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

Abstract Heading: Kainate receptor-mediated LTD

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Key Words

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Abstract

Long-Term Depression (LTD) is well known to be initiated by either NMDA or mGlu receptor activation. Here we report that sustained activation of GluK2 subunit-containing kainate receptors leads to AMPA receptor endocytosis and a novel form of long-term depression (KAR-LTD$_{AMPAR}$) in hippocampal neurons. The KAR-evoked loss of surface AMPA receptors 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 and long lasting depression of AMPA EPSCs 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 (NMDARs), α-Amino-3-Hydroxy-5-Methyl-4-isoxazolepropionic acid receptor (AMPARs) and Kainate receptor (KARs) subtypes, whereas metabotropic glutamate receptors are comprised of 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. Both LTP and LTD are fundamental to learning and memory, and their dysregulation is a prominent feature of cognitive decline in aging, and of neurological and neurodegenerative diseases (Bliss et al., 2014).

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). Moreover, it is now clear that dysfunctional LTP and LTD contribute to a wide range of neurological and neurodegenerative disorders (Cuestas Torres and Cardenas, 2020; Kauer and Malenka, 2007).

While the best characterised 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 a novel form of LTP (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).

Compared to NMDARs and AMPARs, the functional roles of synaptic KARs have been enigmatic, but accumulating evidence has demonstrated that KARs are key regulators of synaptic function (Evans et al., 2019). KARs can be expressed at both the 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, as 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; Ruiz et al., 2005).

Previous work has demonstrated that relatively low level stimulation of KARs (10μM KA for 3 mins) 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). Based on these observations we examined whether transient KAR activation also impacts on AMPARs. We found that metabotropic KAR signalling can enhance AMPAR surface expression, and increases both AMPAR colocalisation with PSD95 and AMPAR mEPSCs in hippocampal neurons and slices, revealing a novel and physiologically relevant form of postsynaptic KAR-dependent, NMDAR-independent, LTP (KAR-LTP_{AMPAR}) (Petrovic et al., 2017).

In contrast to transient KA stimulation, sustained stimulation of KARs (10μM KA for 20 mins) 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 novel 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 (10μM KA for 20 mins) 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 minutes with 1μM tetrodotoxin (TTX), to prevent depolarisation-evoked presynaptic glutamate release, and 40μM GYKI 53655, an AMPAR-specific antagonist (Ashby et al., 2004; Glebov et al., 2015; 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 20μM NMDA and 20μM glycine for 3 minutes, followed by a 17 minute incubation in the absence of NMDA, to allow receptor internalisation {Lee, 1998 #17620). Surface proteins were then labelled by biotinylation, and isolated by streptavidin pulldown. Surface proteins and whole cell lysates (total protein) were then analysed 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 application. 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 desensitising 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 1μM KA, a concentration below the threshold for AMPAR activation (Dai et al., 2001), in the absence of GYKI 53655. 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 10μM KA stimulation, surface levels of GluA2, assessed by surface biotinylation followed by Western blotting, were significantly reduced by 1μM KA application (**Figure 1B**).

KA application decreases surface GluA2 in dendrites

We next used live cell surface staining and confocal imaging to monitor GluA2 surface expression in control and KA treated hippocampal neurons. Neurons were pre-treated with 40μM GYKI 53655 and 1μM TTX for 30 minutes prior to application of 10μM KA for 20 mins, followed by live surface labelling 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 1C**).

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 (Evans et al., 2017; Gurung et al., 2018). 7 days post-transduction, neurons were pre-treated with 40μM GYKI 53655 and 1μM TTX for 30 minutes, before application of 10μM KA for 20 minutes, and subjected to surface biotinylation, streptavidin pulldown and Western blotting (**Figure 2**).

As expected, in neurons expressing the control shRNA, KAR activation decreased GluA2 surface expression. However, the KA-induced reduction in surface GluA2 did not occur in GluK2 knockdown neurons – indeed there was a small but significant increase in GluA2 surface expression – suggesting the KA-mediated reduction in GluA2 surface expression requires the GluK2 KAR subunit.

KA-evoked down-regulation of surface AMPARs requires ionotropic KAR signaling

To investigate the signalling 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-protein inhibitor pertussis toxin (PTx) (Petrovic et al., 2017).

