, I hypothesise that the lack of ‘readily removable’ AMPARs at the cell surface in the presence of H89 or chelerythrine occludes the KA-evoked reduction in surface AMPARs.

I have also tested the effect of phosphatase inhibitors okadaic acid (PP1 and PP2A) and FK506 (calcineurin/PP2B). These too prevented the KA-evoked decrease in surface GluA2-containing AMPARs **[Figure 4-15]**. Interestingly, okadaic acid reduced the basal levels of surface GluA2, indicating roles for the protein phosphatases PP1/PP2A in regulating basal surface expression of AMPARs **[Figure 4-15]**. Together, I interpreted these results to suggest that the dynamics of phosphorylation and dephosphorylation are clearly important both for maintaining the delivery of surface AMPARs, and their removal. Further, I investigated the role of the KAR/AMPAR antagonist CNQX in the KA-induced reduction in surface AMPARs. In conjunction with other inhibitors, CNQX reduced basal levels of AMPARs, and the KA-induced reduction in surface AMPARs was blocked by CNQX **[Figure 4-12]**.

Interestingly, KA treatment significantly increased surface levels of GluA1 AMPARs in the presence of CNQX **[Figure 4-12B]**. Studies using heterologous system have shown that GluK2/GluK5-containing KARs are activated by CNQX in the presence of

a KAR specific agonist (Swanson et al., 2002). Binding of CNQX in the GluK5 subunit of KARs in the presence of KA induces an inward current through the channel rather than an expected blockade. I hypothesise that this inward current thorough the activated KAR channel triggers an increase in the trafficking of GluA1 AMPARs, which are trafficked at a higher rate compared to GluA2 AMPARs **[Figure 4-12B]** (Henley and Wilkinson, 2013). However, this needs to be validated further.

4.3.4 The KAR-induced reduction in surface AMPARs leads to KAR-LTD$_{AMPAR}$

To understand how the KAR-mediated reduction in surface AMPARs impacted synaptic functioning, electrophysiological analysis was performed. In agreement with the biochemical and imaging data, electrophysiology recordings from hippocampal CA1 pyramidal neurons revealed a significant depression in the EPSC$_{AMPA}$ after KA treatment **[Figure 4-17]**. This initial depression in EPSC$_{AMPA}$ were in parallel with a rise in the PPR ratio, which returned to baseline within 10 mins of KA washout, suggesting a presynaptic KAR activation or the involvement of postsynaptic KAR activated retrograde signalling pathway **[Figure 4-18]**. However, importantly, the PPR returned to control levels following an initial short-term change whilst the EPSC$_{AMPA}$ remained depressed, indicating the involvement of postsynaptic KARs in mediating KAR-LTD$_{AMPAR}$. Together this suggest that KA application induces LTD of AMPARs.

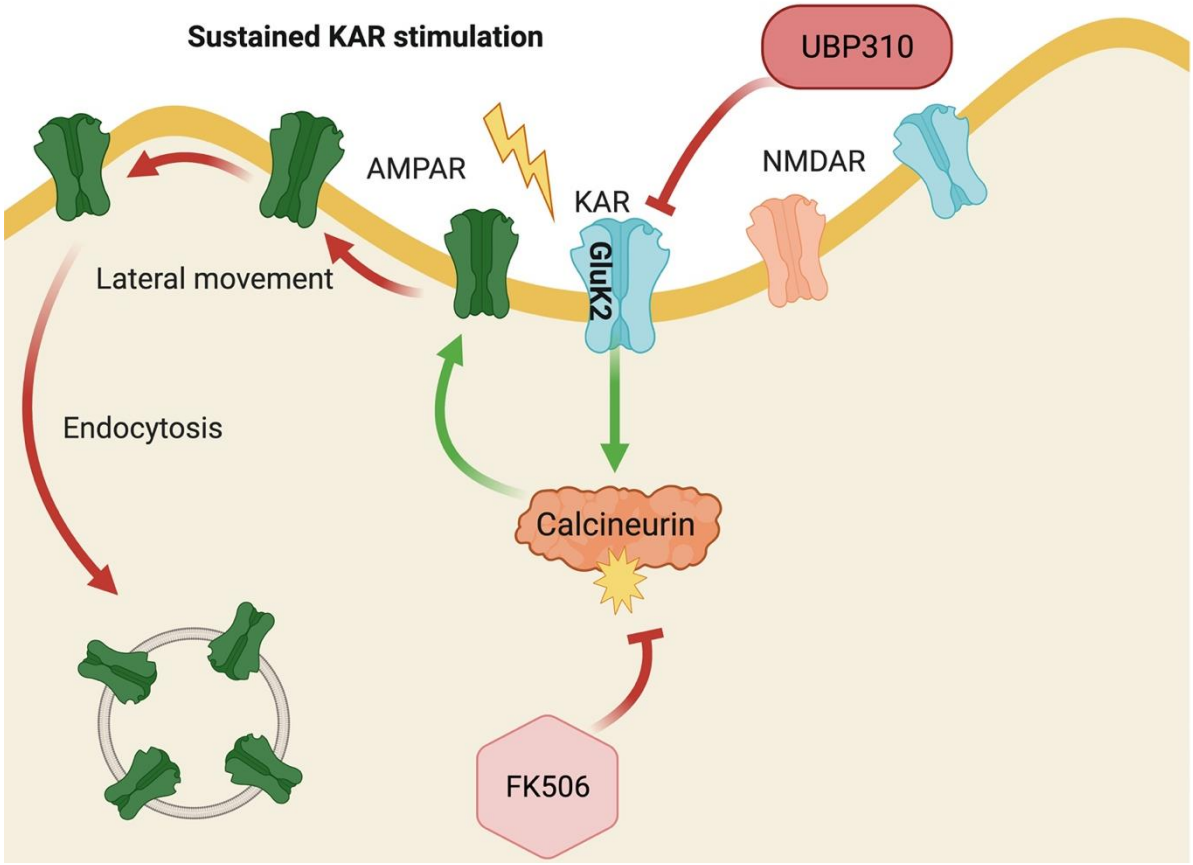


Figure 4-19: Proposed mechanism of KAR-induced reduction of surface AMPARs

Dynamic regulation of exocytosis and endocytosis regulate basal levels of AMPARs at the surface. Sustained stimulation of KARs decreases surface expression of AMPARs. This pathway is dependent on GluK2-containing ionotropic KARs and requires activation calcineurin.

4.4 Conclusion

In this study I report a novel form of LTD of AMPARs in hippocampal neurons that is dependent on ionotropic KAR activation and signalling but is independent of NMDAR or mGluR activation. Depending on activation parameters, activation of postsynaptic KARs bidirectionally regulates AMPAR surface expression and function and evokes bidirectional plasticity of AMPARs **[Figure 4-20]**. In contrast to the KAR-evoked increase in AMPAR surface expression observed in KAR-LTP$_{AMPAR}$, which is mediated via metabotropic GluK2-containing KAR signalling and PKC (but not PKA) (Petrovic et al., 2017a), the KAR-mediated reduction in surface AMPARs requires ionotropic KAR signalling through GluK2-containing KARs and the phosphatase calcineurin. **[Figure 4-19]**

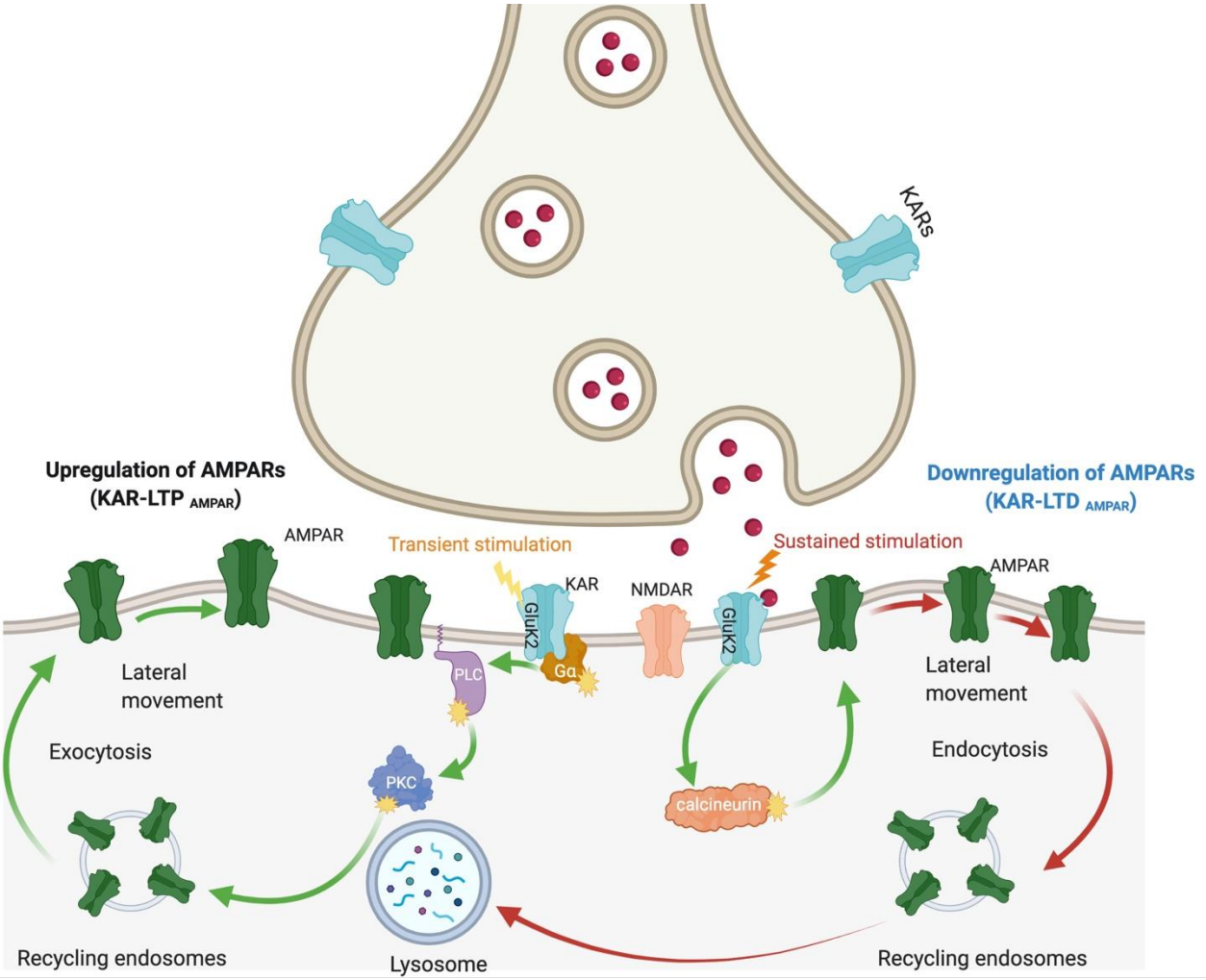


Figure 4-20: KAR activation mediates bidirectional plasticity of AMPARs

Depending upon activation parameters, KARs can bidirectionally regulate the surface expression of AMPARs, thereby inducing novel forms of KAR-dependent plasticity of AMPARs. Transient stimulation of KARs (3 mins) upregulates surface AMPARs and induces KAR-LTP$_{AMPAR}$ via a GluK2-dependent metabotropic signalling mechanism. This pathway is independent of NMDAR activation but requires PKC activation. Sustained stimulation of KARs (20 mins) downregulates surface AMPARs to induce KAR-LTD$_{AMPAR}$ via GluK2-containing ionotropic KARs. This pathway is independent of NMDAR or mGluR activation and requires the phosphatase calcineurin.

Chapter 5. Characterization of GluK2 Q/R editing deficient mice

5.1 Introduction

5.1.1 GluK2 Q/R editing deficient mice

Pre-mRNAs of GluK2 KARs undergo ADAR2 mediated Q/R at the channel pore of the subunit, changing the genetically encoded glutamine(Q) residue to arginine(R) (Seeburg et al., 1998). This conversion is achieved by the hydrolytic deamination adenosine (A) residue in the pre-mRNA to Inosine (I) which is read as guanosine (G) by the translational machinery (Seeburg et al., 1998). An intronic editing complementary site (ECS) is necessary to direct the enzyme for editing prior to splicing (Seeburg et al., 1998). The mice were generated by deleting 600bp in the intronic region corresponding to ECS [**Figure 5-1**] (Vissel et al., 2001a).

The editing of subunits alters the trafficking and functional property of the receptor. GluK2 (R) subunits are less trafficked to the surface compared to un-edited GluK2 (Q) (Ball et al., 2010a, Evans et al., 2017c). This is because of increased ER retention and failure of the edited subunits to oligomerize with other subunits that aid in forward trafficking (Ball et al., 2010a, Pahl et al., 2014). Furthermore, edited subunits are less permeable to Ca^{2+} which may help the neurons to protect themselves from glutamate induced excitotoxic insults (Seeburg et al., 1998, Ball et al., 2010a). This emphasizes the role of GluK2 Q/R editing in maintaining network activity and in neuroprotection from excitotoxic insults (Gurung et al., 2018, Henley et al., 2021).

To study the potential role of GluK2 Q/R editing in various synaptic functions, a knockout mouse was generated by the deletion of ECS from the *GRIK2* gene. A 95% reduction in the Q/R editing of GluK2 pre-mRNA was evident in the knockout mice (Vissel et al., 2001a). However, the remaining 5% editing of GluK2 suggest a mechanism independent of ECS or via ADAR1 enzyme (Vissel et al., 2001a). Further, the mice showed no difference in the expression levels of GluK2 mRNA and editing of I/V and Y/C sites ((Vissel et al., 2001a). The mice displayed altered I-V relationship and enhanced Ca^{2+} permeability further emphasizing Q/R editing in determining channel kinetics. The mutant mice have increased susceptibility to Kainate-induced seizures and displayed normal NMDAR-independent LTP at the medial Perforant-DG synapses suggesting the presence of functional KARs (Vissel et al., 2001a).

Thus, these mice represent a valuable tool to unravel the role of GluK2 Q/R editing in various neuronal functions such as unsilencing of synapses, neuronal development, network formation, modulation of network activity etc. Furthermore, the comparative study between editing deficient and WT mice allows fundamental understanding on the role of GluK2 editing in determining the channel properties, synaptic functioning, trafficking and synaptic expression, network formation and their role in epilepsy.

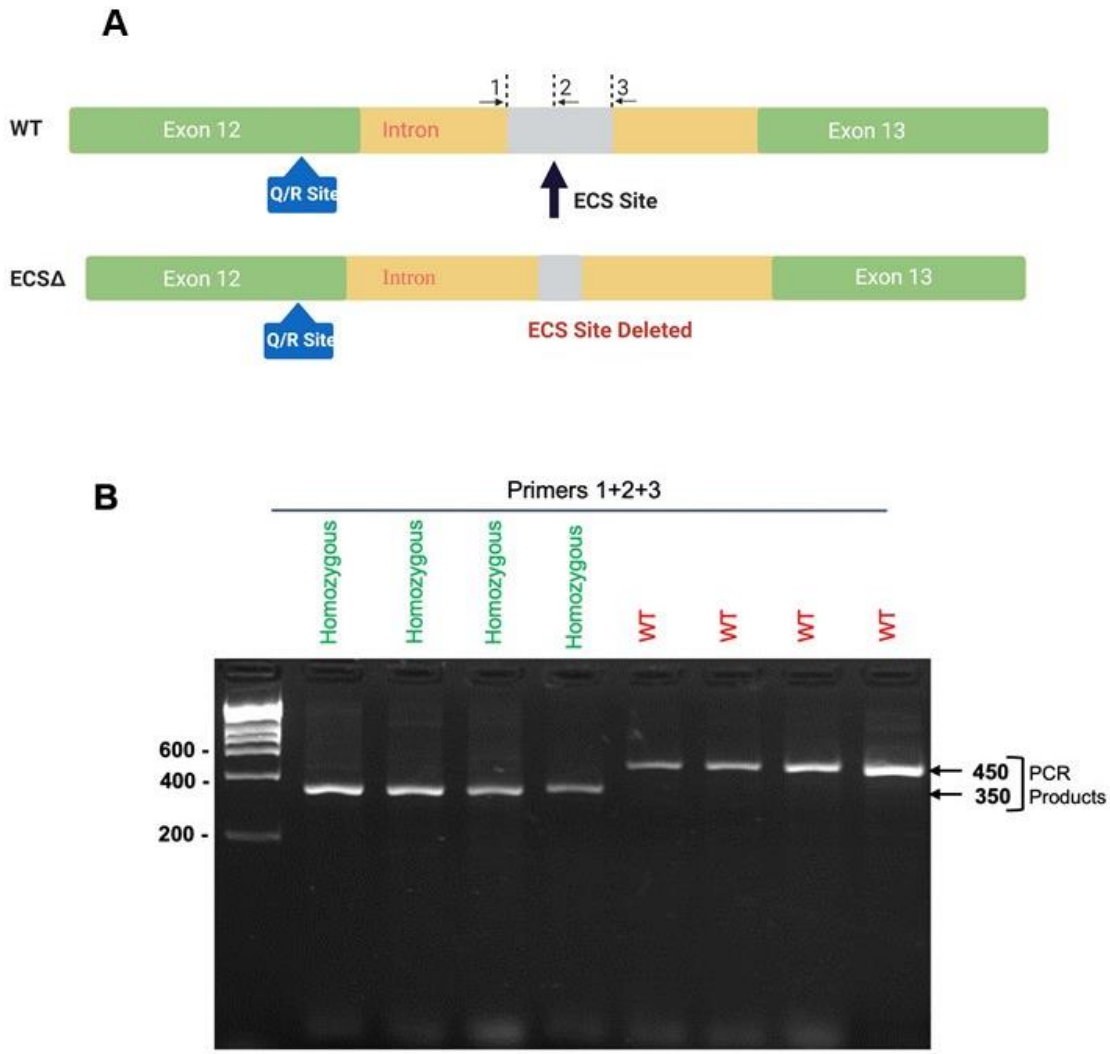


Figure 5-1:Genotyping of GluK2 editing-deficient mice

The Intronic region between exon12 and 13 consists of editing complementary site (ECS), located ~1900nt downstream of exon 12. (A). In the WT mice, this region is intact. However, in homozygous ECSΔ mice, 600bp is deleted in the ECS site. This result in the inability of ECSΔ mice to undergo ADAR2 mediated editing of GluK2 pre-mRNA at Q/R site, leading to the translation of all un-edited GluK2(Q) subunits. Genotyping was performed on mice using primers targeted to the deleted ECS sites to confirm their genetic makeup.

B.). An example of PCR products obtained following genotyping of Homozygous and WT mice. Primers 1,2 and 3 combined to give a 450bp product that corresponds to WT mice and a 350bp (primers 1 and 3) corresponding to Homozygous mice.

5.1.1 Homeostatic plasticity of KARs

In addition to activity-dependent plasticity, KARs can also undergo homeostatic plasticity whereby suppression of network activity by TTX leads to increased surface expression of KARs (Evans et al., 2017c). This mechanism linking neuronal activity to KAR surface expression occurs via changes to the pre-mRNA editing of GluK2 subunits (Gurung et al., 2018). GluK2(Q) subunits form heteromers and are trafficked to the surface more efficiently than GluK2(R) subunits **[Figure 5-2].** This is due to increased retention of receptors containing GluK2(R) within the endoplasmic reticulum, along with reduced ability to oligomerize with other subunits to facilitate forward trafficking (Ball et al., 2010a, Evans et al., 2017c).

Chronic suppression of network activity induces proteasomal degradation of the ADAR2 enzyme, and the consequent reduction in GluK2 Q/R editing leads to an increase in KAR surface expression (Gurung, 2018)(Evans et al., 2017c). These findings suggest that differential mechanisms regulate KAR and AMPAR upscaling (Gurung et al., 2018). Therefore, altering the editing status and scaling of KARs by chronic suppression of synaptic transmission could stabilize network activity and neuronal function. Moreover, the edited GluK2 subunit also confers lower Ca^{2+} permeability to KARs, which may further reduce susceptibility to glutamate induced excitotoxic insults (Seeburg et al., 1998, Ball et al., 2010a).

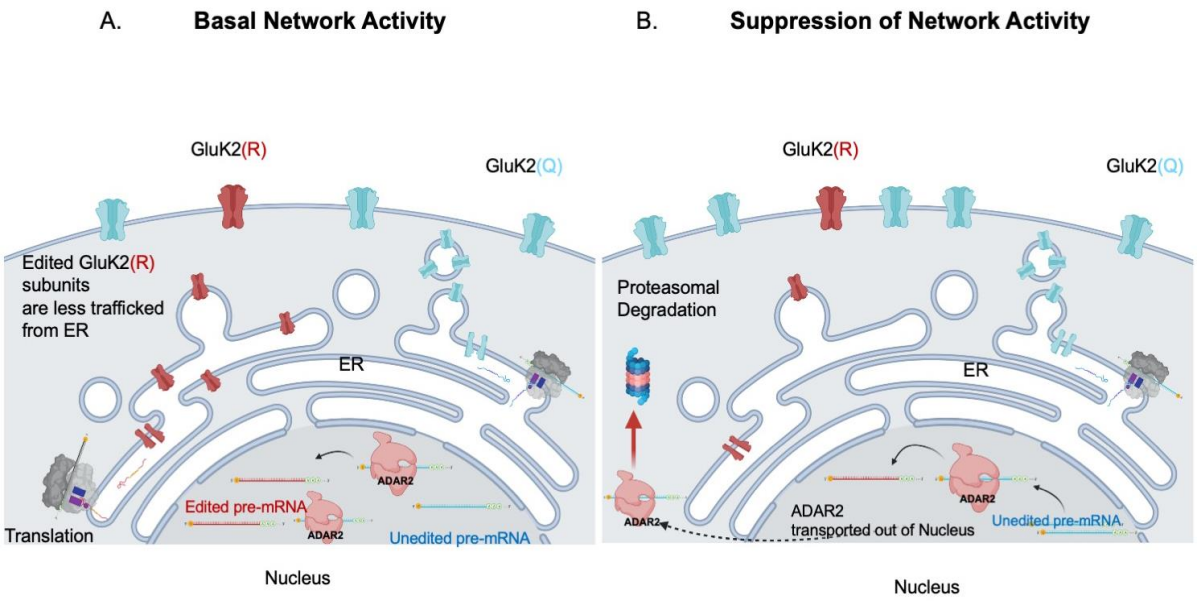


Figure 5-2: Homeostatic plasticity of KARs via ADAR2 mediated editing of GluK2

A) GluK2 undergoes ADAR2 mediated Q/R editing which converts a genetically encoded glutamine (Q) to arginine (R). Translocation of edited GluK2(R) containing KARs to the surface is reduced due to retention within the endoplasmic reticulum. B) Suppression of network activity upregulates KAR surface expression by proteasomal degradation of ADAR2. Degradation of ADAR2 leads to a reduction in GluK2 RNA editing and enhanced abundance of GluK2(Q), which is more effectively transported to the surface membrane. Adapted from (Nair et al., 2021).

5.1.2 Short-term plasticity

Along with long term plasticity, many short forms of plasticity occur in mammalian and invertebrate brains. The short forms of plasticity aid formation of temporary memory, such as adaptations to brief sensory inputs, transient behavioral changes etc.(Zucker and Regehr, 2002) presynaptic short-term plasticity is mediated via the accumulation of Ca^{2+} in the presynaptic terminals by short bursts of activity (Cheng et al., 2018). This increase in presynaptic Ca^{2+} alters the probability of neurotransmitter release by synaptic vesicle fusion and exocytosis (Citri and Malenka, 2008). KARs are known to bidirectionally regulate glutamate release at MF-CA3 synapses resulting in short-term plasticity (Nicoll and Schmitz, 2005, Schmitz et al., 2001, Breustedt and Schmitz, 2004, Contractor et al., 2000, Rodriguez-Moreno and Sihra, 2004).

5.1.3 Paired pulse facilitation and frequency facilitation

Paired Pulse Facilitation (PPF) is a form of short-term plasticity (STP) characterized by an increase in the neurotransmitter release on the second stimuli when two closely paired stimulations are applied in succession with an inter-stimulus interval of 10ms to 1s. The increase of transmitter release depends on the presynaptic receptors including KARs, Ca^{2+} concentration in the terminals and the availability of docked synaptic vesicles (Salin et al., 1996, Nicoll and Schmitz, 2005). Unlike other hippocampal synapses, MF-CA3 show a higher PPF ratio (~2 fold) (Salin et al., 1996). Exogenous application of KA at low concentrations induce PPF at MF-CA3 synapses by enhancing the presynaptic glutamate release (Schmitz et al., 2001).Further, the PPF can also be induced in these synapses by stimulating mossy fibres and is dependent on GluK2 subunits of KARs (Mulle et al., 1998). GluK2$^{-/-}$ show a significant reduction in PPF (Mulle et al., 1998, Schmitz et al., 2001, Breustedt and Schmitz, 2004). Further GluK3 subunits are also implicated in this form of short term plasticity (Contractor et al., 2001, Pinheiro et al., 2007).

Another form of short term plasticity predominant in MF-CA3 pathway is frequency facilitation (FF) which is characterized by an increase in synaptic strength following a change in the frequency of stimulation from low (0.05Hz) to moderate (1Hz) (Creager et al., 1980, Klausnitzer and Manahan-Vaughan, 2008a). A number of receptors including mGluRs, adenosine receptors and KARs have been implicated in this form of plasticity (Nicoll and Schmitz, 2005, Klausnitzer and Manahan-Vaughan, 2008a).

Mice deficient in KAR subunits GluK2$^{-/-}$ and GluK3$^{-/-}$ show impaired FF in MF-CA3 synapses (Pinheiro et al., 2007, Contractor et al., 2001). This form of STP is also reduced in the presence of AMPAR/KAR antagonist CNQX (Contractor et al., 2001). Studies using pharmacological blockers of GluK1 revealed the subunits are key in regulating presynaptic glutamate release resulting in FF (Lauri et al., 2001). Further, mice deficient in either of the high affinity subunits GluK4$^{-/-}$ or GluK5$^{-/-}$ did not show impairments in the STP. However, mice lacking both the subunits exhibited impaired PPF but not FF indicating that PPF and FF might employ diverse mechanisms (Contractor et al., 2003a, Fernandes et al., 2009).

5.1.4 KAR dependent inhibition of I_{sAHP}

In neurons, short bursts of action potentials are followed by a slow long-lasting voltage independent and Ca^{2+}-dependent K^+ current known as the slow after hyperpolarization (I_{sAHP}) **[Figure 5-3]** (Lancaster and Adams, 1986). Activation of I_{sAHP} is proportional to the number and frequency of action potentials. I_{sAHP} lasting for several seconds are therefore key determinants of neuronal excitability and firing patterns (Vogalis et al., 2003). One of the mechanisms underpin the regulation of I_{sAHP} via glutamate receptors is by the metabotropic signaling of KARs (Madison and Nicoll, 1984, Ruiz et al., 2005, Fisahn et al., 2005).

