

ACTIVITY-DEPENDENT REGULATION OF GIRK AND K<sub>v</sub>7 CHANNELS IN  
HOMEOSTATIC PLASTICITY AND EPILEPSY

BY

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DISSERTATION

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## ABSTRACT

Epilepsy is a chronic brain disorder, which will impact about 3% of the general population at some point during their lifetime. Epilepsy manifests as uncontrolled aberrant electrical activity in the brain. Acquired epilepsy can be due to traumatic brain injury from stroke, contusion, or environmental toxins. Temporal lobe epilepsy (TLE), one of the most common forms of focal epilepsy, is often acquired. It is commonly associated with neuronal death, network reorganization, and synaptic sprouting. It is hypothesized that loss of input to the surviving neurons could lead to homeostatic scaling of synaptic strength and intrinsic excitability. Upon the formation of aberrant connections, it may lead to epileptogenesis in TLE.

Homeostatic plasticity acts to stabilize neuronal activity by altering synaptic strength and adjusting intrinsic excitability. Homeostatic scaling of intrinsic excitability refers to the mechanism by which a neuron adjusts its intrinsic membrane properties in response to its environment to maintain a stable firing rate. To achieve this, a neuron must be able to sense its activity level and respond in a negative feedback manner. One way to control excitability is through the regulation of specific neuronal location, surface density, and function of ion channels, which contribute to the electrical properties of a neuron by integrating incoming signals, propagating those signals, and ultimately controlling the firing of an action potential. Here I will focus on how two potassium ( $K^+$ ) channels, G protein-activated inwardly rectifying  $K^+$  channels (GIRK) and voltage-gated *KCNQ/K<sub>v</sub>7* channels, are regulated by neuronal activity and contribute to homeostatic plasticity. We will also examine how they are regulated during hyperexcitability in diseases such as epilepsy.

In Chapter 1, I will introduce GIRK channels and  $K_v7$  channels. I will examine the brain distribution of these channels, their general channel properties, and their associated channelopathies. I will also explore how these features are regulated and what role these channels play in intrinsic excitability, synaptic transmission, and plasticity in the hippocampus. I will then end the discussion with their role in hippocampus-dependent learning and memory.

In Chapter 2, I will summarize my research on the biphasic regulation of GIRK channels upon seizure activity. My research shows that high-frequency epileptiform discharges in cultured hippocampal neurons initially increase surface expression of GIRK1 and GIRK2 within 20 minutes, which homeostatically counteracts excessive seizure-like activity. However, prolonged

epileptic activity (>30 minutes) leads to their caspase-3-dependent cleavage. In addition to identifying the caspase-3 cleavage sites, cleaved GIRK2 loses an ER export motif, thereby abolishing surface and current expression of GIRK2 homomeric channels. Since GIRK1 surface expression depends on GIRK2, GIRK1/GIRK2 heteromeric channels would also lose functional expression upon caspase-3-dependent cleavage. Importantly, kainate-induced status epilepticus causes GIRK1 and GIRK2 cleavage in the hippocampus *in vivo* at 2 hours post kainite injection. These findings suggest the possible role of caspase-3-mediated down-regulation of GIRK channels in aggravating hippocampal neuronal injury during prolonged epileptic seizures when homeostatic regulation fails.

In Chapter 3, I will summarize my research on the regulation of K<sub>v</sub>7 channels upon prolonged activity blockade which our lab has previously shown to induce homeostatic scaling of intrinsic excitability. My research shows that prolonged activity blockade using voltage-gated sodium channel blocker tetrodotoxin (TTX) decreases the activities of brain-derived neurotrophic factor (BDNF) receptor TrkB and its downstream extracellular signal-regulated kinase (ERK1/2) in cultured hippocampal neurons. Both prolonged activity blockade and prolonged pharmacological inhibition of ERK1/2 led to significant reductions in *KCNQ3* and *BDNF* transcripts as well as the levels of K<sub>v</sub>7.3 subunit and ankyrin-G at the axonal initial segments where action potentials are initiated and K<sub>v</sub>7 channels are concentrated by binding to ankyrin-G. These findings suggest that downregulation of both ERK1/2 activity and the AIS expression of K<sub>v</sub>7.3 may mediate homeostatic scaling of intrinsic excitability in hippocampal neurons.

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# **CHAPTER 1: AN INTRODUCTION TO EPILEPSY, HOMEOSTATIC PLASTICITY, AND THE ROLE OF K<sub>v</sub>7 AND GIRK CHANNELS IN NEURONAL PLASTICITY, BEHAVIOR, AND DISEASE**

Epilepsy is a chronic brain disorder, which impacts ~3% of the general population at some point during their lifetime ([www.epilepsy.com](http://www.epilepsy.com)). Epilepsy manifests as uncontrolled aberrant electrical activity in the brain, which manifests as recurrent and unpredictable seizures (Devinsky et al., 2018). Approximately 40% of epilepsy can be attributed to gene mutations (Zhang et al., 2017). Mutations in potassium (K<sup>+</sup>) channels such as GIRK and K<sub>v</sub>7 have been found to induce their dysregulation, resulting in hyperexcitability disorders and seizures (Signorini et al., 1997; Watanabe et al., 2000).

The remaining 60% is considered acquired epilepsy and the cause is relatively unknown. Of acquired epilepsy, about 20% is due to traumatic brain injury from stroke, contusion, or environmental toxins (Reddy and Kuruba, 2013). Temporal lobe epilepsy (TLE) is the most common form of focal epilepsy and is often acquired. It is commonly associated with neuronal death, network reorganization, and synaptic sprouting (Sharma et al., 2007). Loss of input to the surviving neurons could lead to homeostatic scaling of synaptic strength and intrinsic excitability and could form aberrant connections, which may all contribute to epileptogenesis in TLE (Swann and Rho, 2014). It has been shown that 2-day infusion of tetrodotoxin (TTX) into the hippocampus to model activity blockade leads to the development of seizures after 2-4 weeks in an age-dependent manner (Galvan et al., 2000), which involves homeostatic alterations in both intrinsic and synaptic properties (Echegoyen et al., 2007).

Homeostatic plasticity acts to stabilize neuronal activity by altering synaptic strength and intrinsic excitability (Turrigiano, 2011). Homeostatic scaling of intrinsic excitability refers to the mechanism by which a neuron adjusts its intrinsic membrane properties in response to its environment to maintain a stable firing rate (Turrigiano, 2011). To achieve this, a neuron must be able to sense its activity and respond in a negative feedback manner, by counteracting prolonged destabilizing increases or decreases in activity (Turrigiano, 2008). One powerful way to control excitability is through the regulation of specific neuronal location, surface density, and functional regulation of ion channels (Lai and Jan, 2006; Beck and Yaari, 2008; Trimmer, 2015), which contribute to the electrical properties of a neuron by integrating incoming signals, propagating

those signals, and ultimately regulating the firing of an action potential (Debanne et al., 2011). These regulations are achieved through a complex interaction with cytoskeletal scaffolding proteins and signaling proteins, transcriptional and translational modifications, and a variety of posttranslational modifications (Lai and Jan, 2006;Turrigiano, 2008;Grubb et al., 2011;Turrigiano, 2011;Trimmer, 2015;Yamada and Kuba, 2016;Leterrier, 2018).

To understand the role of K<sup>+</sup> channels in homeostatic plasticity and epilepsy, I have been focusing on how GIRK and K<sub>v</sub>7 channels are regulated by neuronal activity in this thesis. This chapter will introduce K<sub>v</sub>7 and GIRK channels and their known roles in neural plasticity and behavior. It is divided into 2 parts: **(1-1)** The role of K<sub>v</sub>7 channels in neural plasticity and behavior, and **(1-2)** The role of GIRK channels in neural plasticity and behavior.

## **THE ROLE OF K<sub>v</sub>7 CHANNELS IN NEURAL PLASTICITY AND BEHAVIOR <sup>1</sup>**

### **1-1 Abstract**

Activity-dependent persistent changes in neuronal intrinsic excitability and synaptic strength are widely thought to underlie learning and memory. Voltage-gated *KCNQ*/K<sub>v</sub>7 K<sup>+</sup> channels have been of great interest as the potential targets for memory disorders due to the beneficial effects of their antagonists in cognition. Importantly, *de novo* dominant mutations in their neuronal subunits *KCNQ2*/K<sub>v</sub>7.2 and *KCNQ3*/K<sub>v</sub>7.3 are associated with epilepsy and neurodevelopmental disorder characterized by developmental delay and intellectual disability. The role of K<sub>v</sub>7 channels in neuronal excitability and epilepsy has been extensively studied. However, their functional significance in neural plasticity, learning, and memory remains largely unknown. Here, we review recent studies that support the emerging roles of K<sub>v</sub>7 channels in intrinsic and synaptic plasticity, and their contributions to cognition and behavior.

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## 1-2 Introduction

Voltage-gate channel K<sup>+</sup> subfamily Q member 1-5 (*KCNQ 1-5*) encodes K<sub>v</sub>7.1-K<sub>v</sub>7.5 channels (Gutman et al., 2005) that are critical regulators of excitability in neurons, muscles, and sensory cells (Soldovieri et al., 2011). All K<sub>v</sub>7 subunits have 6 transmembrane segments (S1-S6) (Robbins, 2001). The S1-S4 comprises a voltage-sensing domain with the S4 being the main voltage-sensor (Robbins, 2001). The pore domain consists of the S5-S6 flanking the pore loop important for K<sup>+</sup> ion selectivity (Sun and MacKinnon, 2017). The gate is formed by the intersection of four S6 segments (Cui, 2016;Sun and MacKinnon, 2017). Upon depolarization, the electric field on the basic residues of S4 promotes its translational rotation and outward displacement, which leads to the opening of the gate (Cui, 2016;Sun and MacKinnon, 2017). All K<sub>v</sub>7 channels require phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) in the plasma membrane for channel opening (Zhang et al., 2003;Suh and Hille, 2008;Zaydman and Cui, 2014), and PIP<sub>2</sub> is proposed to couple the voltage-sensing domain to the pore domain in K<sub>v</sub>7.1 (Zaydman et al., 2013;Sun and MacKinnon, 2020). Each K<sub>v</sub>7 subunit also has a short intracellular N-terminal domain and a long intracellular C-terminal tail that harbors four helices (helix A-D) (Haitin and Attali, 2008). Helices A and B bind to calmodulin (Strulovich et al., 2016;Sun and MacKinnon, 2017), whereas helices C-D mediate subunit assembly (Haitin and Attali, 2008).

In neurons, K<sub>v</sub>7 channels open at subthreshold potentials around -60 mV and produce slowly-activating and non-inactivating outward K<sup>+</sup> currents that potently suppress repetitive and burst firing of action potentials (APs) (Brown and Passmore, 2009). Their functional significance in inhibiting neuronal excitability is underscored by the fact that mutations in their subunits cause epilepsy (Nappi et al., 2020) whereas K<sub>v</sub>7 agonist retigabine inhibits seizures in rodents and humans (Miceli et al., 2008). Importantly, emerging new evidence suggests that K<sub>v</sub>7 channels may contribute to activity-dependent persistent changes in neuronal intrinsic excitability and synaptic strength that are widely thought to underlie learning and memory. This review will summarize the function of K<sub>v</sub>7 channels in the hippocampus and discuss recent studies that investigate their contributions to hippocampal plasticity, cognition, and behavior.

### 1-3 Brain distribution of K<sub>v</sub>7 subunits and their channelopathies

K<sub>v</sub>7.2, K<sub>v</sub>7.3, and K<sub>v</sub>7.5 are the major neuronal K<sub>v</sub>7 subunits (Table 1). K<sub>v</sub>7.2 and K<sub>v</sub>7.3 show strong overlapping expression in the cerebral cortex, hippocampal formation, amygdala, basal ganglia, and hypothalamus (Wang et al., 1998;Cooper et al., 2001;Klinger et al., 2011). K<sub>v</sub>7.5 is highly expressed in the brain stem and to less extent in the cerebral cortex, hippocampus, occipital, frontal, and temporal lobes (Lerche et al., 2000;Schroeder et al., 2000;Tzingounis et al., 2010;Fidzinski et al., 2015). While K<sub>v</sub>7.1 and K<sub>v</sub>7.4 are mainly found in the heart and cochlear hair cells, respectively (Wang et al., 1996;Kubisch et al., 1999), they are also detected at a low level in multiple regions of the brain (Casimiro et al., 2001;Hansen et al., 2006;Goldman et al., 2009;Su et al., 2019) (Table 1).

Importantly, > 300 dominant mutations in *KCNQ2* and *KCNQ3* cause epilepsy including benign familial neonatal epilepsy (BFNE) and epileptic encephalopathy (EE) (Rikee and ClinVar database). *KCNQ2* is the second most frequently mutated gene in neurodevelopmental disorder (Traynelis et al., 2017;Coe et al., 2019) characterized by cognitive and behavioral deficits (Mullin et al., 2013). A few mutations in *KCNQ1*, *KCNQ4*, and *KCNQ5* have been associated with epilepsy, autism, schizophrenia, and developmental disorder (Table 1). Haploinsufficiency in K<sub>v</sub>7 function seems to underlie BFNE variants that cause the transient appearance of neonatal seizures (Soldovieri et al., 2011). EE patients display severe and often drug-resistant neonatal seizures and psychomotor retardation (Weckhuysen et al., 2012), and *de novo* EE mutations in *KCNQ2* and *KCNQ3* induce multiple defects in current and surface expression of K<sub>v</sub>7 channels (Weckhuysen et al., 2012;Milh et al., 2013;Weckhuysen et al., 2013;Miceli et al., 2015;Kim et al., 2018;Zhang et al., 2020).

### 1-4 General properties and regulation of K<sub>v</sub>7 currents

K<sub>v</sub>7.1 assembles with auxiliary  $\beta$  subunit KCNE1 to produce the slow delayed rectifier K<sup>+</sup> current (I<sub>Ks</sub>) important for the repolarization of cardiac APs (Barhanin et al., 1996). Importantly, coassembly with KCNE1 slows the activation kinetics of K<sub>v</sub>7.1 channel, potentiates its current amplitude, and eliminates its voltage-dependent inactivation (Barhanin et al., 1996;Sanguinetti et al., 1996;Tristani-Firouzi and Sanguinetti, 1998). Homomeric K<sub>v</sub>7.2 channels activate at -60 mV

and produce slow-activating and non-inactivating currents (Biervert et al., 1998). In comparison, currents through K<sub>v</sub>7.3 channels are negligible due to an Ala residue in the pore domain (Wang et al., 1998;Gomez-Posada et al., 2010). K<sub>v</sub>7.5 activates at -60 mV with slower kinetics than K<sub>v</sub>7.2 and K<sub>v</sub>7.3 (Schroeder et al., 2000;Gamper et al., 2003). K<sub>v</sub>7.4 activates at -40 mV with slower activation kinetics than other K<sub>v</sub>7 channels (Kubisch et al., 1999).

Neuronal K<sub>v</sub>7 channels are mostly heterotetrameric channels composed of K<sub>v</sub>7.2 and K<sub>v</sub>7.3 and to a lesser extent K<sub>v</sub>7.3 and K<sub>v</sub>7.5 (Wang et al., 1998;Shah et al., 2002) (Table 1). Compared to homomeric channels, significantly larger currents are generated by K<sub>v</sub>7.2/K<sub>v</sub>7.3 channels (Schroeder et al., 1998;Wang et al., 1998;Schwake et al., 2000) and K<sub>v</sub>7.3/K<sub>v</sub>7.5 channels (Schroeder et al., 2000;Gilling et al., 2013). K<sub>v</sub>7.2/K<sub>v</sub>7.3 channels produce M-current ( $I_M$ ) (Wang et al., 1998), which potently suppresses neuronal hyperexcitability (Wang et al., 1998;Yue and Yaari, 2004).  $I_M$  is inhibited by muscarinic acetylcholine receptor activation (Selyanko et al., 2000) and the depletion of PIP<sub>2</sub> (Suh and Hille, 2002;Zhang et al., 2003). K<sub>v</sub>7 channels are also inhibited by other G-protein coupled receptors, including substance P, bradykinin, serotonin, angiotensin, luteinizing hormone-releasing hormone, opioid, and metabotropic glutamate receptors (Marrion, 1997). General properties and diverse regulation of K<sub>v</sub>7 channels are described in detail in a previous review (Soldovieri et al., 2011).