Blocking KAR metabotropic signalling for 1 hr with 1μg/ml PTx prior to KA stimulation did not prevent the KA-induced reduction in GluA2 surface expression (**Figure 3A**). In contrast, 10μM UBP 310 did block the KA-evoked reduction in surface GluA2. These data indicate that KAR channel activity, but not G-protein mediated signalling, is required to initiate the downregulation of AMPAR surface expression.

KA regulation of surface GluA2 requires PKA and PKC phosphorylation

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 signalling 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 signalling (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 (1μM), GYKI 53655 (40μM) and the PKA or PKC specific inhibitors H89 (10μM) or chelerythrine (5μM), respectively. Neurons were then subjected to 20 mins of sustained 10μM KA stimulation. Both inhibitors reduced basal surface levels of GluA2 and occluded the KA-induced reduction in GluA2 surface expression (**Figure 3B**).

Together these data indicate that the KAR-induced decrease in surface GluA2-containing AMPARs requires ionotropic KAR channel opening. Moreover, blockade of PKC or PKA, which are activated downstream of ionotropic as well as by metabotropic signalling, also reduces GluA2 surface expression and occludes the effect of KAR activation.

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 KA (1μM, 10 min) in the presence of 50μM picrotoxin and 50μM D-AP5, to block $GABA_A$ and NMDA receptors, respectively. Consistent with our biochemistry and imaging, AMPAR EPSCs were significantly reduced for 20 mins after KA washout (**Figure 4A**). 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 (STP) (**Figure 4B**) (Lauri et al., 2006). Importantly, however, the PPR returned to baseline levels within 10-20 mins after KA washout, whereas AMPAR EPSCs remained depressed, suggesting the existence of a postsynaptic long-term depression of AMPAR transmission. We interpret these data to indicate that 1μM KA for 10 min mediates both pre- and postsynaptically-expressed depression of AMPAR EPSCs.

Discussion

Both ionotropic and metabotropic KAR signalling 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 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 signalling, 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 signalling was limited to the regulation of KARs themselves or whether KAR-mediated signalling 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 novel KAR-dependent, NMDAR-independent, form of hippocampal LTP (KAR-LTP_{AMPAR}) through a metabotropic signalling pathway (Petrovic et al., 2017).

In this study we now show that prolonged KAR signalling 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 signalling did not prevent KAR-LTD_{AMPAR}, whereas UBP 310, an antagonist of postsynaptic ionotropic signalling through GluK2/GluK5-containing KARs (Grosenbaugh et al., 2018; Petrovic et al., 2017; Pinheiro et al., 2013) blocked the KA-evoked decrease in AMPAR surface expression. Interestingly, we also observed a decrease in the basal surface expression of GluA2 in the presence of metabotropic and/or ionotropic inhibitors, suggesting KARs may also be required for maintaining tonic activity of AMPARs through an as yet unidentified pathway. Note, PTx is inhibits multiple G proteins not only those activated by KARs.

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; 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. However, the

functional implications remain unclear since one study found almost no phosphorylated GluA1 *in vivo* (Hosokawa et al., 2015).

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, favouring 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 long-term depression (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, stabilises 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 calcium- 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.

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). Interestingly, an alternative model has emerged recently suggesting that 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). 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).

Thus, it is possible that phosphorylation is required to overcome an, as 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$_{AMPAR}$.

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 mins of KA washout. However, after this initial short-term change in PPR had 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$_{AMPAR}$ (**Figure 5**). The underlying mechanisms appear to differ since KAR-LTP$_{AMPAR}$ is mediated via a metabotropic signalling pathway, whereas KAR-LTD$_{AMPAR}$ is blocked by the ionotropic KAR signalling 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.