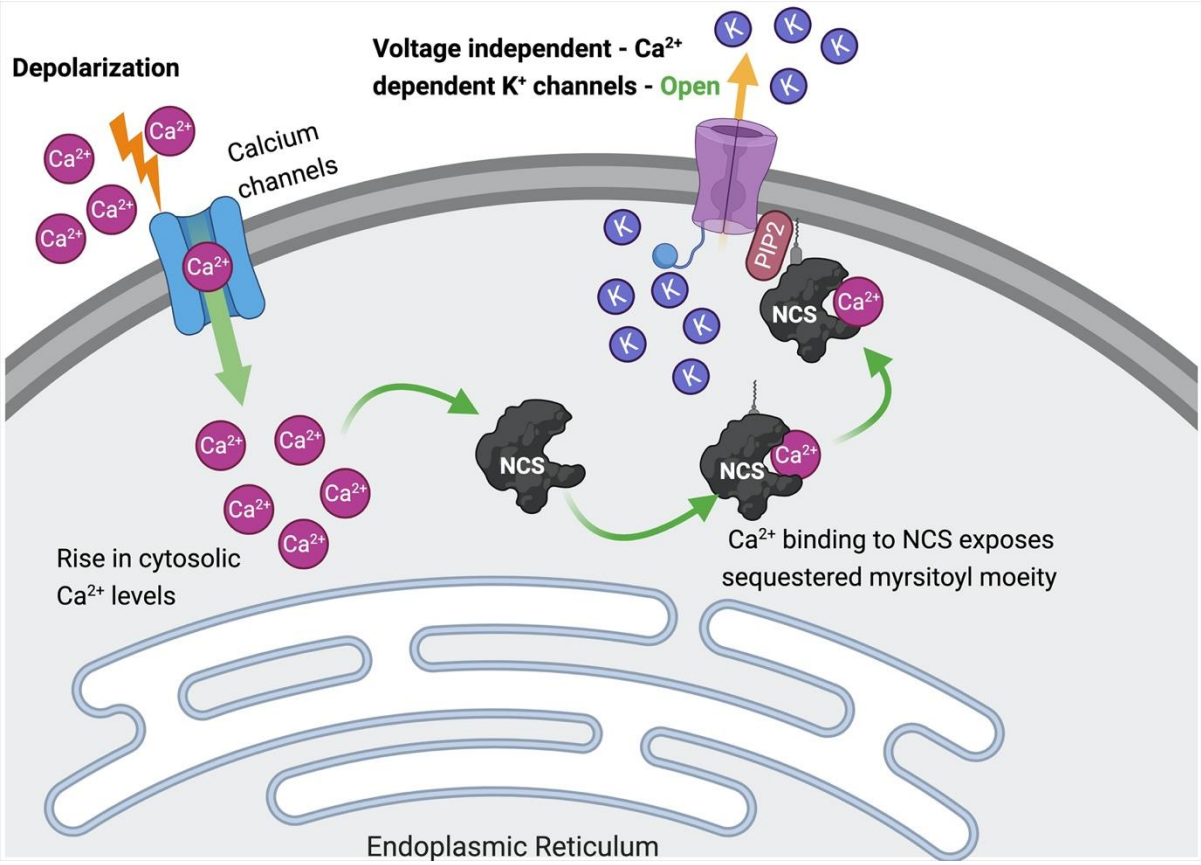


Figure 5-3: Proposed mechanism of I_{sAHP} activation

Short bursts of action potential depolarise the membrane and opening of Ca^{2+} channels. The rise in Ca^{2+} levels activate neuronal calcium sensors (NCS) hippocalcin and neurocalcin. This exposes the sequestered myristol moiety of NCS thereby facilitating their binding to voltage-dependent, Ca^{2+}-dependent K^+ channels inducing I_{sAHP}.

One of the notable features of metabotropic KARs is their ability to regulate neuronal excitability by I_{sAHP} through the activation of $G_{i/o}$ and PKC (Lerma, 2003, Melyan et al., 2002, Fisahn et al., 2005, Melyan et al., 2004, Ruiz et al., 2005). Exogenous

application of KA in CA1 neurons inhibit I_{sAHP} thereby enhancing the neuronal excitability (Melyan et al., 2002). Further, synaptic activation of KARs in MF-CA3 synapses can inhibit both medium AHP (mI_{AHP}) and I_{sAHP}. This feature is absent in GluK2$^{-/-}$ mice but preserved in GluK5$^{-/-}$ mice emphasizing the requirement of GluK2 subunits in inhibition of I_{sAHP} **[Figure 5-4]** (Ruiz et al., 2005, Fisahn et al., 2005). However, the exact subunit dependency for KAR-mediated inhibition of I_{sAHP} remain controversial, see (Ruiz et al., 2005, Fernandes et al., 2009).

Adding on to the complexity, in DRG functional KARs are formed by GluK1 and GluK5 subunits (Mulle et al., 2000). Inhibition of mAHP by KAR in DRG neurons is dependent on GluK1 subunits but not GluK5 subunit of KARs (Rutkowska-Wlodarczyk et al., 2015). Moreover, in CA3 stratum lucidum interneuron synapses of neonatal animals, activation of KARs by ambient glutamate leads to tonic inhibition of mAHP (Segerstråle et al., 2010). This G-protein dependent pathway enhances the firing rate of interneurons and are crucial in shaping network activity and formation of functional hippocampal circuitry (Segerstråle et al., 2010).

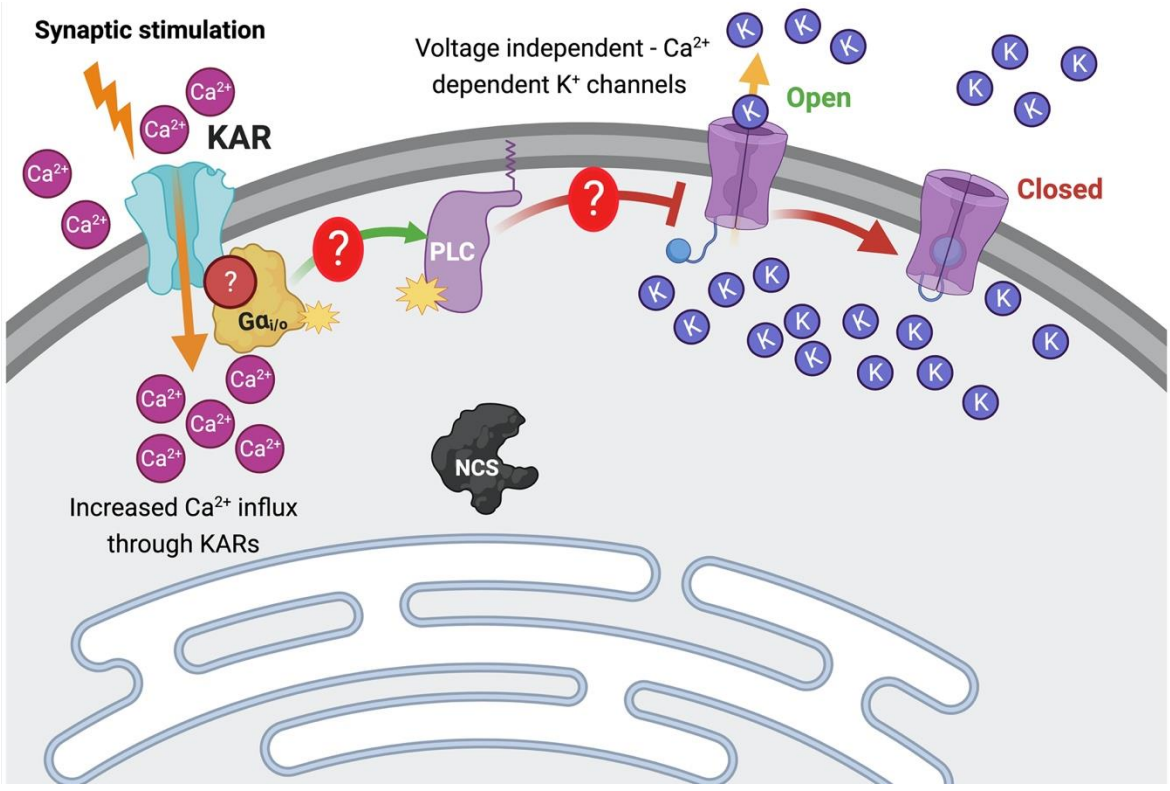


Figure 5-4: Proposed mechanism of metabotropic KAR-dependent inhibition of I_{sAHP}

Postsynaptic metabotropic KARs ($G\alpha_{i/o}$) dependent activation of PKC via currently unknown mechanism inhibit I_{sAHP}.

5.2 Objectives

Network activity can influence the function and trafficking properties of iGluRs by modulating protein-protein interactions and post-transcriptional and post-translational modifications. For example, mRNA editing of subunits by ADAR proteins tune AMPARs and KARs depending on neuronal activity (Wilkinson et al., 2012, Pickering et al., 1995, Jiang et al., 2006, Yokoi et al., 2012).

Accumulating evidence indicates that GluK2 Q/R editing of KARs by ADAR2 plays a key role in modulating the trafficking and surface expression of functional KARs (Gurung et al., 2018, Herb et al., 1996, Henley et al., 2021). For example, previous work from the lab has strongly implicated GluK2 Q/R editing in homeostatic plasticity (Gurung et al., 2018). Chronic suppression of network activity decreases ADAR2-mediated editing of GluK2 subunits leading to enhanced surface expression. On the other hand, chronic enhancement of network activity promotes GluK2 Q/R editing and reduces surface expression of the receptor(Gurung et al., 2018). This ADAR2-dependent modulation of GluK2 Q/R editing may act as a homeostatic mechanism to nullify imbalances in network activity (Gurung et al., 2018, Nair et al., 2021).

Although these findings represent a significant advance, a comprehensive understanding of the physiological implications of GluK2 Q/R editing remain to be determined. Since ~80% of GluK2 is Q/R edited in wildtype rats and mice, one approach to study this question was the use of transgenic mice in which ~95% of GluK2 subunits expressed in the mice are unedited (Vissel et al., 2001a). I therefore explored how downstream functions attributed to KARs are altered in these GluK2-editing deficient mice compared to wildtype mice.

The main objectives for this chapter are to determine differences between GluK2-editing deficient mice and wildtype mice in:

1. presynaptic mossy fibre function
2. postsynaptic ionotropic KAR properties
3. metabotropic KAR properties

5.3 Materials and methods

5.3.1 Artificial cerebrospinal fluid (aCSF)

The below listed reagents were used to prepare aCSF. A 10X stock was prepared and kept at 4°C until further use.

Table 5-1: List of components in aCSF

Reagent	Concentration (mM)
NaCl	124
KCl	3
$NaHCO_3$	24
NaH_2PO_4	1.25
D-Glucose	10

aCSF for the experiment was prepared by diluting 10X stock and adding 4mM $CalC_2$ and 4mM $MgCl_2$. The solution was constantly bubbled with 95% O_2 and 5% CO_2. Slices were perfused at 35ºC.

5.3.2 Sucrose slicing solution

The below listed reagents were used to prepare sucrose solution for slicing hippocampus.

Table 5-2: List of components in sucrose cutting solution

Reagent	Concentration (mM)
Sucrose	205
KCl	2.5
$NaHCO_3$	26
NaH_2PO_4	1.25
D-Glucose	10
$CaCl_2$	0.5
$MgCl_2$	5

1X solution was prepared from 10X stock solution of sucrose slicing solution and was kept in freezer to obtain ice-cold cutting solution prior to slicing. The solution was constantly bubbled with 95% O_2 and 5% CO_2.

5.3.3 Internal solutions

The following reagents were used to prepare cesium (Cs) based internal solution.

Table 5-3: List of components in Cs based whole-cell solution

Reagents	Concentration (mM)
NaCl	8
CsMeSo4	130
HEPES	10
EGTA	0.5
MgATP	4
NaGTP	0.3
QX314.Cl	5
Spermine	0.1

The following reagents were used to prepare potassium based internal solution.

Table 5-4: List of components in K^+ based whole-cell solution

Reagents	Concentration (mM)
NaCl	8
KGluconate	135
HEPES	10
EGTA	0.2
MgATP	2
NaGTP	0.3

pH and osmolarity of the internal solutions were adjusted to 7.4 and 290 mOsm respectively. The solution was aliquoted into 1.5 ml Eppendorf and stored at -20°C until further use.

5.3.4 Acute hippocampal slice preparation

Cervical dislocation followed by decapitation were performed on P14-21 male and female WT and GluK2 editing-deficient mice pups. The brain was removed and placed in ice-cold sucrose slicing solution which was constantly bubbled with 95% O_2 and 5% CO_2. Hippocampi were carefully removed and transverse sections of 400µm thickness were obtained using a vibratome (Leica VT 1200s). Slices were kept for recovery in slice holder containing aCSF constantly bubbled with 95% O_2 and 5% CO_2 at 37°C for 20 mins and later transferred to room temperature and left it for at least 30 mins before performing experiments.

5.3.5 Electrophysiology recordings

Hippocampal slices were placed in a submerged holding chamber continuously perfused with oxygenated aCSF at 36.5^{o}C at a flow rate of 3ml per minute. Hippocampal CA3 pyramidal cells were visually identified using DIC optics and patch-clamped in whole cell configuration using a pulled Harvard borosilicate glass capillary of resistance 5-7M MΩ filled with WCS. The cells were held in voltage clamp mode at -70mV and evoked EPSCs were obtained by stimulating MF pathway with a bipolar stimulating electrode placed in the dentate gyrus hilus layer. 50µm picrotoxin was included in the aCSF to inhibit $GABA_A$ receptors. Cells with series resistance above 30 MΩ or where series resistance changed by >20% were discarded. DCG-IV (1 µM) was bath applied for 5 mins at the end of all experiments to confirm responses arose from mossy fibre stimulation. Recordings were only included in analysis if DCG-IV reduced EPSCs by >70%.

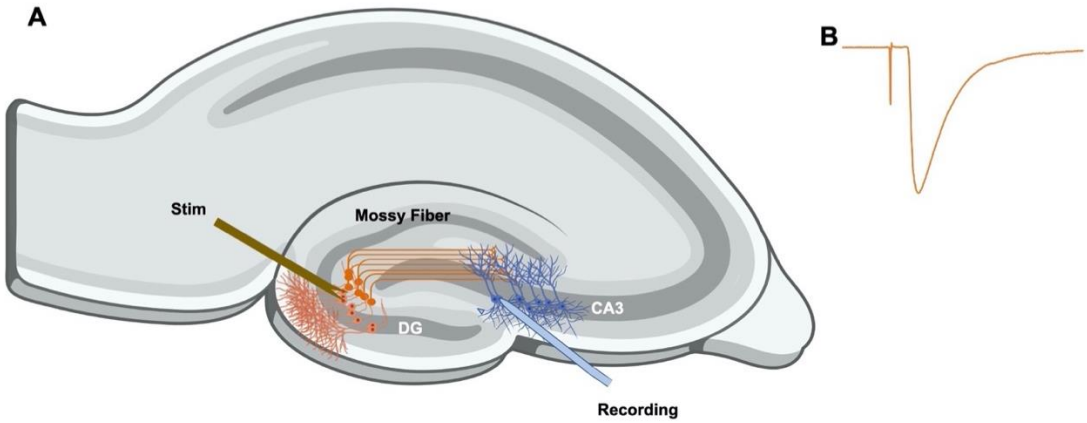


Figure 5-5: Cartoon showing EPSC recording from hippocampal MF-CA3 synapse

A- Schematics representing patch clamp recording on hippocampal CA3 neurons and Mossy Fibre (MF) pathway stimulation.

B- A representative EPSC.

5.3.5.1 Paired pulse and frequency facilitation

Pairs of mossy fibre stimuli (interval 50 ms) were given every 20 s. After recording a 10 mins stable baseline the stimulation interval was reduced to 1 s for 1 min and then switched back to 20 s.

5.3.5.2 Post-synaptic KAR/AMPAR currents

CA3 pyramidal neurons were voltage clamped at -60mV in the presence of 50µM Picrotoxin ($GABA_AR$ antagonist), 50µM D-APV (NMDAR antagonist). Mossy fibres were stimulated with 3 pulses at 167Hz to evoke AMPAR-KAR EPSCs. After achieving a stable baseline, KAR responses were recorded for 20 mins in the presence of GYKI53655(AMPAR antagonist). Amplitude of AMPAR- KAR peaks and KAR peaks were measured individually and ratio of KAR currents to AMPAR-KAR were obtained.

5.3.5.3 Slow afterhyperpolarizations (I_{sAHP})

For I_{sAHP} recordings, CA3 pyramidal neurons were voltage clamped at -50mV in the presence of 50µM picrotoxin ($GABA_AR$ antagonist), 50µM D-APV (NMDAR antagonist), 1µM CGP55845 ($GABA_BR$ antagonist) and GYKI53655 (AMPAR antagonist). In some experiments, UBP310(KAR antagonist) was also included. I_{sAHP}

were induced every 20 s by applying depolarising voltage step to 0mV for 200 ms. Synaptic stimulation of 10 stimuli at 25Hz were induced in mossy fibres to activate KARs before the induction of I_{sAHP}. I_{sAHP} amplitude was measured 300 ms after returning the membrane potential to -50mV to avoid measurement of mAHP.

5.3.6 Data Acquisition

Data were digitized at 10kHz, and low-pass filtered at 2kHz using CED Micro 1401-4 A-D acquisition unit and Axon patch 200B amplifier (Molecular devices). All recordings were obtained using CED Signal 5 software.

5.3.7 Data Analysis

CED Signal acquisition software was used to analyze the recorded data. Mean responses were obtained every minute by averaging consecutive traces. EPSC amplitudes were measured from the averaged traces and normalized to the mean EPSC amplitude of baseline.

KAR/AMPAR EPSC amplitudes were measured by averaging over a period of 5 mins before and after GYKI53655 application. 10-90% rise time for AMPAR and KAR currents after the third stimulus were measured as an interval between 10% and 90% of the EPSC amplitude relative to EPSC baseline (Veruki et al., 2003).

$\boldsymbol{\tau}_{decay}$ for AMPAR and KAR EPSCs after third stimulus were calculated by single exponential curve fitting feature in CED signal software. The equation used is $f(x) = a_0 e^{-x/a_1} + a_2$. Wherein $f(x)$ is regarded as the current as a function of time, a_0 is the amplitude at time 0 and a1 is the time constant ($\boldsymbol{\tau}$) and a_2 is the steady state current.

Paired pulse ratios were obtained by averaging amplitudes of P1 peak to P2 peak. For Frequency facilitation analysis, the ratios were obtained by averaging last 20 frames of P1 amplitude at 0.05Hz with middle 40 frames at 1Hz stimulation.

Percentage of DCGIV block was obtained by the formula

%DCGIV block = (Average baseline P1 peak- Average P1 peak with DCGIV)/Average P1 peak *100)

Data are plotted as mean ± SEM. 'N' - Number of animals used, 'n' - number of cells. ANOVA paired or un-paired student t test were used for statistical analysis and is mentioned in the figure legends with respective p values.

All the graphs and statistical tests were performed using GraphPad Prism version 9.2.

5.4 Results

5.4.1 GluK2 editing-deficient mice shows higher KAR/AMPAR ratio in MF-CA3 synapses

We, and others, have shown previously that disrupting ADAR2-mediated editing of the GluK2 subunit enhances the surface expression, single channel conductance and Ca^{2+} permeability of postsynaptic KARs in cultured neurons (Gurung et al., 2018, Paschen et al., 1997, Swanson et al., 1996). Therefore, I set off by investigating if the GluK2 editing-deficient mice exhibit similar increase in postsynaptic KAR currents.

To assess KAR function and compare between genotypes, the ratio of AMPAR to KAR EPSCs was measured. Mixed AMPAR and KAR EPSCs were first measured by stimulating MF with a frequency of 167Hz every 20 s in the presence of 50µM picrotoxin and 50µM D-APV to block $GABA_A$Rs and NMDARs respectively. Subsequently, KAR EPSCs were isolated by bath application of 40µM GYKI53655 for 10 mins.

A significant increase in postsynaptic KAR EPSCs compared to AMPAR EPSCs were detected in GluK2 editing-deficient mice when compared to age matched WT counterparts (WT= 0.047 ± 0.0051, Tg= 0.086 ± 0.008; p value = 0.0008; mean ± SEM) **[Figure 5-6]**.

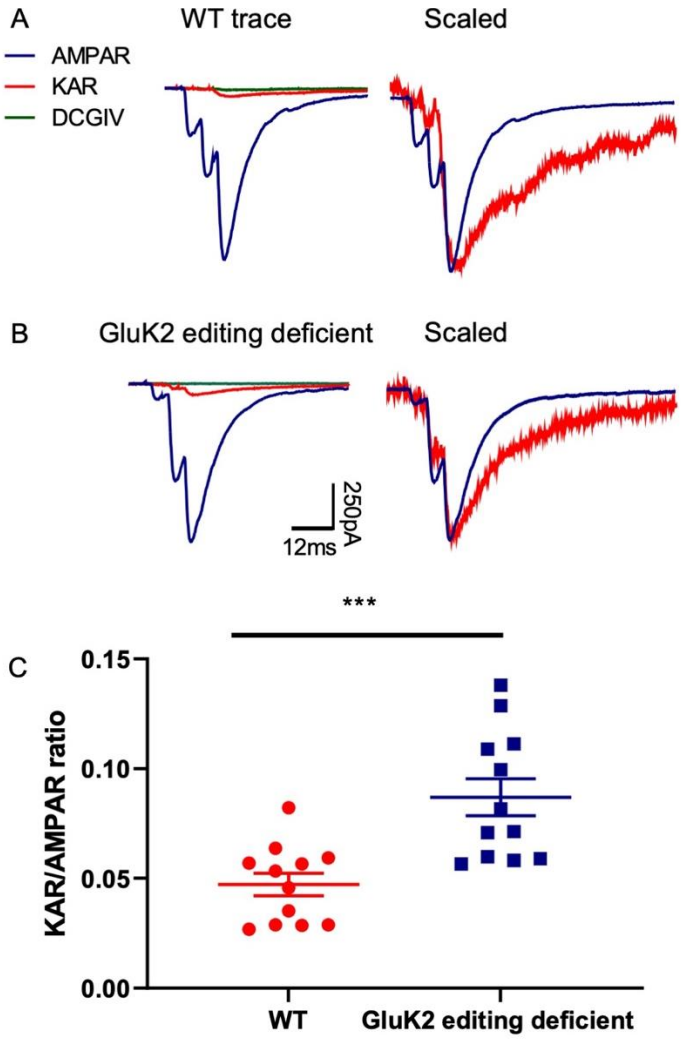


Figure 5-6: GluK2-editing deficient mice show an increase in KAR/AMPAR ratio

Sample traces showing postsynaptic AMPAR and KAR currents in WT(A) and GluK2 editing-deficient mice (B). Quantification of KAR/AMPAR current ratio in WT vs GluK2 editing-deficient mice (C), N=5, n=12 cells, ns p>0.05, *p<0.05, **p<0.01; un-paired t test with Welch's correction. error bar=SEM.

5.4.2 GluK2 editing-deficient mice show faster rise times and decay kinetics

To determine if GluK2 Q/R editing modulates the channel properties, I then investigated the 10-90% rise time kinetics and decay kinetics (τ_{decay}) of KAR EPSCs in both WT and GluK2 editing-deficient mice.

The transgenic mice showed a faster KAR EPSC decay kinetics (τ_{decay}) (22.4 ± 1.2 ms) compared to WT mice (40.3 ± 2.12 ms, p= 0017) [**Figure 5-7B]**. The KAR EPSCs

also had a faster 10-90% rise time kinetics in transgenic mice (1.4±0.10 ms) compared to WT counterparts (2.3 ± 0.20 ms; p= 0.0001) **[Figure 5-7C]**. No significant change was detected in the decay (WT= 10 ± 0.96 ms; Tg= 11.02 ± 0.46; p= 0.43 ms) **[Figure 5-7D]** or the10-90% rise time (WT= 1.33 ± 0.06 ms; Tg= 1.5.088 ± 0.46 ms; p= 0.006) for AMPAR currents between WT and GluK2 editing-deficient mice **[Figure 5-7E]**.