### **1-5 Role of K<sub>v</sub>7 channels in intrinsic excitability and plasticity in the hippocampus**

Brown and Adams have first reported in 1980 that inhibition of  $I_M$  upon stimulation of muscarinic acetylcholine receptor results in repetitive firing of APs in bullfrog sympathetic ganglion neurons (Brown and Adams, 1980). In the hippocampus, strong expression of K<sub>v</sub>7.2, K<sub>v</sub>7.3, and K<sub>v</sub>7.5 is detected in pyramidal neurons (Schroeder et al., 2000;Cooper et al., 2001;Devaux et al., 2004). K<sub>v</sub>7 antagonists XE991 and linopirdine depolarize resting membrane potential (RMP) and reduce AP threshold of hippocampal CA1 pyramidal neurons, resulting in spontaneous AP firing (Aiken et al., 1995;Shah et al., 2008) (Figure 1A). K<sub>v</sub>7 antagonists also increase intrinsic excitability (Yue and Yaari, 2004;2006;Shah et al., 2008), contribute to medium and slow afterhyperpolarization (AHP) currents (Gu et al., 2005), reduce spike frequency adaptation (Aiken et al., 1995), and ultimately lead to an increased AP firing rate (Lezmy et al., 2020) (Figure 1A). Consistent with pharmacologic inhibition, suppression of K<sub>v</sub>7 current by

overexpressing  $K_v7.2$  containing dominant-negative pore mutation G279S enhances intrinsic excitability and reduces spike frequency adaptation and mAHP in CA1 neurons (Peters et al., 2005). Similarly, conditional homozygous deletion of *KCNQ2* increases CA1 excitability due to longer-lasting spike afterdepolarization (ADP) and reduced medium AHP (Soh et al., 2014) (Figure 1A). Thus,  $K_v7$  channels serve as critical “brakes” on neuronal excitability (Soldovieri et al., 2011).

The inhibitory effects of  $K_v7$  currents on neuronal excitability are largely attributed to axonal  $K_v7$  channels.  $K_v7.2/K_v7.3$  channels are preferentially enriched at the axonal plasma membrane compared to the somatodendritic plasma membrane in hippocampal neurons (Chung et al., 2006) with the highest concentration at the axonal initial segments (Maisonpierre et al.) (Chung et al., 2006; Pan et al., 2006) where AP initiates (Clark et al., 2009). CaM binding to  $K_v7.2$  is critical for targeting  $K_v7.2/K_v7.3$  channels to the axonal surface (Cavaretta et al., 2014), whereas disruption of this binding decreases  $I_M$  and increases hippocampal neuronal excitability (Shahidullah et al., 2005). Furthermore, disrupting the enrichment of  $K_v7$  channels at the AIS by blocking their interaction with ankyrin-G results in spontaneous firing of CA1 neurons by depolarizing RMP and reducing AP threshold (Shah et al., 2008).

In contrast to the well-documented function of axonal  $K_v7$  channels discussed above, the existence and role of dendritic  $K_v7$  channels are still in debate. Non-inactivating  $K_v7$  current sensitive to muscarinic agonist is detected in the distal apical dendrites of CA1 neurons (Chen and Johnston, 2004). Dendritic  $K_v7$  current can increase the threshold for initiating  $Ca^{2+}$  spikes and induce spike bursts only in hyperexcitable conditions that promote  $Ca^{2+}$  electrogenesis in these dendrites (Yue and Yaari, 2006). However, XE991 and linopridine do not affect input resistance of CA1 dendrites (Shah et al., 2008) and focal inhibition of dendritic  $I_M$  has no effect on the excitatory postsynaptic potential (EPSP) summation and excitability of CA1 neuron (Hu et al., 2007), indicating a very low level of dendritic  $K_v7$  current.

There is accumulating evidence for activity-dependent modulation of  $K_v7$  channels and their contribution to persistent changes in intrinsic excitability termed “intrinsic plasticity”. In the pilocarpine model of temporal lobe epilepsy, reduced  $K_v7$  function and expression may contribute to muscarinic-dependent ictogenesis (Maslarova et al., 2013). However, acute induction of seizures increases *KCNQ2* and *KCNQ3* transcripts in the hippocampi as a homeostatic response to suppress neuronal hyperexcitability, and this regulation requires activation of L-type voltage-gated

Ca<sup>2+</sup> channels (Zhang and Shapiro, 2012). Enhancing neuronal activity by K<sub>v</sub>7 inhibition with XE991 also results in homeostatic suppression of firing rate over 48 hours (Lezmy et al., 2020). In contrast, prolonged blockade of neuronal activity or NMDAR increases firing rate and reduces in *KCNQ3* transcript and K<sub>v</sub>7 current in hippocampal neurons (Lee and Chung, 2014; Lee et al., 2015). In the avian cochlear neurons, depriving afferent inputs induces a switch from fast activating K<sub>v</sub>1 to slow activating K<sub>v</sub>7.2 channels at the AIS, resulting in enhanced excitability (Kuba et al., 2015). This activity-dependent regulation of K<sub>v</sub>7 transcript and distribution offers a powerful means to control intrinsic excitability.

### **1-6 Role of K<sub>v</sub>7 channels in synaptic transmission and plasticity in the hippocampus**

Since the discovery of long-term potentiation (LTP) in the dentate gyrus of the hippocampus (Bliss and Lomo, 1973), persistent modification in synaptic strength termed “synaptic plasticity” has attracted significant attention as the cellular correlate of learning and memory (Nicoll, 2017). LTP at excitatory synapses can exert destabilizing influence on neural circuits by generating unconstrained synaptic strengthening (Turrigiano, 2012). Homeostatic plasticity counteracts such destabilizing conditions by allowing neurons to adjust their synaptic strength (Turrigiano, 2012). While activity-dependent modulation of glutamate release and glutamate receptors serves as key mechanisms for LTP expression (Turrigiano, 2012; Humeau and Choquet, 2019), K<sub>v</sub>7 channels and upstream muscarinic acetylcholine receptors have emerged as important regulators of excitatory synaptic transmission and plasticity.

Synaptic functions of K<sub>v</sub>7 channels have been extensively studied at the excitatory synapses formed by hippocampal CA1 and CA3 pyramidal neurons (Figure 1A). These neurons show strong expression of K<sub>v</sub>7.2 and K<sub>v</sub>7.3 (Cooper et al., 2001; Pan et al., 2006). Conditional deletion of *KCNQ2* and *KCNQ3* increases the frequency of spontaneous excitatory postsynaptic currents (EPSC) in CA1 neurons (Soh et al., 2018), suggesting enhanced presynaptic release at CA1-CA3 synapses. Consistent with this notion, application of K<sub>v</sub>7 antagonist XE991 increases whereas K<sub>v</sub>7 agonist Flupirtine decreases miniature EPSC frequency in CA1 neurons (Sun and Kapur, 2012). Furthermore, K<sub>v</sub>7 inhibition with linopirdine and XE991 treatment also increases neurotransmitter release (Martire et al., 2004; Peretz et al., 2007). While K<sub>v</sub>7 current restrains AP-evoked Ca<sup>2+</sup> influx into the presynaptic terminal and decreases the paired-pulse ratio of evoked

EPSCs at the mossy fiber–CA3 synapses (Martinello et al., 2019), paired-pulse facilitation of EPSP is higher at CA1-CA3 synapses in XE991-treated mice (Fontan-Lozano et al., 2011), suggesting differential roles of K<sub>v</sub>7 channels in short-term plasticity at two different synapses.

K<sub>v</sub>7.2 and K<sub>v</sub>7.3 are expressed in GABAergic neurons including parvalbumin (PV)- and somatostatin (SST)-positive interneurons in the hippocampus (Cooper et al., 2001;Lawrence et al., 2006). Application of XE991 abolishes *I<sub>M</sub>*, depolarizes RMP, increases AP firing in SST+ interneurons (Lawrence et al., 2006), and enhances intrinsic excitability of PV+ interneurons (Soh et al., 2018) (Figure 1A). Furthermore, conditional deletion of *KCNQ2* and *KCNQ3* from PV+ interneurons increase their firing and spontaneous inhibitory postsynaptic current (sIPSC) frequency of CA1 neurons in the hippocampus (Soh et al., 2018).

These studies highlight the presynaptic influence of K<sub>v</sub>7 channels at glutamatergic and GABAergic synapses. Given that increased firing rate and burst firing can enhance neurotransmitter release probabilities (Hansen et al., 2008), K<sub>v</sub>7 inhibition may increase neurotransmitter release as a consequence of increased axonal excitability (Devaux et al., 2004;Shah et al., 2008;Klinger et al., 2011). Indeed, when CA3 neurons are depolarized upon elevating extracellular K<sup>+</sup> concentration, XE991 enhances EPSP amplitude in CA1 neurons as a consequence of increasing spike ADP and burst firing of CA3 neurons (Vervaeke et al., 2006). Alternatively, K<sub>v</sub>7 channels at the presynaptic terminals (Cooper et al., 2001;Martire et al., 2004;Regev et al., 2009) may directly counteract the depolarization of the presynaptic membrane necessary for synaptic vesicle fusion and neurotransmitter release.

The postsynaptic role of K<sub>v</sub>7 channels is unclear. A recent electron microscopy study shows that K<sub>v</sub>7.2, K<sub>v</sub>7.3, and K<sub>v</sub>7.5 colocalize with muscarinic acetylcholine receptors at dendritic spines in layer III pyramidal neurons of the primate prefrontal cortex (Galvin et al., 2020), although the specificity of the immunolabeling needs to be further validated. In the CA1-CA3 synapses, the mEPSC amplitude is unaltered by agonist nor antagonists of K<sub>v</sub>7 channels (Sun and Kapur, 2012), suggesting their negligible role in regulating postsynaptic glutamate receptor function at this synapse (Figure 1A).

Nonetheless, accumulating evidence suggests that K<sub>v</sub>7 channels regulate hippocampal synaptic plasticity. At CA1-CA3 synapses, XE991 induces LTP by subthreshold theta-burst stimulation (Petrovic et al., 2012). Systemic administration of XE991 also decreases the threshold for LTP induction in the hippocampal CA1 area *in vivo* without affecting the field EPSP amplitude

(Song et al., 2009;Fontan-Lozano et al., 2011). Lastly, a homeostatic increase in excitatory synaptic transmission in CA1 neurons has been observed upon conditional deletion of *KCNQ2* and *KCNQ3* from GABAergic interneurons (Soh et al., 2018), suggesting the contribution of  $K_v7$  channels in synaptic scaling.

### **1-7 Role of $K_v7$ channels in hippocampus-dependent learning and memory**

Hippocampal LTP occurs during hippocampus-dependent learning and memory (Bliss et al. 2018) and its reduction is linked to memory loss in mouse models of Alzheimer's disease (Mango et al., 2019). Facilitation of LTP induction by XE991 (Song et al., 2009;Fontan-Lozano et al., 2011;Petrovic et al., 2012) suggests that pharmacologic  $K_v7$  inhibition may enhance learning and memory. Indeed, linopirdine enhances the performance of rats in a hippocampus-dependent active avoidance test (Cook et al., 1990). XE991 improves memory in object recognition task in wild-type mice and mouse models of dementia induced by cholinergic depletion and neurodegeneration (Fontan-Lozano et al., 2011;Ballinger et al., 2016;Dennis et al., 2016), despite its ability to induce seizures at a higher dose (Fontan-Lozano et al., 2011) (Figure 1B). In contrast,  $K_v7$  agonists have yielded mixed results on affecting memory in rodents (Li et al., 2014;Frankel et al., 2016).

The cognition-enhancing effect of linopirdine is correlated with the increased release of acetylcholine in the hippocampus (Fontana et al., 1994), and stimulation of muscarinic acetylcholine receptor inhibits  $I_M$  in hippocampal neurons (Shah et al., 2002). Consistently, muscarinic agonist improves whereas anticholinergic agent scopolamine impairs performance in hippocampus-dependent memory tasks (Fontana et al., 1994;Fontan-Lozano et al., 2011). Muscarinic acetylcholine receptors in the prefrontal cortex also modulate working memory in primates via  $K_v7$  channels (Galvin et al., 2020). Since cholinergic depletion and dysfunction in the hippocampus and prefrontal cortex are implicated in age-related cognitive decline and Alzheimer's disease (Ballinger et al., 2016;Haam and Yakel, 2017), these studies support the therapeutic potential for  $K_v7$  antagonists as cognitive enhancers.

Surprisingly, genetic inhibition or reduction of  $K_v7$  currents induces an opposite effect on memory (Figure 1B). Deficits in hippocampal-dependent spatial memory and spontaneous seizures are observed in mice with conditional transgenic expression of dominant-negative mutant

K<sub>v</sub>7.2-G279S (Peters et al., 2005) and heterozygous knock-in mice for K<sub>v</sub>7.2 containing epileptic encephalopathy loss-of-function variant T274M (Milh et al., 2020). Considering that K<sub>v</sub>7 channels are critical for development and inhibition of neonatal brain (Peters et al., 2005;Soh et al., 2014), the memory impairment in these genetic models could be attributed to abnormal hippocampal morphology and/or hyperexcitability (Peters et al., 2005;Milh et al., 2020).

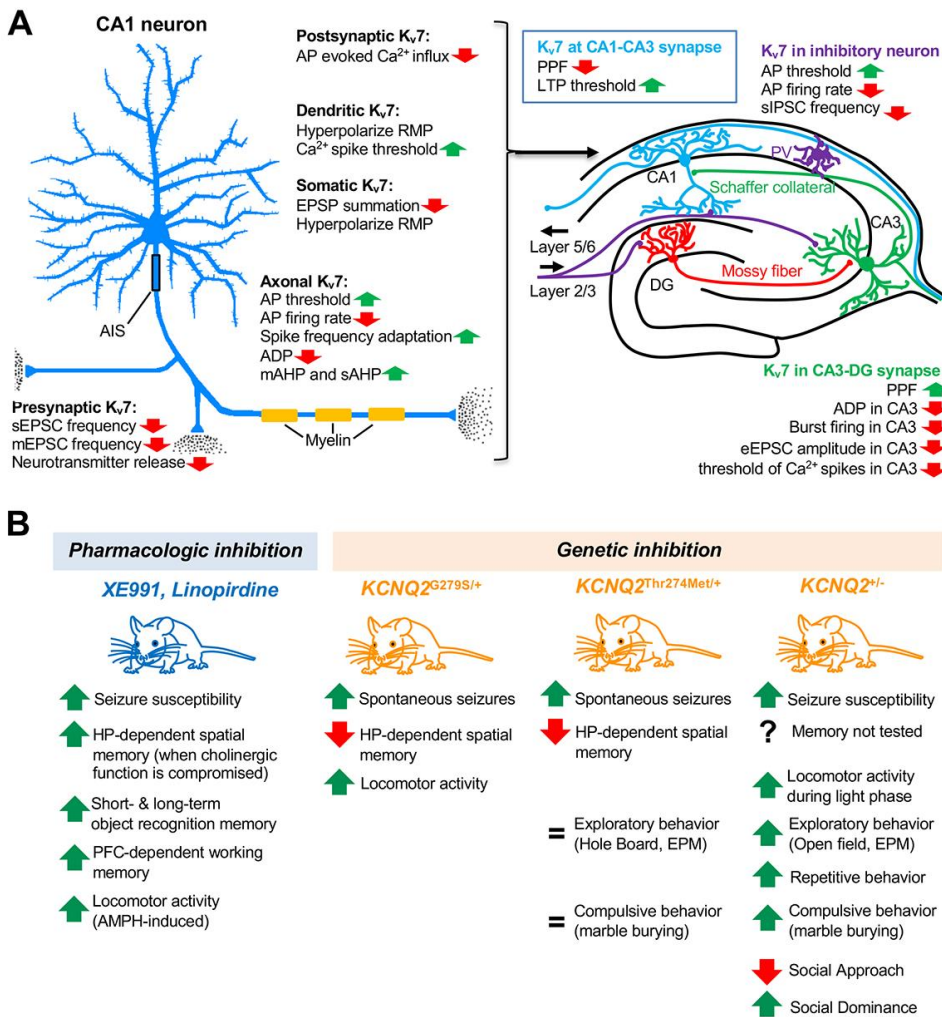
K<sub>v</sub>7 channels also regulate multiple behaviors (Figure 1B). Behavioral phenotyping of the global or conditional homozygous *KCNQ2* knock-out mice has not been possible due to their early postnatal lethality or premature death, respectively (Watanabe et al., 2000;Soh et al., 2014). However, heterozygous *KCNQ2* knock-out mice are viable and display increased locomotor activity and exploratory behavior (Kim et al., 2020), consistent with behavioral hyperactivity induced by transgenic suppression of K<sub>v</sub>7 currents (Peters et al., 2005) and amphetamine and XE991 (Sotty et al., 2009). These mice also show decreased sociability and increased repetitive and compulsive behavior (Kim et al., 2020), reminiscent of autism seen in some EE patients with dominant *KCNQ2* mutations (Weckhuysen et al., 2012;Milh et al., 2013;Weckhuysen et al., 2013). However, the precise circuitries responsible for these abnormal behaviors remain unknown.