Materials and Methods

Drugs used

Drugs	Concentration (μM)	Diluted in	Supplier	Catalogue Number
Chelerythrine	5	DMSO	Sigma	C2932
CNQX	10	DMSO	Sigma	C127
D-AP5	50	ddH2O	Tocris	0106
Glycine	20	ddH_2O	Sigma	G8790
GYKI 53655	40	ddH_2O	Tocris	2555
H89	10	ddH_2O	Tocris	2910
Kainate (KA)	10	ddH_2O	Tocris	0222
NMDA	20	ddH_2O	Tocris	0114
PTx	1μg/ml	ddH_2O	Tocris	3097
TTX	1	ddH_2O	Tocris	1069
UBP310	20	DMSO	Tocris	2079
Picrotoxin	5	ddH20	Sigma	P1675

Primary Antibodies used

Antibody Used	Species	Supplier	Catalogue number	Dilution	Type
GluA1 (WB)	Rabbit	Millipore	AB1504	1:1000	Polyclonal
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
EGFR (WB)	Rabbit	Abcam	Ab52894	1:3000	Monoclonal
GAPDH (WB)	Mouse	Abcam	Ab8245	1:10000	Monoclonal

Dissociated primary Neuronal Culture

Primary hippocampal cultures were prepared from E18 Han Wistar rats as previously described (Fletcher-Jones et al., 2019; Konopacki et al., 2011). The cells were plated in varying densities for biochemistry and imaging experiments and placed in incubator for 17-18 days. Neuronal plating media containing Neurobasal (Gibco) supplemented with 10% Horse Serum, B27, 2mM Glutamax and Penicillin-Streptomycin was changed to feeding media devoid of Horse serum after 24 hrs.

Lentivirus production and transduction

For GluK2 knockdown experiments, shRNA sequences targeting GluK2 were cloned into a modified pXLG3-GFP vector (Rocca et al., 2017) under the control of a H1 promoter. The shRNA target sequences were: Control, non-targeting shRNA: AATTCTCCGAACGTGTCAC; GluK2-targeting shRNA GCCGTTTATGACACTTGGA: The viruses were produced in HEK293T cells as reported previously (Rocca et al., 2017), harvested and added to hippocampal neurons at 9–10 days *in vitro* (DIV) for 7 days before use.

Sustained KA stimulation

500,000 cells per well of hippocampal neurons were plated in a 6 well culture dish and left in 37°C incubator until DIV 17-18. On DIV 17, the cells were pre-treated with 1μM TTX (Tocris), GYKI 53655 (Tocris) and 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. All washes for surface biotinylation were performed with ice cold Earle's Buffer.

Neuronal Surface Biotinylation

Using cell impermeant Sulfo-NHS-SS-Biotin (Thermo Fisher, Cat No.21331), live hippocampal neurons were surface biotinylated post KA and drug treatment on ice, as described previously (Martin and Henley, 2004).

Immunocytochemistry

Live cell antibody labelling was performed as described previously (Jaafari et al., 2013). Following viral transduction and/or pretreatment with drugs the neurons were stimulated with KA, washed and then incubated with GluA2 N-Terminal Antibody (MAB397, Millipore, 1:70)

for 20 mins at 4°C. The cells were washed and fixed in prewarmed 2% formaldehyde (Thermo Fisher) and 2% sucrose for 12 mins. Cells were permeabilized and blocked with 3% BSA containing 0.1% Triton X100 in PBS for 20 mins at RT and labelled with anti-mouse Cy3 antibody (Jackson ImmunoResearch, 1:400) for 1 hr at RT. The coverslips were mounted using Fluoromount-GTM with DAPI (Thermo Fisher) before visualizing on a Leica SP5-II confocal laser scanning microscope.

Acute slice preparation

Postnatal day 13-15 male and female Han Wistar rats were anaesthetised with 4% isolflurane 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.

Electrophysiology

Hippocampal slices were placed in a submerged holding chamber and perfused with 30°C oxygenated aCSF at 2ml min^{-1}. Excitatory post-synaptic 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 minimise 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 digitised at 10 kHz using a Axopatch 200B amplifier (Molecular Devices) and WinLTP v1.11 acquisition software (Anderson and Collingridge, 2007).

Statistical Analysis

All graphs were generated, and statistical tests performed, using GraphPad Prism version 8.0. 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. The n (number of cells) and N (number of independent dissections/Number of Animals) are also mentioned.

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Author contributions

JDN performed all the biochemistry and neuronal live surface staining experiments with help from RS and BPY. EB performed the electrophysiology guided by ZIB. DNA constructs were made by KAW. JMH and KAW supervised the study. JDN, KAW and JMH wrote, and all authors contributed to reading and editing the manuscript.