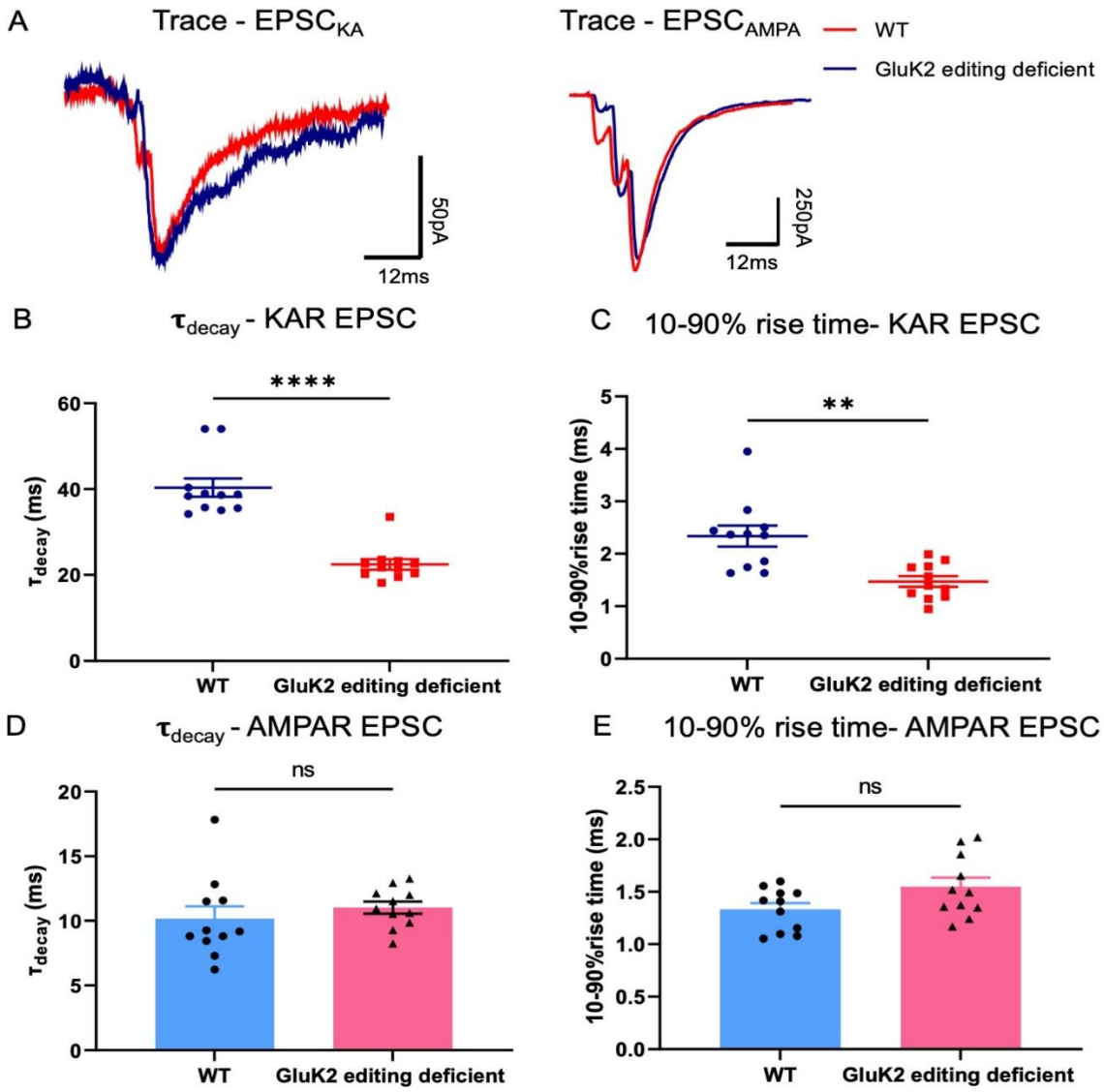


Figure 5-7: GluK2 editing-deficient mice display altered KAR EPSC 10-90% rise time and decay kinetics

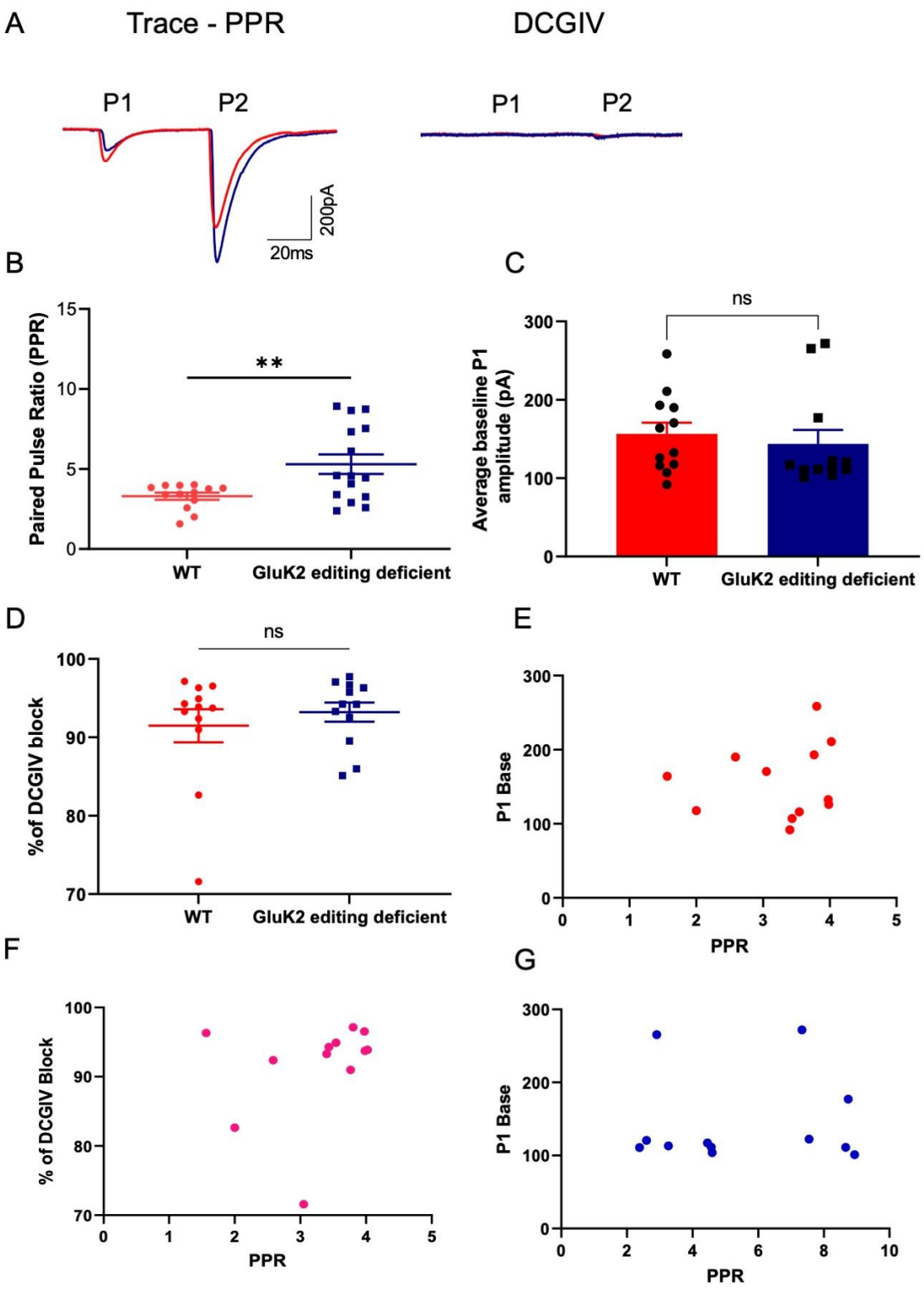
Sample traces showing 10-90%rise time and decay kinetics of KAR and AMPAR currents in WT and GluK2 editing-deficient mice (A). Quantification of τ_{decay} for KAR currents in WT vs GluK2 editing-deficient (B) and 10-90%rise time for KAR currents (C). Quantification of τ_{decay} for AMPAR currents (D) and10-90%rise time for AMPAR currents (E). N=5, n=12 cells, ns p>0.05, *p<0.05, **p<0.01; ****p<0.0001; un-paired t test with Welch's correction).

5.4.3 GluK2 editing-deficient mice show a higher paired-pulse ratio

To investigate how presynaptic KAR function is altered in the GluK2 editing-deficient, I next compared the PPR (paired-pulse ratio) of GluK2 editing-deficient mice with their WT counterparts. To achieve this in acutely prepared hippocampal slices, CA3 neurons were voltage clamped at -70mV and EPSCs were evoked by MF stimulations (paired stimulations 50 ms apart, every 20 s) in the presence of 50µM picrotoxin for 10 mins. No significant change in the basal P1 amplitude was detected for WT or GluK2 editing-deficient mice (WT= 156.5 ± 14.39 pA, Tg= 143.854 ± 17.74 pA; p value= 0.5830) **[Figure 5-8C]**. DCGIV was applied for 10 mins after the PPR recordings to confirm the activation of MF- CA3 pathway.

Paired-pulse facilitation was evident in both WT and GluK2 editing-deficient mice, but the degree of facilitation was greater in the GluK2 editing-deficient mice (WT= 3.3 ± 0.22 pA, Tg= 5.3 ± 0.61 pA; p value = 0.0067) **[Figure 5-8B]**.

To understand whether there are changes in the number of mossy fibres in GluK2 editing-deficient mice compared to WT counterparts, percentage of DCGIV block was calculated. However, no significant change was observed for both the phenotypes (WT= 91.47 ± 2.1%, Tg= 93.20 ± 1.2%; p value = 0.4879) **[Figure 5-8D]**. Further, No significant change in correlation between PPR and basal EPSC amplitude (WT, r= 0.127,R^2= 0.0163, p value= 0.692; Tg= 0.03834, R^2 = 0.00147, p value= 0.906; 95% confidence interval) or DCGIV block (WT, r= 0.2774, R^2= 0.07694, p value= 0.383; Tg= -0.2801,R^2 = 0.07846, P value= 0.378; 95%confidence interval) in both the phenotypes further confirmed that the change in PPR is not dependent on any confounding factors such as changes in the baseline amplitude etc. **[Figure 5-8E-H]**.


A
Trace - PPR
DCGIV
P1
P2
P1
P2
200pA
20ms
B
Paired Pulse Ratio (PPR)
15
10
5
0
**
WT
GluK2 editing deficient
C
ns
Average baseline P1 amplitude (pA)
300
200
100
0
WT
GluK2 editing deficient
D
ns
%of DCGIV block
100
90
80
70
WT
GluK2 editing deficient
E
P1 Base
300
200
100
0
0
1
2
3
4
5
PPR
F
% of DCGIV Block
100
90
80
70
0
1
2
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PPR

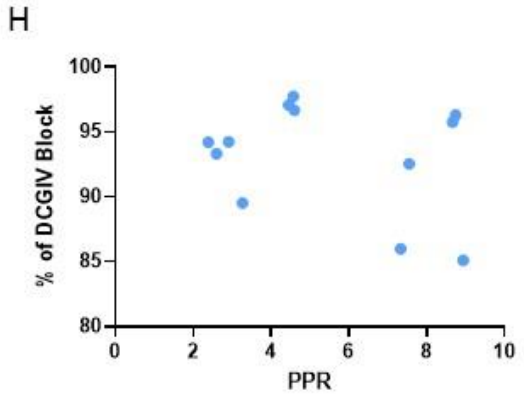

Figure 5-8: Paired-pulse ratio is higher in GluK2 editing-deficient mice

Representative trace showing paired pulse EPSCs of both WT vs GluK2 editing-deficient mice (A). Quantification of average paired-pulse ratio in WT vs GluK2 editing-deficient mice (B). Average baseline P1 amplitude for both the phenotypes (C). Percentage of DCGIV block in both the phenotypes (D). WT, N=7, n=13 cells, Tg, N=8, n=15 cells ns $p>0.05$, *$p<0.05$, **$p<0.01$; un-paired t test with Welch's correction. Pearson's correlation between PPR and P1 baseline amplitude (E). PPR and percentage of DCGIV block in WT (F). Pearson's correlation between PPR and P1 baseline amplitude (G). PPR and percentage of DCGIV block in GluK2 editing-deficient mice (H),

5.4.4 GluK2 editing-deficient mice show an increase in Frequency-Facilitation

I next determined if frequency-facilitation (FF) differed between GluK2 editing-deficient mice and its WT counterparts. In acutely prepared hippocampal slices, CA3 neurons were voltage clamped at -70mV and EPSCs were evoked by MF stimulations of 0.05Hz every 20 s for 10 mins and then stimulation frequency was increased to 1Hz, every 1 s for 1 min. The frequency was brought back to 0.05Hz and DCGIV was applied to confirm the MF pathway. The recordings were made in the presence of 50µM picrotoxin.

In slices of both WT and GluK2 editing-deficient mice, increasing frequency from 0.05Hz to 1Hz enhanced the EPSC amplitudes. Interestingly, however, the enhancement in EPSC amplitude with 1Hz stimulation was higher in GluK2 editing-deficient mice (WT= 4.5 ± 0.44 pA, Tg= 6.6 ± 0.62 pA; p value = 0.0114) **[Figure 5-9]**.

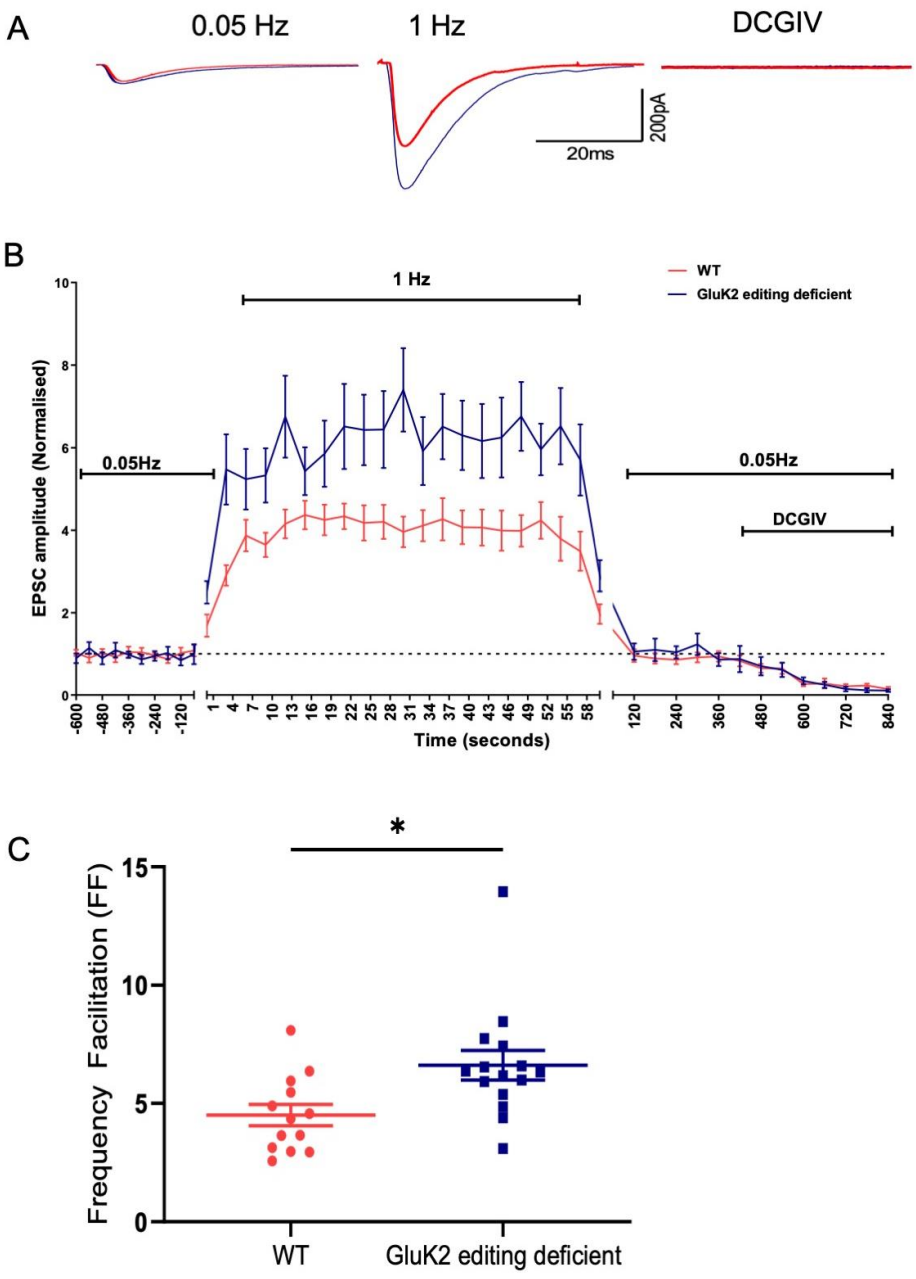

Figure 5-9: Frequency-facilitation is higher in GluK2 editing-deficient mice

Traces showing frequency facilitation of WT vs GluK2 editing-deficient mice (A). Timeline of frequency facilitation experiments in WT and Tg mice (B). Quantification of average paired-pulse ratio in WT vs GluK2 editing-deficient mice (C). WT, N=7, n=13 cells, Tg, N=8, n=15 cells ns $p>0.05$, *$p<0.05$, **$p<0.01$; un-paired t test with Welch's correction.

5.4.5 GluK2 editing-deficient mice display less inhibition of I_{sAHP}

Given that KARs signal via both ionotropic and metabotropic pathways, I next investigated if metabotropic functions of KARs is impaired in GluK2 editing-deficient mice by measuring the inhibition of I_{sAHP} by synaptic KARs in acute hippocampal slices. To investigate this, I_{sAHP} currents were evoked in whole cell voltage clamped CA3 pyramidal cells by depolarizing the membrane potential to 0mV from -50mV holding potential for 200 ms. The experiment was performed in the presence of 50µM picrotoxin, 50µM D-APV, 40µM GYKI53655 and 1µM CGP55845 to inhibit $GABA_A$Rs, NMDARs, AMPARs and $GABA_B$Rs, respectively. This stimulation produced a robust and stable I_{sAHP} (WT= 66.95 ± 7.98 pA; Tg= 63.09 ± 6.62 pA, p= 0.712) **[Figure 5-10]** (Chamberlain et al., 2013). No changes in the baseline amplitudes were evident for both the phenotypes.

Synaptic KARs were activated by MF stimulations (10 stimuli at 25Hz every 20 s for 10 mins). This synaptic stimulation produced KAR EPSCs which depressed I_{sAHP} in WT mice similar to previous reports (Chamberlain et al 2013). However, synaptic KAR activation produced a much smaller reduction in I_{sAHP} in GluK2 editing-deficient mice (WT= 45.05 ± 3.72, Tg= 26.68 ± 2.69%; p= 0006;) suggesting reduced metabotropic signaling despite increased ionotropic function **[Figure 5-10].**

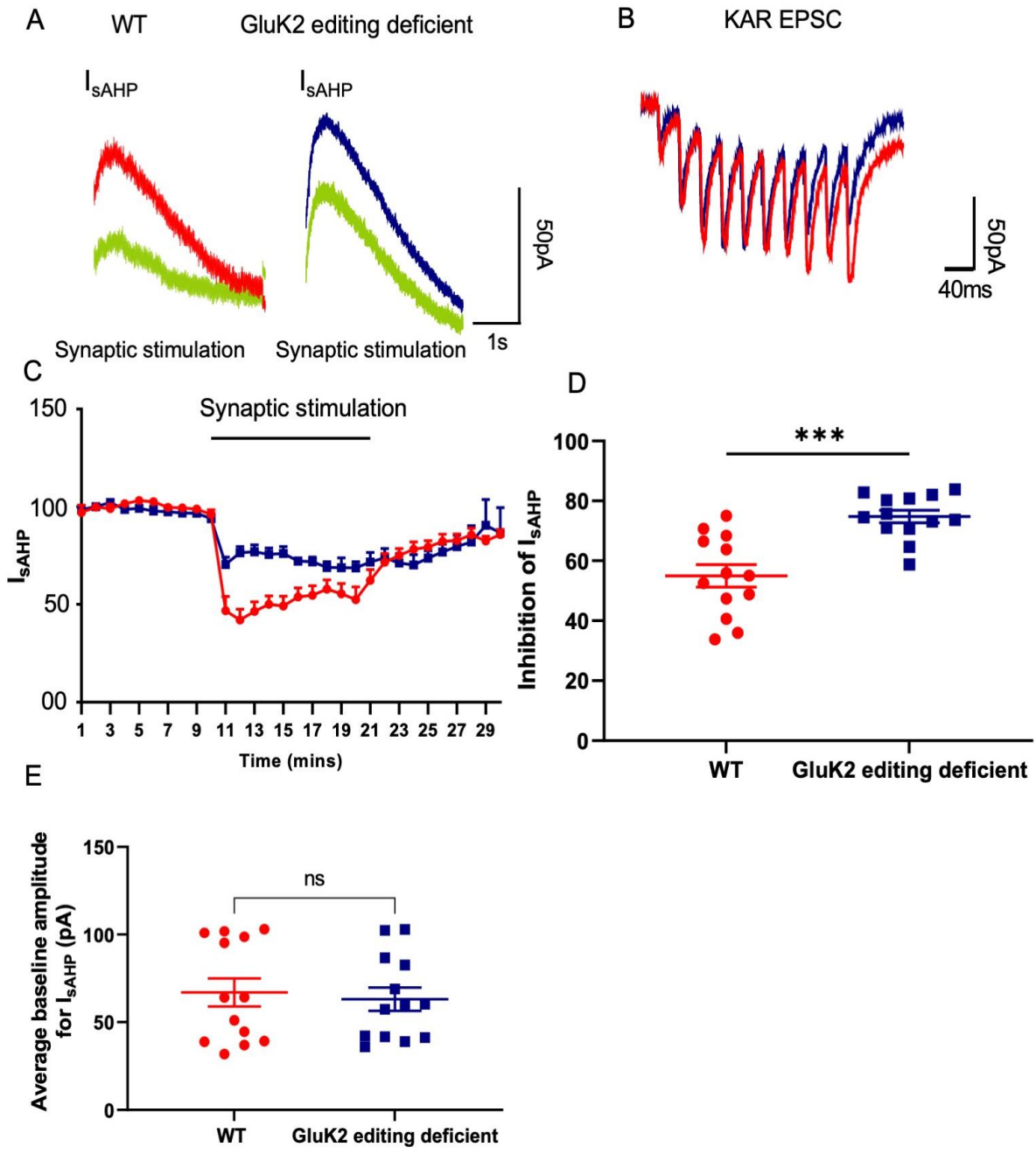

Figure 5-10:GluK2 editing-deficient mice display altered metabotropic functions

Sample traces showing I_{sAHP} before (WT and GluK2 editing-deficient mice) and with synaptic stimulation (A) traces of KAR EPSCs (B). Timeline showing depression of I_{sAHP} by synaptic KARs (C). Quantification of inhibition of I_{sAHP} in WT vs GluK2 editing-deficient upon KAR activation (D). Quantification of average baseline amplitude (E); N=4 animals, n=13 cells, ns $p>0.05$, **$p<0.0001$; un-paired t test with Welch's correction.

5.4.6 KAR-dependent inhibition of I_{sAHP} is blocked by UBP310

To test if the increased ionotropic function of KARs in the GluK2 editing-deficient mice somehow reduced the metabotropic function, I attempted to separate the ionotropic and metabotropic KAR functions using UBP310 in WT mice. This compound is reported to selectively inhibit the ionotropic function of GluK2/GluK5 containing KARs and a previous study demonstrated that I_{sAHP} inhibition by metabotropic KARs are not inhibited in the presence of UBP310 (Pinheiro et al., 2013).

I_{sAHP} currents were evoked in whole cell voltage clamped CA3 pyramidal cells of acutely prepared hippocampal slices by depolarizing the membrane potential to 0mV from -50mV holding potential for 200 ms. The experiments were performed in the presence of 50µM picrotoxin, 50µM D-APV,40µM GYKI53655, 1µM CGP55845, to inhibit $GABA_A$Rs, NMDARs, AMPARs, $GABA_B$Rs respectively in the presence or absence of 10µM UBP310 **[Figure 5-11**].

Synaptic KARs were stimulated by MF stimulations with 10 stimuli at 25Hz every 20 s for 10 mins. This synaptic stimulation produced KAR EPSCs which depressed I_{sAHP} in control (WT mice without UBP310). Unexpectedly, however, in the presence of UB310, KAR-mediated inhibition of I_{sAHP} was prevented (control= 45.05 ± 3.72%; UBP310 =14.44 ± 1.89%; p= <0.0001) indicating that UBP310 also inhibits metabotropic KAR function **[Figure 5-11F]**.

No change in the basal amplitudes of I_{sAHP} was detected either in the presence or absence of UBP310 (Control= 66.95 ± 7.98 pA; UBP310= 66.0 6± 7.38 pA, p= 0.9354) further confirming the effect is independent of changes in basal amplitude **[Figure 5-11 E]**.

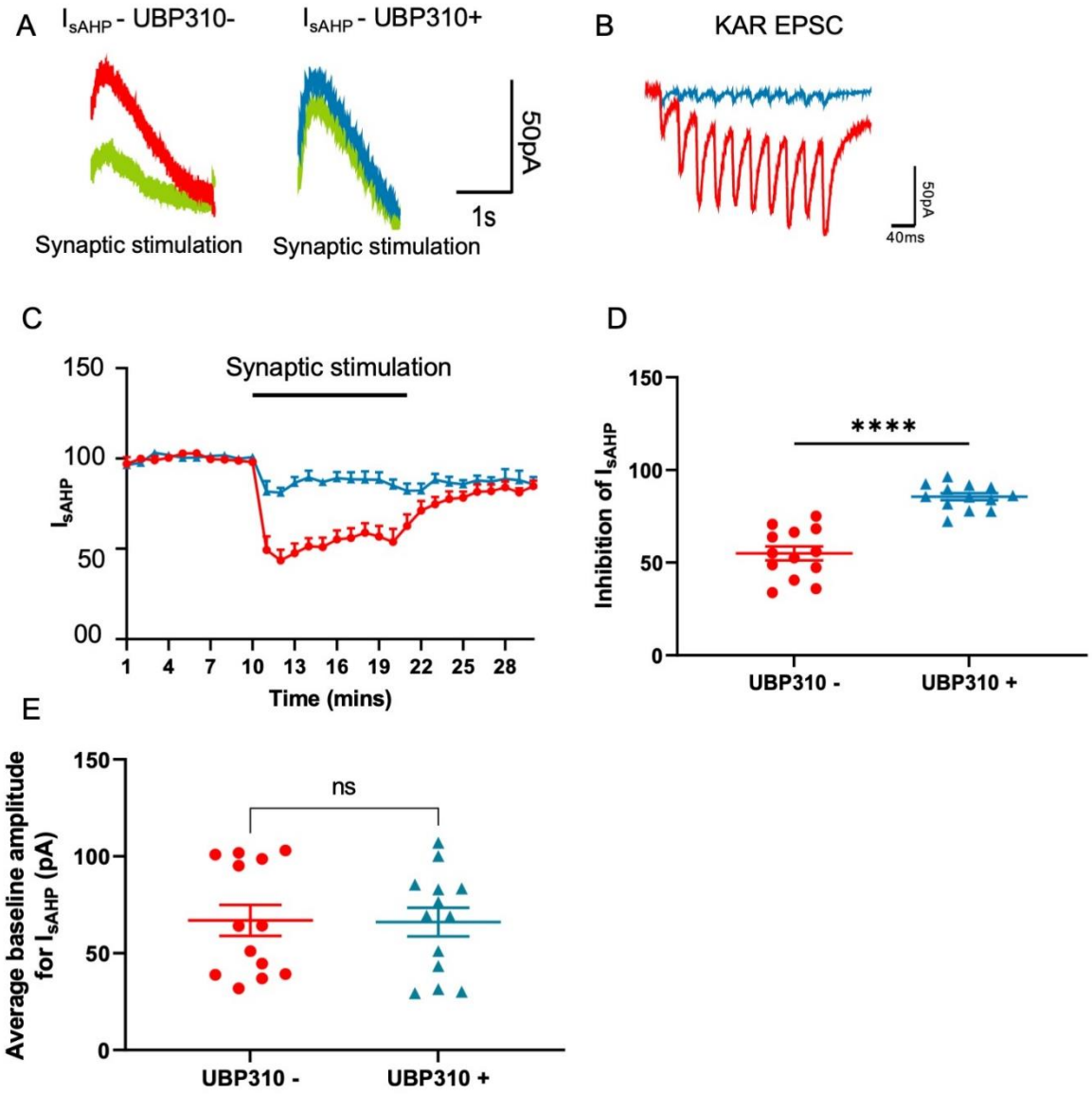

Figure 5-11: Investigating ionotropic KAR activity in KAR-dependent inhibition of I_{sAHP}

Sample traces showing I_{sAHP} before (UBP310- ; UBP310+) and with synaptic KAR stimulation in WT slices (A) traces of KAR EPSCs (B). Timeline showing depression of I_{sAHP} by synaptic KARs without UBP310 and with UBP310 (C). Quantification of inhibition of I_{sAHP} in WT slices in the presence or absence of UBP310 with KAR activation (D). Quantification of average baseline amplitude (E). N=4 animals, n=13 cells, ns $p>0.05$, **$p<0.001$; un-paired t test with Welch's correction; Error bar=SEM.