## **1-8 Future perspectives**

The studies discussed in this review support the emerging concept that K<sub>v</sub>7 channels contribute to neural plasticity, memory, and behavior. However, there is a significant knowledge gap in our understanding of the underlying molecular and cellular mechanisms. Future studies should continue to investigate structure-function and subcellular targeting of K<sub>v</sub>7 channels, which will provide mechanistic insights for developing specific modulators of their function and trafficking. Generation of mouse models in which deletion of a K<sub>v</sub>7 subunit from specific neurons and subcellular localization with temporal control will be critical to delineate cell- and circuit-specific function of K<sub>v</sub>7 channels in neural plasticity, cognition, and behavior.

# 1-9 Figure

Figure 1.1



## Figure Legends

**Figure 1.1 The role of  $K_v7$  channels in hippocampal neurons, memory, and behavior.** (A) Function of  $K_v7$  channels in excitatory pyramidal neuron and GABAergic inhibitory neuron in the hippocampus. Abbreviations used: AP (action potential), RMP (resting membrane potential), EPSP (excitatory postsynaptic potential), ADP (Afterdepolarization), mAHP (medium afterhyperpolarization), sAHP (slow afterhyperpolarization), sEPSC (spontaneous excitatory postsynaptic current), mEPSC (miniature excitatory postsynaptic current), PPF (Paired-pulse facilitation), LTP (Long-term potentiation), sIPSC (spontaneous inhibitory postsynaptic current), PV (parvalbumin), and eEPSC (evoked excitatory postsynaptic current). (B) Effects of pharmacological or genetic inhibition of  $K_v7$  channels on memory and behavior. Abbreviations used: HP (hippocampus), PFC (Pre-frontal Cortex), AMPH (Amphetamine), and EPM (elevated plus maze).

**1-10 Table**  
**Tables 1.1**

**Table 1.1 Distribution of Kv7 subunit in the brain and the diseases associated with its pathogenic variants.**

| Gene         | Protein            | Primary Location | Distribution in the Brain                  |                                                                                                                                         | Pathogenic variants                                                                                 |                               |
|--------------|--------------------|------------------|--------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|-------------------------------|
|              |                    |                  | Regions                                    | Reference                                                                                                                               | Associated Diseases                                                                                 | Reference                     |
| <i>KCNQ1</i> | K <sub>v</sub> 7.1 | Heart            | CTX, HPF, MB, CB, BS                       | Casimiro et al., 2001; Goldman et al., 2009. AIBS, THPA                                                                                 | Long QT syndrome 1, JLNS, Familial Atrial Fibrillation Epilepsy, SUDEP, ASD, Developmental disorder | ClinVar<br>LOVD<br>denovo-db  |
| <i>KCNQ2</i> | K <sub>v</sub> 7.2 | Nervous System   | CTX, HPF, A, HY, TH, OA, MD, SN, P, MY, CB | Wang et al., 1998; Cooper et al., 2001; Devaux et al., 2004; Klinger et al., 2011; D'Este et al., 2016; Galvin et al., 2020. AIBS, THPA | BFNE, EE, ASD, Intellectual disability, Developmental disorder, Sporadic infantile spasm syndrome   | ClinVar<br>RIKEE<br>denovo-db |
| <i>KCNQ3</i> | K <sub>v</sub> 7.3 | Nervous System   | CTX, HPF, A, HY, TH, OA, MD, SN, P, MY, CB | Wang et al., 1998; Devaux et al., 2004; Klinger et al., 2011; Galvin et al., 2020. AIBS, THPA                                           | BFNE, EE, ASD, Intellectual disability, Developmental disorder                                      | ClinVar<br>RIKEE<br>denovo-db |
| <i>KCNQ4</i> | K <sub>v</sub> 7.4 | Inner Ear        | BS, OA, MD, RN, NA, MY, VTA, P             | Hansen et al., 2006; Su et al., 2019. AIBS, THPA                                                                                        | DFNA2, ASD                                                                                          | ClinVar<br>denovo-db          |
| <i>KCNQ5</i> | K <sub>v</sub> 7.5 | Nervous System   | CTX, HPF, BS, CB                           | Schroeder et al., 2000a; Lerche et al., 2000; Tzingounis et al., 2010; Fidzinski et al., 2015; Galvin et al., 2020. AIBS, THPA          | EE, ASD, Intellectual disability, Schizophrenia                                                     | ClinVar<br>RIKEE<br>denovo-db |

Brain regions: Cortex (CTX), Olfactory areas (OA), Hippocampal formation (HPF), Amygdala (A) Nucleus accumbens (NA), Brain stem (BS), Thalamus (TH), Hypothalamus (HY), Midbrain (MB), Raphe Nuclei (RN), Substantia nigra (SN) Ventral tegmental area (VTA), Pallidum (PAL), Hindbrain (HB), Cerebellum (CB), Pons (P), Medulla (MY).

K<sub>v</sub>7 channelopathies: Jervell and Lange-Nielsen syndrome (JLNS), Sudden Unexpected Death in Epilepsy (SUDEP), Benign Familial Neonatal Epilepsy (BFNE), Epileptic Encephalopathy (EE), Autism Spectrum Disorder (ASD), Nonsyndromic Sensorineural Deafness Type 2 (DFNA2). Database website: Allen Institute for Brain Science (AIBS, <https://alleninstitute.org/what-we-do/brain-science/>). The Human Protein Atlas (THPA, <https://www.proteinatlas.org/>). ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>), Leiden Open Variation Database (LOVD, <https://research.cchmc.org/LOVD2/home.php>), denovo-db (<http://denovo-db.gs.washington.edu/denovo-db/index.jsp>), Rational Intervention for KCNQ2/3 Epileptic Encephalopathy (RIKEE, <https://www.rikee.org/>).

# THE ROLE OF GIRK CHANNELS IN NEURAL PLASTICITY AND BEHAVIOR

## 1-11 Abstract

Activity-dependent changes in neuronal intrinsic excitability and synaptic strength are widely thought to underlie learning and memory. G protein-activated inwardly rectifying potassium ( $K^+$ ) (GIRK1-4) channels have become of interest as they are located near excitatory synapses and respond to various neurotransmitters via their G protein-coupled receptors (GPCRs). GIRK channels become activated during long-term potentiation (LTP) stimulation contributing to a slow hyperpolarizing current. Interestingly, some disorders including epilepsy, Down syndrome, and Parkinson's disease appear to implicate GIRK dysfunction, offering novel targets for these disorders. The function of GIRK channels in neural plasticity, learning, and memory remains to be completely uncovered, however, it is clear from animal studies that GIRK channels play a role in long term-depression (LTD) and depotentiation of LTP whereas their loss of function leads to numerous neurologic disorders.

## 1-12 Introduction

Inwardly-rectifying  $K^+$  channels subfamily J (*KCNJ*) encodes for Kir3.x channels, which are also referred to as G protein-activated inwardly rectifying  $K^+$  channels (GIRK) (Kubo et al., 2005). *KCNJ3*, *KCNJ6*, *KCNJ9*, and *KCNJ5* encode GIRK1-4, which are also named Kir3.1-3.4, respectively. These channels are referred to as “Inwardly-rectifying” due to the change in the current-voltage relationship at the reversal potential for  $K^+$  caused by intracellular  $Mg^{2+}$  and polyamines blocking the central pore. Due to these properties, GIRK channels produce a small outward  $K^+$  current when the membrane potential is above the equilibrium potential for  $K^+$ . These channels are present in multiple cell types including the brain, heart, kidney, endocrine cells, ears, and retina (Kubo et al., 2005). The primary expression of GIRK subunits in the brain includes heterotetramers GIRK1/GIRK2, GIRK2/GIRK3, and GIRK1/GIRK3, as well as homotetramers of GIRK2 (Luscher and Slesinger, 2010). These GIRK channels have been identified in hippocampal pyramidal neurons, dopaminergic and GABAergic interneurons in the ventral tegmental area, substantia nigra, thalamus, hypothalamus, amygdala, olfactory bulb, locus coeruleus, nucleus basalis, nucleus accumbens, spinal cord, raphe nuclei, medial prefrontal cortex, and cerebral cortex

(Jeremic et al., 2021). Homotetramers of GIRK2 appear in the substantia nigra. While, GIRK4 is found mostly in the heart but has also been detected in the brain restricted to fewer brain regions than other GIRK subunits (Wickman et al., 2000).

All GIRK subunits have two transmembrane (M1-M2) domains connected by a poor loop and contain the K<sup>+</sup> selectivity filter upon heterotetramer formation. The second transmembrane domain (M2) and the central pore (G-loop) compose two gating mechanisms (Jin et al., 2002; Pegan et al., 2005; Lujan et al., 2009). The main gating mechanism involves the G-loop which opens through a conformational rotation due to Gβγ binding (Whorton and MacKinnon, 2013). The secondary gating mechanism involving the M2 requires PIP<sub>2</sub> binding, which causes a modest conformational rotation of M2, resulting in widening the pore at the inner helix gate (Niu et al., 2020). PIP<sub>2</sub> is a necessary co-factor to open GIRK channels (Huang et al., 1998). Gβγ binding induces a structural change in the N- and C-terminal domains (Riven et al., 2003), which facilitates the opening of the gate and mediates K<sup>+</sup> currents. Only GIRK2 can form homomeric channels in the CNS, however, these channels have a shorter channel opening time than heteromeric channels (Luscher and Slesinger, 2010). Homomeric GIRK1 channels are non-functional because they lack an ER export motif. GIRK1 needs GIRK2 or GIRK4 which contains an ER export motif in order to make functional channels that transport to the plasma membrane (Ma et al., 2002). Interestingly, GIRK3 also contains a unique lysosomal targeting motif that has been shown to promote the degradation of GIRK channels (Ma et al., 2002). However, GIRK3 can form functional channels with GIRK1 suggesting the ER export motif is not the only requirement for channel trafficking to the cell membrane (Lujan et al., 2014).

In the brain, GIRK channels regulate neuronal excitability by activating numerous G-protein-coupled receptors in response to various neurotransmitters including acetylcholine, dopamine, opioids, serotonin, somatostatin, adenosine, and GABA (Luscher and Slesinger, 2010). The binding of these neurotransmitters to their respective G-protein coupled receptors (GPCRs) causes the Gβγ to dissociate from the pertussis toxin (PTX)-sensitive Gα<sub>i</sub> and Gα<sub>o</sub> subunits. The Gβγ directly binds to intracellular domains of the GIRK channel, allowing them to open (Reuveny et al., 1994; Huang et al., 1995). Upon activation of GPCRs by their ligand, they undergo a conformational change and promote the exchange of GDP/GTP associated with the Gα subunit. The G<sub>q</sub>-coupled GPCRs regulate GIRK channels through stimulation of phospholipase C (PLC) which depletes levels of PIP<sub>2</sub> and desensitizes GIRK current (Kobrinisky et al., 2000). Interestingly,

PLC can stimulate PKC activity which has also been found to decrease GIRK activity (Stevens et al., 1999).

GIRK1 and GIRK2 are the major subunits of neuronal GIRK channels that mediate membrane hyperpolarization and synaptic inhibition in the brain (Luscher and Slesinger, 2010). The functional importance of GIRK channels inhibiting neuronal excitability can be seen in GIRK2 homozygote knock-out mice (GIRK2<sup>-/-</sup>) and GIRK2 and GIRK3 double homozygote knockout mice (GIRK2<sup>-/-</sup>GIRK3<sup>-/-</sup>) which display spontaneous and lethal seizures (Signorini et al., 1997;Torrecilla et al., 2002). Application of GIRK inhibitor tertiapin causes pro-convulsive effects in Wistar rats (Mazarati et al., 2006) while ML297, a selective activator of GIRK1 channels had an antiseizure effect in mice (Kaufmann et al., 2013).

Evidence suggests that GIRK channels may contribute to activity-dependent persistent changes in neuronal intrinsic excitability and synaptic strength that are widely thought to underlie learning and memory. This review will summarize the function of GIRK channels in the hippocampus and discuss recent studies that investigate their contributions to hippocampal plasticity, cognition, and behavior.

### **1-13 Brain distribution of GIRK subunits and their channelopathies**

GIRK1-3 subunits are expressed in many areas of the brain, whereas GIRK4 is expressed only sparsely (Karschin et al., 1996;Wickman et al., 2000). GIRK1-3 have been found in the hippocampus, substantia nigra, locus coeruleus, nucleus basalis, ventral tegmental area, and the medial prefrontal cortex, whereas GIRK4 appears largely restricted to the hypothalamus, midbrain, pons, medulla, and spinal cord (Luscher and Slesinger, 2010;Jeremic et al., 2021). Although GIRK1-3 subunits display widespread expression throughout the brain, there still appears to be subunit-specific localization. For example, GIRK channels formed by heteromeric assembly of GIRK2 with GIRK3 are found in midbrain dopamine neurons, whereas GABAergic neurons in the midbrain express GIRK1-3 (Labouebe et al., 2007). The importance of these subunit combinations in specific brain regions remains to be completely understood.

Subcellularly, neuronal GIRK channels are preferentially localized in the soma and dendrites of hippocampal neurons (Kulik et al., 2006;Fernandez-Alacid et al., 2011) and have been found in numerous excitatory and inhibitory neurons (Lujan et al., 2009). Specific neurons in

thalamocortical presynaptic nerve terminals have also been reported to express GIRK channels (Ponce et al., 1996). Macromolecular complexes containing GIRK channels and their associated GPCRs have been identified, such as GABA<sub>B</sub> receptors which uniquely co-localize on dendritic spines (Kulik et al., 2006). Muscarinic receptors have been found to dimerize with GABA<sub>B</sub> receptors creating an even larger heteromeric complex with GIRK channels (Boyer et al., 2009). Electron microscopy has shown that GIRK channels are localized to the dendritic shafts, perisynaptic, and extrasynaptically in hippocampal neurons (Kulik et al., 2006). The exact composition of subunits remains elusive due to multiple splice variants and the possibility of asymmetric channels containing 3 or more different subunits. Differences in the N- and C- terminal targeting of different GIRK subunits convey diverse targeting of GIRK channels (Ma et al., 2002). GIRK2 and GIRK3 channels have been observed in the postsynaptic density, whereas GIRK1 and GIRK3 channels expressed perisynaptic in the cerebellum (Fernandez-Alacid et al., 2009).

Human mutations in the *KCNJ3*/GIRK1 and *KCNJ5*/GIRK4 have been associated with Schizophrenia. *KCNJ9*/GIRK3 has been associated with EAST syndrome characterized by epilepsy, ataxia, hearing impairment, and renal tubulopathy. Mutations in *KCNJ6*/GIRK2 can result in Keppen-Lubinsky syndrome characterized by severe developmental delay and intellectual disability. *KCNJ5*/GIRK4 and *KCNJ6*/GIRK2 have been associated with long QT syndrome characterized by arrhythmia in the heart (Jeremic et al., 2021) (<https://www.ncbi.nlm.nih.gov/clinvar/>).

The *KCNJ5* gene encoding GIRK4 has been shown to cause long QT syndrome which results in cardiac arrhythmias that can result in death (Yang et al., 2010; Wang et al., 2013b). This is in line with its lower CNS expression compared to other GIRK channels (Lujan et al., 2014). *KCNJ3*/GIRK1 has been associated with schizophrenia as a possible gene candidate (Yamada et al., 2011; Yamada et al., 2012). Humans with mutation of the *KCNJ6* gene have been reported to have Keppen-Lubinsky syndrome which is characterized by severe developmental delay and intellectual disability (Masotti et al., 2015).