Declaration of Interests

The authors declare no competing interests.

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Figure Legends

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

A) DIV 18 cultured hippocampal neurons were pre-treated for 30 minutes with 1μM TTX, 40μM GYKI 53655 before treatment with vehicle or 10μM KA for 20 minutes. For NMDA treatment neurons were pre-treated with 1μM TTX for 30 mins followed by 3 mins of treatment with 20μM NMDA and 20μM glycine. Surface proteins were biotin labelled and isolated by streptavidin pulldown, and lysates and surface fractions Western blotted.
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 normalised to 100% of control for GluA2 (Aii), GluA1 (Aiii), GluK2 (Aiv) and EGFR (Av). 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) 1μM KA also 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 % of control. N=4 experiments from independent dissections, **$p<0.01$, Unpaired t test with Welch's correction, error bar = SEM.

C) Live confocal immunostaining of DIV18 hippocampal neurons shows a significant reduction in GluA2 surface expression after 10μM KA for 20 minutes. Ci) Representative images of control and KA-treated neurons showing proximal and first branch dendrites. Scale bar = 20μm. Cii) Expanded images of ROIs indicated in boxes in Ci) Scale bar = 10μm. Ciii) Quantification of intensity of surface GluA2 staining in proximal dendrites. Civ) Quantification of 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.

Figure 2: GluK2 containing KARs mediate the reduction in surface expression of AMPARs

A) DIV10 hippocampal neurons were transduced with lentivirus expressing either control or GluK2 shRNA. After 7 days neurons were treated 10μM KA or vehicle and surface proteins isolated and Western blotted. Representative Western blots of surface and total levels of GluA2 and GluK2. B) Quantification of the surface to total ratio of GluA2 expressed as a % of

control. N=4 independent dissections, ns=p>0.05, *p<0.05, **p<0.01, Two-way ANOVA with Dunnett's multiple comparisons test, error bars = SEM.

Figure 3: KA-induced decreases in AMPAR surface expression require ionotropic KAR signaling, PKA and PKC.

A) DIV18 hippocampal neurons were pre-treated for 1 h with 1μg/ml PTx, or 30 minutes with 10μM UBP 310 then 20 min with vehicle or 10μM 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, *p<0.05, ****p<0.0001, Two-way ANOVA with Dunnett's multiple comparisons test, error bars = SEM.

B) As A, except neurones were pre-treated 40μM GYKI 53655 and either 10μM H89 or 5μM Chelerythrine prior to 10μM KA. Bi) Representative blots of GluA2. Bii) – Quantification of the surface to total ratio of GluA2 expressed as a % of control. N=3 independent dissections, ns p>0.05, *p<0.05, **p<0.01, ***p<0.001; Two-way ANOVA with Dunnett's multiple comparisons test, error bars = SEM.

Figure 4: Sustained KAR activation induces depression of AMPAR EPSCs at hippocampal CA1 synapses.

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

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

C) Quantification of mean AMPAR EPSC amplitudes in the initial 5 mins after KA washout. Two-way ANOVA with Tukey's multiple comparisons test, error bars = SEM.

D) Quantification of mean AMPAR EPSC amplitudes 20 mins after KA washout. N=14 and 18 cells for control and KA respectively from at least 4 different animals Un-paired t test with Welch's correction.

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

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

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

Figure 5

Schematic showing KAR-induced LTD of AMPARs (KAR-LTD$_{AMPAR}$).

Dynamic regulation of exocytosis and endocytosis regulate basal levels of AMPARs at the surface. Inhibition of PKA or PKC activity by specific inhibitors reduces basal surface expression of GluA2-containing AMPARs. Sustained activation of GluK2-containing KARs triggers canonical ionotropic signalling of KARs and leads to a reduction in surface AMPARs.