5.5 Discussion

5.5.1 GluK2 editing-deficient mice show enhanced PPF and FF and postsynaptic KAR/AMPAR ratio.

ADAR2 mediated editing of GluK2 subunits alters the trafficking and functional properties of both GluA2 AMPARs and GluK2 KARs. Edited subunits are less trafficked to the surface compared to un-edited subunits (Filippini et al., 2017)(Ball et al., 2010a, Pahl et al., 2014). Editing also dramatically reduces Ca^{2+} permeability through the ion channel (Seeburg et al., 1998, Ball et al., 2010a). To study the potential role of GluK2 Q/R editing site, a transgenic mouse line was generated by deletion of the ECS from the *GRIK2* gene (Vissel et al., 2001a). This prevented the editing of GluK2 subunits by ADAR2, resulting in 95% of GluK2 subunits being unedited (Vissel et al., 2001a). However, the remaining 5% editing of GluK2 suggests there also exists a mechanism independent of the ECS, or which occurs via the ADAR1 enzyme (Vissel et al., 2001a).

KARs are present in both pre and postsynaptic sites of MF-CA3 synapses where they modulate the release of glutamate from the presynapse and mediate a component of the postsynaptic EPSC (Darstein et al., 2003). I investigated how presynaptic and postsynaptic KAR function is altered in the editing deficient mice by measuring the PPF-FF and postsynaptic KAR/AMPAR ratio. GluK2 editing-deficient mice showed an increase in PPR **[Figure 5-8]**, FF **[Figure 5-9]** and KAR/AMPAR ratio **[Figure 5-6]**. This could be attributed to either an increase in the number of KARs, or permeability for divalent cations, at pre and postsynaptic sites.

5.5.2 GluK2 editing-deficient mice have a faster 10-90% rise time and decay kinetics

KARs channel kinetics is determined by factors including the subunit assembly, post-transcriptional and post-translational modifications, and various auxiliary subunits such as Neto1 and Neto2 (Li et al., 2019, Fisher and Mott, 2012, Cui and Mayer, 1999, Fisher and Fisher, 2014). $Neto1^{-/-}$ mice displayed faster decay and rise time kinetics of $EPSC_{KA}$ at MF-CA3 and associational/commissural-CA3 synapses (Straub et al., 2011a).

I compared the 10-90% rise time and decay kinetics of postsynaptic KAR currents in WT and GluK2 editing-deficient mice. The KAR EPSCs from GluK2 editing-deficient mice showed faster 10-90% rise time and decay kinetics (τ_{decay}) **[Figure 5-7]**. While the precise cause of these changes in biophysical parameters have not yet been determined, one potential cause could be reduced interaction with Neto1 protein and this needs to be experimented further.

5.5.3 Metabotropic function of KARs is reduced in GluK2 editing-deficient mice

KARs are key players in regulating neuronal excitability. Activation of metabotropic KARs can induce hyperexcitability in neurons by inhibiting I_{sAHP} (Madison and Nicoll, 1984, Ruiz et al., 2005, Fisahn et al., 2005, Chamberlain et al., 2013). In CA1 pyramidal neurons, brief KA application induces long-lasting inhibition of I_{sAHP} and is blocked by the G-protein inhibitor pertussis toxin, NEM or PKC-specific inhibitors (Melyan et al., 2002). Despite the efforts of various groups to identify the subunit involved in this non-canonical signaling of KARs, it still not free from controversies (Ruiz et al., 2005, Fernandes et al., 2009).

Despite these controversies revolving around the subunit requirement, it is well accepted that KARs inhibit I_{sAHP} via metabotropic function (Ruiz et al., 2005, Fernandes et al., 2009, Lerma, 2003, Chamberlain et al., 2013).

To better understand how GluK2 Q/R editing impacts metabotropic signalling of KARs, I analysed the ability of KARs to inhibit I_{sAHP} in WT and GluK2 editing-deficient mice. Interestingly, GluK2 editing-deficient mice showed only ~20% inhibition of I_{sAHP} compared to ~50% inhibition in WT animals. This effect is most likely due to reduced metabotropic signaling in GluK2 editing-deficient mice **[Figure 5-10]**. A reduction in the $EPSC_{KA}$ along with inhibition of the I_{sAHP} was evident in the presence of the GluK2/GluK5 antagonist UBP310 in WT mice **[Figure 5-11]**. This is contradictory to previous findings where the KAR-mediated inhibition of I_{sAHP} was insensitive to UBP310 (Pinheiro et al., 2013).

However, the exact mechanism underlying reduced inhibition of I_{sAHP} in GuK2 editing deficient mice or inhibition of I_{sAHP} by UBP310 is currently unknown. An attractive, but yet untested, hypothesis is that the reduced inhibition of I_{sAHP} in GluK2 editing-deficient

mice might be due to un-edited GluK2 subunits being unable to form metabotropic KARs.

5.6 Conclusion

In this chapter, I researched the physiological implications of GluK2 Q/R editing in various synaptic responses using GluK2 editing-deficient mice. The mutant mice are viable and breed normally, however they are more susceptible to seizures (Vissel et al., 2001a). My data shows for the first time that the mice display higher PPF-FF and KAR/AMPAR ratio. Moreover, the KAR currents in these mice have a faster 10-90% rise time and decay kinetics (τ_{decay}) compared to their wildtype counterparts. Perhaps most interestingly, the GluK2 editing-deficient mice show impaired metabotropic KAR function evidenced by reduced inhibition of I_{sAHP}. These results demonstrate that GluK2 Q/R editing is a determining factor in the biophysical properties and signalling of KARs.

Chapter 6. General Discussion

6.1 Summary of my research

Building on previous observations in the lab that KARs can induce AMPAR LTP (Petrovic et al., 2017c), the overall aim of my PhD was to determine if, and how, KARs can also induce long term depression (LTD) of AMPARs. As a first step, I explored changes in the phosphorylation status of GluA1 S845, a well-studied marker of synaptic plasticity, upon sustained KAR stimulation. Previous studies indicated that the basal phosphorylation of GluA1 S845 is reduced in NMDAR dependent chem-LTD (Esteban et al., 2003). However, contrary to this, the KAR-LTD protocol increased phosphorylation of GluA1 S845. This was partially blocked by AMPAR antagonist GYKI53655. Although the exact mechanism underpinning this KAR-mediated increase in phosphorylation is unknown, the simplest explanations for this is either an increase in glutamate release by the activation of presynaptic KARs or non-specific activation of AMPARs by higher concentration of KA.

My subsequent experiments demonstrated a novel form of NMDAR- and mGluR-independent, KAR-mediated LTD of AMPARs. This novel form of KAR-LTD$_{AMPAR}$ requires signaling through GluK2-containing ionotropic KARs and the phosphatase calcineurin. This, in conjunction with previous studies from the lab, highlight the relevance of KARs in inducing bidirectional plasticity of AMPARs.

Finally, I investigated the physiological relevance of Q/R editing of the GluK2 KAR subunit using the GluK2-editing deficient mouse model. My results show that Q/R editing of GluK2 subunits alter the pre and postsynaptic signaling and kinetics of the KAR currents.

6.1.1 KAR stimulation increases phosphorylation of GluA1 S845

Synaptic plasticity usually requires altering the numbers of surface AMPARs at the postsynaptic membrane. Increased or decreased synaptic AMPARs modulates synaptic strength during LTP or LTD induction. In *in vitro* conditions, the trafficking and expression of surface AMPARs are regulated by multiple protein-protein interactions and post-translational modifications (Huganir and Nicoll, 2013, Henley and Wilkinson, 2013, Henley et al., 2021). Phosphorylation of various subunits of AMPARs are crucial in regulating the trafficking and channel conductance of AMPARs (Huganir and Nicoll, 2013). The C-terminal tail of AMPAR subunits undergo phosphorylation at several

sites by discrete protein kinases as indicated below (Roche et al., 1996, Chung et al., 2000, Lussier et al., 2015, Hayashi and Huganir, 2004).

Table 6-1: Table showing phosphorylation at the C-terminus of various AMPAR subunits by discrete protein kinases

AMPAR Subunit	Target site	Protein kinase	Reference
GluA1	S845	PKA	(Esteban et al., 2003)
GluA1	S831	PKC/CaMKII	(Boehm et al., 2006, Lee et al., 2007)
GluA1	S818, T840	PKC	(Boehm et al., 2006, Lee et al., 2007)
GluA2	S863	PKC	(Boehm et al., 2006, Lee et al., 2007)
GluA2	S880	PKC	(Boehm et al., 2006, Lee et al., 2007)
GluA1	S567	CaMKII	(Boehm et al., 2006, Lee et al., 2007)
GluA4	S842	cGMP dependent protein kinases (PKG)	(Diering et al., 2018)
GluA1	S579	Casein kinase II	(Lussier et al., 2014)
GluA2	S588	Casein kinase II	(Lussier et al., 2014)
GluA1	S863	p21-activated protein kinase (PAK3)	(Hussain et al., 2015)
GluA1	Y876	Src family of tyrosine kinases	(Roche et al., 1996)

However, the best-studied sites for the phosphorylation of AMPAR subunits are at GluA1 S845 and S831. Previous reports indicated that phosphorylation of GluA1 S845 and S831 regulate the trafficking of AMPAR subunits and facilitates their incorporation at synapses (Huganir and Nicoll, 2013). Under basal conditions, only 15% of AMPAR subunits are phosphorylated. However, during the induction of LTP, phosphorylation

of GluA1 S845 surges to 60%. In contrast, LTD is characterized by a significant decline in levels of phosphorylation to ~10% (Oh et al., 2006).

Initially, to assess the mechanism of KAR activation in LTD of AMPARs, I was keen to determine the changes in phosphorylation of GluA1 S845 with KAR activation. In particular, I set out to define whether KAR and NMDAR activation share the same signaling pathways and change in the phosphorylation status. Intriguingly, rather than an expected decrease in the phosphorylation of GluA1 S845, an increase in the phosphorylation was evident with KA treatment. This rise in the phosphorylation of GluA1 S845 can largely be interpreted as KAR- and NMDAR-mediated downregulation of AMPARs following distinct pathways.

6.1.2 The KA-induced increase in phosphorylation of GluA1 might be mediated by direct agonism of AMPAR by KA

I further investigated the mechanism underpinning the increase in phosphorylation of GluA1 S845 with KAR stimulation. 10μM KA can nonspecifically activate AMPARs (Levchenko-Lambert et al., 2011, Ruiz et al., 2005). Therefore, to exclude the possibility of nonspecific activation of AMPARs by a comparatively high KA concentration, I repeated the experiments with 3μM KA, a concentration that is below the threshold for AMPAR activation (Massey et al., 2001). In agreement with the higher KA concentration (10μM), 3μM KA treatment also increased the phosphorylation of GluA1 S845.

To eliminate the contribution of activation of NMDARs and AMPARs directly or indirectly by presynaptic KARs, the experiments were repeated in the presence of D-APV (NMDAR antagonist) and GYKI53655 (AMPAR antagonist). Surprisingly, AMPAR antagonist partially blocked the effect. This is most likely due to enhanced presynaptic release via the activation of KARs or by non-specific activation of AMPARs by higher concentration of KA.

KARs can signal via canonical ionotropic and non-canonical metabotropic pathways (Rodrigues and Lerma, 2012, Contractor et al., 2011, Petrovic et al., 2017b, Nair et al., 2021, González-González et al., 2012, Henley et al., 2021). To discern the signaling pathway, I used the $G_{i/o}$-protein inhibitor PTx and the ionotropic KAR inhibitor UBP310. Neither PTx nor UBP310 inhibited the KAR-mediated increase in the

phosphorylation of GluA1 S845. The simplest explanation for this is KAR signaling via receptors devoid of GluK2/GluK5 subunits. However, the AMPAR/KAR antagonist CNQX completely blocked the effect, further emphasizing the requirement for activation of KARs or AMPARs.

I next questioned the involvement of protein kinase PKA in KA-induced phosphorylation of GluA1 S845. In the presence of H89 (PKA inhibitor), KA treatment failed to cause an increase in GluA1 S845 phosphorylation.

While the effect of KA in increasing the phosphorylation of GluA1 S845 is interesting, the contrasting direction of change in phosphorylation at the same site (GluA1 S845) with chem-LTD protocol for NMDAR versus sustained KA treatment poses significant challenges. In addition, the lack of specific inhibitors to block KAR-evoked neurotransmitter release led me to discontinue this aspect of the study and carry forward with investigation of the surface expression of AMPARs with biotinylation and imaging experiments.

6.1.3 KARs directly or indirectly regulate GluA1 trafficking

ShRNA-mediated knock down of GluK2 in hippocampal cultures significantly reduced surface levels of GluA1 alongside an increase in total levels of GluA1. No significant change in the surface or total GluA2 levels were detected. This altered levels of GluA1 AMPARs signify the role of GluK2-containing KARs in maintaining the tone of AMPARs via a direct or indirect mechanism that is currently unknown.

6.1.4 KARs induce a novel form of KAR-LTD$_{AMPAR}$ that requires ionotropic signaling through GluK2-containing KARs

Previous studies from the lab have shown that depending upon activation parameters, KARs mediate bidirectional regulation of their own surface expression in neurons (Martin et al., 2008, Henley, 2004). More recently, we demonstrated that transient KAR activation leads to an increase in the surface expression of AMPARs and subsequently induces KAR-LTP$_{AMPAR}$ (Petrovic et al., 2017c). This form of KAR-dependent plasticity of AMPARs requires metabotropic signaling via GluK2-containing KARs, activation of PKC and recruitment of Rab11-dependent transferrin positive endosomes to the spine (Petrovic et al., 2017c, Gonzalez-Gonzalez and Henley, 2013).

In contrast to transient stimulation, which enhances KAR surface expression, sustained agonist stimulation of KARs promotes their endocytosis and degradation (Henley, 2004). I therefore hypothesized that sustained KAR stimulation might also induce LTD of AMPARs by downregulating the surface expression of AMPARs.

Consistent with this hypothesis, sustained KAR stimulation significantly reduced surface expression of both GluA1 and GluA2 AMPAR subunits, as well as the GluK2 KAR subunit. This reduction is independent of NMDAR, AMPAR or mGluR activation. Electrophysiological recordings from CA1 pyramidal neurons revealed a significant depression in $EPSC_{AMPA}$ for at least 20 mins after bath application of KA, leading to LTD induction. Intriguingly, PPR data revealed a dual form of plasticity with an initial presynaptic KAR dependent short-term depression (STD) followed by the postsynaptic KAR-mediated LTD of AMPARs.

I next investigated the signaling pathways underpinning $KAR\text{-}LTD_{AMPARs}$. The KAR antagonist UBP310 abolished the KAR-mediated reduction in AMPARs emphasizing the role of ionotropic KAR signaling. Comparable with $KAR\text{-}LTP_{AMPAR}$, $KAR\text{-}LTD_{AMPAR}$ requires the GluK2 subunit of the receptor. Knocking down GluK2 in neurons using shRNA abolished the reduction in surface levels of AMPARs post KAR stimulation. Further, inhibiting calcineurin with FK506 blocked the KA-induced reduction in surface AMPARs, indicating the requirement of the phosphatase calcineurin. In addition, a significant decrease in basal GluA1 and GluA2 surface levels in the presence of UBP310 suggests KARs may also be required for maintaining tonic activity of AMPARs through a yet unidentified pathway and may indicate that UBP310 occludes LTD by resulting in a loss of ‘removable’ AMPARs from the cell surface such that they can no longer be removed by KA. Further, both PKA and PKC inhibitors occluded the KA-induced reduction in surface AMPARs and reduced their basal levels without KA treatment.

Interpreting my results in terms of a ‘slot availability’ model suggests that constitutive activity of kinase(s) may be required to maintain slots, and thus inhibiting these leads to a reduction in the number of slots, which may explain why blocking PKC and PKA occludes $KAR\text{-}LTD_{AMPAR}$. However, I note that my data demonstrate that PKA/PKC signalling is required to sustain surface GluA2 levels, and I do not directly address

whether these kinases regulate incorporation of GluA1 and GluA2 into synaptic slots, although I do show that synaptic GluA2 levels are decreased by KA stimulation.

Further, I propose that the reduction in the basal surface AMPAR levels by the pharmacological inhibitor CNQX, and by inhibitors of PKA and PKC, suggest that these inhibitors are blocking the pathway that delivers AMPARs to the surface, leading to a depletion in the levels of 'removable AMPARs' at the surface.

Nevertheless, this study, together with KAR-LTP$_{AMPAR}$, reveals a novel form of KAR-mediated bidirectional plasticity of AMPARs that may occur in physiological conditions could play critical roles in learning and memory formation and be defective in neurological disorders.

6.1.5 GluK2 editing is a crucial determinant in signaling and channel kinetics of KARs

ADAR2-meditated Q/R editing of GluK2 subunits of KARs is a developmentally regulated process (Gurung et al., 2018, Cenci et al., 2008, Jin et al., 2009). 80% of GluK2 subunits expressed in adult brain contain the edited subunit (GluK2 (R)) (Belcher SM, 1997, Paschen et al., 1997, Bernard et al., 1999). Our lab has reported studies in primary neuronal cultures that revealed the importance of GluK2 editing in regulating the trafficking of KARs (Gurung et al., 2018, Evans et al., 2017c)

More than 20 years ago a transgenic mouse line was generated with deletion of the ECS site, resulting in expression of almost exclusively the edited GluK2 (R) subunit (Vissel et al., 2001a). These transgenic mice showed 95% less editing of GluK2 subunits compared to WT counterparts (Vissel et al., 2001b) but further analyses were not performed. On our request these mice were reanimated and shipped to us from Australia. Once breeding colonies were established, I investigated a range of KAR-mediated responses from the MF-CA3 pathway, where KARs are well characterized at both pre and postsynaptic sites, in both GluK2 editing-deficient mice and their WT counterparts (Vissel et al., 2001a) .

GluK2 editing-deficient mice showed enhanced PPF-FF and postsynaptic KAR/AMPAR ratio compared to WT counterparts. The enhanced PPF and KAR/AMPAR ratio are most likely due to either an increase in channel conductance or the number of functional KARs, or both. Intriguingly, the KAR currents in GluK2

editing-deficient mice showed a faster 10-90% rise time and decay kinetics (τ_{decay}). Moreover, there were clear impairments in KAR metabotropic function in the GluK2 editing-deficient mice compared to WT. I speculate that the reduction in metabotropic KAR function in GluK2 editing-deficient mice may be due to the conformational changes in structure of un-edited GluK2 KARs. Further, my studies showed that UBP310 can inhibit the KAR-evoked inhibition of I_{sAHP}.

In summary, my results are indicative of ADAR2-mediated GluK2 editing being a key player in regulating the function and channel kinetics of the receptor.

6.2 Future work

The findings made raise many interesting questions, resolution of which could lead to better understanding of KAR function and trafficking. Some of the questions are outlined below.

6.2.1 Do GluK2 KARs regulate trafficking of GluA1 AMPARs?

KARs induce plasticity of AMPARs by modulating the surface expression of AMPARs. However, to my knowledge, there is no clear evidence for direct interaction between KARs and AMPARs. Knocking down GluK2 subunits in primary hippocampal neuronal cultures significantly reduced surface GluA1 while increasing total GluA1 levels. This change in the surface and total levels of GluA1 AMPARs in the GluK2 KD neurons strongly suggests that GluK2-containing KARs are required for trafficking and/or docking of AMPARs at the synapse.

To study the direct or indirect interactions of GluK2 with GluA1 subunit of AMPARs, various techniques such as pull down of native subunits, co-localization studies or fluorescence resonance energy transfer (FRET) can be employed. Depending on the results obtained there are a wide range of future avenues that could be explored to define the mechanisms and consequences of the interplay between GluK2 KARs and GluA1 AMPARs.

6.2.2 How does GluK2 Q/R editing alter homeostatic scaling?

GluK2 Q/R editing is key in determining the trafficking of the receptor to the surface. Un-edited GluK2 subunits are trafficked more compared to the edited ones (Evans et al., 2017c). This is because the edited subunits are retained more at the ER due to their relative inability to co-assemble with other KAR subunits (Ball et al., 2010b, Gurung et al., 2018, Evans et al., 2017b).

Previous studies from the lab have shown that chronic inhibition of network activity by TTX leads to reduced ADAR2-mediated Q/R editing of GluK2 KARs and upscaling of the receptor. Alternatively, chronic enhancement of network activity by bicuculline promotes GluK2 editing and downregulates surface KARs (Gurung et al., 2018, Gurung, 2018). Another logical question is how do completely edited or unedited GluK2 subunits respond to KAR scaling? This can be addressed by employing molecular replacement strategies in primary neuronal cultures with GluK2(Q)/GluK5 or GluK2(R)/GluK5 recombinant receptors.

Secondly, the forward trafficking of edited vs un-edited subunits of KARs are well characterized (Gurung et al., 2018, Evans et al., 2017c). Nonetheless, there is little information on the removal of these subunits from the surface. This raises the question on how efficiently these subunits are removed from the surface and whether they follow the same endocytic/degradation pathways?

6.2.3 Is I_{sAHP} affected by KAR scaling?

I_{sAHP} are outward potassium currents generated in response to successive trains of action potentials (Lancaster and Adams, 1986). These long-lasting K^+ currents are neuroprotective as they prevent a neuron from being hyper-excitable (Chamberlain et al., 2013). Metabotropic KARs modulate neuronal excitability by suppressing I_{sAHP} thus making the neurons more susceptible to over-excitation (Chamberlain et al., 2013, Fisahn et al., 2005). Consistent with this, GluK2 editing-deficient mice are characterized by reduced inhibition of I_{sAHP} by synaptic KAR activation. This implies that GluK2 Q/R editing is likely to have an effect on regulating the excitability of a neuron.

24 hr TTX treatment mediates chronic suppression of network activity and upregulates KARs, whereas bicuculline treatment downregulates KARs, by altering GluK2 Q/R

editing (Gurung et al., 2018). It would be interesting to know if the inhibition of I_{sAHP} by KARs is altered upon chronic suppression or enhancement of network activity? I expect that results obtained would highlight the role of GluK2 Q/R editing in maintaining network activity by altering the excitability of neurons.

6.2.4 Is GluK2 editing a key determinant of KAR signaling?

An unusual and intriguing aspect of KARs is their ability to activate both ionotropic and metabotropic signaling pathways (Henley and Wilkinson, 2013, Henley et al., 2021, Nair et al., 2021, Lerma and Marques, 2013, Rodrigues and Lerma, 2012, Rozas et al., 2003). Although both signaling mechanisms have been investigated, it is currently unknown what determines the mode of signaling of KARs.

GluK2 editing-deficient mice have impaired metabotropic function compared to their WT counterparts. This could indicate that GluK2 Q/R editing is a key determinant of ionotropic/metabotropic signaling. Addressing this question is particularly interesting and has the potential to open entirely new avenues of research.

Inhibition of I_{sAHP} by KARs in cultured neurons with CRISPR-Cas9 or shRNA mediated-knockdown of endogenous KARs and subsequent replacement with GluK2(Q)/GluK5 or GluK2(R)/GluK5, alongside measurement of postsynaptic KAR currents could provide novel insights into whether, and how, GluK2 Q/R editing determines the mode of KAR signaling. The study can be extended to rodents by injecting virus/using *in utero* electroporation techniques to knock down and replace KAR subunits to closely mimic physiological conditions. Addressing this question using GluK2 editing-deficient mice, as well as GluK2 knockout mice, will also provide key information as to the roles of KAR channels formed by subunits other than GluK2.

6.2.5 Does GluK2 editing alter mossy fibre development and the number of synapses?

KARs are crucial in the formation of glutamatergic synapses by regulating neurite growth cones and filopodial formations (Ibarretxe et al., 2007, Joseph et al., 2011, Tashiro et al., 2003, Ryazantseva et al., 2020). Functional presynaptic and postsynaptic KARs are perhaps best characterized at MF-CA3 synapses. Further, mossy fibres represent a site for seizure formation with aberrant mossy fibre sprouting (Epsztein et al., 2005, Hendricks et al., 2019, Danzer, 2017, Peret et al., 2014, Mulle

et al., 1998). It would be interesting to understand if there is aberrant mossy fibre sprouting in GluK2 editing-deficient mice that might explain the pathophysiology of the GluK2 editing-deficient mice being prone to seizures. This can be addressed by labelling mossy fibres with specific markers such as ArfGAP1, synpatophorin (SPO) etc. prior to confocal imaging experiments.

6.3 Conclusion and significance

An increasing number of studies are beginning to appreciate the role of KARs in regulating various synaptic functions such as synaptic plasticity and modulation of network activity. In the initial part of my study, I report that KA application increases phosphorylation of GluA1 S845. This is in contrary to the well-studied NMDAR-dependent chem-LTP/LTD-mediated phosphorylation of GluA1 S845, suggesting the involvement of a distinct signaling pathway. In conjunction, I report that pharmacological activation of ionotropic GluK2-containing KARs induces a novel form of LTD of AMPARs that is independent of NMDAR and mGluR activation. This form of KAR-LTD $_{AMPAR}$ is dependent on calcineurin. This, together with the previously reported KAR-LTP$_{AMPAR}$, emphasize the importance of KARs in inducing the bidirectional plasticity of AMPARs that precedes learning and memory formation. Further, I have emphasized the relevance of GluK2 subunits in maintaining the tone of GluA1-containing AMPARs. In addition, I have highlighted the physiological significance of GluK2 Q/R editing by characterizing various KAR-mediated responses in GluK2 editing-deficient mice. Although my studies were restricted mainly to understand pre- /post-synaptic ionotropic and metabotropic functions of KARs, understanding the significance of GluK2 Q/R editing in determining KAR-mediated signaling mechanisms and plasticity, and their role in temporal lobe epilepsy, will stand as an extensive and ambitious topic for future research.

6.4 Limitations of the study

Although this study revealed many novel and interesting results relating to KAR signalling and plasticity, I am mindful that the pharmacological inhibitors used primarily in chapter 3 and chapter 4 have limitations in assessing synaptic function of KAR signalling due to their wider effects. In chapter 3, stimulation with KA significantly increased phosphorylation of GluA1 S845, a well-established marker of synaptic

plasticity (Diering et al., 2018, Huganir and Nicoll, 2013). However, rather than the expected decrease in phosphorylation associated with LTD, as reported previously with NMDA mediated chem-LTD (Lee et al., 1998, Glebov et al., 2015, Ashby et al., 2004), sustained KA stimulation increased GluA1 S845 phosphorylation but decreased surface AMPARs. A partial decrease in the extent of phosphorylation was evident in the presence the AMPAR-specific antagonist. Based on these data I hypothesised that presynaptic KAR-dependent glutamate release via a TTX insensitive pathway or non-specific activation of AMPARs by higher concentration of KA could underpin the increase in phosphorylation of GluA1 S845. However, the lack of specific inhibitors to block the presynaptic release in this TTX insensitive pathway posed a greater challenge to this study. Future work with specific inhibitors of presynaptic release to investigate this KAR-dependent phosphorylation in trafficking and regulating functions of AMPARs are therefore required.