GIRK channels are implicated in the pathophysiology of multiple diseases. As mentioned previously GIRK2 homozygote knockout mice (GIRK2<sup>-/-</sup>) and GIRK2 and GIRK3 double homozygote knockout mice (GIRK2<sup>-/-</sup>GIRK3<sup>-/-</sup>) display spontaneous and lethal seizures (Signorini et al., 1997; Torrecilla et al., 2002). ML297, a selective activator of GIRK1 channels had an antiseizure effect in mice models exposed to maximal electroshock model displaying increased

latency to seizures and pentylenetetrazol (PTZ) model of epilepsy which showed a reduced percentage of mice displaying seizures as well as increased survival (Kaufmann et al., 2013). No single mutation of GIRK1-4 have been shown to cause epilepsy clinically. GIRK2<sup>-/-</sup> mice and GIRK2<sup>-/-</sup> GIRK3<sup>-/-</sup> total knock-out mice display sporadic seizures, increased seizure susceptibility, and a reduced lifespan (Signorini et al., 1997;Torrecilla et al., 2002), demonstrating that loss of GIRK function can lead to excessive neuronal excitability resulting in epilepsy. Consistently, tertiapin, which blocks GIRK channels, has a convulsive effect (Mazarati et al., 2006). In contrast, ML297, a selective GIRK agonist, delays seizure onset, reduces the percentage of mice that experience seizures, and increases survival in the maximal electroshock and pentylenetetrazol models of epilepsy (Kaufmann et al., 2013). Activation of GIRK channels prevents kindling-induced epileptogenesis (Mazarati et al., 2006). These studies together demonstrate the protective role that GIRK channels play in preventing seizures. Interestingly, the seizures themselves can regulate GIRK channels. Electroconvulsive shock leads to increased GIRK expression (Pei et al., 1999). My thesis research has shown that seizures induced by kainite injection cause GIRK cleavage *in vivo* (Baculis et al., 2017).

In the *Weaver* mice, a spontaneous missense mutation in the GIRK2 gene (p.Gly156Ser) results in a loss of the K<sup>+</sup> selectivity of GIRK channels (Slesinger et al., 1996) and allows an aberrant Na<sup>+</sup> influx that can trigger cell death in the hippocampus, substantia nigra, and cerebellum (Hess, 1996). Additionally, this mutation results in a complex phenotype including learning deficits, epileptic seizures, and motor disturbances (Schmidt et al., 1982;Eisenberg and Messer, 1989;Derenne et al., 2007;Luscher and Slesinger, 2010). *Weaver* mice, which have been used to model Parkinson's disease, show progressive degeneration of dopaminergic neurons in the substantia nigra and cerebellar granular neurons, causing causes gait instability, poor limb coordination, and tremors (Schmidt et al., 1982;Harkins and Fox, 2002).

As mentioned previously GIRK channels can be found in the mesocorticolimbic dopamine system consisting of the ventral tegmental area, nucleus accumbens, and medial prefrontal cortex. This circuitry underlies reward and addiction (van Huijstee and Mansvelder, 2014). In addition, GIRK signaling is altered by numerous drugs of abuse. GABA<sub>B</sub>R–GIRK currents are enhanced by ethanol (Federici et al., 2009). Ethanol has also been found to bind to GIRK channels directly to activate these channels (Aryal et al., 2009). In humans, single-nucleotide polymorphism in the *KCNJ6* was associated with alcohol dependence (Clarke et al., 2011). GIRK2/3<sup>-/-</sup> knockout mice

displayed reduced self-administration of cocaine compared to wild-type controls and GIRK2<sup>-/-</sup> or GIRK3<sup>-/-</sup> single knockouts (Morgan et al., 2003). This decrease in self-administration could be due to the greater effect of cocaine on GIRK2<sup>-/-</sup> as observed in their enhanced locomotor responses to cocaine (Arora et al., 2010). These findings have been confirmed with GIRK knockout in only dopaminergic neurons (GIRK2<sup>DAT</sup>) enabling the precise role of GIRK2 in dopaminergic neurons to be investigated (McCall et al., 2019). GIRK channels are coupled to  $\mu$ -,  $\kappa$ -, and  $\delta$ -opioid receptors (Nagi and Pineyro, 2014), and GIRK2/3 channels in VTA dopamine neurons were found to be required for the motor-stimulatory effect of morphine exposure (Kotecki et al., 2015), similar to that found due to cocaine. In humans, single nucleotide polymorphisms in *KCNJ6*/GIRK2 were associated with increased opioid requirements for analgesia and a lack of opioid withdrawal symptoms (Lotsch et al., 2010). Interestingly, GIRK1<sup>-/-</sup> knockout mice have shown reduced antinociceptive effects of opioids (Marker et al., 2004).

Duplication of human chromosome 21 causes Down's syndrome. Since the human chromosome 21 contains the *KCNJ6* gene, *KCNJ6*/GIRK2 has been thought to contribute in part to the disorder (Luscher and Slesinger, 2010). Using the Ts65Dn mouse model of Down syndrome, evidence has been found that GIRK2 signaling is enhanced and LTP is reduced resulting in poorer performance on novel object tests as well as a failure to produce short-term and long-term plasticity in the dentate gyrus (Kleschevnikov et al., 2017). Weaver mice which also contain a point mutation in the *KCNJ6* gene have observed symptoms of Parkinson's disease including degeneration of dopaminergic neurons, gait instability, tremors, and learning deficits (Schmidt et al., 1982;Derenne et al., 2007). Weaver mice have also been observed to display increased motor activity and seizures like GIRK2 homozygote knock-out mice (GIRK2<sup>-/-</sup>) (Schmidt et al., 1982;Eisenberg and Messer, 1989).

## **1-14 General properties and regulation of GIRK currents**

Inward rectification refers to the change in the slope of the current-voltage relationship of the channel at the reversal potential, in this case, it is the reversal potential of a K<sup>+</sup> ion. At membrane potential that is depolarized than K<sup>+</sup> equilibrium potential, inwardly rectifying K<sup>+</sup> channels conduct a small outward current. At membrane potentials that are below K<sup>+</sup> equilibrium potential, these channels conduct inward K<sup>+</sup> currents. These channel characteristics were found to

be due to  $Mg^{2+}$  and polyamines blockage of the pore (Yamada et al., 1998). GIRK channels produce a small outward  $K^+$  current when the membrane potential is above the equilibrium potential for  $K^+$ . Upon membrane potential below the equilibrium potential for  $K^+$ , there is a larger inward  $K^+$  current.

Various neurotransmitters, such as acetylcholine, dopamine, opioids, serotonin, somatostatin, adenosine, and GABA, regulate GIRK channels by stimulating their GPCRs.  $G\beta\gamma$  dimers released from  $G_i$ - or  $G_o$ -coupled GPCRs bind directly to GIRK channels, causing them to open.  $G\beta\gamma$  binding opens GIRK channels through direct binding and closes the channel upon  $G\beta\gamma$  dissociation (Whorton and MacKinnon, 2013; Niu et al., 2020). The binding of  $G\alpha$  to GIRK channels has been found to suppress basal activity (Peleg et al., 2002). A second inhibitory pathway through  $G_q$ - and  $G_{11}$ -coupled GPCR stimulate phospholipase C (PLC) which depletes plasma membrane levels of  $PIP_2$ , leading to desensitizing GIRK currents (Kobrinisky et al., 2000). Activation of PLC also leads to the stimulation of PKC. PKC-dependent phosphorylation decreases, whereas PKA-dependent phosphorylation enhances channel activity (Luscher and Slesinger, 2010). PKC phosphorylation sites in GIRK have been identified as Ser-185 in GIRK1 and Ser-191 in GIRK4 (Mao et al., 2004). GIRK1/GIRK4 contains a strong PKA consensus site on the c- terminal (Mullner et al., 2000), however, whether this site is directly phosphorylated by PKA or modulates GIRK through an alternative substrate remains to be determined. GIRK channels are also thought to be regulated by tyrosine kinase,  $Ca^{2+}$  calmodulin-dependent protein kinase 2, protein phosphatase 1, and protein phosphatase 2A (Luscher and Slesinger, 2010).

GIRK currents can also be regulated by the changes in their surface expression. GIRK2 and GIRK4 have various trafficking signals in their intracellular N- and C-terminal domains that regulate ER export (Ma et al., 2002). The C-terminal tail of GIRK2 containing an ER export motif <sup>396</sup>ELETEEE<sup>403</sup> allows for efficient surface expression of heterotetrameric channels (Ma et al., 2002; Mirshahi and Logothetis, 2004). The ER export motif controls surface expression by regulating GIRK channels export from the ER. GIRK1 and GIRK3 subunits, on the other hand, lack an ER export signal and must assemble with GIRK2 or GIRK4 subunits (Ma et al., 2002). GIRK3 subunit contains an additional lysosomal-targeting motif which reduces surface expression (Ma et al., 2002). Among the GIRK subunits, GIRK2 and GIRK3 possess C-terminal PDZ-binding motifs that regulate endosomal trafficking of GIRK channels (Lunn et al., 2007). Neuronal activity and specifically NMDA receptor activation in hippocampal neurons have been found to elevate

surface expression of the GIRK channels within 15 minutes. This increase in surface expression was dependent on protein phosphatase-1 which mediated dephosphorylation of a serine residue (Ser-9) preceding the GIRK2 Val-13/Leu-14 internalization motif (Chung et al., 2009b).

GIRK channel subunit composition can alter channel function. As previously mentioned, GIRK1 and GIRK3 form heterotetrameric channels while GIRK2 can form homotetramers and heterotetramers (Ma et al., 2002;Luscher and Slesinger, 2010). GIRK2 homotetrameric channels have shorter open times compared to heterotrimeric channels including GIRK1/GIRK2, GIRK1/GIRK3, GIRK1/GIRK4 (~1–2 ms mean open times) (Kofuji et al., 1995;Lesage et al., 1995;Slesinger et al., 1995;Jelacic et al., 1999;Luscher and Slesinger, 2010). Single-channel conductance (~30 pS) appears similar between most heteromeric GIRK subunits (Kofuji et al., 1995;Lesage et al., 1995;Jelacic et al., 1999;Luscher and Slesinger, 2010). In addition to subunit-specific kinetics, GIRK2/GIRK3 heteromeric channels had a lower half-maximal effective concentration for G $\beta\gamma$  activation than GIRK1/GIRK3 heteromeric channels (Jelacic et al., 2000). This difference in G $\beta\gamma$  binding could have major implications on these channel functions as they rely on passive G $\beta\gamma$  diffusion for activation. The functional implication of the differences in specific subunit channel activity is not completely understood.

### **1-15 Role of GIRK channels in intrinsic excitability and plasticity in the hippocampus**

GIRK channels contribute to the resting potential of neurons, hyperpolarizing the membrane voltage by ~ 8 mV in hippocampal neurons (Luscher et al., 1997). In addition, activation of GIRK channels hyperpolarizes neurons in response to activation of many different GPCRs (Pfaffinger et al., 1985;Andrade et al., 1986;Christie and North, 1988;Inoue et al., 1988). When GIRK channels activation by their PTX-sensitive (G $\alpha_i$  and G $\alpha_o$ ) G $\alpha$  subunit then causes the G $\beta\gamma$  to dissociate and directly bind to the GIRK channels intracellular domains allow channel opening (Reuveny et al., 1994;Huang et al., 1995). When membrane potentials are higher than the equilibrium potential for potassium, these channels slowly conduct outward K $^+$  ions. However when the membrane potential is lower than the equilibrium potential for K $^+$ , the Mg $^{2+}$  and polyamine block is removed, and there is a greater inward current than the outward current of K $^+$  (Nishida and MacKinnon, 2002). GIRK channel activation decreases excitability by inhibiting both sodium and calcium spikes (Lippiello et al., 2020).

A<sub>1</sub> Adenosine receptors couple with GIRK channels in CA3-CA1 excitatory neurons in the hippocampus where they contribute to resting membrane conductance and hyperpolarization (Kim and Johnston, 2015). GIRK channels can regulate the integration of excitatory inputs in CA1 dendrites, inhibiting the generation of plateau potentials and constricting the window for temporal summation from Schaffer collateral stimulation onto CA1 neurons (Malik and Johnston, 2017). GIRK activation has also been found to control the excitability of neurons through GIRK-mediated self-inhibition in which neurotransmitter stimulation of G<sub>i</sub>- and G<sub>o</sub>-coupled receptor on dendrites CA1 neurons can activate GIRK channels which then give rise to slow inhibitory current (Luscher et al., 1997; Takigawa and Alzheimer, 2002; Chen and Johnston, 2005).

### **1-16 Role of GIRK channels in synaptic transmission and plasticity in the hippocampus**

GIRK channels give rise to slow inhibitory postsynaptic current (IPSC) (Luscher et al., 1997). The generation of slow IPSCs is important for regulating the rhythmic gamma oscillations activity of the network (Pietersen et al., 2009). In addition to postsynaptic activation, GABA<sub>B</sub> receptor-mediated presynaptic inhibition contains a component that is sensitive to a GIRK-specific antagonist tertiapin, suggesting a presynaptic role for GIRK channels in inhibiting neurotransmitter release (Jin and Lu, 1998; Ladera et al., 2008; Michaeli and Yaka, 2010). Adenosine receptor activation of GIRK channels in the hippocampus has been found to play a role in the suppressing effects of adenosine in response to seizure activity (Hill et al., 2020). Serotonin receptor, 5-HT<sub>1A</sub>, are co-expressed with GIRK channel in the hippocampus, cerebral cortex, septum, and dorsal raphe nucleus (Saenz del Burgo et al., 2008) and upon 5-HT<sub>1A</sub> activation of GIRK, this induces hyperpolarization (Beck et al., 1992) and mediates cell inhibition and ultimately results in a decrease in firing rate (Johnston et al., 2014). Dopamine metabotropic receptors have a different effect on GIRK channels depending on their subtype. For example, D<sub>1</sub> receptor activation reduces GIRK currents (Witkowski et al., 2008), while D<sub>2</sub> autoreceptors activate GIRK channels (Ford, 2014).

LTP stimulation leads to a simultaneous potentiation of GIRK-dependent slow IPSC which requires GABA<sub>B</sub> stimulation (Huang et al., 2005). GIRK channels activation by adenosine A<sub>1</sub> receptors can increase slow IPSC in an NMDAR-dependent manner (Huang et al., 2005). LTP stimulation leading to GIRK activation directly implicates GIRK channel activation in the

maintenance of hippocampal NMDAR-dependent LTP. Activation of NMDARs in hippocampal cultured neurons increased the surface expression of GIRK1 and GIRK2 channels in their soma and dendrites (Chung et al., 2009b). GIRK2<sup>-/-</sup> knockout acute slices were unable to undergo depotentiation of LTP at CA1 synapses in hippocampal neurons (Chung et al., 2009a). Similarly, wild-type acute slices treated with GIRK antagonist tertiapin, impaired the depotentiation of LTP at CA1 synapses in hippocampal neurons (Chung et al., 2009a). Dendritic GIRK channels have more recently been found to contribute to the threshold for LTP in the dorsal but not ventral pole of the hippocampus CA1 neurons (Malik and Johnston, 2017). Recent Alzheimer's disease mouse models demonstrate that GIRK signaling can restore LTP/LTD threshold change responsible for disruption of synaptic plasticity and impaired hippocampal-dependent memory performance (Sanchez-Rodriguez et al., 2020). A recent knock-in in which the GIRK1 subunit was fluorescently tagged (GIRK1-YFP) resulted in a functional knockdown display similar impairments as GIRK1<sup>-/-</sup> knockout mice, resulting in impaired depotentiation following LTP (Mett et al., 2021). ML297, a GIRK channel agonist, application has been found to decrease basal fEPSP amplitude, while tertiapin-Q (T-Q), a selective GIRK channel inhibitor, significantly increased fEPSP amplitude (Djebari et al., 2021). Using high-frequency stimulation of the Schaffer collateral pathway, it was found that ML297 blocked LTP and caused LTD, while T-Q allowed for LTP at 30 minutes but was lost by 50 minutes post-stimulation (Djebari et al., 2021). In vivo injection of either ML297 or T-Q induced depression of the synaptic response following high frequency stimulation of Schaffer collaterals showing GIRK channels are critical for the induction and maintenance of hippocampal CA3-CA1 synapses (Djebari et al., 2021).