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

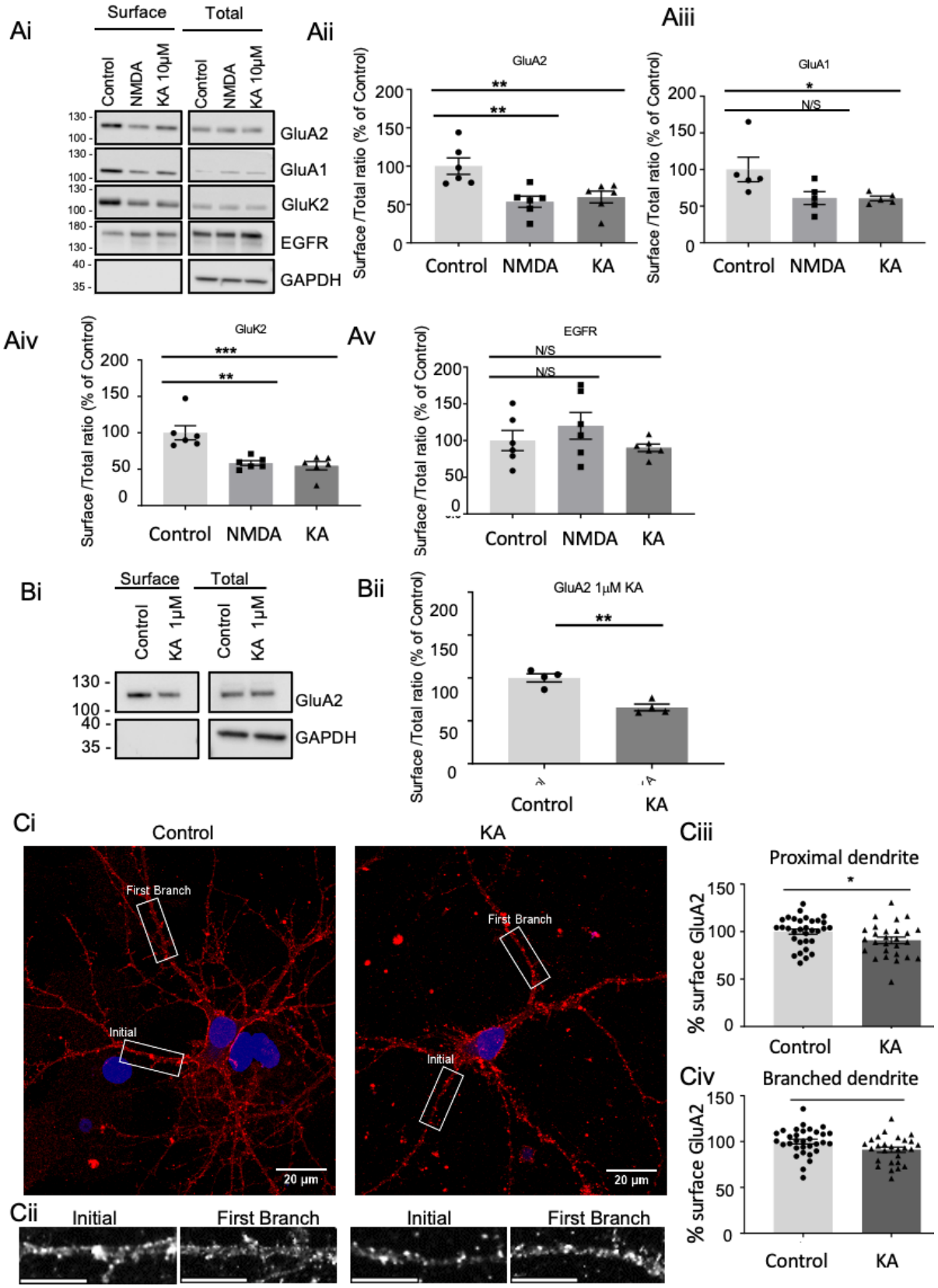
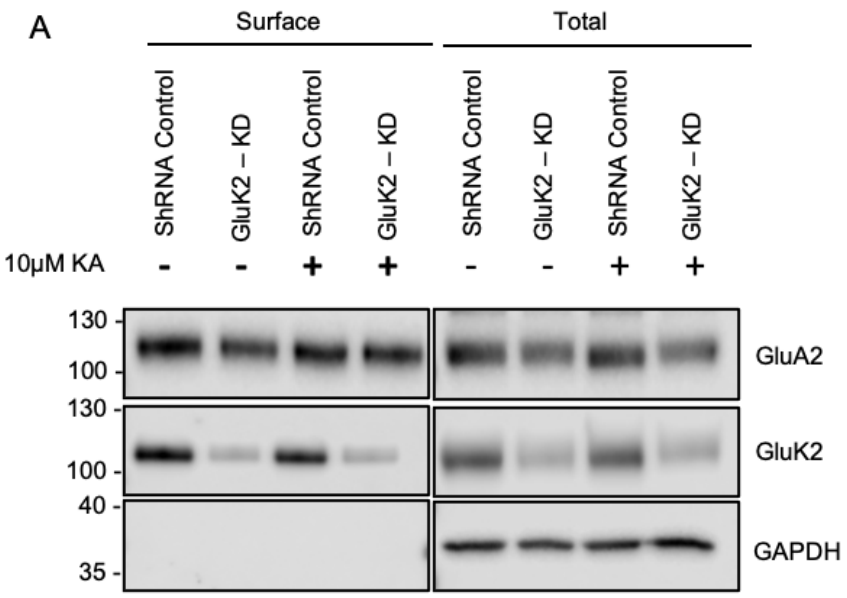