Similarly, it is difficult to rule out possible wider effects of the pharmacological inhibitors of metabotropic or ionotropic signalling through KARs, as well as the inhibition of PKC (chelerythrine), PKA (H89), PP1 and PP2A (okadaic acid) and Calcineurin/PP2B (FK506) in chapter 4. Nonetheless, the key observation is that these drugs have distinct effects on the KA-evoked changes in surface GluA1 and GluA2, strongly implicating their target proteins in the mechanisms underpinning KAR-evoked LTD_{AMPAR}. However, blockade of several distinct pathways led to a decrease in surface AMPARs under basal conditions (Man et al., 2007a), suggesting that interference with these pathways could occlude the KA-induced loss of surface AMPARs. An intriguing possibility is that such pathways could be involved in 'priming' KAR-LTD_{AMPAR} by suppling removable AMPARs to the cell surface, rather than in the direct mechanism of KAR-mediated AMPAR removal. Further work will therefore be required to distinguish between these possibilities.

Notwithstanding these potential caveats, I believe my results provide compelling evidence that agonist activation of GluK2-containing KARs mediates a novel form of LTD. While we have not yet identified the physiological/pathological conditions under which KAR-LTD_{AMPAR} occurs in intact hippocampal slices or *in vivo*, future experiments to define the precise physiological induction conditions, roles, and consequences of

KAR-LTD$_{AMPAR}$, and the relationship of this to other forms of LTD represent exciting avenues for future investigation.

In chapter 5, I use electrophysiology to show that KAR-dependent signalling is altered in GluK2 editing-deficient mice, emphasising the relevance of GluK2 Q/R editing. Future work will aim to analyse the subunit composition and the number of KARs expressed in these mice using biochemical tools to better understand the mechanisms underlying the altered kinetics and signalling of KARs. In addition, we have not yet explored how KAR-LTP$_{AMPAR}$ is affected in the GluK2 editing-deficient mice. Finally, because so little has been reported on these mice, they also offer the future opportunity to investigate how different aspects of learning and behaviour are altered by GluK2 editing. Clearly, although we have made significant progress, the tools, and systems we have used provide exciting avenues for future research into the importance of KARs in synaptic function, and their dysfunction in various neurological disorders.

Chapter 7. References

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Appendix- i: Published papers

Appendix - i

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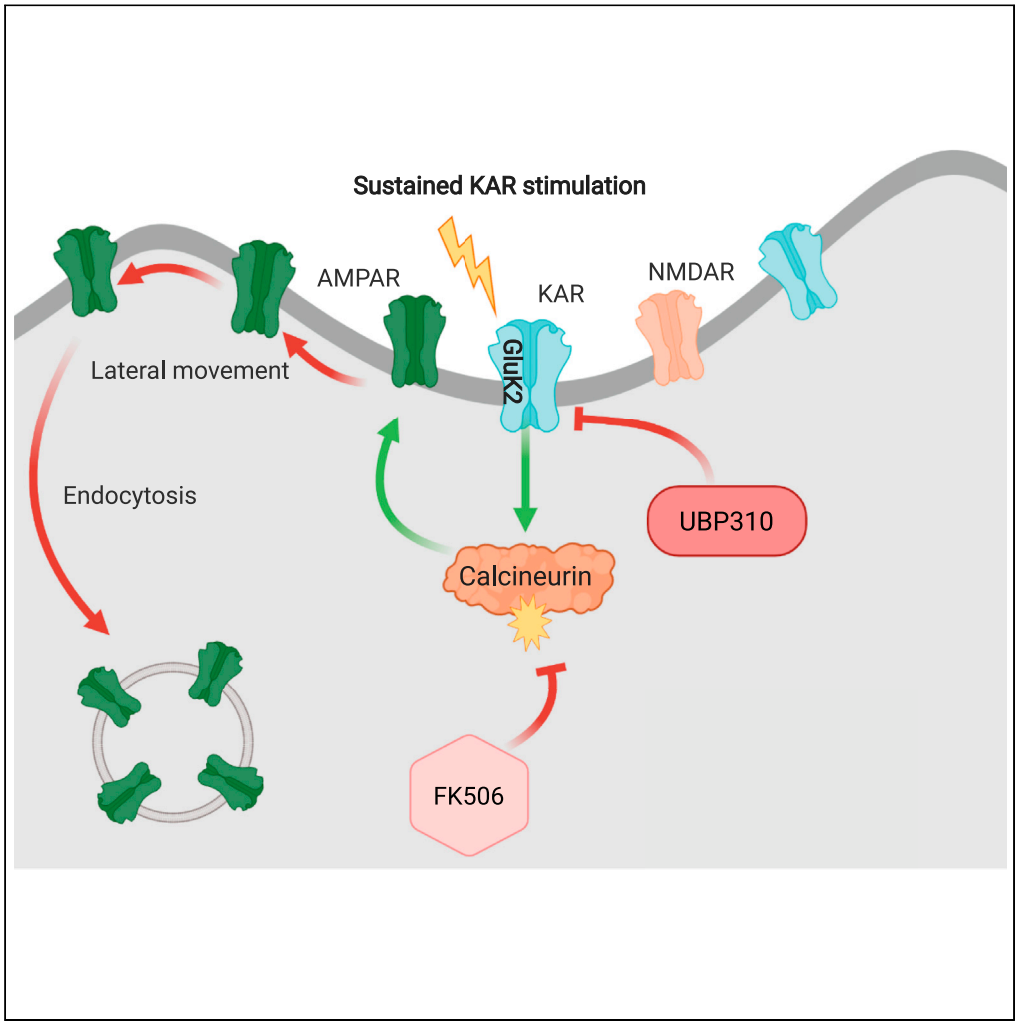
Article

Sustained postsynaptic kainate receptor activation downregulates AMPA receptor surface expression and induces hippocampal LTD



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Highlights

Sustained kainate receptor (KAR) activation downregulates surface AMPARs

KAR ionotropic signaling induces AMPAR LTD (KAR-LTD$_{AMPAR}$)

KAR-LTD$_{AMPAR}$ is dependent on the GluK2 KAR subunit and calcineurin

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Article

Sustained postsynaptic kainate receptor activation downregulates AMPA receptor surface expression and induces hippocampal LTD

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SUMMARY

It is well established that long-term depression (LTD) can be initiated by either NMDA or mGluR activation. Here we report that sustained activation of GluK2 subunit-containing kainate receptors (KARs) leads to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) endocytosis and induces LTD of AMPARs (KAR-LTD$_{AMPAR}$) in hippocampal neurons. The KAR-evoked loss of surface AMPARs is blocked by the ionotropic KAR inhibitor UBP 310 indicating that KAR-LTD$_{AMPAR}$ requires KAR channel activity. Interestingly, however, blockade of PKC or PKA also reduces GluA2 surface expression and occludes the effect of KAR activation. In acute hippocampal slices, kainate application caused a significant loss of GluA2-containing AMPARs from synapses and long-lasting depression of AMPAR excitatory postsynaptic currents in CA1. These data, together with our previously reported KAR-LTP$_{AMPAR}$, demonstrate that KARs can bidirectionally regulate synaptic AMPARs and synaptic plasticity via different signaling pathways.

INTRODUCTION

By activating distinct classes of both ionotropic and metabotropic receptors, glutamate mediates the overwhelming majority of excitatory neurotransmission in the mammalian central nervous system (CNS) and is critical for nearly all aspects of brain function. Ionotropic glutamate receptors comprise N-methyl-D-aspartate receptor (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), and kainate receptor (KAR) subtypes, whereas metabotropic glutamate receptors comprise 3 subfamilies of 8 mGluRs (groups I-III; mGluR1-8) (Collingridge et al., 2009).

Each of these receptor subtypes has been implicated in forms of synaptic plasticity that enhance or decrease the efficiency of synaptic transmission; long-term potentiation (LTP) and long-term depression (LTD), respectively. LTP and LTD occur at many different synapses across the CNS and are critical throughout life for processes ranging from synaptic formation and maturation during development, through to high level cognitive functioning and multiple aspects of learning and memory (Citri and Malenka, 2008; Malenka and Bear, 2004).

In part due to their fundamental roles in learning and memory, dysregulation of LTP and LTD is a prominent feature of cognitive decline in aging and a wide range of neurological and neurodegenerative disorders (Cuestas Torres and Cardenas, 2020; Kauer and Malenka, 2007). While the best characterized forms of LTP and LTD are initiated by activation of postsynaptic NMDARs (Collingridge et al., 2013; Hardingham, 2019), other induction pathways also exist. For example, there are mGluR-dependent forms of both LTP and LTD (Anwyl, 2009; Gladding et al., 2009), and more recently, it has been shown that activation of KARs can induce of LTP of AMPARs (Petrovic et al., 2017).

Regardless of the induction pathway, changes in the numbers and properties of surface-expressed postsynaptic AMPARs, and the consequent persistent strengthening or weakening of AMPAR-mediated transmission, underpin LTP and LTD (Henley and Wilkinson, 2016; Malenka and Nicoll, 1999). In summary, increases in surface-expressed synaptic AMPARs lead to functional and structural LTP, whereas decreases in surface-expressed synaptic AMPARs lead to LTD (Diering and Huganir, 2018; Huganir and Nicoll, 2013).

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Compared with NMDARs and AMPARs, the functional roles of synaptic KARs have been enigmatic, but accumulating evidence demonstrates that KARs are key regulators of synaptic function (Evans et al., 2019). KARs can be expressed at both pre- and postsynaptic sites throughout the brain, where they contribute to the regulation of transmission, neuronal excitability, and network activity (Lerma and Marques, 2013). Intriguingly, in addition to operating as an ionotropic receptor, KARs can also signal through a non-canonical pertussis toxin-sensitive G protein-dependent pathway that is independent of ion flow through the channel (Melyan and Wheal, 2011; Rodriguez-Moreno and Sihra, 2007).

Previous work has demonstrated that relatively low level stimulation of KARs (10 μM KA for 3 min) in cultured neurons and hippocampal slices increases surface expression of KARs (Carta et al., 2013; Martin et al., 2008; Rivera et al., 2007; Selak et al., 2009) and leads to spine growth mediated by changes in post-endocytic sorting and enhanced recycling (Gonzalez-Gonzalez and Henley, 2013). Building on these observations, we examined whether transient KAR activation also impacts on AMPARs. We found that metabotropic KAR signaling can promote AMPAR surface expression and increases both AMPAR colocalization with PSD95 and AMPAR mEPSCs in hippocampal neurons and slices, revealing a physiologically relevant form of postsynaptic KAR-dependent, NMDAR-independent, LTP (KAR-LTP$_{AMPAR}$) (Petrovic et al., 2017).

In contrast with transient KA stimulation, sustained stimulation of KARs (10 μM KA for 20 min) leads to their long-lasting loss from the cell surface (Gonzalez-Gonzalez and Henley, 2013; Martin et al., 2008; Martin and Henley, 2004). However, whether sustained KAR stimulation can also induce plasticity of AMPARs has not been investigated. Here we report a form of AMPAR LTD that can be induced by sustained stimulation of KARs (KAR-LTD$_{AMPAR}$).

RESULTS

Sustained KA treatment decreases surface levels of both AMPARs and KARs

Most AMPARs are heterotetramers of GluA1/GluA2 subunits or GluA2/GluA3 subunits (Zhao et al., 2019). Therefore, we first tested the effects of sustained KA application (10 μM KA for 20 min) on surface expression levels of the AMPAR subunits GluA1 and GluA2, and the KAR subunit GluK2, in cultured hippocampal neurons. Neurons were pre-treated for 30 min with 1 μM tetrodotoxin (TTX), to prevent depolarization-evoked presynaptic glutamate release, and 40 μM GYKI 53655, an AMPAR-specific antagonist (Partin and Mayer, 1996; Paternain et al., 1995), to prevent direct activation of AMPARs by KA. In parallel, on neurons from the same dissection, we used a well-established NMDAR-mediated chem-LTD protocol comprising 20 μM NMDA and 20 μM glycine for 3 min, followed by a 17-min incubation in the absence of NMDA, to allow receptor internalization (Ashby et al., 2004; Glebov et al., 2015; Lee et al., 1998). Surface proteins were then labeled by biotinylation and isolated by streptavidin pulldown. Surface proteins and whole-cell lysates (total protein) were analyzed by Western blotting for GluA2, GluA1, and GluK2 (Figure 1A). Surface expression of GluA2, GluA1, and GluK2 were all significantly reduced to similar levels by both KA and NMDA treatment. Importantly, these effects are selective since there was no change in the surface expression of EGFR, a non-iGluR protein used as a control.

KA is a partial, weakly desensitizing agonist at AMPARs (Levchenko-Lambert et al., 2011). Therefore, to further validate that activation of KARs, rather than direct KA agonism of AMPARs, evokes the loss of surface AMPARs we also tested 1 μM KA, a concentration below the threshold for AMPAR activation (Dai et al., 2001) so inclusion of the AMPAR inhibitor GYKI 53655 was not required. In these, and subsequent experiments, we focused on surface expression of GluA2, which is routinely used as a reporter for AMPAR endocytosis (Evers et al., 2010; Nishimune et al., 1998). As for 10 μM KA stimulation, surface levels of GluA2, assessed by surface biotinylation followed by Western blotting, were significantly reduced by 1 μM KA application (Figure 1B).

KA application decreases surface GluA2 in dendrites

We next used live cell surface staining and fixed confocal imaging to monitor GluA2 surface expression in control and KA-treated hippocampal neurons. Neurons were pre-treated with 40 μM GYKI 53655 and 1 μM TTX for 30 min prior to application of 10 μM KA for 20 min, followed by live surface labeling of AMPARs with an N-terminal anti-GluA2 antibody. Consistent with the biochemical data, our imaging showed a significant reduction in the surface levels of GluA2 in segments of both proximal and branched secondary dendrites (Figure 2A).

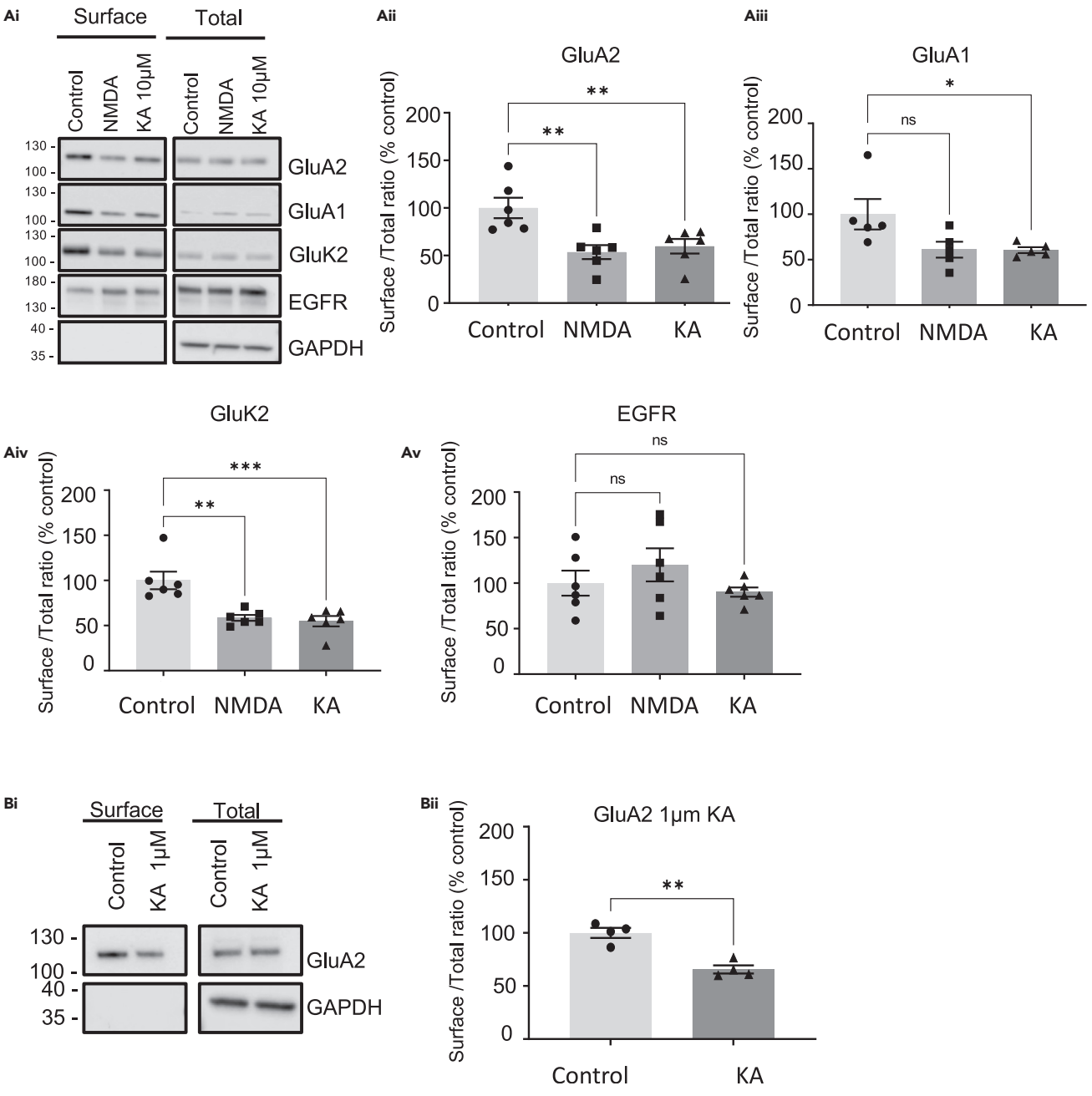

Figure 1. KAR activation reduces surface expression of AMPARs and KARs

DIV 18 cultured hippocampal neurons were pre-treated for 30 min with 1 μM TTX and 40 μM GYKI 53655 before treatment with vehicle or 10 μM or 1 μM KA for 20 min. For NMDA treatment neurons were pre-treated with 1 μM TTX for 30 min followed by 3 min of treatment with 20 μM NMDA and 20 μM glycine. Surface proteins were biotin labeled and isolated by streptavidin pulldown, and lysates and surface fractions Western blotted.

(A) (Ai) Representative Western blots of surface and total levels of GluA2, GluA1, GluK2, and EGFR. EGFR was used as a non-glutamate receptor expressed on the neuronal surface. GAPDH was used as a control to ensure no internal proteins were biotinylated. The surface to total ratio was calculated and expressed as a percentage of the control for (Aii) GluA2, (Aiii) GluA1, (Aiv) GluK2, and (Av) EGFR. N = 6 experiments from independent dissections, *p < 0.05, **p < 0.01, ***p < 0.001; one-way ANOVA with Dunnett's multiple comparisons test, error bars = SEM.

(B) 1 μM KA effectively reduces surface expression of GluA2-containing AMPARs. (Bi) Representative Western blots of surface and total levels of GluA2. (Bii) Quantification of the surface to total ratio of GluA2 expressed as a percentage of control. N = 4 experiments from independent dissections, **p < 0.01, Un-paired t test with Welch's correction, error bar = SEM.

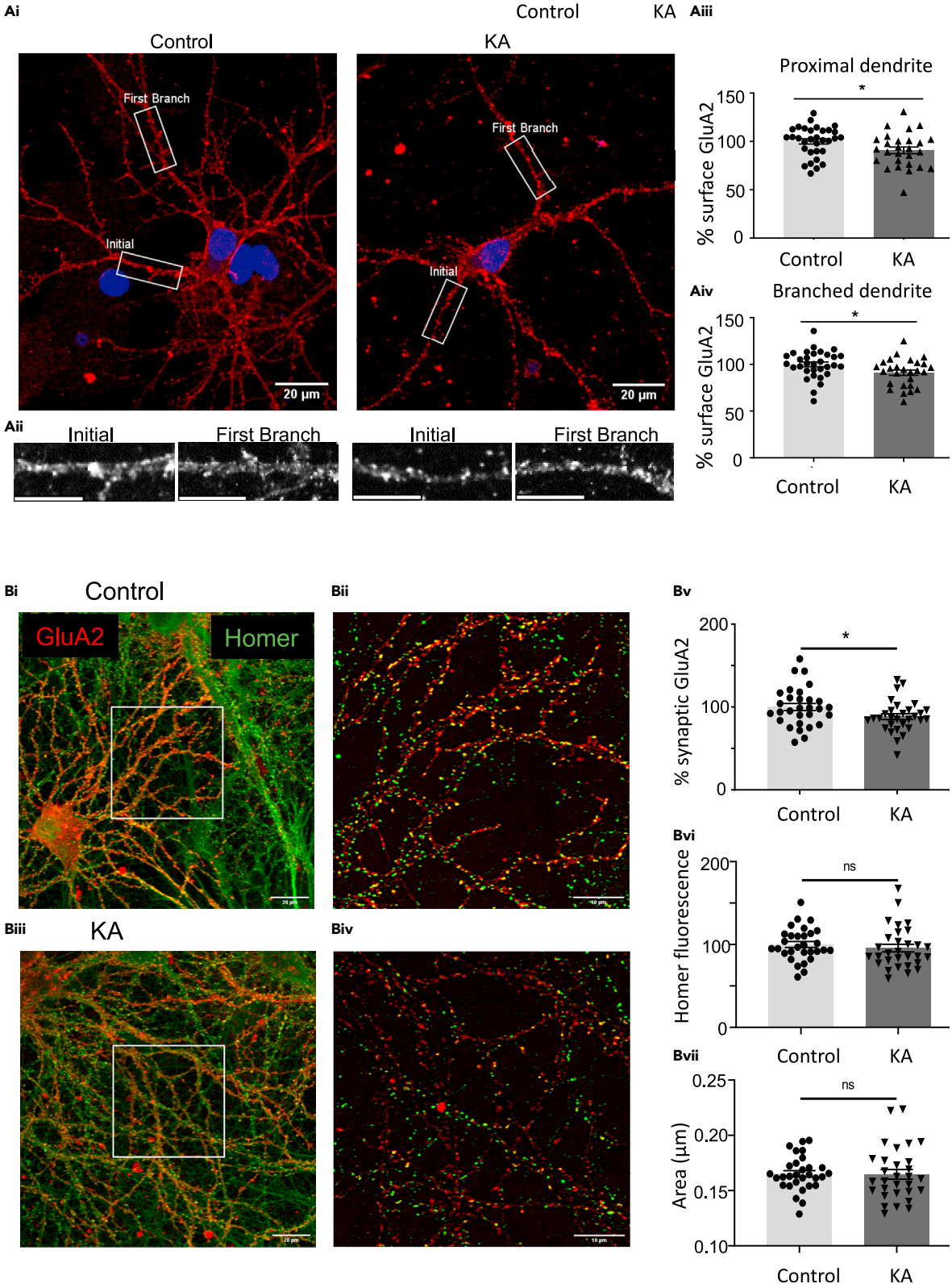
Ai
Control
KA
First Branch
Initial
20 μm
Aii
Initial
First Branch
Aiii
Proximal dendrite
% surface GluA2
Control
KA
Aiv
Branched dendrite
Bi
Control
GluA2
Homer
Bii
Biii
KA
Biv
Bv
% synaptic GluA2
Bvi
Homer fluorescence
ns
Bvii
Area (μm)

Figure 2. Imaging of DIV 18 hippocampal neurons shows a significant reduction in GluA2 surface expression after 10 μM KA for 20 min

(A) Surface GluA2 in dendrites. (Ai) Representative images of control and KA-treated neurons showing proximal and first branch dendrites. Scale bar = 20 μm. (Aii) Expanded images of ROIs indicated in boxes in (Ai). Scale bar = 10 μm. (Aiii) Quantification of the intensity of surface GluA2 staining in proximal dendrites. (Aiv) Quantification of the intensity of surface GluA2 staining after the first dendritic branch. In all cases n = 32 cells, N = 3 independent dissections, *p < 0.05, Un-paired t test with Welch's correction, error bars = SEM.

(B) GluA2 surface expressed at synapses. Representative images of control and KA-treated neurons of surface GluA2 colocalised with the postsynaptic marker Homer. (Bii and Biv) are zooms of the ROI areas indicated in (Bi) and (Biii). Scale bars = 20 μm and 10 μm, respectively. (Bv) Quantification of the number of surface-expressed synaptic GluA2 ± KA treatment expressed as percentage of control. (Bvi) quantification of Homer (marker of synapse) fluorescence expressed as percentage of control ± KA treatment. (Bvi) area of Homer fluorescence ± KA treatment. In each condition n = 10 ROIs, each containing between 47 and 461 particles, were analyzed and averaged. Data from N = 3 independent dissections, *p < 0.05, Un-paired t test, error bars = SEM.

KA application decreases synaptic surface-expressed GluA2

To determine if the decrease in GluA2-containing AMPARs occurred at synapses, we monitored surface GluA2 expression specifically at puncta positive for the synaptic marker Homer (Hayashi et al., 2009; Tao-Cheng et al., 2014). As expected, the mean fluorescence of surface-expressed GluA2 specifically at Homer puncta was a significantly decreased (Figures 2Bi–Biv). Quantification of the data show that surface GluA2-containing AMPARs are decreased at synapses, but that the mean fluorescence and area of Homer puncta are not altered by KAR activation (Figures 2Bv–Bvii).

KA-induced decreases in AMPAR surface expression are independent of NMDARs and mGluRs

To exclude any indirect effect mediated by either NMDARs or mGluRs, we pre-treated neurons with the NMDAR antagonist D-APV (50 μM) (Morris, 1989) or a combination of the mGluR5 antagonist (MPEP) (Lea and Faden, 2006) and the mGluR1 antagonist YM298198 (Kohara et al., 2005) (10 μM and 1 μM, respectively). As shown in Figure 3, 10 μM KA still caused a significant decrease in GluA2 surface expression in the presence of these drugs, whereas there was no change in surface EGFR levels under any condition, indicating the KA-induced decrease in surface GluA2 occurs in the absence of NMDAR and mGluR activity.

GluK2 is required for the KA-evoked decrease in AMPAR surface expression

We have shown previously that the GluK2 KAR subunit is required for induction of KAR-LTP$_{AMPAR}$ in the hippocampus (Petrovic et al., 2017). We therefore explored the role of GluK2-containing KARs in the KA-dependent reduction of surface GluA2 levels. Neurons were infected with lentiviruses either expressing a GluK2-targeting shRNA or a control virus expressing a non-targeting control shRNA. Consistent with our previous reports (Evans et al., 2017; Gurung et al., 2018), the GluK2 shRNA reduced total GluK2 levels by ~70% (Figures 4A and 4B), but there was no significant difference in levels of total or surface GluA2 between control and GluK2 knockdown conditions (Figures 4A and 4C).

7 days post-transduction, neurons were pre-treated with 40 μM GYKI 53655 and 1 μM TTX for 30 min, prior to application of 10 μM KA for 20 min. Following the KA challenge neurons were subjected to surface biotinylation, streptavidin pulldown, and Western blotting (Figures 4A and 4D). As expected, in neurons expressing the control shRNA, KAR activation significantly decreased GluA2 surface expression. The KA-induced reduction in surface GluA2 did not occur in GluK2 knockdown neurons, indicating that the KA-mediated reduction in GluA2 surface expression requires the GluK2 KAR subunit.