### **1-17 Role of GIRK channels in hippocampus-dependent learning and memory**

Knockout rodent models have been critical in our understanding of GIRK channels' role in learning and memory-related behaviors. GIRK1<sup>-/-</sup> knockout mice displayed reduced anxiety with signs of hyperactivity (Blednov et al., 2001; Pravetoni and Wickman, 2008). (GIRK1-YFP) functional knockout mice exhibit impaired spatial learning and memory showing delay in learning the location of the escape platform in the Morris water maze as well as impairments in long-term memory formation displaying reduced time spent in the correct quadrant during the probe test compared to controls (Mett et al., 2021). As previously mentioned, GIRK2<sup>-/-</sup> and GIRK2<sup>-/-</sup>GIRK3<sup>-/-</sup>

<sup>-/-</sup> double knockout mice display sporadic seizures and a reduced lifespan (Signorini et al., 1997; Torrecilla et al., 2002). GIRK2<sup>-/-</sup> knockout mice also appear hyperactive and showed reduced anxiety-related behavior (Pravetoni and Wickman, 2008). GIRK2<sup>-/-</sup> knockout mice self-administer cocaine less than wildtype (Morgan et al., 2003) which corresponds with the reduced antinociceptive effect of opioids (Marker et al., 2004). To test the effect of GIRK2<sup>-/-</sup> knock out on learning and memory, CaMKII Cre mice were crossed with GIRK<sup>fl/fl</sup>, resulting in a loss of GIRK in forebrain pyramidal neurons. These mice have impaired contextual and cued fear conditioning, meaning they have impaired associative learning. These mice also displayed enhanced LTD and no depotentiation of LTP in CA1 synapse upon stimulation of the Schaffer collateral pathway in the dorsal hippocampus (Victoria et al., 2016). GIRK4<sup>-/-</sup> knockout mice have also been found to display impaired performance in the Morris water maze (Wickman et al., 2000), meaning they show impaired performance on spatial learning and memory tasks. Interestingly, GIRK3<sup>-/-</sup> knockout mice appear relatively normal on most behavioral tasks, however opioid-induced hyperpolarization in the locus coeruleus was found to be mediated by GIRK3 indicating its potential involvement in behaviors related to addiction (Torrecilla et al., 2002). Additionally, GIRK3<sup>-/-</sup> knockout mice have been found to result in increased alcohol consumption and binge-like drinking (Mayfield et al., 2016) as well as reduced withdrawal from ethanol (Kozell et al., 2009). GAT1508 has higher selectivity for GIRK1/2 channels has been shown to promote extinction of fear memories in mice (Xu et al., 2020). VU0466551 is a potent GIRK activator that shows anxiolytic and analgesic effects (Abney et al., 2019). Due to VU0466551 activation of non GIRK1 channels, this is an interesting drug for studying addiction since dopaminergic neurons in the VTA region express only GIRK2 and GIRK3 subunits. GiGA1 is a novel GIRK activator that directly binds to the alcohol-binding pocket of GIRK1/2 (Zhao et al., 2020). T-Q is a synthetic, stable derivate of tertiapin and is currently so far the most selective GIRK channel inhibitor (Jin and Lu, 1999). T-Q has been shown to impair LTD in the dorsal hippocampus (Malik and Johnston, 2017). A recent study examining intracerebroventricular injection of ML297 or T-Q on C57BL/6 male mice found that either treatment led to an impairment of memory retrieval on the novel object recognition task as well as learning an associative operant task (Djebari et al., 2021).

## 1-18 Future perspectives

These studies discussed above support the idea that GIRK channels contribute to neural plasticity, memory, and behavior. Whereas dysregulation of GIRK channels can lead to diseases such as epilepsy. More selective inhibitors/activators for specific GIRK subunit/subunit combinations could offer additional insights into their complex contribution to behavior.

### SUMMARY

GIRK and K<sub>v</sub>7 channels have emerged as important regulators of neuronal activity and plasticity while their dysregulation has been found to result in hyperexcitability disorders and neurodegenerative disease (Signorini et al., 1997; Watanabe et al., 2000).

In Chapter 2, we report the role of GIRK in response to seizures within the hippocampus. Epileptic seizures cause progressive hippocampal sclerosis, which is associated with caspase-3 activation and NMDAR-dependent excitotoxicity (Henshall, 2007; Thom, 2014). However, the identity of caspase-3 substrates that contribute to seizure-induced hippocampal atrophy remained largely unknown. Here, we have shown that prolonged high-frequency epileptiform discharges in cultured hippocampal neurons lead to a caspase-dependent cleavage of GIRK1 and GIRK2. We found that prolonged seizure activity caused caspase-dependent cleavage and dysfunction of GIRK channels *in vivo*. When the GIRK2 C-terminal was cleaved they display reduced binding to Gβγ and could not coassemble with GIRK1. We found that this cleavage ultimately led to a reduction in GIRK surface expression and ultimately a loss of current. These results suggest the possible role of caspase-3 mediated down-regulation of GIRK channel function and expression in hippocampal neuronal injury during prolonged epileptic seizures. This work was published on *Scientific Reports*, (Baculis et al., 2017).

In Chapter 3, we report the effect of prolonged activity blockade and ERK1/2 inhibition on K<sub>v</sub>7 channels in cultured hippocampal neurons. Prolonged activity blockade for 48 hours leads to a homeostatic increase in action potential firing rate of hippocampal neurons in dissociated culture, slice culture, and *in vivo* (Aptowicz et al., 2004; Echegoyen et al., 2007; Lee et al., 2015). This homeostatic scaling of intrinsic excitability in cultured hippocampal neurons is associated with decreases in the transcripts of potassium channels critical for regulating intrinsic membrane properties (Lee et al., 2015). However, whether voltage-gated KCNQ/K<sub>v</sub>7 potassium channels

decrease at the AIS was unknown. Here we have shown that prolonged activity blockade in cultured hippocampal neurons decreased phosphorylation of TrkB and ERK1/2 along with reducing *BDNF* and *KCNQ3* transcripts and AIS expression of K<sub>v</sub>7.3 and ankyrin-G. ERK1/2 inhibition revealed a similar reduction in AIS expression of K<sub>v</sub>7.3 and ankyrin-G. These findings suggest ERK1/2 is a key signaling pathway that mediates homeostatic downregulation of K<sub>v</sub>7 channels at the AIS. This work has been submitted for publication (Baculis et al., submitted).

## CHAPTER 2: PROLONGED SEIZURE ACTIVITY CAUSES CASPASE DEPENDENT CLEAVAGE AND DYSFUNCTION OF G-PROTEIN ACTIVATED INWARDLY RECTIFYING POTASSIUM CHANNELS <sup>2</sup>

### 2-1 Abstract

Recurrent high-frequency epileptic seizures cause progressive hippocampal sclerosis, which is associated with caspase-3 activation and NMDA receptor-dependent excitotoxicity. However, the identity of caspase-3 substrates that contribute to seizure-induced hippocampal atrophy remains largely unknown. Here, we show that prolonged high-frequency epileptiform discharges in cultured hippocampal neurons leads to caspase-dependent cleavage of GIRK1 and GIRK2, the major subunits of neuronal G protein-activated inwardly rectifying potassium K<sup>+</sup> (GIRK) channels that mediate membrane hyperpolarization and synaptic inhibition in the brain. We have identified caspase-3 cleavage sites in GIRK1 (<sup>387</sup>ECLD<sup>390</sup>) and GIRK2 (<sup>349</sup>YEVD<sup>352</sup>). The YEVD motif is highly conserved in GIRK2-4, and located within their C-terminal binding sites for Gβγ proteins that mediate membrane-delimited GIRK activation. Indeed, the cleaved GIRK2 displays reduced binding to Gβγ and cannot coassemble with GIRK1. Loss of an ER export motif upon cleavage of GIRK2 abolishes surface and current expression of GIRK2 homotetrameric channels. Lastly, kainate-induced status epilepticus causes GIRK1 and GIRK2 cleavage in the hippocampus in vivo. Our findings are the first to show direct cleavage of GIRK1 and GIRK2 subunits by caspase-3, and suggest the possible role of caspase-3 mediated down-regulation of GIRK channel function and expression in hippocampal neuronal injury during prolonged epileptic seizures.

### 2-2 Introduction

Epilepsy is a chronic brain disorder characterized by recurrent epileptic seizures indicative of neuronal hyperexcitability (Fisher et al., 2014). Temporal lobe epilepsy (TLE) is the most

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common epilepsy in adults (Tellez-Zenteno and Hernandez-Ronquillo, 2012) and is associated with progressive hippocampal sclerosis, cognitive decline, and drug-resistant seizures (Sharma et al., 2007;Tellez-Zenteno and Hernandez-Ronquillo, 2012;Thom, 2014). Studies in human TLE and kainate-induced rodent models of TLE suggest that both excitotoxicity induced by excessive activation of N-methyl-D-aspartate receptors (NMDARs) and apoptotic mechanisms including activation of cysteinyl aspartate-specific protease (caspase) family proteins may contribute to hippocampal atrophy following chronic epileptic seizures (Henshall, 2007;Thom, 2014). However, the identity of caspase substrates that contribute to seizure-induced neuronal death remains largely unknown.

G protein-activated inwardly rectifying potassium (GIRK) channels belong to the Kir3.x subfamily of inwardly rectifying potassium channels, which potently inhibit neuronal excitability (Luscher and Slesinger, 2010). Most neuronal GIRK channels are heterotetramers of GIRK1 and GIRK2 subunits (Liao et al., 1996), and to a lesser extent heterotetramers of GIRK3 and GIRK2 (Jelacic et al., 2000), although some neuronal populations contain GIRK2a or GIRK2c homotetrameric channels (Inanobe et al., 1999), or GIRK4 (Murer et al., 1997). Neuronal GIRK channels are preferentially localized in the soma and dendrites of hippocampal neurons (Kulik et al., 2006;Fernandez-Alacid et al., 2011) where they mediate hyperpolarization induced by activation of adenosine A<sub>1</sub> and GABA<sub>B</sub> receptors (Takigawa and Alzheimer, 2002;Chen and Johnston, 2005) and give rise to slow inhibitory postsynaptic current upon GABA<sub>B</sub> receptor activation (Luscher et al., 1997). Importantly, mice deficient in GIRK2 display decreased GIRK1 protein expression, sporadic seizures, increased susceptibility to the convulsant agents, and a shortened lifespan due to spontaneous lethal seizures (Signorini et al., 1997), suggesting that the loss of GIRK channel function contribute to pathologic hyperexcitability. In contrast, GIRK1 agonists delay seizure onset, prevent convulsions, and reduce lethality in animal models of epilepsy (Kaufmann et al., 2013), further confirming the protective role of GIRK channels against seizures.

We have previously reported that NMDARs can be activated by synaptically released glutamate in cultured hippocampal neurons upon removal of the chronic pharmacological NMDAR blockade in the absence of magnesium (Mg<sup>2+</sup>) (Chung et al., 2009b). Such activation of synaptic NMDARs causes high frequency burst discharges and increases surface expression of GIRK1 and GIRK2 within 15 min, basal GIRK current, and GIRK activation by adenosine A<sub>1</sub>

receptors (Chung et al., 2009a; Chung et al., 2009b), suggesting that this initial increase in GIRK surface density may serve as a homeostatic response to dampen membrane excitability.

In this study, we discovered that prolonged epileptiform seizure activity in cultured primary rat hippocampal neurons for >30 min resulted in caspase dependent cleavage of surface and intracellular GIRK1 and GIRK2 subunits in their cytoplasmic C-terminal tails. Similarly, kainate-induced status epilepticus in rats also induced C-terminal cleavage of GIRK1 and GIRK2 in their hippocampi. We found that caspase-3 directly cleaved GIRK1 at <sup>387</sup>ECLD<sup>390</sup> and GIRK2 at <sup>349</sup>YEVD<sup>352</sup>. Mutant GIRK2-Y353X, which mimics truncated GIRK2 upon caspase-3 mediated cleavage, displayed reduced binding to Gβγ and GIRK1. Furthermore, truncated GIRK2-Y353X channels failed to express on the plasma membrane and display K<sup>+</sup> current. Given that hippocampal neuronal apoptosis induced by prolonged high-frequency epileptiform discharges is mediated by activation of NMDARs and caspase-3 family proteins (Furshpan and Potter, 1989; Meller et al., 2003), our data suggest that this novel down-regulation of GIRK channels by caspase-3 may contribute to NMDAR-dependent hippocampal atrophy following chronic epileptic seizures.

## 2-3 Results

### **Caspase-3 induces C-terminal cleavage of GIRK channels in hippocampal neurons upon prolonged seizure activity**

To test whether prolonged epileptic seizures can regulate GIRK channels, we first treated high-density rat hippocampal neuronal culture with the NMDAR antagonist 2-amino-5-phosphonovaleric acid (APV) for 2-3 days at 10–11 days in vitro (DIV) when mature synapses appear (Rao et al., 1998). Then the cultured neurons were incubated in fresh artificial cerebrospinal fluid (ACSF) bath solution containing NMDAR co-agonist glycine, GABA<sub>A</sub> receptor antagonist picrotoxin, glycine receptor antagonist strychnine, but not Mg<sup>2+</sup> and APV (Chung et al., 2009a; Chung et al., 2009b) (Fig. 1a). Such activation of NMDAR by glycine and synaptically released glutamate upon APV withdrawal induced high-frequency burst firing of action potentials and sustained depolarization (Fig. 1a), similar to those seen in well-established hippocampal neuronal culture models for epileptic seizure activity (Meller et al., 2003; Mangan and Kapur, 2004; Goodkin et al., 2005; Blair et al., 2006; Deshpande et al., 2007), as well as epileptic seizures

seen in vivo (Mody et al., 1988; Gabriel et al., 2004). This APV withdrawal treatment increased action potential firing rates and burst firing which lasted up to 90 min (Supplementary Fig. S1). In contrast, neurons incubated in fresh ACSF solution containing APV and  $Mg^{2+}$  (APV control) displayed occasional spontaneous burst firing but not high-frequency epileptiform discharges in the presence of picrotoxin and strychnine (Fig. 1a).

Surface biotinylation revealed that APV withdrawal increased surface expression of endogenous GIRK1 and GIRK2 within 30 min (Fig. 1b), consistent with our previous report demonstrating a similar increase within 15 min (Chung et al., 2009b). Surprisingly, APV withdrawal over 30-90 min led to a progressive increase in truncated GIRK1 and GIRK2 proteins on the plasma membrane and total lysate while decreasing surface and total expression of full length proteins (Fig. 1b-d). While smears or ladders of truncated GIRK1 proteins from 50 kD band were detected upon prolonged APV withdrawal, a distinct immunoblot band of truncated GIRK2 proteins were observed around 40 kD (Fig. 1b-d). This cleavage occurred in their C-terminal domains because GIRK subunits were immunoblotted with antibodies that recognize their intracellular N-termini (Fig. 1b-d, Supplementary Fig. S2).

Induction of high-frequency epileptic seizure activity in cultured hippocampal neurons causes activation of caspases and apoptosis (Meller et al., 2003). Compared to APV control-treated neurons which displayed high levels of full-length GIRK1 and GIRK2 and low levels of truncated subunits, APV withdrawal for 60 min and 120 min significantly decreased the levels of full-length subunits, and increased the levels of truncated subunits (Fig. 2a-d). This C-terminal cleavage of surface and total GIRK1 and GIRK2 was blocked by pretreatment with cell-permeable irreversible pan-caspase inhibitor ZVAD-fmk (Fig. 2a, c-d). To identify which caspases were responsible for GIRK1 and GIRK2 cleavage, we repeated the experiments with a widely used caspase-3 inhibitor DEVD-fmk that also inhibits caspase-6, 7, 8 and 10. Pretreatment with DEVD-fmk blocked the C-terminal cleavage of GIRK subunits and inhibited the reduction in the level of full-length subunits induced by prolonged APV withdrawal (Fig. 2b-d). In contrast, seizure activity-induced cleavage of GIRK subunits was unaffected by pretreatment with YVAD-cmk that inhibits caspase-1, 4 and 5 (Fig. 2b-d).

## Caspase-3 directly cleaves GIRK1, GIRK2, and GIRK4 in vitro

Our results from Figure 2b-d suggest that GIRK1 and GIRK2 cleavage upon prolonged seizure activity may likely be mediated by caspases which cleave their substrates at DEVD, EXXD, or XEXD motifs after aspartate (D) residue (Fischer et al., 2003;Mahrus et al., 2008). One of the primary caspases in this family is caspase-3, which is the main executioner of apoptosis (Fischer et al., 2003;Mahrus et al., 2008) and has been implicated in hippocampal sclerosis after epileptiform activity (Furshpan and Potter, 1989;Meller et al., 2003). Sequence alignment of GIRK subunits with known caspase-3 substrates revealed the presence of <sup>387</sup>ECLD<sup>390</sup> in GIRK1 similar to EXXD motif (Fischer et al., 2003;Mahrus et al., 2008), and YEVD motifs in GIRK2 and GIRK4 that matched XEXD motif (Fig. 3a). In vitro cleavage assay with purified caspases revealed that caspase-3 cleaved GIRK1 and GIRK2A while caspase-1 did not (Fig. 3b, d). These results are consistent with our previous results that caspase-3 inhibitor DEVD-fmk but not caspase-1 inhibitor YVAD-cmk, blocked seizure-induced GIRK1 and GIRK2 cleavage in cultured hippocampal neurons (Fig. 2b-d).