Figure 2. GluK2 containing KARs mediate the reduction in surface expression of AMPARs



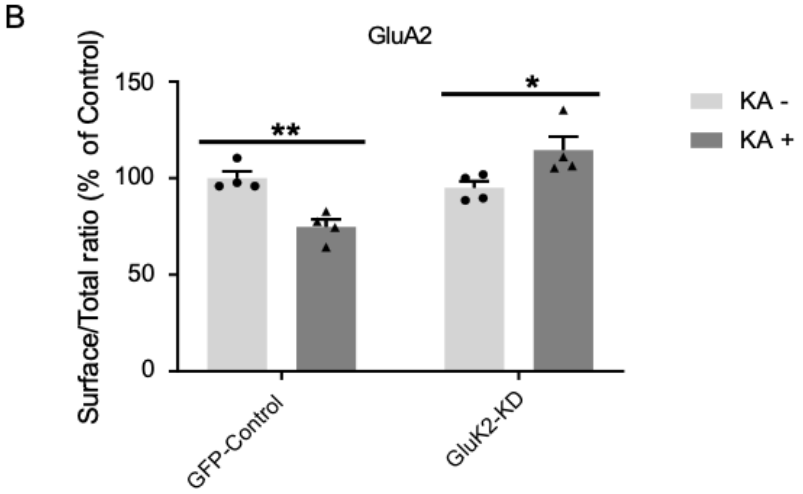


Figure 3. KA-induced decreases in AMPAR surface expression require ionotropic KAR signaling, PKA and PKC.