KA-evoked downregulation of surface AMPARs requires ionotropic KAR signaling

To investigate the signaling pathway required for the KAR-induced decrease in surface GluA2-containing AMPARs, we used the ionotropic KAR blocker UBP 310 (Dolman et al., 2005; Grosenbaugh et al., 2018; Petrovic et al., 2017; Pinheiro et al., 2013) and the G$\alpha_{i/o}$ protein inhibitor pertussis toxin (PTx) (Petrovic et al., 2017). In contrast to KAR-LTP$_{AMPAR}$, blocking KAR metabotropic signaling for 1 hr with 1 μg/mL PTx prior to KA stimulation did not prevent the KA-induced reduction in GluA2 surface expression (Figures 5A and S1). However, 10 μM UBP 310, an antagonist of postsynaptic ionotropic signaling through GluK2/GluK5-containing KARs did block the KA-evoked reduction in surface GluA2. Thus, our data indicate that KAR channel activity, but not G-protein mediated signaling, is required to initiate the downregulation of AMPAR surface expression. We note, however, that UBP 310 caused a significant reduction in surface GluA2 levels in the absence of KA stimulation, suggesting KAR activity may be required to maintain surface AMPAR

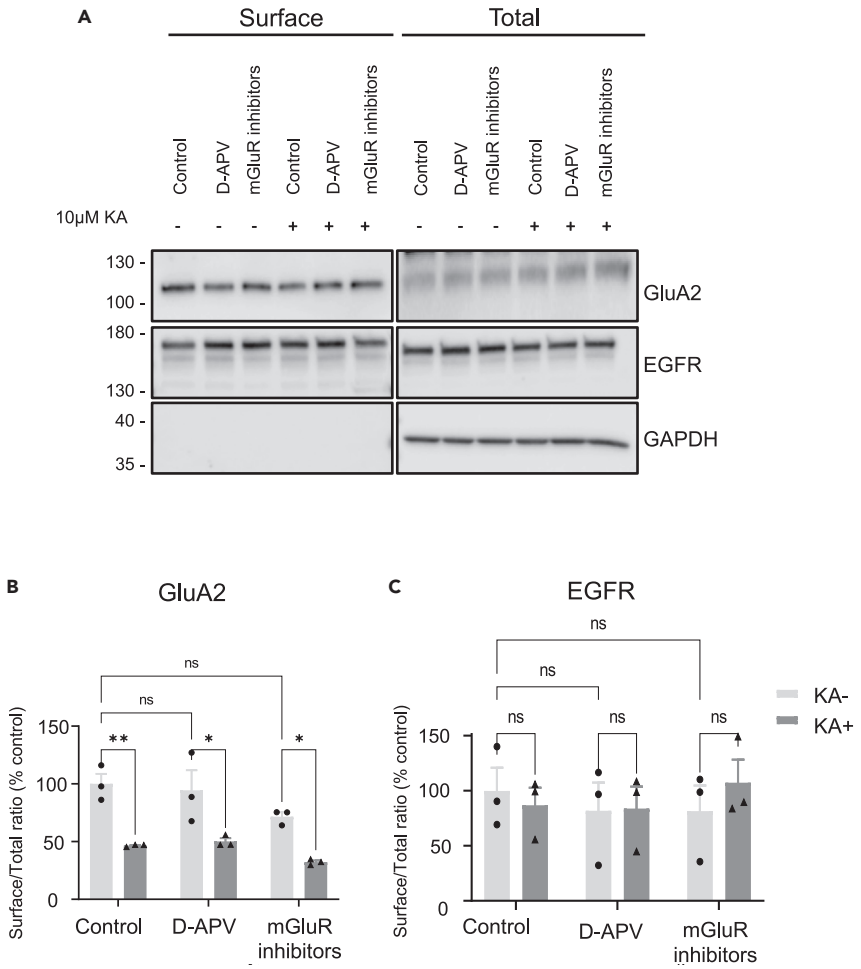

Figure 3. KA-induced decreases in AMPAR surface expression are independent of NMDAR or mGluR1/mGluR5 activation

DIV18 hippocampal neurons were pre-treated for 30 min with 50 μM D-APV (NMDAR antagonist) or 10 μM MPEP (mGluR5 antagonist) along with 1 μM YM298198 (mGluR1 antagonist) in addition to 1 μM TTX and 40 μM GYKI53655. Neurons were then incubated for a further 20 min with vehicle or 10 μM KA. Surface proteins were biotinylated, isolated, and Western blotted.

(A) Representative blot of surface and total levels of GluA2 and EGFR. GAPDH was used as a control to ensure no internal proteins were biotinylated.

(B and C) Quantification of the surface to total ratio of GluA2 (B) and EGFR (C) expressed as a percentage of control. N = 3 independent dissections, ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$; two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

expression. Consequently, the lack of KA-induced loss of surface GluA2 in the presence of UBP 310 may represent an occlusion effect.

Effects of PKA and PKC on KA regulation of surface GluA2

Phosphorylation cascades play key roles in all identified forms of synaptic plasticity (Lu and Roche, 2012), and kinase activation is an integral downstream component of both ionotropic and metabotropic KAR signaling pathways (Evans et al., 2019). We therefore explored the role of the protein kinases PKA and PKC in modulating surface expression of GluA2-containing AMPARs in response to KA treatment because of their well-established roles in AMPAR trafficking and KAR signaling (Gonzalez-Gonzalez and Henley, 2013; Konopacki et al., 2011; Martin et al., 2008; Martin and Henley, 2004). AMPAR surface expression was assessed by surface biotinylation after 30 min pre-treatment of cultured hippocampal neurons with TTX (1 μM), GYKI 53655 (40 μM), and the PKA or PKC-specific inhibitors H89 (10 μM) or chelerythrine

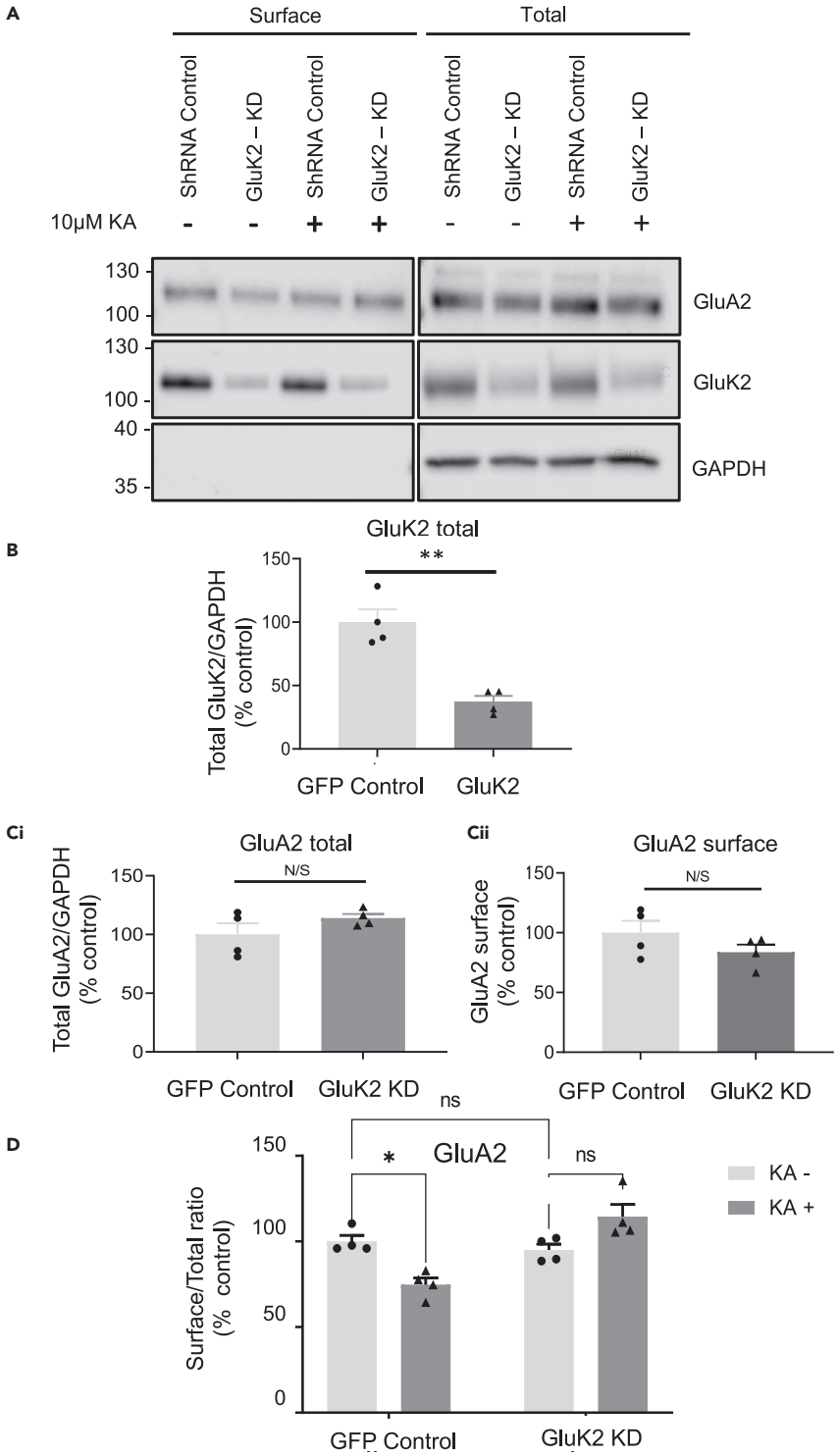

Figure 4. GluK2-containing KARs mediate the reduction in surface expression of AMPARs

(A) DIV 10 hippocampal neurons were transduced with lentivirus expressing either control or GluK2 shRNA. After 7 days neurons were pre-treated for 30 min with 1 μM TTX and 40 μM GYKI 53655. Neurons were then treated with 10 μM KA or vehicle for another 20 min. Surface proteins were biotinylated, isolated, and Western blotted. Representative Western blots of surface and total levels of GluA2 and GluK2

(B) Total GluK2 levels following infection with GluK2 shRNA knockdown lentivirus.

Figure 4. *Continued*
(C) Total (Ci) and surface (Cii) expressed GluA2 levels following GluK2 knockdown lentivirus. N = 4 independent dissections, ns = $p > 0.05$, **$p < 0.01$, Un-paired t test with Welch's correction, error bars = SEM.
(D) Quantification of the surface to total ratio of GluA2 expressed as a percentage of control. N = 4 independent dissections, ns = $p > 0.05$, *$p < 0.05$, **$p < 0.01$, two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

(5 μM), respectively. Neurons were then subjected to 20 min of sustained 10 μM KA stimulation. Both kinase inhibitors trended toward reduced basal surface levels of GluA2 suggesting that PKA/PKC signaling pathways support the surface expression of GluA2-containing AMPARs. We hypothesize that H89 and chelerythrine, like UBP 310, may lead to the loss of 'removable' AMPARs from the neuronal surface to a 'floor level' so that KA stimulation cannot decrease them any further. Thus, blocking PKA or PKC occludes the KA-induced reduction in GluA2 surface expression (Figure 5B).

In addition to kinases, the protein phosphatases PP1, PP2A, and PP2B (calcineurin) have been reported to be involved in forms of LTD (Belmeguenai and Hansel, 2005; Groth et al., 2003). We therefore investigated the effects of pre-treatment with inhibitors of these phosphatases (Figure 5C). We tested the effects of 1 μM okadaic acid to inhibit PP1 and PP2A (Schnabel et al., 2001), or 50 μM FK506 to inhibit calcineurin/PP2B (Beattie et al., 2000), on the KA-evoked decrease in GluA2 surface expression. Okadaic acid reduced surface levels of GluA2 in non-stimulated conditions, indicating roles for the protein phosphatases PP1/PP2A in regulating basal surface expression of AMPARs. These data suggest that okadaic acid occludes, whereas FK506 blocks the KA-evoked decrease in GluA2 surface expression.

Together, these data indicate that the KAR-induced decrease in surface GluA2-containing AMPARs does not occur in the absence of KAR channel opening. Moreover, blockade of PKC or PKA, which are activated downstream of ionotropic as well as metabotropic signaling, also reduces GluA2 surface expression and occludes the effect of KAR activation, demonstrating that PKA/PKC signaling support the surface targeting of GluA2. Interestingly, blocking dephosphorylation with phosphatase inhibitors also prevents the KAR-mediated decrease in GluA2 surface expression. The multiple phosphatase pathways involved in plasticity are complex (Foley et al., 2021), and our results indicate that both phosphorylation and dephosphorylation play key roles both in supplying surface AMPARs and allowing their removal in response to KA.

KAR stimulation induces both short-term and long-term synaptic depression

We next investigated the effects of KA stimulation on synaptic function by monitoring AMPAR excitatory postsynaptic currents (EPSCs) in the CA1 region of acute rat hippocampal slices. Here, where key circuitry remains intact, we bath applied 1 μM KA for 10 min to avoid non-specific activation of AMPARs without the need for perfusion of GYKI 53655. Also included in the perfusate was 50 μM picrotoxin to prevent any confounding effect of KAR-induced GABA release from inhibitory interneurons and 50 μM D-AP5 to block NMDARs. Consistent with our biochemistry and imaging, AMPAR EPSCs were significantly reduced for 20 min after KA washout (Figure 6A-D). These results demonstrate that sustained KA application causes a long-term, NMDAR-independent reduction in synaptic AMPAR currents at CA1 synapses.

We also measured the paired pulse ratio (PPR) following KA stimulation to assess any changes in the probability of glutamate release (Debanne et al., 1996; Manabe et al., 1993). Immediately following KA stimulation, we observed an increase in PPR, indicating a decrease in the release probability characteristic of presynaptic short-term depression (Figure 6E-G) (Lauri et al., 2006). Importantly, however, the PPR returned to baseline levels within 10–20 min after KA washout, whereas AMPAR EPSCs remained depressed, suggesting the existence of a postsynaptic LTD of AMPAR transmission. We interpret these data to indicate that 1 μM KA for 10 min mediates both pre- and postsynaptically expressed depression of AMPAR EPSCs.

DISCUSSION

Both ionotropic and metabotropic KAR signaling pathways are important regulators of excitatory and inhibitory neurotransmission (Evans et al., 2019). We have shown previously that sustained KAR activation causes a PKC-dependent, PKA-independent, internalization of GluK2-containing KARs (Martin and

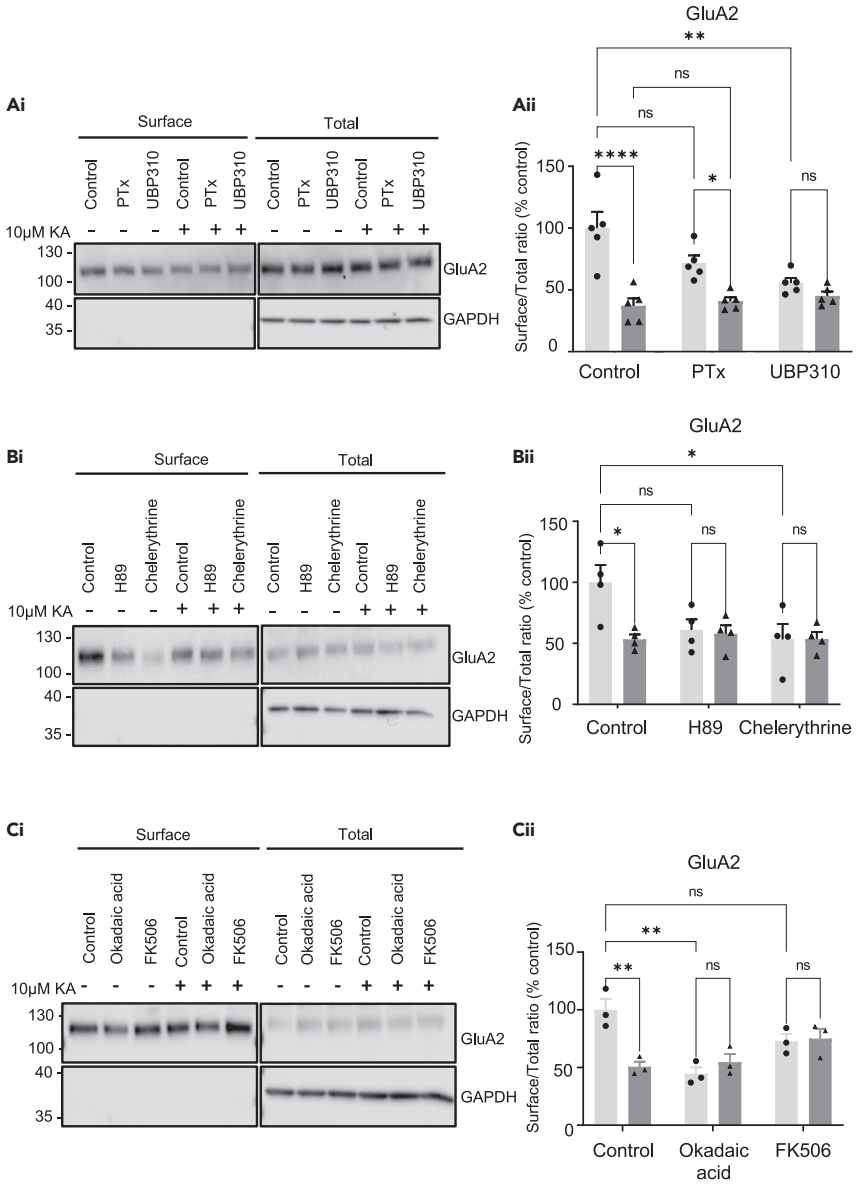

Figure 5. KA-induced decreases in AMPAR surface expression require ionotropic KAR signaling, PKA, and PKC

(A) DIV 18 hippocampal neurons were pre-treated for 1 hr with 1 μg/mL PTx, or 30 min with 10 μM UBP 310 in addition to 1 μM TTX and 40 μM GYKI53655, then for a further 20 min with vehicle or 10 μM KA. Surface proteins were biotinylated, isolated, and Western blotted. (Ai) Representative blot of surface and total levels of GluA2. GAPDH was used as a control to ensure no internal proteins were biotinylated. (Aii) Surface to total ratio of GluA2. N = 5 independent dissections. See also Figure S1.

(B) As (A), except neurons were pre-treated 40 μM GYKI 53655 and either 10 μM H89 or 5 μM Chelerythrine prior to 10 μM KA. (Bi) Representative blots of GluA2. (Bii) Quantification of the surface to total ratio of GluA2 expressed as a percentage of control. N = 4 independent dissections.

(C) As (A), except neurons were pre-treated were pre-treated for 30 min with 1 μM Okadaic acid (PP1A and PP2A inhibitor) or 50 μM FK506 (calcineurin inhibitor) prior to 10 μM KA. (Ci) Representative blots of GluA2. (Cii) Quantification of the surface to total ratio of GluA2 expressed as a percentage of control. N = 3 independent dissections. In all cases ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ***$p < 0.0001$; two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

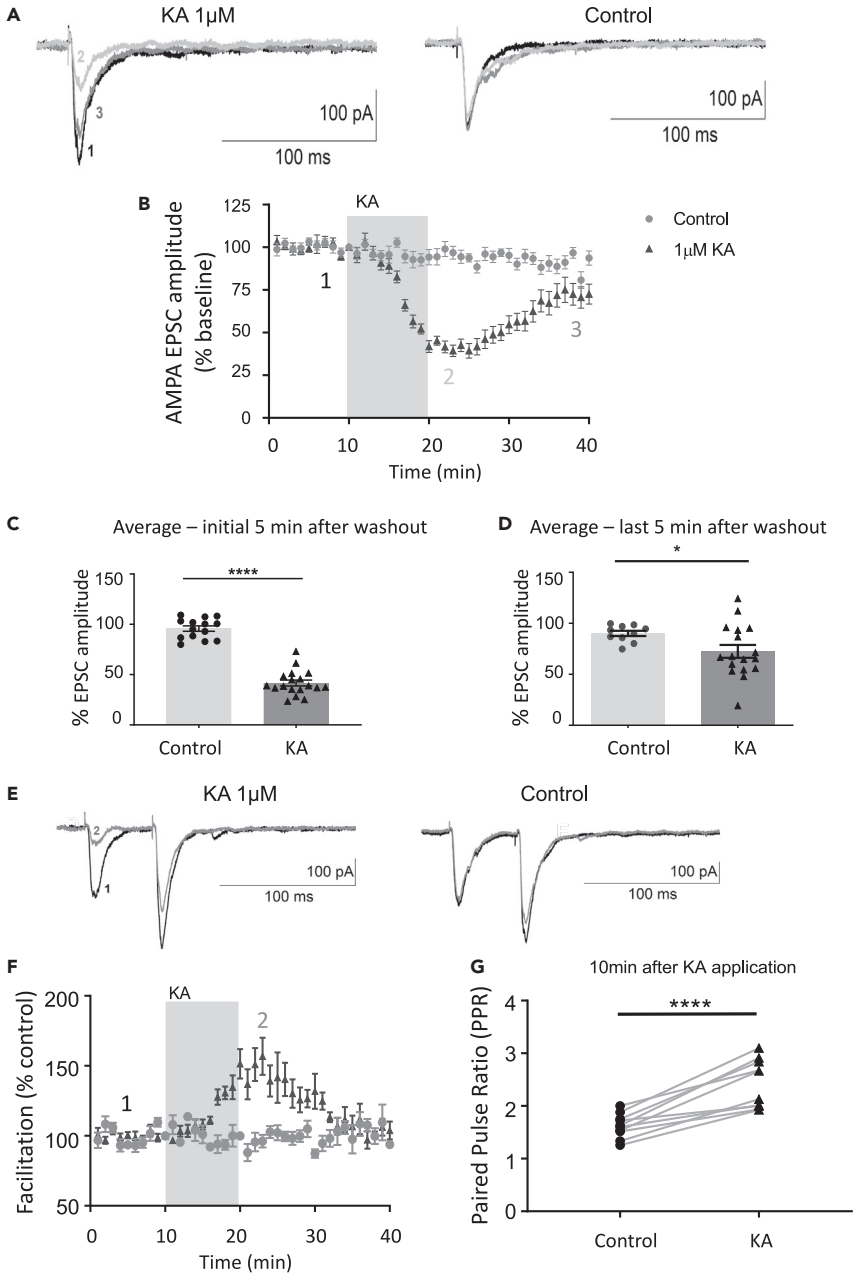

Figure 6. Sustained KAR activation induces depression of AMPAR EPSCs at hippocampal CA1 synapses

(A) Example AMPAR EPSC traces in the presence or absence of 1 μM KA recorded at time points indicated in (B) in the CA1 region of acute hippocampal slices.

(B) Plot of AMPAR EPSC amplitude. EPSCs were normalized to baseline corresponding to an initial 10 min prior to KA application. The traces were recorded in the presence of 50 μM D-AP5 and 50 μM picrotoxin. n = 14 and 18 cells for control and KA, respectively, from at least 4 different animals.

(C and D) Quantification of mean AMPAR EPSC amplitudes in 5 min (C) and 20 min (D) after KA washout. Un-paired t test with Welch's correction, error bars = SEM. n = 14 and 18 cells for control and KA, respectively, from at least 4 different animals.

(E) Example AMPAR paired pulse EPSC traces in the presence or absence of 1 μM KA, recorded at time points indicated in (F).

Figure 6. *Continued*

(F) Plot of paired pulse facilitation expressed as a percentage of control after 10 min of sustained stimulation with 1 μM KA. N = 10 cells from at least 4 different animals. Error bars = SEM.

(G) Quantification of paired pulse ratio after 10 min of stimulation with 1 μM KA. N = 10 cells, ns = $p > 0.05$, *$p < 0.05$, **$p < 0.01$,***$p < 0.001$, ****$p < 0.0001$, paired t test.

Henley, 2004). Surprisingly, however, we observed that following an initial loss of surface KARs, a short kainate application elicited a long-lasting increase in surface-expressed KARs to levels significantly greater than those prior to the agonist challenge (Martin et al., 2008). We further showed that after initial endocytosis, transient agonist activation evokes increased KAR exocytosis via metabotropic signaling, which recruits Rab11-dependent, transferrin-positive endosomes to synapses and increases KAR recycling (Gonzalez-Gonzalez and Henley, 2013). Overall, these data suggest that KARs are subject to bidirectional regulation, allowing neurons to adapt their physiological responses to changes in synaptic activation.

These findings raised the question as to whether the KAR-initiated signaling was limited to the regulation of KARs themselves or whether KAR-mediated signaling also impacts on other synaptic proteins. Therefore, building on our KAR work we investigated if transient KAR activation also affected AMPARs. We demonstrated that both pharmacological and synaptic activation of KARs induced increased surface expression of AMPARs and evoked a KAR-dependent, NMDAR-independent, form of hippocampal LTP (KAR-LTP_{AMPAR}) through a metabotropic signaling pathway (Petrovic et al., 2017).

In the current study, we show that prolonged KAR signaling can also downregulate AMPAR surface expression and evoke KAR-dependent, NMDAR-independent LTD (KAR-LTD_{AMPAR}). Moreover, the KA-evoked reduction in surface AMPARs did not occur in GluK2 knock down neurons, indicating that GluK2-containing KARs mediate this effect. In contrast to KAR-LTP_{AMPAR}, inhibition of metabotropic signaling did not prevent KAR-LTD_{AMPAR}, whereas UBP 310, an antagonist of postsynaptic ionotropic signaling through GluK2/GluK5-containing KARs (Grosenbaugh et al., 2018; Petrovic et al., 2017; Pinheiro et al., 2013), did. The basal surface expression of GluA2 appeared to be reduced in the presence of metabotropic and/or ionotropic inhibitors, although this was not significant for PTx. It should be noted that PTx inhibits the signaling of multiple G protein-coupled receptors in addition to the metabotropic actions of KARs. The significant decrease in basal GluA2 surface levels in the presence of UBP 310 suggests KARs may also be required for maintaining tonic activity of AMPARs. Thus, through a yet unidentified pathway, UBP 310 can occlude LTD by decreasing the population of "endocysosable" AMPARs at the cell surface thereby diminishing the pool available for removal by KAR activation.

The mechanisms and roles of AMPAR subunit phosphorylation have been an area of intense study for many years (for reviews see Lu and Roche (2012) and Wang et al. (2014)). Phosphorylation of the GluA1 subunit, which contains phosphorylation sites for PKC and PKA, as well as sites for other kinases, has been most extensively investigated. The functional implications of AMPAR subunit phosphorylation have been questioned by one study which reported almost no phosphorylated GluA1 *in vivo* (Hosokawa et al., 2015). However, a subsequent study directly refuted that finding, showing that 12%–50% of the total population of GluA1 is phosphorylated under basal and stimulated conditions *in vitro* and *in vivo* (Diering et al., 2016). Moreover, it has been reported previously that activation of PKA with forskolin promotes surface trafficking of GluA1-containing AMPARs without affecting rates of endocytosis, resulting in increased AMPAR surface expression (Man et al., 2007). Our experiments using the PKA inhibitor H89 decreased AMPAR surface expression, consistent with reduced surface trafficking but unaffected endocytosis. Thus, similar to our data using UBP 310, we propose that a lack of 'readily endocytosable' AMPARs at the cell surface in the presence of H89 or chelerythrine occludes the KA-evoked reduction in surface AMPARs.