Caspases cleave substrates with 20,000-fold preference for aspartate over glutamate (E) residue (Seaman et al., 2016). Caspase-3 failed to cleave mutant GIRK1 in which the aspartate residue in <sup>387</sup>ECLD<sup>390</sup> is mutated to glutamate (D390E) but not the GIRK1 containing D393E mutation (Fig. 3c). Caspase-3 cleaved mutant GIRK2A containing D346E mutation located upstream of <sup>349</sup>YEVD<sup>352</sup> motif but not GIRK2A harboring D352E mutation within this motif (Fig. 3d). Similarly, D347E mutation in <sup>344</sup>YEVD<sup>347</sup> motif of GIRK4 abolished caspase-3 mediated cleavage of GIRK4 (Fig. 3e). Although recent publication has shown that caspases can cleave at glutamate residue with slower kinetics in synthetic peptide substrates (Seaman et al., 2016), a complete absence of caspase-3 mediated cleavage of GIRK1-D390E, GIRK2-D352E, and GIRK4-D347E in 90 min reaction indicates that <sup>387</sup>ECLD<sup>390</sup> of GIRK1, <sup>349</sup>YEVD<sup>352</sup> in GIRK2, and <sup>344</sup>YEVD<sup>347</sup> in GIRK4 are caspase-3 cleavage motifs.

## GIRK2A truncated at <sup>349</sup>YEVD<sup>352</sup> motif shows decreased binding to Gβγ and GIRK1

GIRK channels are gated by direct binding of Gβγ in response to neurotransmitters and neuromodulators that activate G protein-coupled receptors (GPCR) coupled to pertussis toxin-

sensitive Gi/o proteins (Glaaser and Slesinger, 2015; Logothetis et al., 2015). GIRK2 homotetrameric channels possess four G $\beta\gamma$  binding sites at the well-conserved cytoplasmic interfaces between adjacent subunits that contribute to formation of the extended cytoplasmic pore (Whorton and MacKinnon, 2013). Specifically, the G $\beta\gamma$  contact site in GIRK2 is formed by secondary  $\beta$  sheet structure elements  $\beta$ K,  $\beta$ L,  $\beta$ M and  $\beta$ N from one subunit and by elements  $\beta$ D and  $\beta$ E from an adjacent subunit (Whorton and MacKinnon, 2013). Sequence alignment of the distal C-terminal tails of all GIRK subunits revealed that caspase-3 cleavage site <sup>387</sup>ECLD<sup>390</sup> of GIRK1 is located distal to its G $\beta\gamma$  contact site whereas caspase-3 cleavage sites (YEVD) of GIRK2, GIRK3, and GIRK4 are located at their secondary  $\beta$ M sheet structure elements within the G $\beta\gamma$  contact site (Fig. 4a-b). Since <sup>349</sup>YEVD<sup>352</sup> motif of GIRK2 falls within and near the G $\beta\gamma$  contact sites (Fig. 4c-d), caspase-3 cleavage of GIRK2 may disrupt GIRK2 interaction with G $\beta\gamma$ . To test this, co-immunoprecipitation of G $\beta\gamma$  was performed with wild type GIRK2A or truncated GIRK2A-Y353X in which Y353 residue right after <sup>349</sup>YEVD<sup>352</sup> motif was mutated to a stop codon. Thus, GIRK2A-Y353X mimics GIRK2A cleaved by caspase-3. We first found that the level of GIRK2A-Y353X proteins was consistently lower compared to wild type GIRK2A proteins although an equal amount of each plasmid was transfected in HEK293T cells (Fig. 5a). To increase the expression level of GIRK2A-Y353X to a similar extent as wild type GIRK2A for coimmunoprecipitation, we doubled the amount of GIRK2-Y353X plasmid for transfection compared to wild type GIRK2 plasmid (Fig. 5b). Immunoprecipitation of G $\beta_1\gamma_2$  tagged with yellow fluorescent proteins (YFP) resulted in coimmunoprecipitation of wild type GIRK2A (Fig. 5b), consistent with the previous reports demonstrating their interactions (Huang et al., 1995; Whorton and MacKinnon, 2013). In contrast, coimmunoprecipitation of GIRK2A-Y353X with YFP-G $\beta_1$  and YFP-G $\gamma_2$  was decreased to 50% of wild-type GIRK2A (Fig. 5b), indicating that truncated GIRK2A-Y353X showed decreased binding to G $\beta\gamma$ .

We also noticed a significant decrease in GIRK1 expression when this subunit is coexpressed with GIRK2A-Y353X compared to wild type GIRK2A (Fig. 5a). Since GIRK1 heteromerization with GIRK2 stabilizes GIRK1 expression (Ma et al., 2002; Mirshahi and Logothetis, 2004), we hypothesized that caspase-3 cleavage of GIRK2 may disrupt GIRK2 interaction with GIRK1. To test this, we first doubled the amount of GIRK2A-Y353X plasmid for transfection compared to wild type GIRK2A plasmids, and performed immunoprecipitation using the same amount of anti-GIRK2 N-terminal antibodies that could immunoprecipitate a fraction but

not all of transfected GIRK2A subunits from the lysate. Although the same amount of wild type GIRK2A and GIRK2A-Y353X proteins were immunoprecipitated, GIRK1 co-immunoprecipitated only with wild type GIRK2A but not GIRK2A-Y353X (Fig. 5c), indicating that truncated GIRK2A-Y353X could not coassemble with GIRK1.

### **GIRK2A truncated at <sup>349</sup>YEVD<sup>352</sup> motif does not express at the plasma membrane**

Trafficking of GIRK channels is tightly regulated by multiple amino acid sequence motifs within their subunits that control their forward trafficking from the endoplasmic reticulum (ER) as well as post-ER endocytic trafficking (Ma et al., 2002; Mirshahi and Logothetis, 2004; Lunn et al., 2007; Chung et al., 2009b). GIRK1 subunits are retained in the ER when expressed alone, but GIRK1 assembly with GIRK2 containing ER export motif <sup>396</sup>ELETEEE<sup>403</sup> allows efficient surface expression of heterotetrameric channels (Ma et al., 2002; Mirshahi and Logothetis, 2004). The C-terminal tail of GIRK2 distal to its <sup>349</sup>YEVD<sup>352</sup> motif contains this ER export motif (Fig. 4b, 6a) (Ma et al., 2002). To test if caspase-3 cleavage of GIRK2 and subsequent loss of its ER export motif disrupts surface expression of GIRK2 channels or GIRK1/GIRK2 channels, surface immunostaining was performed in COS7 cells transfected with GIRK1 or GIRK2A, which were tagged with an extracellular hemagglutinin (HA) epitope (Fig. 6a-b, e). While wild type HA-GIRK2A channels and HA-GIRK1/GIRK2A channels displayed robust surface expression, truncated HA-GIRK2A-Y353X channels or HA-GIRK1/GIRK2A-Y353X channels failed to express on the plasma membrane (Fig. 6c-d, 6f-g). Lower total expression of HA-GIRK1 was observed in the cells cotransfected with GIRK2A-Y353X compared to the cells expressing wild type GIRK2A (Fig. 6f), similar to our western blot analyses in HEK293T cells (Fig. 5a).

### **GIRK2A channels truncated at <sup>349</sup>YEVD<sup>352</sup> motif do not express K<sup>+</sup> current**

To test if truncated GIRK2A-Y353X channels are functional, two-electrode voltage-clamp recording was performed to examine macroscopic K<sup>+</sup> currents of wild type GIRK2A or truncated GIRK2A-Y353X homotetrameric channels from *Xenopus* oocytes coexpressing Gβ<sub>1</sub>γ<sub>2</sub> subunits (Fig. 7). Wild type GIRK2 channels displayed very low level of basal K<sup>+</sup> currents in the absence of Gβ<sub>1</sub>γ<sub>2</sub>, but produced significantly larger K<sup>+</sup> currents upon coexpression of Gβ<sub>1</sub>γ<sub>2</sub> (Fig. 7a, c),

consistent with previous studies reporting  $G\beta\gamma$ -dependent activation of GIRK channels (Huang et al., 1995; Bichet et al., 2006). In contrast, GIRK2-Y353X channels produced negligible  $K^+$  currents whether  $G\beta_1\gamma_2$  proteins were coexpressed or not (Fig. 7a, c). Because this lack of  $K^+$  currents (Fig. 7a, c) could be due to very low protein expression of GIRK2A-Y353X compared to wild type GIRK2A (Fig. 7b), we repeated the recording in the oocytes injected with 20 ng of GIRK2A-Y353X cRNA. Although these oocytes expressed increased level of GIRK2A-Y353X especially in the presence of  $G\beta_1\gamma_2$  coexpression compared to the oocytes injected with 5 ng of cRNA (Fig. 7b), they failed to produce  $K^+$  currents in the presence or absence of  $G\beta_1\gamma_2$  (Fig. 7a, c). None of the tested oocytes produced  $Na^+$  currents, indicating that  $K^+$  selectivity is intact in wild type and truncated GIRK2A channels.

### **Kainate-induced status epilepticus induces C-terminal cleavage of GIRK channels in the hippocampus**

To test if prolonged epileptic seizures in vivo (i.e. status epilepticus) lead to the C-terminal cleavage of GIRK channels, we used a well-established rodent model of TLE in which status epilepticus was induced by intraperitoneal (i.p.) injection of kainate (Fig. 8a), a potent agonist for ionotropic glutamate receptors (Sharma et al., 2007; Levesque and Avoli, 2013). We chose the kainate model of TLE due to hippocampus-restricted injuries and histopathological correlates of hippocampal sclerosis (Sharma et al., 2007; Levesque and Avoli, 2013) associated with increased level and activation of caspase-3 (Ferrer et al., 2000; Engel and Henshall, 2009). Furthermore, kainate-induced seizures have been shown to induce expression of an immediate-early gene c-Fos which is involved in dampening excitability and promoting survival of hippocampal neurons (Zhang et al., 2002), as well as another immediate early gene c-Jun which is a primary substrate of c-Jun N-terminal kinases (JNKs) important for excitotoxic neuronal apoptosis in the hippocampus following status epilepticus (Spigolon et al., 2010).

Injection of kainate (9 mg/kg) but not vehicle control ( $H_2O$ ) induced behavioral hyperactivity within 20 min and recurrent stage 4-5 seizures (Racine scale) and status epilepticus within 1 hour (h) in Sprague Dawley rats. Kainate-induced status epilepticus significantly increased the level of C-terminally cleaved GIRK2 subunits in rat hippocampi at 8 h post injection (Fig. 8b-c). There was an increasing trend in c-Jun expression at 8 h post kainate injection, but this

trend did not reach statistical significance (Fig. 8b-c). In separate experiments, CD001 rat hippocampal lysate was prepared at 3 h post injection with vehicle control (saline) or kainate (20 and 30 mg/kg) (Fig. 8d-e). There was a low level of cleaved GIRK1 and GIRK2 proteins in the hippocampi of saline-treated CD001 rats (Fig. 8d). Injection of 20 mg/kg kainate caused stage 2-3 seizures which did not last after 1 h post injection, and did not further induce C-terminal cleavage of GIRK1 and GIRK2 at 3 h post injection (Fig. 8d). However, injection of 30 mg/kg kainate induced recurrent stage 4-5 seizures in CD001 rats which significantly increased the levels of c-Fos and C-terminally cleaved GIRK1 proteins but not the levels of C-terminally cleaved GIRK2 proteins within 3 h post injection (Fig. 8d-e). Similar to rats, C57BL/6J mice injected with 15 mg/kg and 30 mg/kg kainate displayed recurrent stage 3-4 seizures and 4-5 seizures respectively, and displayed enhanced c-Fos and c-Jun expression and increased C-terminal cleavage of GIRK2 (Supplementary Fig. S8).

## 2-4 Discussion

The intracellular N- and C-terminal tails of GIRK channel subunits provide multifunctional regions that form the cytoplasmic pore of the channels and the interacting domains for G $\beta$  $\gamma$  and other signaling proteins that regulate their function and trafficking (Luscher and Slesinger, 2010; Dascal and Kahanovitch, 2015). Here, we demonstrate a novel mode of GIRK channel regulation: caspase-3 cleavage of neuronal GIRK channel subunits GIRK1 and GIRK2 which occurs during prolonged seizure activity (Fig. 1-2). Our surface biotinylation experiment showed that prolonged seizure activity induced by APV withdrawal resulted in caspase-3 dependent C-terminal cleavage of endogenous GIRK1 and GIRK2 subunits at the plasma membrane (Fig. 1, 2). Furthermore, such cleavage of GIRK subunits was also observed in total neuronal lysate which contained both surface and intracellular GIRK subunits (Fig. 1, 2). Since our previous report has shown that 7% total GIRK1 proteins and 19% total GIRK2 proteins were on the cell membrane of cultured hippocampal neurons (Chung et al., 2009b), cleavage of almost 50% total GIRK1 and GIRK2 proteins (Fig. 2) suggests that intracellular GIRK1 and GIRK2 were also cleaved by caspase-3.

To date, our studies are the first to identify caspase-3 cleavage sites in GIRK subunits (Fig. 3). The "YEVD" caspase-3 cleavage motifs and the surrounding amino acid sequences are highly

conserved in the intracellular C-terminal tails of GIRK2-4 (Fig. 4) and located upstream of specific motifs that control their surface expression, intracellular trafficking, and protein-protein interaction (Ma et al., 2002; Mirshahi and Logothetis, 2004; Lunn et al., 2007; Chung et al., 2009b). Indeed, GIRK2A-Y353X, which mimics GIRK2A cleaved by caspase-3, failed to express on the plasma membrane of COS7 cells due to the loss of its ER export motif <sup>396</sup>ELETEEE<sup>403</sup> (Fig. 6). Although we cannot completely exclude the possibility that caspase cleavage at YEVD motif may directly destroy the channel activity, impairment in surface expression most likely underlies the inability of truncated GIRK2A-Y353X channels to produce current in oocytes (Fig. 7, 9). Similarly, the loss of forward trafficking motif in GIRK4 by caspase-3 cleavage of intracellular GIRK4 (Fig. 4b) is expected to reduce GIRK4 surface expression. Since GIRK3 lacks an ER export motif and therefore requires GIRK2 for its surface expression (Jelacic et al., 2000; Ma et al., 2002), caspase-3 cleavage of GIRK2 and GIRK3 subunits at the ER is expected to block the ER export of GIRK2/GIRK3 channels.

Interestingly, we observed a significantly lower expression of truncated GIRK2A-Y353X than wild-type GIRK2A (Fig. 5a, 6b, 7b), suggesting the degradation of cleaved GIRK2 proteins retained in the ER. Furthermore, a marked decrease in GIRK1 surface expression was also observed in cells expressing GIRK2A-Y353X (Fig. 6) which were unable to interact with GIRK1 (Fig. 5), consistent with the report that surface expression of GIRK1 requires its interaction with GIRK2 (Jelacic et al., 2000; Ma et al., 2002). Reduced total expression of GIRK1 (Fig. 5) and the ladder of cleaved GIRK1 proteins in the immunoblots of cultured hippocampal neurons (Fig. 1, 2) points to the possibility of ubiquitination-mediated degradation of truncated GIRK1. These observations suggest the instability of cleaved channel portions of GIRK1 and GIRK2, although the fate of much shorter C-terminal fragments of GIRK1 and GIRK2 remains unclear.

Importantly, caspase-3 cleavage of GIRK2-4 at "YEVD" motif removes the secondary  $\beta$ M and  $\beta$ N sheet structural elements from the channel contact surface for G $\beta$  $\gamma$  proteins (Fig. 4), which mediate membrane-delimited activation of GIRK channels (Dascal and Kahanovitch, 2015). Since truncated GIRK2A-Y353X coimmunoprecipitated with G $\beta$  $\gamma$  significantly less than the wild type GIRK2A (Fig. 5), caspase-3 mediated cleavage of GIRK2 is predicted to decrease G $\beta$  $\gamma$  binding to GIRK1/GIRK2 channels at the plasma membrane that constitute most neuronal GIRK channels (Liao et al., 1996), as well as GIRK2/GIRK3 or GIRK4 channels that are expressed in a subset of neurons (Murer et al., 1997; Inanobe et al., 1999; Jelacic et al., 2000). Caspase-3 cleavage site in

GIRK1<sup>387</sup>ECLD<sup>390</sup> (Fig. 3) is located far distal to the G $\beta\gamma$  contact surface of GIRK1 (Fig. 4), and thus cleavage would have minimal effect on G $\beta\gamma$  binding to GIRK1. However, inability of truncated GIRK2A-Y353X to interact with GIRK1 (Fig. 5) suggests that caspase-3 cleavage of GIRK2 would cause disassembly of GIRK1/GIRK2 channels, further contributing to their dysfunction (Fig. 8f).