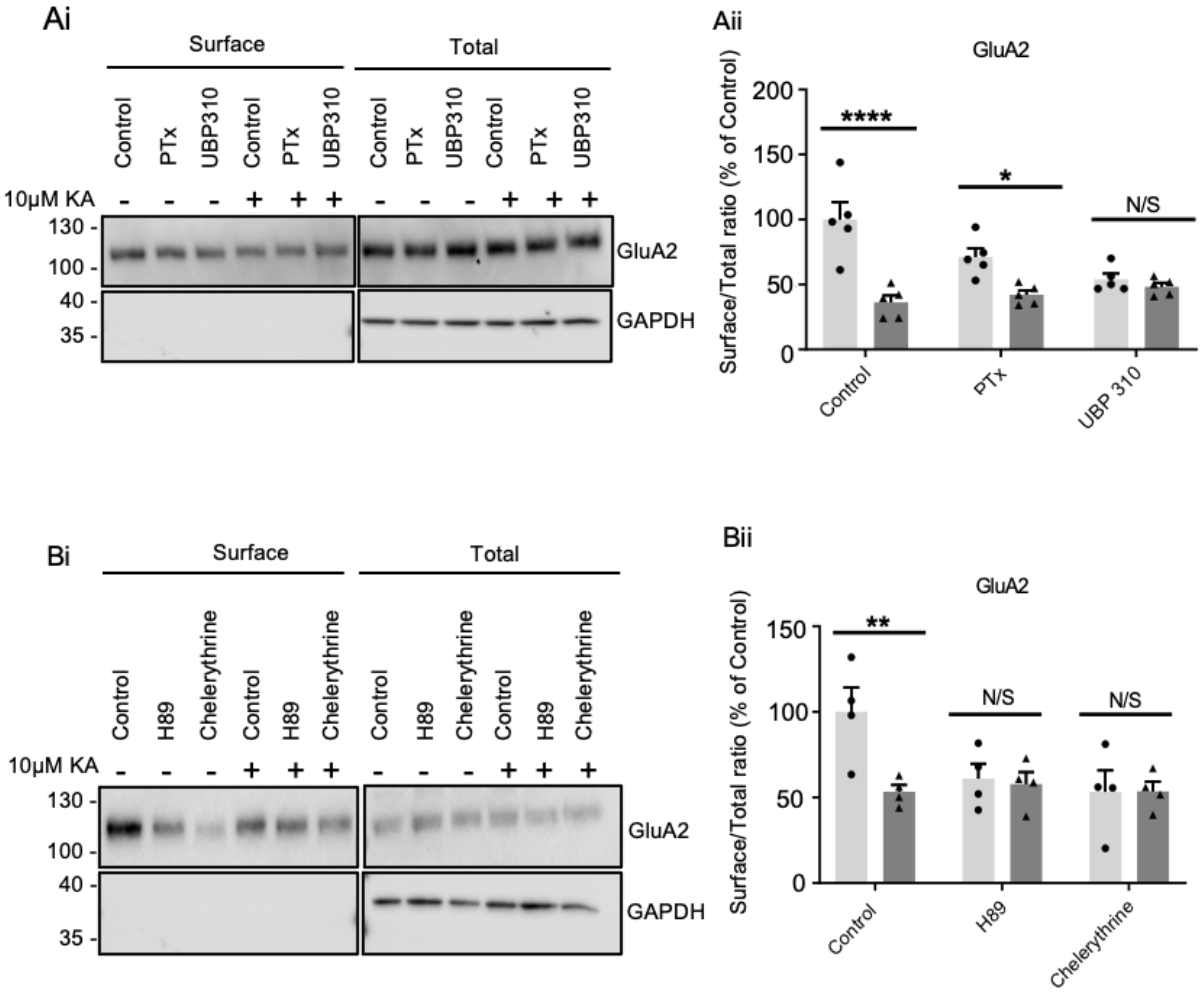


Figure 4. Sustained KAR activation induces depression of AMPAR EPSCs at hippocampal CA1 synapses.