The GluA2 subunit contains a PKC site at Ser880 (Matsuda et al., 1999), located within the PDZ ligand at the extreme C-terminus, which selectively controls interactions with the PDZ domain containing protein GRIP1 (glutamate receptor-interacting protein) but not PICK1 (protein interacting with C kinase 1), which also binds the GluA2 PDZ ligand (Chung et al., 2000). It has been reported that PKC phosphorylation of Ser880 releases GRIP1, favoring PICK1 binding and leading to internalization of surface GluA2 (Chung et al., 2000). Moreover, a phosphomimetic mutant of Ser880 excludes GluA2-containing AMPARs from

synapses, depresses transmission, and partially occludes LTD. Conversely, a phosphonull mutation at Ser880 reduces LTD (Seidenman et al., 2003). However, the situation is complex, and it has also been reported that GRIP, rather than holding AMPARs at the surface, stabilizes internalized receptors in an intracellular pool, and prevents them from recycling back to the plasma membrane or entering a degradative pathway (Braithwaite et al., 2002; Daw et al., 2000). Moreover, KAR endocytosis involves PKC activation, whereas PKC phosphorylation promotes surface expression of GluA1-containing AMPARs, and these effects have been linked to regulation of interactions of GluK2 and GluA1 subunits with the cytoskeletal adaptor protein 4.1N (Copits and Swanson, 2013; Shen et al., 2000).

Our data suggest that blocking PKC or PKA reduce GluA2-containing AMPAR surface expression and occlude KAR-evoked LTD suggesting tonic PKC and PKA activity are required to maintain surface AMPAR levels under control conditions. Moreover, our results raise the possibility that KAR activation potentially causes LTD by reducing PKC/PKA activity. However, it is important to note that our experiments blocking PKC and PKA do not specifically target GluA2, and the effects we observe could be mediated via altering phosphorylation of other core or auxiliary subunits in the AMPAR complex and/or by actions on interacting proteins. Interestingly, it has been reported recently that KAR activation causes a Ca^{2+}- and PKC-dependent endocytosis of glycine receptors (GlyRs) and reduced GlyR-mediated synaptic activity (Sun et al., 2014). The proposed mechanism involves deSUMOylation of PKC and it is possible that an analogous PKC-deSUMOylation pathway may exist for the cross talk between KARs and AMPARs.

We also tested the effect of phosphatase inhibitor okadaic acid (PP1A and PP2A) and FK506 (calcineurin/PP2A). These too prevented the KA-evoked decrease in surface GluA2-containing AMPARs. Interestingly, okadaic acid reduced the basal levels of surface GluA2, indicating roles for the protein phosphatases PP1/PP2A in regulating basal surface expression of AMPARs. Together, we interpret these results to suggest that the dynamics of phosphorylation and dephosphorylation are clearly important both for maintaining the delivery of surface AMPARs and in their KAR-initiated removal.

The current consensus is that AMPAR surface expression and plasticity is mediated via phosphorylation (and other modifications) of the C-terminal domains of AMPAR subunits regulating the trapping or release of receptors at pre-existing "slots" in the postsynaptic density (PSD) (Hayashi et al., 2000; Henley and Wilkinson, 2016). However, in conditional knockout experiments in which GluA1, GluA2, and GluA3 subunits were ablated and replaced with either GluA1 or GluA2 subunits lacking a C-terminal domain, basal AMPAR trafficking and LTP were shown to be normal (Granger et al., 2013) (but see Zhou et al. (2018)). Most recently, a knock-in mouse study in which the C-terminal domain of endogenous GluA1 was truncated reported no deficit in basal synaptic transmission, LTP, or spatial memory (Diaz-Alonso et al., 2020). These authors propose an alternative model in which it is the number of slots rather than the C-terminal domains of the AMPAR subunits themselves that regulate the extent of AMPAR surface expression and plasticity at synapses (Diaz-Alonso et al., 2020). Thus, it is possible that phosphorylation is required to overcome a yet unidentified, negative modulatory effect, which is lacking in phosphodeficient AMPAR subunits. Overall, the currently available evidence suggests that plasticity involves the formation/unmasking or removal/masking of slots in the PSD to snare or release passively diffusing AMPARs, potentially irrespective of their phosphorylation or other posttranslational modifications (Diaz-Alonso et al., 2020).

In terms of our results, what then does this "slot availability" model predict? We suggest that constitutive PKC and PKA activity may be required to maintain slots, and thus blocking these kinases leads to a decrease in slot number, which may explain why blocking PKC and PKA occludes KAR-LTD_{AMPAR}. We note, however, that our data demonstrate that PKA/PKC signaling is required to sustain surface GluA2 levels, and we do not directly address whether these kinases regulate incorporation of GluA2 into synaptic slots, although we do show that synaptic GluA2 levels are decreased by KA stimulation.

Consistent with these biochemical data, synaptic electrophysiology revealed two phases to the depression of AMPAR EPSC responses following KA activation and washout. The initial depression in EPSCs correlated with a rise in the PPR, which returned to baseline after 10 min of KA washout. However, after this initial short-term change in PPR returned to control levels, AMPAR EPSCs remained depressed, indicating a postsynaptic locus of KAR-LTD_{AMPAR}. Thus, KA application reduces synaptic activity via an initial presynaptic

mechanism which contributes to the early stages of depression in AMPAR EPSCs, but KAR-mediated loss of postsynaptic surface-expressed AMPARs maintains KAR-LTD$_{AMPAR}$.

In summary, we show that in addition to transient KAR activation evoking KAR-LTP$_{AMPAR}$, sustained KAR activation can induce KAR-LTD$_{AMPAR}$. The underlying mechanisms appear to differ since KAR-LTP$_{AMPAR}$ is mediated via a metabotropic signaling pathway, whereas KAR-LTD$_{AMPAR}$ is blocked by the ionotropic KAR signaling blocker UBP 310. The physiological/pathological roles of KAR-mediated regulation of AMPAR surface expression at synapses and synaptic plasticity, and how they fit into the larger picture of NMDAR- and mGluR-mediated forms of plasticity remain to be elucidated. However, since KAR abundance and dysfunction is strongly linked to epilepsy (Crepel and Mulle, 2015; Peret et al., 2014), it is tempting to speculate that dysfunctional KAR-mediated plasticity of AMPARs, either directly or through affecting the availability of AMPAR slot proteins, could play important roles in neurological diseases.

Limitations of the study

We are mindful that the pharmacological inhibition of metabotropic (pertussis toxin) or ionotropic (UBP 310) signaling through KARs, as well as the inhibition of PKC (chelerythrine), PKA (H89), PP1A and PP2A (okadaic acid), and calcineurin/PP2B (FK506), although selective for their targets, will have wider effects than on solely KAR signaling. Nonetheless, we contend that the key point is that these drugs have distinct effects on the KA-evoked changes in surface GluA2, strongly implicating their target proteins in the underpinning mechanisms of KAR-evoked LTD$_{AMPAR}$. However, as noted above, inhibition of several of these pathways leads to a decrease in surface AMPARs under basal conditions, suggesting their blockade may occlude the KA-induced loss of surface GluA2. Potentially, these pathways could be involved in "priming" KAR-LTD$_{AMPAR}$ by suppling removable AMPARs to the cell surface, rather than in the direct mechanism of KAR-mediated AMPAR removal. Further work will therefore be required to distinguish between these possibilities.

We believe the data we present provide compelling evidence that GluK2-containing KARs can mediate LTD of AMPARs in cultured neuronal systems. This work, however, is restricted to pharmacological approaches using the application of kainate to induce this form of plasticity. We have not yet identified the physiological/pathological conditions under which KAR-LTD$_{AMPAR}$ occurs in intact hippocampal slices or *in vivo*. Experiments to define the precise physiological induction conditions, roles, and consequences of KAR-LTD$_{AMPAR}$, and the relationship of this to other forms of LTD represent exciting avenues for future investigation.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

J.D.N. performed all the biochemistry and neuronal live surface staining experiments with help from A.F.-J., R.S., and B.P.Y. E.B. performed the electrophysiology guided by Z.I.B. DNA constructs were made by K.A.W. J.M.H. and K.A.W. supervised the study. J.D.N., K.A.W., and J.M.H. wrote, and all authors contributed to reading and editing the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit Polyclonal Anti-Glutamate receptor 1	Millipore	AB1504
Mouse Monoclonal Anti-Glutamate receptor 2, extracellular clone,6C4	Millipore	MAB397
Rabbit Monoclonal Anti-GluR6/7, clone NL9	Millipore	04-921
Mouse Monoclonal Anti-Glutamate Receptor	BD Pharmingen	556341
Rabbit Monoclonal Anti EGFR	Abcam	Ab52894
Mouse Monoclonal Anti GAPDH	Abcam	Ab8245
Donkey Anti-Mouse Cy3	Jackson immuno	715-165-150
Chicken Polyclonal Anti-Homer 1	Synaptic Systems	160 006
Chemicals, Peptides, and Recombinant Proteins		
Chelerythrine	Sigma-Aldrich	C2932, CAS Number: 3895-92-9
CNQX	Sigma-Aldrich	C127, CAS Number: 115066-14-3
D-AP5	Tocris	Catalog Number: 0106
Glycine	Sigma-Aldrich	G8790, CAS Number: 56-40-6
GYKI 53655	Tocris	Catalog Number:2555
H89	Tocris	Catalog Number: 2910
Kainate	Tocris	Catalog Number: 0222
NMDA	Tocris	Catalog Number: 0114
Pertussis Toxin (PTx)	Tocris	Catalog Number: 3097
TTX	Tocris	Catalog Number: 1069
UBP 310	Tocris	Catalog Number: 2079
Picrotoxin	Sigma-Aldrich	P1675, CAS Number124-87-8
EZ-Link™ Sulfo-NHS-SS-Biotin	Thermo Fisher	Catalog Number: 21331
Streptavidin-Agarose from *Streptomyces Avidinii*	Sigma-Aldrich	S1638-5ML
Experimental models: cell lines		
Primary Rat Hippocampal Neuronal Cell cultures	(Fletcher-Jones et al., 2019; Konopacki et al., 2011).	N/A
Experimental Models: Organisms/Strains		
Wistar rat	University of Bristol Animal Services	N/A
Recombinant DNA		
GluK2 shRNA targeting: GCCGTTTATGACACTTGGA (pXLG3-GFP vector)	(Rocca et al., 2017)	N/A
Non-targeting shRNA: AATTCTCCGAACGTGTCAC (pXLG3-GFP vector)	(Rocca et al., 2017)	N/A
Software and Algorithms		
WinLTP v1.11 acquisition software	(Anderson and Collingridge, 2007)	https://www.winltp.com/
GraphPad Prism version 8.0	This paper	https://www.graphpad.com/scientific-software/prism/
LI-COR Biosciences ImageStudio Lite Version 5.2	(Evans et al., 2019)	https://www.licor.com/bio/image-studio-lite/download

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jeremy Henley (j.m.henley@bristol.ac.uk).

Materials availability

This study did not generate new unique reagents.

Dissociated primary neuronal culture

Pregnant E18 (Embryonic Day 18) Han Wistar rats were anesthetised with isoflurane and humanely sacrificed using schedule 1 method (cervical dislocation) after checking for reflexes. Brains of male and female embryos were removed under a dissection microscope, and the hippocampi excised for hippocampal cultures and the rest of the cortex was used for preparation of cortical cultures.

Dissected hippocampi and cortices were transferred to 15ml and 50ml falcon tubes respectively under a laminar airflow chamber and cortices were further dissociated using a sterile scalpel blade. Tissues were washed three times with 10ml HBSS for hippocampi and 30ml for cortices. Cortices were incubated for 15 mins in 30ml HBSS containing 0.005% trypsin/EDTA and hippocampi for 9 mins in 30ml HBSS containing 0.005% trypsin/EDTA at 37°C with frequent inverting. The trypsinised tissues were washed three times with HBSS and one wash with 1ml plating media for hippocampi and 5ml plating media for cortices. The cell suspensions were triturated with 1ml pipette for hippocampus and 5ml serological pipette for cortical tissues. Hippocampal and cortical cell suspension were diluted up to 4ml and 20ml respectively with plating media.

The cells were counted in a hemocytometer. For biochemistry experiments, 500,000 cells per well and for imagining 110,000 cells per well were plated in pre-treated 6 well tissue culture dish containing plating media and incubated in a 37°C incubator. 2 hrs after seeding the cells, the plating media was replaced with 2ml of feeding media (Neurobasal® medium supplemented with 2% B27 and 1% glutamax) and replaced in a 37°C incubator. 7 days later the neurones were fed with a further 1ml of feeding media and stored until use.

Acute hippocampal slice preparation from postnatal male and female Han Wistar rats for electrophysiology

Postnatal day 13-15 male and female Han Wistar rats were anaesthetized with 4% isoflurane and decapitated. Brains were rapidly removed and placed in 4°C oxygenated (95% O_2, 5% CO_2) sucrose solution (in mM: sucrose 189, D-glucose 10, $NaHCO_3$ 26, KCl 3, $MgSO_4$ 5, $CaCl_2$ 0.1 and NaH_2PO_4 1.25). Parasagittal hippocampal slices 400μm thick were prepared using a vibratome (7000smz-2, Campden Instruments). Slices were kept in a slices holder containing artificial cerebrospinal fluid (aCSF in mM): NaCl 124, $NaHCO_3$ 26, KCl 3, NaH_2PO_4 1, $MgSO_4$ 1, D-glucose 10 and $CaCl_2$ 2; and incubated for 30 mins at 35°C and then for a further 30 mins at room temperature before use.

Data and code availability

- **Data**: All data reported in this paper will be shared by the lead contact upon request.
- **Code**: This paper does not report original code.
- **Experimental model and subject details**: The following animals were obtained from animal services facility at the University of Bristol.

All the animal experiments and procedures were performed in compliance with the UK Animal Scientific Procedures act (1986) and were guided by the Home Office Licensing Team at the University of Bristol. All animal procedures relating to this study were approved by the Animal Welfare and Ethics Review Board at the University of Bristol.

METHOD DETAILS

Lentivirus production and transduction

DMEM was filter sterilized using a 0.2μM syringe filter and 2.5ml of plain DMEM was mixed with 20μl of XLG viral vector consisting of shRNA expressed under H1 promoter (pXLG3-100bp stuffer), 5μg pDMD2.G

packaging vector (Addgene) and 15μg of p8.91 helper vector (Addgene). The shRNA target sequences were Control, non-targeting shRNA: AATTCTCCGAACGTGTCAC; GluK2-targeting shRNA: GCCGTTTAT GACACTTGGA. 2.5ml of plain DMEM media was mixed with 4.8% 1mg/ml polyethylenimide (PEI) in a sterile 15ml falcon tube. The mixture was mixed thoroughly, and filter sterilized into a fresh 15ml falcon tube with a 0.2μM syringe filter. This was left at RT for 2-3 mins with occasional mixing. The transfection media was prepared by mixing PEI-DMEM with the DNA mixture. Culture plates containing HEK293T cells were washed twice with 6ml of plain media and 5ml of transfection media was slowly added and left for 4 hours at 37°C. After incubation, the transfection media was aspirated and supplied with 7ml of pre-warmed neuronal feeding media or DMEM. The cells were placed back in the incubator for 2-3 days to produce virus. Media containing the virus was transferred into a fresh 15ml falcon tube and centrifuged at to 3000g for 10 minutes at 4°C to pellet cellular debris. The virus containing supernatant was syringe filtered (0.45μM) into a fresh 15ml falcon, aliquoted and stored at -80°C until further use.

Sustained KA stimulation

Hippocampal neurons were plated in a 6 well culture dish (500,000 cells per well) and maintained in a 37°C incubator. On DIV 17-18, the neurons were pre-treated with 1μM TTX (Tocris) and GYKI 53655 (Tocris), with or without additional drugs in Earle's Buffer (140mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgCl_2$, 25mM HEPES, 0.9g/L D-Glucose, pH 7.4) and placed back in the incubator for 30 mins. After the incubation, 10μM KA was added to the wells and incubated for 20 mins at 37°C.

Neuronal surface biotinylation

After KA stimulation, neurones were cooled down to 4°C post treatment to prevent further trafficking of receptors. The wells were washed twice with 2ml of cold Earle's Buffer (EBS) (140mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgCl_2$, 25mM HEPES and 0.9g/L D-glucose). The surface proteins were tagged with 1.5ml of Sulfo-NHS-SS-Biotin (0.3mg/ml diluted in 1X EBSS) (Thermo Fisher, Cat No.21331), for 10 mins with gentle movement every 2 mins and washed three times with 2ml of EBSS. 2ml of 100mM NH_4Cl was added to scavenge the free biotin for 1 min. The cells were washed with 2ml of EBSS and were lysed in 200 μL lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1% triton X-100, 0.1% SDS, 1X protease inhibitor in ddH_2O), scraped and transferred to a 1.5ml eppendorf. The cells were sonicated briefly with 3 pulses and were incubated on ice for 30 mins. After incubation cells were centrifuged at 20,000g at 4°C for 30 mins to get rid of cell debris.

The surface proteins were pulled down with 150μl Pierce™ Streptavidin UltraLink™ resin beads. The beads were washed twice with Lysis buffer devoid of protease inhibitor cocktail by centrifuging at 1500g at 4°C for 2 mins followed by aspirating the buffer containing supernatant. 80μl of lysate was added to the beads and mixed on a rotating wheel at 4°C for 1 hr. Following incubation, the beads were washed three times in lysis buffer and the supernatant was aspirated. The beads were re-suspended in 2X sample buffer and were heated at 95°C for 10 min on a heating block before western blotting.

Immunocytochemistry

Cells were incubated for 20 min in the primary antibody at RT. GluA2 N-Terminal Antibody (MAB397, Millipore, 1:70) was mixed in a 1.5mL Eppendorf containing 100μL of conditioned media (per coverslip). 90μL of the antibody containing media pipetted onto parafilm and the coverslips were gently placed with cells facing down on to the primary antibody. After incubation, the coverslips were placed back into a 6 well plate containing 2ml of DPBS (cells side facing up). The coverslips were washed 3-5 times in DPBS and fixed with 1ml of pre-warmed 4% formaldehyde + 5% sucrose for 12 mins. The cells were again washed 3 times in DPBS followed by a wash with 1ml of 100mM glycine dissolved in DPBS to quench residual formaldehyde. To remove glycine, the coverslips were again washed 3 times with DPBS. The cells were permeabilised and blocked using 3% BSA in DPBS containing 0.1% Triton-X 100 for 20 mins with gentle shaking at RT. Anti-mouse Cy3 antibody (Jackson ImmunoResearch, 1:400) secondary antibodies were diluted 1:400 in 3% BSA in 1XDPBS and were incubated for 1 hr (same as primary antibody incubation). After the incubation, the coverslips were placed back into the wells and were washed 3 times with DPBS. 40μL of mounting media (Fluoromount-GTM with DAPI (Thermo Fisher)) was pipetted on to slides and the coverslips were mounted (cells facing down) after gently dipping into ddH2O (to prevent salt crystal formation). The slides were left overnight to dry before imaging or storage at 4°C.

To measure the dendritic GluA2 distribution, random but clearly selectable and isolated dendrites were selected from initial dendrites (starting after cell body to first branch point) and first branch dendrites (from initial branch point to the subsequent branch point). For both categories, at least 4 dendrites were selected, and the branches were traced using the 'Simple Neurite Tracer' tool, filled automatically to the thickness of the neurite to create masks of the dendritic structure within the Region of Interest (ROI). The mean values of surface GluA2 intensity were measured from the masks by using Fiji software and the intensity values were averaged for each ROI of each neuron and analyzed in Graph Prism version 9.1.2.

For assessment of synaptic GluA2, cells were additionally stained for the postsynaptic marker Homer (anti-Homer, Synaptic Systems, 1:500) for 1 hr at RT following fixation and permeabilisation. Confocal imaging was carried out on a Leica SP8 with a 100x objective, 2x zoom, 10 ROIs per condition. Homer-stained ROIs were background subtracted, filtered (median), and auto thresholded (Otsu) in FIJI. Particles 20 pixels or larger were analyzed for mean GluA2 and Homer fluorescence. For each ROI, between 47 and 461 particles were analyzed and averaged together. 10 ROIs per independent experiment, 3 independent dissections.

Electrophysiology

Hippocampal slices were placed in a submerged holding chamber and perfused with 30°C oxygenated aCSF at 2ml min^{-1}. Excitatory postsynaptic currents (EPSCs) of AMPA transmission were evoked at -70mV by stimulating the Schaffer collateral pathway and recorded from CA1 pyramidal neurons. Pyramidal neurons were patch-clamped in the whole-cell configuration using borosilicate glass (Harvard Apparatus) electrodes with a resistance of 2-5 MΩ and were backfilled with a solution containing (in mM): $CsMeSO_4$ 130, NaCl 8, Mg-ATP 4, Na-GTP 0.3, EGTA 0.5, HEPES 10, QX-314-Cl 5; pH 7.2. The CA3 area of the hippocampal slices was removed using a scalpel blade in order to minimize epileptic activity. D-AP5 (50μM) and picrotoxin (50μM) were bath applied to isolate AMPA-mediated EPSCs. Cells in which the series resistance changed above 20 MΩ or deviated by 20% were discarded.

After a 10 min stable baseline was achieved, 1μM kainic acid was bath applied for 10 mins followed by a 30 min washout period.

Signals were low-pass filtered at 2 kHz and digitized at 10 kHz using a Axopatch 200B amplifier (Molecular Devices) and WinLTP v1.11 acquisition software (Anderson and Collingridge, 2007).

QUANTIFICATION AND STATISTICAL ANALYSIS

For each experiment, the signal for each condition was divided by the mean overall signal from that experiment. This analysis was performed for each replicate experiment, and for presentation purposes, the mean of the control condition set to 100.

All graphs were generated, and statistical tests performed, using GraphPad Prism version 9.1.2. Our sample sizes correspond to previous published results and no statistical tests were performed to predetermine the sample size (Gonzalez-Gonzalez and Henley, 2013; Petrovic et al., 2017). The details of the statistical tests performed on each experiment are explained in the figure legend along with p-values and error bars. Number of cells = n and number of independent dissections/number of animals = N.

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Invited Review

Kainate receptors and synaptic plasticity

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ABSTRACT

Synaptic plasticity has classically been characterized to involve the NMDA and AMPA subtypes of glutamate receptors, with NMDA receptors providing the key trigger for the induction of long-term plasticity leading to changes in AMPA receptor expression. Here we review the more subtle roles played by kainate receptors, which contribute critical postsynaptic signalling as well as playing major presynaptic auto-receptor roles. We focus on two research areas: plasticity of kainate receptors themselves and the contribution they make to the plasticity of synaptic transmission.

This article is part of the special issue on Glutamate Receptors - Kainate receptors.

1. Introduction

The mammalian brain is a supremely adaptable dynamic system. Throughout life, events and experiences cause changes to neural circuits that modulate emotional and behavioural responses. These functional adaptations arise primarily as a result of synaptic plasticity, the process by which synapses undergo changes in their efficacy to relay information between neurons and that represents the central mechanism for learning and memorizing new information (Martin et al., 2000; Abraham et al., 2019; Magee and Grienberger, 2020). Activity-dependent long-lasting changes in synaptic function can lead to an increase (Long-Term Potentiation; LTP) or a decrease (Long-Term Depression; LTD) in the strength of synaptic transmission and occur at synapses throughout the CNS and across the entire lifespan. Given its critical role in brain function, disruption to synaptic plasticity is a fundamental underlying factor in neurodevelopmental, neuropsychiatric and neurological disorders (Citri and Malenka, 2008; Bliss et al., 2014; Takeuchi et al., 2014).

The most common forms of long-term synaptic plasticity at glutamatergic synapses are triggered by NMDA receptor (NMDAR) activation. This causes changes in the numbers and subunit compositions of postsynaptic AMPA receptors and remodelling of postsynaptic morphology (Shepherd and Huganir, 2007; Bliss et al., 2014; Nicoll, 2017). However, other glutamate receptors, such as metabotropic glutamate receptors and kainate receptors (KARs), can also contribute to both the induction and expression of long- and short-term forms of synaptic plasticity (Isaac et al., 2004; Lerma and Marques, 2013; Evans et al., 2017a).

KARs belong to the family of ionotropic glutamate receptors (iGluRs), with functional KARs comprised of hetero-tetrameric assemblies of combinations of 5 core subunits (GluK1-5) (previously named GluR5, GluR6, GluR7, KA1 and KA2) encoded by genes *GRIK 1–5*, respectively (Chittajallu et al., 1999; Lerma, 2003; Pinheiro and Mulle, 2006; Collingridge et al., 2009). Interestingly, KARs can signal through both canonical ionotropic signalling and non-canonical metabotropic signalling [Fig. 1] (Frerking et al., 2001; Rozas et al., 2003; Fernandes et al., 2009; Lerma and Marques, 2013), a feature that has also been characterized for NMDARs and AMPARs (Valbuena and Lerma, 2016). Ionotropic KARs require the inclusion of GluK1-3 subunits whereas the mechanisms and therefore subunits required for metabotropic signalling remain unclear (Valbuena and Lerma, 2016). Functional KARs can be located at pre-, post- and/or extra-synaptic sites where they perform functions ranging from modulation of neurotransmitter release to postsynaptic depolarization and, via their metabotropic signalling, regulation of ion channels and intrinsic excitability (Chittajallu et al., 1996; Castillo et al., 1997; Vignes and Collingridge, 1997; Frerking and Nicoll, 2000; Melyan et al., 2002; Lerma, 2003; Isaac et al., 2004; Pinheiro and Mulle, 2006).