There is emerging evidence for a macromolecular complex on the plasma membrane consisting of GIRK channels, GPCRs, G-protein subunits, and signaling proteins such as regulators of G protein signaling (Clancy et al., 2005; Kulik et al., 2006; Fowler et al., 2007; Labouebe et al., 2007) that support membrane-delimited opening of GIRK channels upon activation of GPCRs (Dascal and Kahanovitch, 2015). Furthermore, G $\beta\gamma$  binding is shown to strengthen the interaction between GIRK channels and phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>), an essential co-factor for channel gating (Huang et al., 1998; Logothetis et al., 2015). We speculate that caspase-3 cleavage of GIRK2-4 on the plasma membrane and subsequent reduction in G $\beta\gamma$  and GIRK1 binding would destabilize this macromolecular complex and PIP<sub>2</sub> interaction, decoupling the activation of GPCRs from the membrane-delimited gating of GIRK channels.

What are the physiologic consequences of caspase-3 mediated cleavage of GIRK subunits? GIRK channels regulate resting membrane potential and excitability in hippocampal neurons by mediating inhibitory effects of GPCRs for neurotransmitters and neuromodulators, including adenosine A1 receptors and GABA<sub>B</sub> receptors (Luscher and Slesinger, 2010). Our current study has observed that surface expression of GIRK1 and GIRK2 is increased within 15-30 min of seizure activity (Fig. 1, 2). These results are consistent with our previous studies (Chung et al., 2009a; Chung et al., 2009b), which report that this regulation involves enhanced recycling of GIRK channels to the plasma membrane, and is associated with increased basal GIRK current and GIRK channel activation induced by adenosine A1 receptors but not GABA<sub>B</sub> receptors (Chung et al., 2009a; Chung et al., 2009b). Such initial upregulation of GIRK surface density may likely provide homeostatic defense to reduce neuronal excitability against seizure activity.

However, when this seizure activity persisted for >30 min, we discovered a novel finding that surface and total GIRK1 and GIRK2 proteins are cleaved by caspases sensitive to potent caspase-3 inhibitor DEVD-fmk with *K<sub>i</sub>* of 0.2 nM (Fig. 2). Since DEVD-based peptide inhibitor also blocks other apoptosis executioner caspase-7 (*K<sub>i</sub>* = 1.6 nM), as well as upstream apoptosis initiators caspase-8 (*K<sub>i</sub>* = 0.9 nM) and caspase-10 (*K<sub>i</sub>* = 12 nM) (Garcia-Calvo et al., 1998), the

identity of caspases that cleave GIRK1 and GIRK2 during prolonged seizure activity in hippocampal neurons remains unclear. Nonetheless, we demonstrated that GIRK2-Y353X, which mimics GIRK2 cleaved at YEVD motif by caspase-3, had decreased binding to G $\beta\gamma$  and GIRK1 and failed to display surface and current expression (Fig. 4-7). Based on our findings and the reports of increased spontaneous sporadic and lethal seizures in GIRK2 knock-out mice (Signorini et al., 1997), we propose that caspase-mediated cleavage and subsequent down-regulation of GIRK channels may decrease their basal and GPCR-activated K<sup>+</sup> current and disrupt their ability to dampen excitability (Fig. 8f). Future studies are needed to determine the role of caspase-mediated cleavage of GIRK in neuronal excitability.

The possible involvement of apoptosis initiators caspase-8 and 10 that can also cleave at DEVD and XEXD (Fischer et al., 2003;Mahrus et al., 2008) motifs is intriguing since earlier cleavage of GIRK1 and GIRK2 by these caspases could potentially alter neuronal physiology before cell death. Furthermore, in addition to a key role of caspase-3 in terminal apoptosis execution events (Utz and Anderson, 2000), recent studies have revealed apoptosis-independent roles of caspase-3 in neurons such as synaptic plasticity (D'Amelio et al., 2012;Snigdha et al., 2012) including NMDAR-dependent long-term depression of excitatory synapses(Li et al., 2010;Jiao and Li, 2011). Interestingly, we observed a low level of cleaved GIRK1 and GIRK2 subunits in cultured hippocampal neurons under APV control condition (Fig. 1, 2) and in the hippocampi of vehicle-treated control rats (Fig. 8), which could be caused by caspase-8 and 10 or apoptosis-independent caspase-3 activity. GIRK channels and GABA<sub>B</sub> receptors are detected in dendritic spines that harbor the majority of excitatory synapses in hippocampal neurons (Kulik et al., 2006) where their activation provides slow inhibitory postsynaptic currents (Luscher et al., 1997). In addition to postsynaptic roles, activation of GIRK channels by GABA<sub>B</sub> receptors in presynaptic terminals has been shown to inhibit neurotransmitter release (Ladera et al., 2008;Fernandez-Alacid et al., 2009). Furthermore, both GIRK channels and A<sub>1</sub> receptors reside on the dendritic spines and shafts (Luscher et al., 1997;Ochiishi et al., 1999;Rebola et al., 2003;Kulik et al., 2006) where GIRK activation by adenosine attenuates the excitatory postsynaptic potentials (Takigawa and Alzheimer, 2002) and contributes to depotentiation of NMDAR-dependent long-term potentiation (Chung et al., 2009a). Therefore, it is tempting to speculate that caspase-mediated cleavage of GIRK1 and GIRK2 may disrupt GIRK-mediated synaptic inhibition and/or plasticity before neurons are fully committed to apoptosis.

We have also discovered that kainate-induced status epilepticus in rats leads to C-terminal cleavage of GIRK1 and GIRK2 in their hippocampi (Fig. 8) where GIRK1-3 subunits are expressed with overlapping distribution patterns (Lujan and Aguado, 2015). Studies in human TLE and kainate rodent models of TLE indicate that excessive glutamate release during status epilepticus causes hippocampal neuronal death by a combination of necrosis and apoptosis depending on the seizure intensity and the cellular energy levels (Henshall, 2007;Thom, 2014). In addition, activation of caspase-2, 3, 6, 7, and 8 has been observed in the hippocampi of human TLE patients and rodent models of TLE where activity of caspase-3 and 7 is highly correlated with hippocampal sclerosis (Engel and Henshall, 2009). In cultured hippocampal neurons, apoptosis induced by prolonged high-frequency epileptiform discharges is evident by DNA fragmentation and activation of caspase-3 family proteins (Furshpan and Potter, 1989;Meller et al., 2003), and is blocked by pharmacological inhibition of NMDARs (Meller et al., 2003). Indeed, one important culprit for glutamate-induced neuronal death has been shown to be massive accumulation of intracellular  $\text{Ca}^{2+}$  upon overstimulation of NMDARs (Lau and Tymianski, 2010). Though highly speculative, caspase-mediated cleavage and down regulation of neuronal GIRK channels may cause sustained depolarization and overstimulation of NMDARs, creating pathologic positive feedback mechanisms that amplify  $\text{Ca}^{2+}$  overload and sensitize the neurons to  $\text{Ca}^{2+}$ -induced excitotoxic death. This drastic measure could allow the brain to eliminate the neurons that persistently produce epileptiform discharges when initial homeostatic mechanisms fail to dampen their excitability, and may in part underlie sclerosis at or close to the seizure foci in the hippocampi of human TLE patients (Thom, 2014). Testing this hypothesis warrants future studies. Lastly, GIRK channels may likely represent one of many caspase-3 targets, and therefore future research shall include a more comprehensive identification of other caspase-3 substrates involved in seizure-induced hippocampal neuronal injury.

## 2-5 Methods

**Experimental animals.** All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign and the University of California San Francisco (UCSF) in accordance with the guidelines of the U.S National Institutes of Health (NIH).

**Induction of seizure activity in hippocampal neuronal culture.** Primary dissociated hippocampal neuronal cultures at high density (330 cells/mm<sup>2</sup>) were prepared from Sprague-Dawley rat embryos at E18 as described (Chung et al., 2009b). Cultured neurons (10-11 DIV) were treated for 2-3 days with DL-APV (200  $\mu$ M, Tocris). To induce seizure activity, APV-treated neurons were incubated with "APV withdrawal" ACSF solution containing (in mM): 10 HEPES, 145 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 10 Dextrose, 0.1 glycine, 0.1 picrotoxin, and 0.005 strychnine (Sigma, Tocris) as described (Chung et al., 2009b). For control, APV-treated neurons were incubated with "APV control" ACSF containing (in mM): 10 HEPES, 145 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 Dextrose, 0.1 picrotoxin, 0.005 strychnine, 0.2 DL-APV (pH 7.4, 305-315 mOsm). Neuronal lysates were prepared in RIPA buffer and immunoblotted with antibodies against GIRK1 and GIRK2 N-termini (1:200) (Chung et al., 2009b) (Supplementary Fig. S2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1,000). ImageJ software (NIH, <http://rsb.info.nih.gov/ij/>) was used to measure background-subtracted immunoblot band intensities of full-length GIRK, cleaved GIRK, and GAPDH. The ratio of GIRK/GAPDH from APV control samples (Fig. 1c-d, 2c-d) were taken as 100% and the ratio of APV withdrawal samples were normalized to the ratio of APV control samples to obtain % APV control.

**Whole cell patch clamp recordings.** Current clamp recordings of spontaneous firings from hippocampal pyramidal neurons were carried out at 23-25 °C using gap free mode as described (Chung et al., 2009a) first in "APV control" ACSF for 10 min, and then in "APV control" or "APV withdrawal" ACSF for 15 min. Recording pipettes had a resistance of 3-5 M $\Omega$  when filled with internal solution containing (in mM): 130 K-Gluconate, 7 KCl, 2 NaCl, 10 HEPES, 1 MgCl<sub>2</sub>, 0.1 EGTA and 2 ATP-Mg, 0.3 Na-GTP (pH 7.3, 285-295 mOsm). Recordings were performed using a Multiclamp 700B amplifier, Digidata 1440A, and the pClamp 10.6 (Molecular Devices), filtered at 2 kHz, digitized at 10 kHz, and analyzed using Clampfit 10.6 (Molecular Devices).

**Surface biotinylation.** After APV control or withdrawal, surface biotinylation was performed with Sulfo-NHS-SS (or LC)-Biotin (1 mg/mL, Pierce) in ACSF on ice as described (Chung et al., 2009b). Biotinylated proteins were precipitated using 50% NeutraAvidin agarose (200  $\mu$ L Pierce) and eluted in SDS sample buffer upon heating at 85 °C for 30 min. For inhibition of caspases,

APV-treated neurons were preincubated for 2 h with ZVAD-fmk (100  $\mu$ M, Enzyme Systems), YVAD-cmk (20  $\mu$ M, Clontech), or DEVD-fmk (20  $\mu$ M, Calbiochem) before APV control or withdrawal.

**DNA constructs and mutagenesis.** The plasmids pcDNA3 containing GIRK1 (NM\_031610.3), GIRK2A (NM\_010606.2), GIRK4A (NM\_017297), GIRK1 with an extracellular HA tag (HA-GIRK1), and pCMV5 containing GIRK2A with an extracellular HA tag (HA-GIRK2A) have been described (Chung et al., 2009b) (kind gifts from Dr. Lily Y. Jan, UCSF). Mutations in GIRK1 (D390E, D393E), GIRK2A (D346E, D352E, Y353X), and GIRK4 (D347E) were generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent), and verified by sequencing the entire cDNA construct. Mutagenesis oligonucleotides were: GIRK1-D390E (sense 5'CAATTCTGTGGAGTGCTTAGAGGGACTA GATGACATTAGC<sup>3'</sup>, antisense 5'GCTAATGTCATCTAGTCCCTCTAAGCACTCCACAGA ATTG<sup>3'</sup>), GIRK1-D393E (sense 5'GTGCTTAGATGGACTAGAGGACATTAGCACAAAA CTTCC<sup>3'</sup>, antisense 5'GGAAGTTTTGTGCTAATGTCTCTAGTCCATCTAAGCA<sup>3'</sup>), GIRK2-D346E (sense 5'GTCCTAACGCTGGAAGAAGGGTTCTACGAAGTTG<sup>3'</sup>, antisense 5'CAAC TTCGTAGAACCCCTTCTTCCAGCGTTAGGAC<sup>3'</sup>), GIRK2-D352E (sense 5'CGGGTTCTAC GAAGTTGAGTACAACAGCTTCCATG<sup>3'</sup>, antisense 5'CATGGAAGCTGTTGTACTCAAC TTCGTAGAACCCG<sup>3'</sup>), GIRK2-Y353X (sense 5'GTTCTACGAAGTTGACTAGAACAGC TTCCATGAGAC<sup>3'</sup>, antisense 5'GTCTCATGGAAGCTGTTCTAGTCAACTTCGTAGAAC<sup>3'</sup>), and GIRK4-D347E (sense 5'GGCTTCTATGAGGTGGAGTACAACACTTTCCACG<sup>3'</sup>, antisense 5'CGTGGAAGTGTGTACTCCACCTCATAGAAGCC<sup>3'</sup>). The underlined bases indicate amino acid substitution mutations in the sense and antisense sequences.

**In vitro cleavage reaction.** The pcDNA3 plasmids containing wild type or mutant GIRK cDNA (1  $\mu$ g) were incubated with the TnT coupled Reticulocyte Lysate System (50  $\mu$ L, Promega) and L-[<sup>35</sup>S]-Methionine (>1,000 Ci/mmol at 10 mCi/ml) (Amersham Biosciences) for 90 min at 30 °C. This system utilizes crude reticulocyte lysate which could contain microsomes, and has been widely used to synthesize membrane proteins *in vitro* in a cell-free system (Sachse et al., 2014). Translated proteins were subjected to *in vitro* cleavage reaction at 37 °C for 2 h with 0.2  $\mu$ g caspase-1 (Calbiochem) or caspase-3 (BD Biosciences) in a buffer containing (in mM): 20 PIPES,

100 NaCl, 10 DTT, 1 EDTA, 0.1% CHAPS, and 10% sucrose. Products were separated by electrophoresis and visualized by exposure to autoradiographic BioMax MS films (Kodak).

**Visual molecular dynamics.** Crystal structures of GIRK2 alone or GIRK2 complexed with G $\beta\gamma$  (protein data bank ID code 4kfm) (Whorton and MacKinnon, 2013) were rendered with Visual Molecular Dynamics software (Humphrey et al., 1996) to create molecular surface representation of GIRK2 in blue and G $\beta\gamma$  in green.

**Coimmunoprecipitation.** For coimmunoprecipitation (coIP) of GIRK2A and G $\beta\gamma$ , HEK293T cells were transfected using FuGENE6 transfection reagent (Promega) with pcDNA3.1-YFP-G-protein G $\beta_1$  (0.4  $\mu$ g), pcDNA3.1-YFP-G-protein-G $\gamma_2$  (0.4  $\mu$ g) (kind gifts from Narasimhan Gautam, Addgene plasmids #36397, #36102) and pcDNA3-GIRK2A wild-type (0.8 or 1  $\mu$ g) or Y353X (0.8 or 2  $\mu$ g). Immunoprecipitation (IP) was performed at 24 h post transfection as described (Cavaretta et al., 2014) with Protein A/G agarose beads (50  $\mu$ L) and mouse anti-GFP antibodies (5  $\mu$ g, Cell Signaling). After washing, the beads were incubated with SDS sample buffer at 90 °C for 5 min. The lysates and IP eluates were immunoblotted with antibodies for GFP, GIRK2 N-term and GAPDH (1:500 - 1:1,000). For coIP of GIRK2A and HA-GIRK1, HEK293T cells were transfected with pcDNA3.1-HA-GIRK1 (1  $\mu$ g), and pcDNA3-GIRK2A wild type (1  $\mu$ g) or Y353X (2  $\mu$ g). IP was performed with rabbit anti-GIRK2 N-terminal antibodies (5  $\mu$ g, Cell Signaling) and analyzed by immunoblotting with anti-HA, anti-GIRK2 N-term, anti-GAPDH antibodies (1:500 - 1:1,000) (Cell Signaling). To quantify coIP, background-subtracted IP band intensity ratios of GIRK2A/ YFP- G $\beta_1$  or HA-GIRK1/ GIRK2A from GIRK2A Y353X samples were normalized to the ratios from GIRK2A WT samples to obtain % GIRK2A WT (Fig. 5b) or % HA-GIRK1 + GIRK2A WT (Fig. 5c).