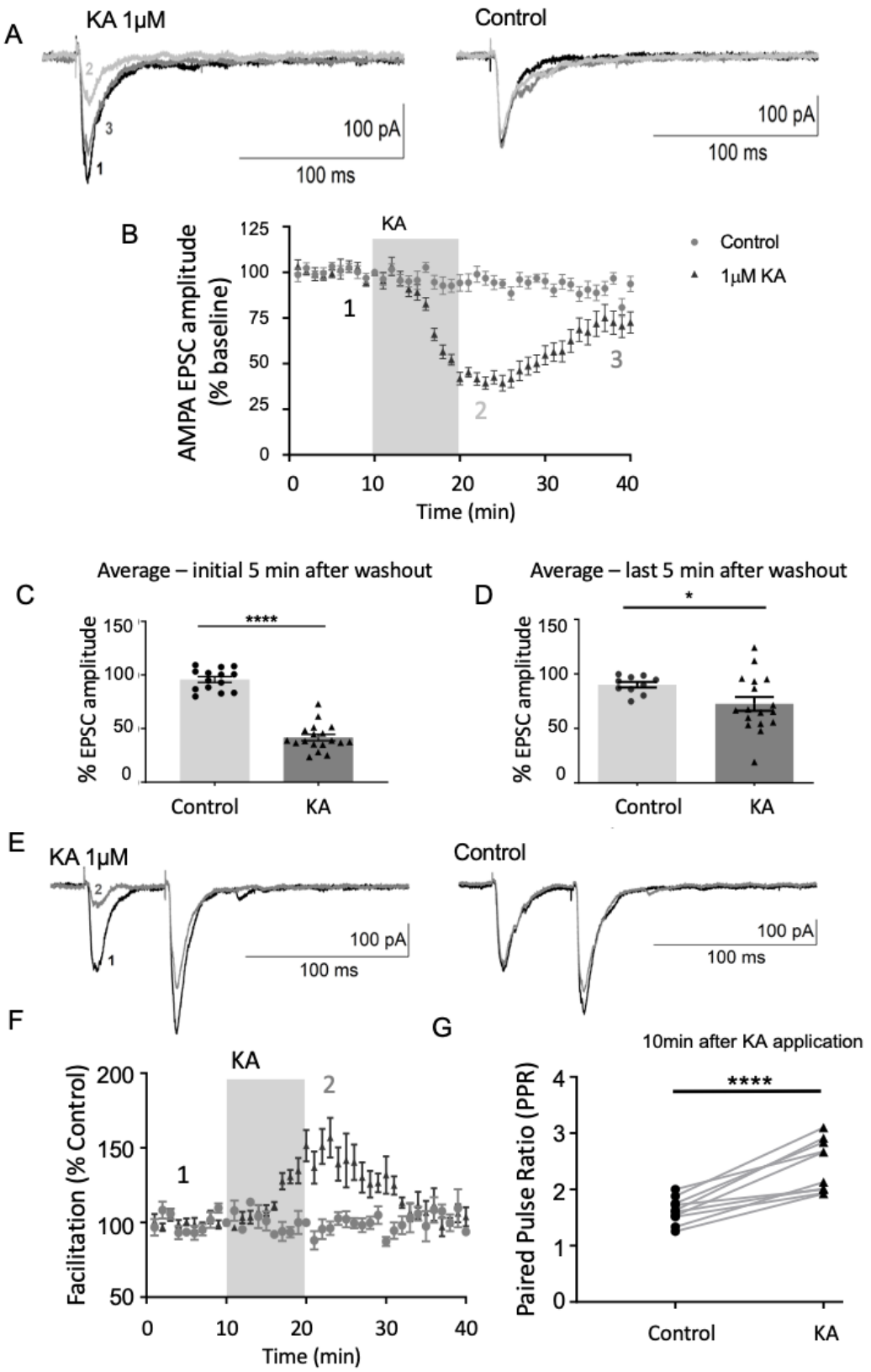
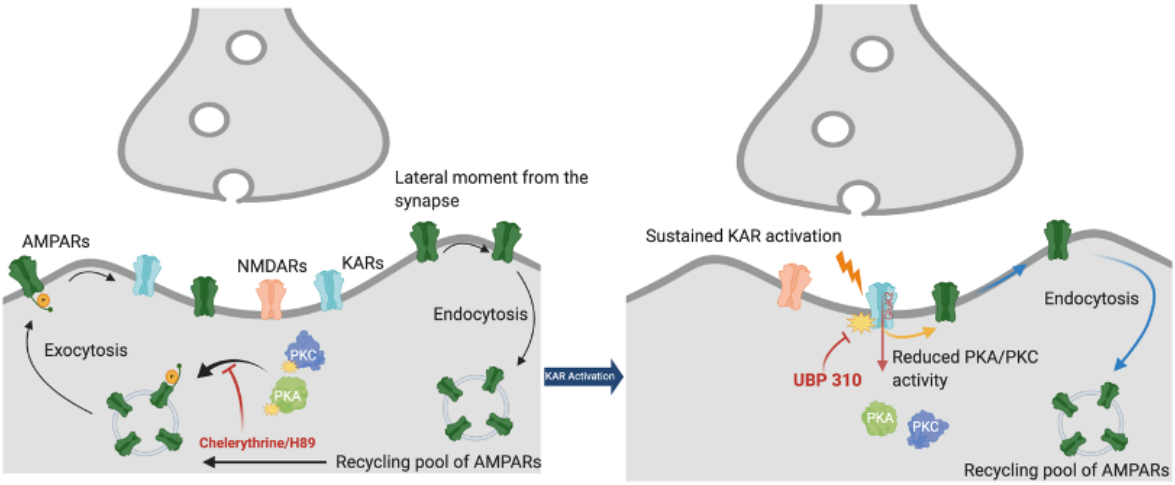



Figure 5. Schematic showing KAR-induced LTD of AMPARs (KAR-LTD$_{AMPAR}$).