During CNS development and maturation KAR expression patterns are tightly regulated, resulting in developmentally regulated synaptic

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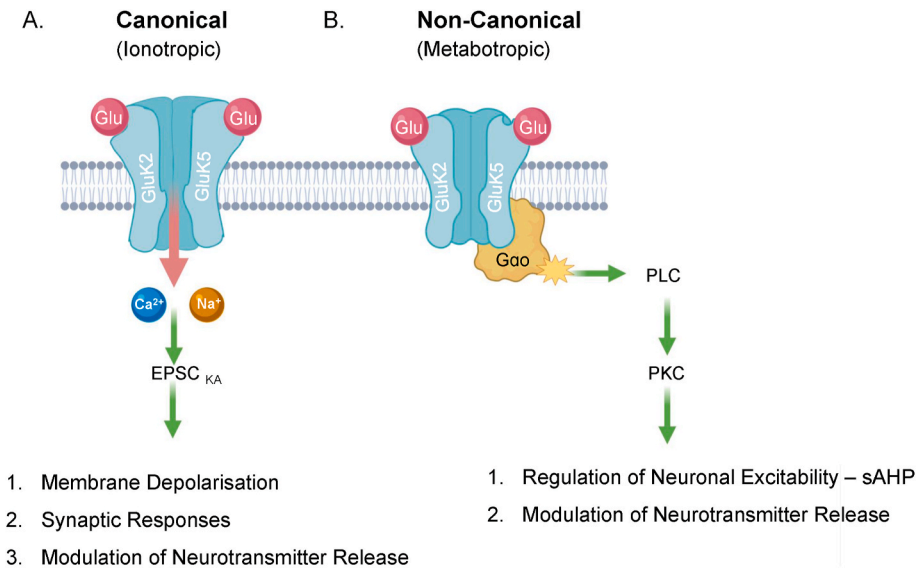

Fig. 1. Kainate receptor signalling pathways.
KARs have dual canonical ionotropic and a non-canonical metabotropic signalling pathways. A) Activation of KARs by endogenous glutamate opens the channel pore by inducing conformational changes in the subunits leading to influx of mono and divalent cations. Canonical KAR signalling is reported to (i) depolarize the postsynaptic membrane resulting in (ii) synaptic responses, (iii) regulate neurotransmitter release and (iv) regulate glutamate receptor trafficking. B) Non-canonical signalling of KARs activates PLC and PKC via a metabotropic G-protein dependent pathway. regulating (i) neuronal excitability by inhibiting the slow afterhyperpolarization (sAHP), (ii) neurotransmitter release and (iii) glutamate receptor trafficking.

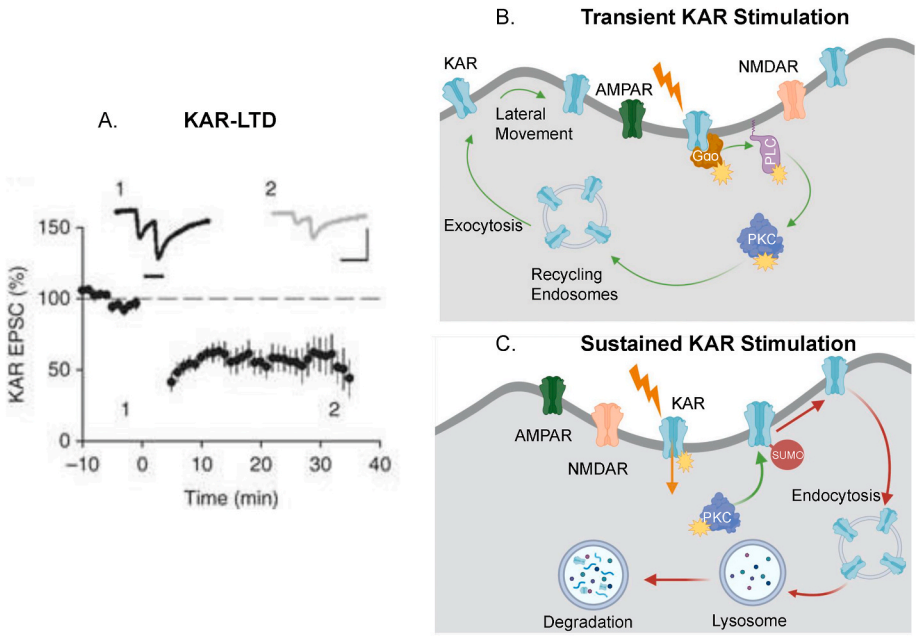

Fig. 2. Bidirectional regulation of kainate receptor surface expression.
A) Sustained low frequency (black bar, 1 Hz for 5 min) stimulation of hippocampal mossy fiber synapses induces KAR-LTD. Numbers represent timepoints for example kainate responses (Adapted from (Chamberlain et al., 2012)). B) Transient stimulation of KARs leads to enhanced surface expression of KARs via activation of metabotropic KARs, PLC, PKC and via recruitment of Rab 11-positive endosomes. C) Sustained stimulation of KARs leads to activation of PKC and increased SUMOylation of GluK2. Down-regulation of surface KARs occurs via endocytosis and lysosomal degradation.

plasticity (Kidd and Isaac, 1999; Kidd et al., 2002; Lauri et al., 2006; Lanore et al., 2012). This strict developmental regulation implies KARs play key roles in neuronal and network maturation, a point that is further underlined by genetic and functional data linking disrupted KAR signalling to neurodevelopmental disorders including Autism Spectral Disorders (ASD) and epilepsy (Lerma and Marques, 2013; Peret et al., 2014; Uzunova et al., 2014; Aller et al., 2015; Xu et al., 2017; Arora et al., 2018).

In this review we focus on the role played by KARs in synaptic plasticity and distinguish between plasticity of KARs, where the response of KARs adapts to changing neural activity patterns, and KAR regulation of both short- and long-term forms of synaptic plasticity.

2. Plasticity of kainate receptors

Synaptic plasticity at glutamatergic synapses is underpinned by alterations in the number and complement of glutamate receptors in the postsynaptic membrane. This is achieved through protein-protein interactions and post-translational modifications that stabilize or destabilise receptor complexes at the postsynaptic density (Henley et al., 2021). Each glutamate receptor subtype has its own complement of interacting proteins that enable synapses to selectively control the synaptic surface expression of individual glutamate receptors. KAR subunits have a unique set of PDZ and non-PDZ ligand mediated protein-protein interactions, including with the NETO proteins, cadherins, C1ql 2/3, PICK1 and GRIP1 (Hirbec et al., 2003; Zhang et al., 2009; Straub et al., 2011, 2016; Matsuda et al., 2016; Polenghi et al., 2020), and post-translational modifications including phosphorylation and SUMOylation that regulate their activity-dependent trafficking and surface expression (Jaskolski et al., 2005; Martin et al., 2007; Konopacki et al., 2011; Chamberlain et al., 2012; González-González et al., 2012; Copits and Swanson, 2013; Lerma and Marques, 2013; Gurung et al., 2018).

Activation of KARs bidirectionally regulates their own surface

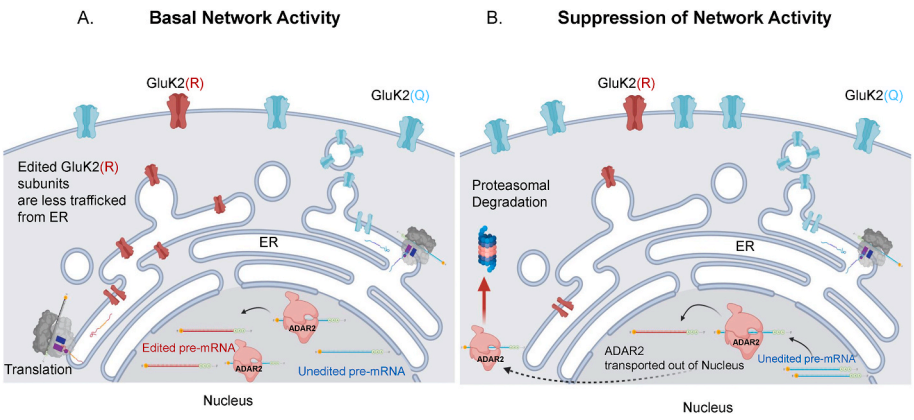

Fig. 3. Homeostatic plasticity of kainate receptors via ADAR2 mediated editing of GluK2.
A) Under basal network activity, GluK2 undergoes ADAR2 mediated Q/R editing which converts a genomically encoded glutamine (Q) to arginine (R). Translocation of edited GluK2(R) containing KARs to the surface is reduced due to retention within the endoplasmic reticulum. B) Suppression of network activity upregulates KAR surface expression by proteasomal degradation of ADAR2. Degradation of ADAR2 leads to a reduction in GluK2 RNA editing and enhanced abundance of GluK2(Q), which is more effectively transported to the surface membrane.

expression, as well as that of other receptors [Fig. 2]. Transient KAR stimulation (10 μM kainate for 3 min) increases the surface expression of GluK2 containing heteromeric KAR complexes in the postsynaptic membrane (Martin et al., 2008). This kainate-induced increase in surface expressed KARs is sensitive to the G-protein inhibitor pertussis toxin, indicating mediation by metabotropic KAR signalling. It also requires Ca^{2+}, PKC and PLC, and results in recycling of KARs and recruitment into spines via Rab 11-dependent endosomal pathways, promoting the upregulation of postsynaptic KARs (Gonzalez-Gonzalez and Henley, 2013). These findings strongly predict that KARs will undergo LTP (KAR-LTP) in response to brief bursts of activity at glutamatergic synapses, but this has yet to be demonstrated.

Conversely, sustained KAR stimulation (10 μM kA for 10 min) decreases the surface expression of postsynaptic KARs by enhancing GluK2 SUMOylation, leading to endocytosis and targeting to lysosomes for degradation (Martin and Henley, 2004; Martin et al., 2007, 2008; Konopacki et al., 2011). This pathway is dependent on phosphorylation of GluK2 by PKC, but not PKA. Activation of NMDARs can also trigger internalisation of KARs via a Ca^{2+}, PKA and PKC dependent pathway, however, this pathway is independent of SUMOylation (Martin and Henley, 2004; Martin et al., 2008).

SUMOylation of GluK2 at K886, promoted by prior phosphorylation at S868, triggers internalisation of the receptor during the induction of KAR-LTD at mossy fiber synapses in the hippocampus, a phenomenon resulting from sustained low frequency stimulation of synapses releasing glutamate to activate postsynaptic KARs (Chamberlain et al., 2012). KAR-LTD also requires GluK5 participation in a complex with the PDZ proteins PICK1 and GRIP1, and the tSNARE complex protein SNAP25, which has been reported to recruit KARs into vesicles for endocytosis (Selak et al., 2009). In CA3 neurons, blockade of SNAP25 increased KAR EPSCs, while cells over-expressing SNAP25 exhibited increased intracellular accumulation of GluK5 containing KARs, suggesting a role for SNAP25 in destabilising GluK5 containing KARs at the cell surface and thereby promoting their internalisation (Selak et al., 2009). In addition, the lateral diffusion of KARs into and out of the synapse is stabilized by glutamate binding, which enhances KAR interactions with N-cadherin and reduces the lateral diffusion of desensitized KARs (Polenghi et al., 2020).

KAR-LTD at mossy fiber synapses can also be induced by brief high frequency stimulation that mimics natural spike patterns in dentate gyrus granule cells, illustrating the physiological relevance of KAR plasticity. KAR-LTD induced by high frequency stimulation requires coactivation of adenosine A2A receptors, in addition to activation of mGluR5, which is also required for sustained low frequency induced KAR-LTD (Chamberlain et al., 2012, 2013). Surprisingly, the major consequences of KAR-LTD do not result from the reduced synaptic current, even though these small but prolonged currents display significant influence on cellular spiking (Sachidhanandam et al., 2009), but instead arise from the reduced ability to inhibit channels mediating slow afterhyperpolarizations (sAHP), leading to a reduction in cell excitability (Melyan et al., 2002; Chamberlain et al., 2013). This in turn offsets the excitatory effect of NMDAR-LTP at mossy fiber synapses, which is also induced by brief high frequency stimulation, potentially protecting CA3 neurons from excitotoxic insults (Kwon and Castillo, 2008; Rebola et al., 2008; Chamberlain et al., 2013).

KAR-LTD also exists at synapses onto layer II/III neurons in the perirhinal cortex, where 5 Hz synaptic stimulation induces LTD of KAR mediated transmission, and at developing thalamocortical synapses in somatosensory cortex (Kidd and Isaac, 1999; Park et al., 2006). KAR-LTD in perirhinal cortex has many mechanistic similarities to mossy fiber KAR-LTD, relying on postsynaptic Ca^{2+} levels, mGluR5, PKC activation and PICK1 PDZ domain interactions (Park et al., 2006). In layer IV cells of somatosensory cortex, postsynaptic KARs undergo activity-dependent LTD and are replaced by AMPARs during the first postnatal week (p3-p7), consistent with an important developmental role for KAR-LTD (Lauri et al., 2021; Kidd and Isaac, 1999).

2.1. *Homeostatic plasticity of kainate receptors*

In addition to activity-dependent plasticity, KARs can also undergo homeostatic plasticity whereby suppression of network activity by TTX or blockade of AMPARs leads to increased surface expression or function of KARs illustrating the importance of KARs in maintaining balanced network activity (Yan et al., 2013; Evans et al., 2017b). There are two mechanisms proposed by which this may occur. The first is via increasing the abundance of the high affinity GluK5 subunit to enhance KAR function (Yan et al., 2013). The second mechanism links neuronal activity to KAR surface expression via changes to the mRNA editing of GluK2 subunits (Evans et al., 2017b). GluK2 pre-mRNA undergoes ADAR2 mediated Q/R editing, changing a genetically encoded glutamine (Q) residue to Arginine (R) at the channel pore of the subunit (Seeburg et al., 1998). This conversion is achieved by the hydrolytic deamination of an adenosine nucleotide in the pre-mRNA to inosine, which is read as guanosine by the translational machinery. GluK2(Q) subunits form heteromers and are trafficked to the surface more efficiently than GluK2(R) subunits [Fig. 3]. This is due to increased retention of receptors containing GluK2(R) within the endoplasmic reticulum, along with reduced ability to oligomerize with other subunits to facilitate forward trafficking (Ball et al., 2010; Henley et al., 2021).

Chronic suppression of network activity induces proteasomal degradation of the ADAR2 enzyme, and the consequent reduction in GluK2 Q/R editing leads to an increase in KAR surface expression (Gurung et al., 2018). Although the GluA2 subunit of AMPARs is also edited by ADAR2, and the suppression of network activity by TTX also causes upscaling of AMPARs, the editing status of AMPARs is less sensitive to alterations in ADAR2 activity (Evans et al., 2017b). These findings suggest that differential mechanisms regulate KAR and AMPAR upscaling (Gurung et al., 2018). Therefore, altering the editing status

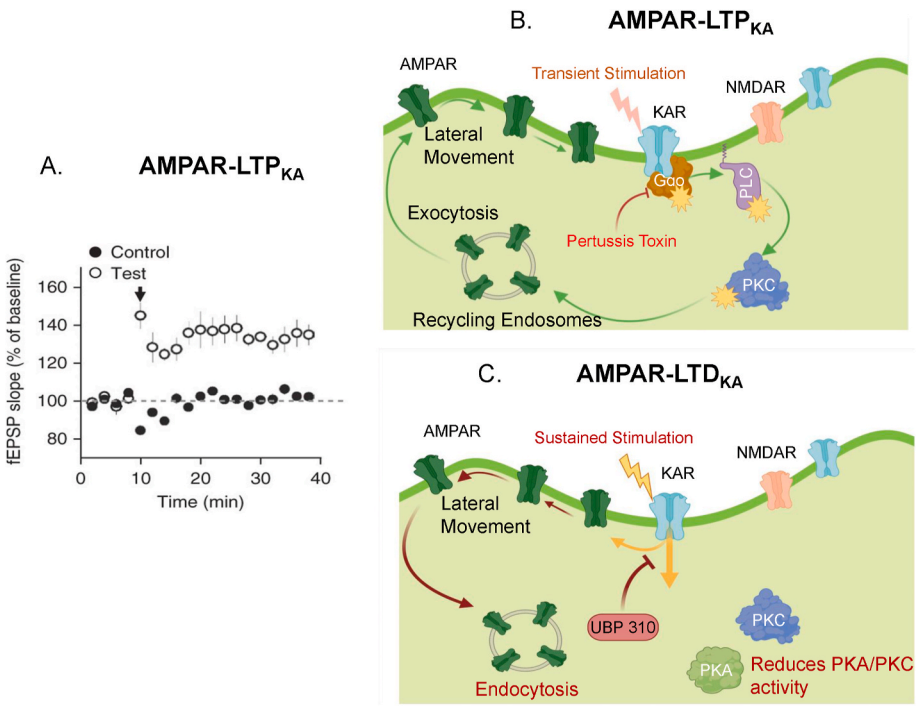

Fig. 4. Bidirectional regulation of AMPA receptor surface expression by kainate receptors.
A) High frequency stimulation (4 bursts of 100 stimuli at 200 Hz) of Schaffer collateral synapses induces AMPAR-LTP$_{KA}$ (adapted from (Petrovic et al., 2017)). B) Transient stimulation of KARs induces increased surface expression of AMPARs. The increase in surface AMPAR expression requires metabotropic signalling of GluK2 containing KARs and activation of PKC. C) Conversely, sustained stimulation of KARs decreases surface expression of AMPARs. This is mediated by ionotropic KAR signalling and is dependent on GluK2 containing KARs, PKA and PKC.

and scaling of KARs by chronic suppression of synaptic transmission could stabilize network activity and neuronal function. Moreover, the edited GluK2 subunit also confers lower Ca^{2+} permeability to KARs, which may further reduce susceptibility to glutamate induced excitotoxic insults (Seeburg et al., 1998; Ball et al., 2010).

3. Kainate receptor regulation of synaptic plasticity

The contributions of KARs to synaptic plasticity are not restricted to direct modulation of KAR function themselves but can also include regulation of synaptic function more generally. This includes regulation of both postsynaptic AMPAR expression and presynaptic release of neurotransmitter.

3.1. Kainate receptor regulation of postsynaptic AMPA receptors

KARs can induce bidirectional plasticity of AMPARs depending upon the activation parameters. Transient activation of GluK2 containing KARs induces LTP of AMPARs in hippocampal CA1 neurons (Petrovic et al., 2017) [Fig. 4]. This KAR-dependent AMPAR plasticity (AMPAR-LTP$_{KA}$) requires metabotropic signalling and PKC activation that cause increased surface expression of AMPARs at synapses via exocytosis from recycling endosomes (Petrovic et al., 2017). When combined with KAR-LTD, AMPAR-LTP$_{KA}$ may provide a mechanism for the observed developmental switch in glutamate receptor expression at several synapses where KARs are replaced by AMPARs during specific developmental windows (Kidd and Isaac, 1999; Lauri et al., 2006; Lanore et al., 2012).

In contrast, sustained stimulation of KARs decreases surface expression of AMPARs, inducing a novel form of AMPAR-LTD$_{KA}$ in primary hippocampal cultures and acute slices (Nair et al., 2020). Consistent with AMPAR-LTP$_{KA}$, AMPAR-LTD$_{KA}$ is dependent on the GluK2 subunit, and its absence abolishes KAR induced down-regulation of surface AMPARs. Unlike AMPAR-LTP$_{KA}$, AMPAR-LTD$_{KA}$ requires activity of both PKC and PKA, and KAR induced internalisation of AMPARs is dependent on the ionotropic rather than metabotropic signalling of KARs (Nair et al., 2020). Thus, KARs can regulate network activity by altering the surface expression of AMPARs, alongside regulating their own surface expression.

3.2. Kainate receptor regulation of presynaptic neurotransmitter release

KARs are expressed on presynaptic terminals of both glutamatergic and GABAergic synapses throughout the brain (Chittajallu et al., 1996; Min et al., 1999; Darstein et al., 2003; Lerma, 2003). These presynaptic KARs can lead to increases or decreases in the probability of neurotransmitter release, principally by altering the presynaptic Ca^{2+} concentration. A number of mechanisms by which KARs may regulate presynaptic Ca^{2+} have been reported, including: (i) direct depolarization of presynaptic axons and terminals altering voltage-gated Ca^{2+} channel (VGCC) activity (Schmitz et al., 2000, 2001; Semyanov and Kullmann, 2001; Kidd et al., 2002), (ii) Ca^{2+} permeable KARs (Lauri et al., 2003; Scott et al., 2008), and (iii) metabotropic signalling, which can regulate VGCCs and vesicle release (Rodriguez-Moreno and Lerma, 1998; Rodriguez-Moreno and Sihra, 2004). Where KARs are located presynaptically at glutamatergic synapses they can act as auto-receptors and consequently contribute to short-term plasticity dynamics (Valbuena and Lerma, 2021). In addition, presynaptic KARs can also contribute to long-term plasticity at glutamatergic synapses, such as hippocampal mossy fiber synapses, where LTP and LTD are expressed by presynaptic mechanisms (Nicoll and Schmitz, 2005; Pinheiro and Mulle, 2008; Lerma and Marques, 2013).

At developing thalamocortical synapses in the somatosensory cortex, activation of presynaptic kainate auto-receptors by physiologically relevant high frequency stimulation induces depression of synaptic transmission on a short-term and rapid timescale (Kidd et al., 2002). This regulation of presynaptic release is not seen after the first postnatal week and may be crucial in the maturation of the sensory network and high frequency information transfer to the cortex by thalamocortical neurons (Kidd et al., 2002).

At mossy fiber synapses between dentate gyrus granule cells and CA3 pyramidal cells in the hippocampus KARs are located presynaptically as well as postsynaptically. In contrast to their function at thalamocortical synapses, kainate auto-receptors at mossy fiber synapses (and cerebellar parallel fiber synapses) can both depress and facilitate glutamate release depending on the concentration of agonist, with lower concentrations

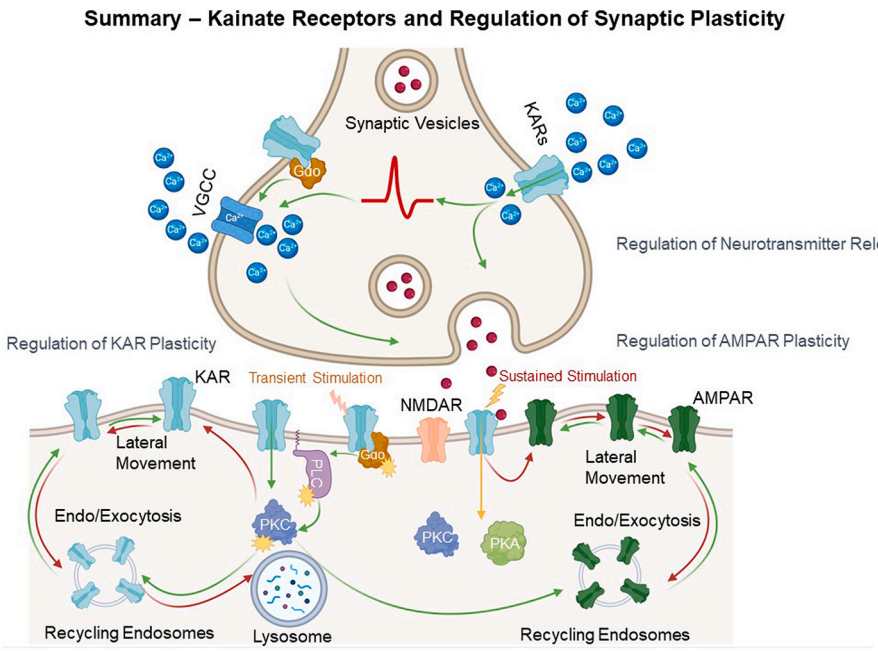

Fig. 5. Role of kainate receptors in synaptic plasticity.
Presynaptic KARs bidirectionally regulate the release of glutamate. Proposed mechanisms include regulation of presynaptic Ca^{2+} by depolarization, Ca^{2+}-permeable KARs and metabotropic signalling. Postsynaptic KARs bidirectionally regulate the surface expression of KARs and AMPARs via metabotropic signalling and regulation of PKC. Transient activation of KARs leads to increased surface expression of both KARs and AMPARs whereas sustained activation leads to decreases in surface expression.

resulting in facilitation and higher concentrations resulting in depression (Schmitz et al., 2000, 2001; Contractor et al., 2001; Delaney and Jahr, 2002). Synaptic glutamate release generally results in lower KAR activation and therefore synaptic facilitation. This decrease in KAR opening is a major component of the observed facilitation of synaptic transmission in response to repetitive synaptic stimulation, for stimulation frequencies ranging from ~1 Hz–100 Hz. However, longer high frequency bursts of synaptic activity can release sufficient glutamate to depress subsequent glutamate release (Schmitz et al., 2001).

The receptor subunit complement of the presynaptic KARs at mossy fiber synapses was initially thought to comprise predominantly GluK1 (Bortolotto et al., 1999; Lauri et al., 2001), but subsequent reports supported the inclusion of GluK2, GluK3, GluK4 and GluK5 (Contractor et al., 2003; Darstein et al., 2003; Schmitz et al., 2003; Pinheiro et al., 2007). These presynaptic KARs can regulate vesicle release by several reported mechanisms. The depolarization of presynaptic terminals and axons causes a broadening of the action potential, leading to enhanced Ca^{2+} influx through VGCCs and increased likelihood of vesicle release (Schmitz et al., 2001), although this has not been measured (Scott et al., 2008). This mechanism relies on ionotropic KAR function and can therefore act rapidly, on the millisecond timescale found with high frequency synaptic stimulation (Schmitz et al., 2001; Kidd et al., 2002). On an equally fast timescale, increases in presynaptic Ca^{2+} could result from Ca^{2+} influx through Ca^{2+} permeable KARs that in turn can promote release from Ca^{2+} stores (Lauri et al., 2003; Scott et al., 2008). However, there are also reports of metabotropic signalling by presynaptic KARs to regulate neurotransmitter release (Rodriguez-Moreno and Lerma, 1998; Frerking et al., 2001). These would necessarily act on a slower timescale where G-protein coupled signalling might regulate presynaptic VGCCs or directly interact with vesicle exocytosis.

Hippocampal mossy fiber synapses also exhibit a presynaptically expressed form of long-term plasticity that is dependent on presynaptic Ca^{2+} (Nicoll and Schmitz, 2005). Since presynaptic KARs regulate short-term plasticity principally via the influx of Ca^{2+} they can therefore also regulate the induction of this form of long-term plasticity. Initially, KARs were thought to be directly required for mossy fiber long-term potentiation (mfLTP) (Bortolotto et al., 1999), but subsequently they were shown to alter the threshold for mfLTP induction in line with their role in short-term plasticity (Schmitz et al., 2003). In addition, the receptor subunits important for mfLTP (including GluK2, GluK3 and GluK5) mirror those underlying short-term plasticity (Contractor et al., 2001, 2003; Schmitz et al., 2003; Pinheiro et al., 2007).

4. Summary

The role of KARs in synaptic plasticity contrasts starkly with the other ionotropic glutamate receptors, AMPARs and NMDARs. KARs play more subtle and modulatory roles that nonetheless are critical to control the adaptation of circuits to environmental requirements. This is both through the regulation of KAR expression, localization and subunit composition, and the role that KARs play in modulating synaptic strength, dynamically responding to ongoing glutamatergic activity [Fig. 5]. Perturbations to this feedback provided by KAR regulation of synaptic plasticity and the expression of KARs has important consequences for the maintenance of stable and functional neuronal network activity. Further work to establish the generalizability and ubiquity of KAR plasticity across multiple brain regions and to define the function and dysfunction of these processes could have far reaching implications for understanding a wide range of neurological, psychiatric and neurodegenerative disorders at neuronal and network levels.

Author statement

JDN, JRM wrote, and KAW, JMH contributed to content and editing of the manuscript.

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