**Immunocytochemistry.** At 24 h post transfection with pCMV5-HA-GIRK2A (for GIRK2A channels), or pcDNA3-HA-GIRK1 with pcDNA3-GIRK2A (for HA-GIRK1/GIRK2A channels), COS7 cells were fixed with 4% formaldehyde/ 4% sucrose in PBS for 8 min and incubated with anti-HA antibodies (1:300, Cell Signaling) followed by incubation with Alexa488- or Alexa594-conjugated secondary antibodies, respectively (1:200, Invitrogen). In a separate experiment, biotin-conjugated secondary antibodies were used followed by incubation with Alexa488- or

Alexa594-conjugated streptavidin, respectively. After fixation and permeabilization with 0.2% Triton X-100 in PBS for 10 min, total HA-GIRK2A, HA-GIRK1, or GIRK2A proteins were labeled with anti-HA or anti-GIRK2 antibodies (1:300, Cell Signaling), followed by Alexa594-, Alexa488-, or Alexa680-conjugated secondary antibodies, respectively. High-resolution grayscale images of immunostained cells were acquired using the same exposure time under a Zeiss Axiovert 200M inverted microscope equipped with AxioCam HRm Camera and Axiovert software. Background-subtracted mean fluorescence intensities of immunostained cells were measured using ImageJ software as described (Chung et al., 2009b; Cavaretta et al., 2014).

**Two electrode voltage clamp recordings.** After linearizing the plasmids pLin-GIRK2A, pFroggy-G $\beta_1$ , pFroggy-G $\gamma_2$  (Bichet et al., 2006; Chung et al., 2009b), capped cRNA were synthesized using the Amplicap T7 or Sp6 High Yield Message Maker kit (Epicentre Technologies). Stage V–VI *Xenopus laevis* oocytes were injected with cRNA (2 ng for G $\beta_1$  and G $\gamma_2$ , 5 ng for GIRK2A, 5 and 20 ng for GIRK2A-Y353X), and maintained at 16 °C in ND96 solution containing (in mM): 96 NaCl, 2 KCl, 1 MgCl<sub>2</sub>, 5 HEPES (pH 7.4). Macroscopic currents were recorded from oocytes at 23–25 °C using GeneClamp 500B (Axon Instruments). Electrodes filled with 3 M KCl had a resistance of 0.4–1 M $\Omega$ . To assess the resting potential and leakiness of the cell, initial and final recordings were performed in "90 mM NaCl" external bath solution containing (in mM): 90 NaCl, 2 MgCl<sub>2</sub>, and 10 HEPES (pH 7.4). To record K<sup>+</sup> current, "90 mM NaCl" solution was changed to "90 mM KCl" bath solution containing (in mM): 90 KCl, 2 MgCl<sub>2</sub>, and 10 HEPES (pH 7.4) using a ValveLink 16 perfusion system (AutoMate Scientific).

**Kainate-induced status epilepticus.** To elicit status epilepticus, male rodents were subjected to intraperitoneal (i.p.) injection of vehicle control (autoclaved H<sub>2</sub>O or saline) or kainate (abcam) as described (Levesque and Avoli, 2013) at 9 mg/kg for Sprague-Dawley rats weighing 200–250 g (Fig. 8b–c), 20 and 30 mg/kg for CD001 rats weighing 110–160 g (Fig. 8d–e), 15 and 30 mg/kg for C57BL/6J mice weighing 20 g (Supplementary Fig. S8). Rodents were returned to their home cage and video monitored. At 3 h and 8 h post injection, 2 hippocampi per rat was homogenized in modified RIPA buffer (1 mL) containing 1% SDS and protease inhibitors, sonicated briefly, and centrifuged at 14,000 rpm. Two hippocampi per mouse was homogenized as described (Jang et al., 2016b) to isolate crude homogenate (S1), soluble (S2) and membrane (P2) protein fractions.

Hippocampal lysates were immunoblotted with antibodies for GIRK1 and GIRK2 N termini (1:200, Supplementary Fig. S2), c-Fos, c-Jun, anti- $\beta$ -tubulin, or GAPDH (1:500-1:1,000, all Cell Signaling). Several hippocampal lysates from vehicle-treated rats did not show any immunoblot bands for cleaved GIRK (Fig. 8b-e). Therefore, background-subtracted immunoblot band intensity ratios of cleaved GIRK / loading controls ( $\beta$ -tubulin or GAPDH) from kainate-injected rats were taken as 100%. The ratio from vehicle-injected rats were then normalized to the ratio from kainate-injected rats to obtain % KA.

**Statistical Analyses.** Using Origin 9.1 (Origin Lab), the Student's *t*-test and one-way ANOVA with Fisher's multiple comparison test were performed to identify the statistically significant difference with a priori value (*p*) < 0.05 between 2 groups and for >3 groups, respectively.

## 2-6 Acknowledgements

We thank Dr. Lily Y. Jan (University of California San Francisco) for providing reagents and two-electrode voltage clamp set-up for us to do electrophysiological recording. We thank Dr. James A. Wells (University of California San Francisco) for providing his expertise in caspases and his critical comments on our manuscript. We thank Dr. Claudio Grosman (University of Illinois at Urbana Champaign) for providing his help with VMD modeling and his critical comments on our manuscript. We thank Laura Barker and Evera Wong for their technical assistance. This research was supported by the Research Project Grant #R01NS083402 from the NIH National Institute of Neurological Disorders and Stroke (PI: Chung).

## 2-7 Supplementary Information:

### Supplementary Methods:

**Multi-electrode array (MEA) recordings.** Primary dissociated rat hippocampal neurons ( $1.5 \times 10^5$  cells) were plated onto poly D-lysine (0.1 mg/mL, Sigma)-coated MEA electrode area containing 64 electrodes per plate (Axion Biosystem). Neurons were fed twice a week by removing half of the medium and adding fresh medium. At 11 days in vitro (DIV), neurons were treated for 2 days with DL-APV (200  $\mu$ M). At DIV 13, field potentials at each MEA electrode relative to a

ground electrode were recorded with a sampling rate of 25 kHz using Axion Muse 64 channel system inside a MEA recording incubator (5% CO<sub>2</sub>, 37°C) as described (Jewett et al, 2016). To start, DL-APV (200 µM), PTX (100 µM), and strychnine (5 µM) were added to the original medium in the MEA plate in bio-safety cabinet. The plate was immediately placed in the MEA recording incubator for the "APV control" recording for 30 min. The MEA plate was removed from the recording chamber, and all medium was replaced with the saved original medium from the last feeding, which were supplied with glycine (100 µM), PTX (100 µM), and strychnine (5 µM). The MEA plate was returned to the recording incubator for "APV withdrawal " recording for additional 90 min. Data was analyzed using Axion Integrated studio (AxIS) as described<sup>1</sup>. Raster plots were generated using NeuralMetricTool software.

**References:**

Jewett, K.A. *et al.* Feedback modulation of neural network synchrony and seizure susceptibility by Mdm2-p53-Nedd4-2 signaling. *Mol Brain*. 9:32. doi: 10.1186/s13041-016-0214-6 (2016).

## 2-8 Figures

**Figure 2.1**

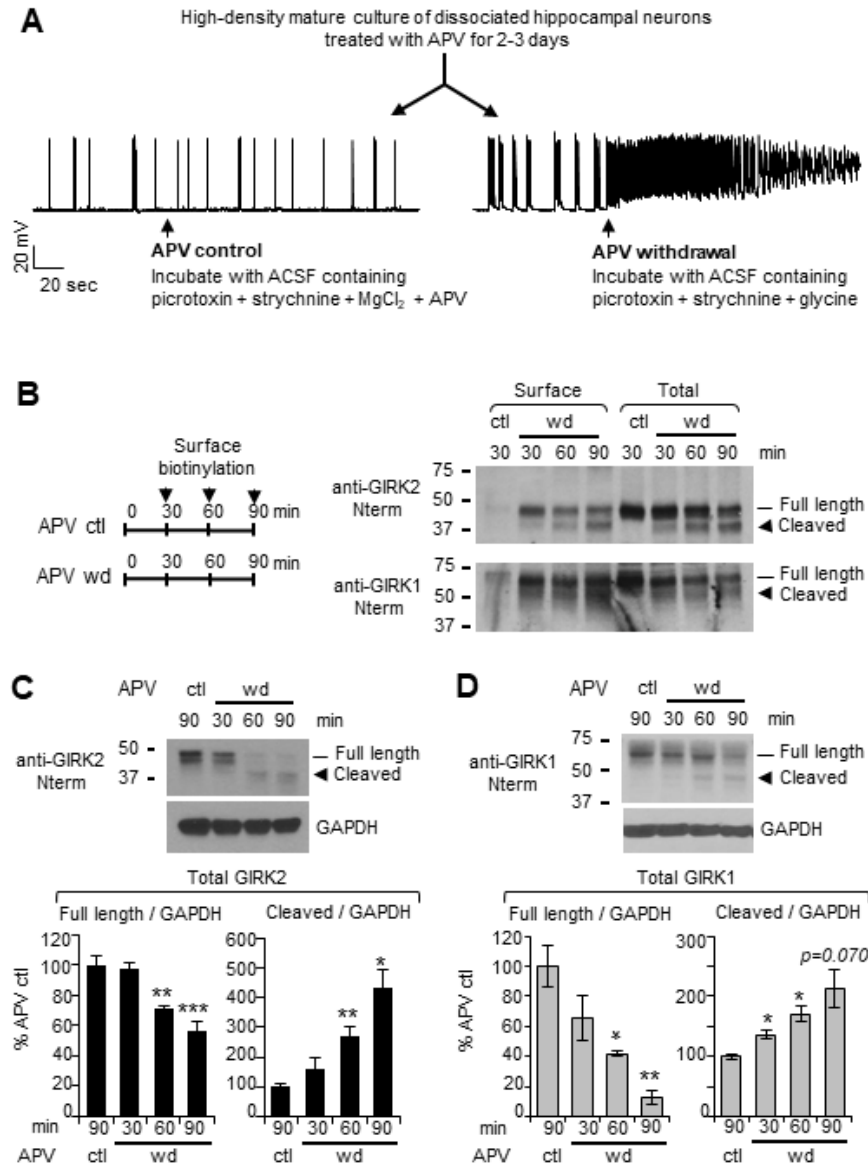


**Figure 2.1 Prolonged seizure activity induces C-terminal cleavage of GIRK channels in cultured hippocampal neurons.** (A) Whole-cell patch clamp recording of spontaneous action potentials in cultured hippocampal neurons (12-13 DIV, pretreated with 200  $\mu$ M DL-APV for 2-3 days at 10-11 DIV) before and after APV control (left representative trace) and APV withdrawal (right representative trace). Activation of synaptic NMDAR upon APV withdrawal induced high frequency burst firing of action potentials and sustained depolarization. (B) Surface biotinylation of cultured hippocampal neurons after APV control (ctl) or APV withdrawal (wd) was analyzed by immunoblotting with antibodies recognizing intracellular N-termini of GIRK1 and GIRK2. Prolonged APV withdrawal for 30-90 min resulted in the C-terminal cleavage of GIRK1 and GIRK2 proteins that were biotinylated (Surface) and in the lysates (Total). (C-D) Quantitative immunoblot analyses of total GIRK2 (C) and GIRK1 (D) expression in cultured hippocampal neurons after APV control for 90 min (ctl,  $n = 4-5$ ) or APV withdrawal (wd) for 30-90 min ( $n = 3-4$  each time point). GAPDH served as a loading control. Data shown represent the mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ . The cropped gray-scale blots are displayed. Full-length blots are included in the Supplementary Fig. S3.

**Figure 2.2**

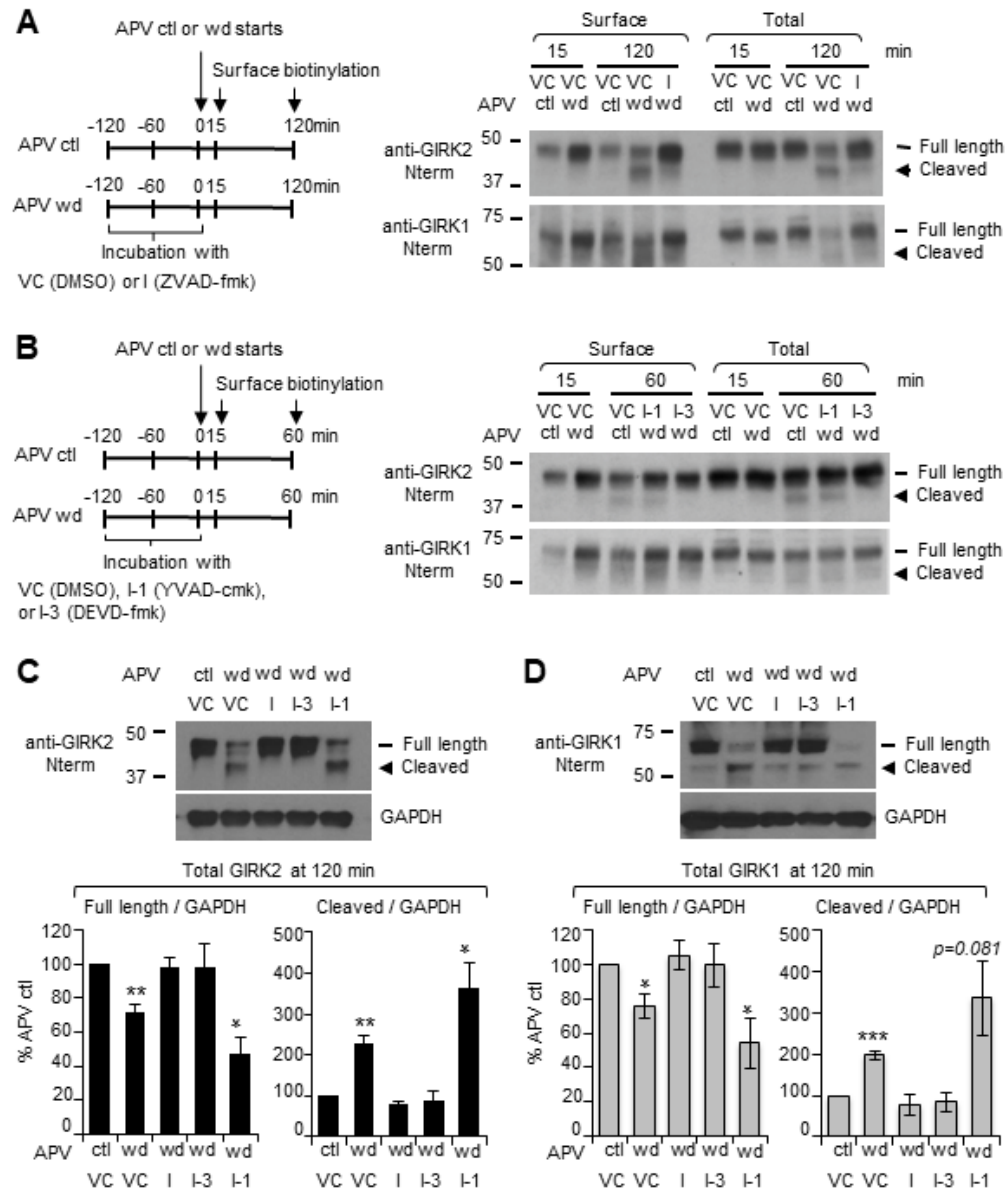


**Figure 2.2 Caspase-3 mediates C-terminal cleavage of GIRK channels in cultured hippocampal neurons upon prolonged seizure activity.** (A-B) Surface biotinylation was performed after APV control (ctl) or APV withdrawal (wd) in cultured hippocampal neurons pretreated for 2 h with vehicle control (VC, 0.1% DMSO), pan caspase inhibitor ZVAD-Fmk (I, 100  $\mu$ M) (A), caspase-1 inhibitor YVAD-cmk (I-1, 20  $\mu$ M), or caspase-3 inhibitor DEVD-fmk (I-3, 20  $\mu$ M) (B). Surface and Total GIRK1 and GIRK2 proteins were examined by immunoblotting with antibodies recognizing intracellular N-termini of GIRK1 and GIRK2. (C-D) Quantitative immunoblot analyses of total GIRK2 (c) and GIRK1 (d) expression after APV control (ctl) or APV withdrawal (wd) for 120 min in cultured hippocampal neurons which were pretreated for 2 h with vehicle control (VC), ZVAD-Fmk (I), YVAD-cmk (I-1), or DEVD-fmk (I-3). GAPDH served as a loading control. Pretreatment with ZVAD-Fmk and DEVD-fmk but not YVAD-cmk blocked C-terminal cleavages of GIRK1 and GIRK2 induced by prolonged APV withdrawal. Data shown represent the mean  $\pm$  SEM (n = 4 per treatment). \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.005. The cropped gray-scale blots are displayed. Full-length blots are included in the Supplementary Fig. S4.

**Figure 2.3**

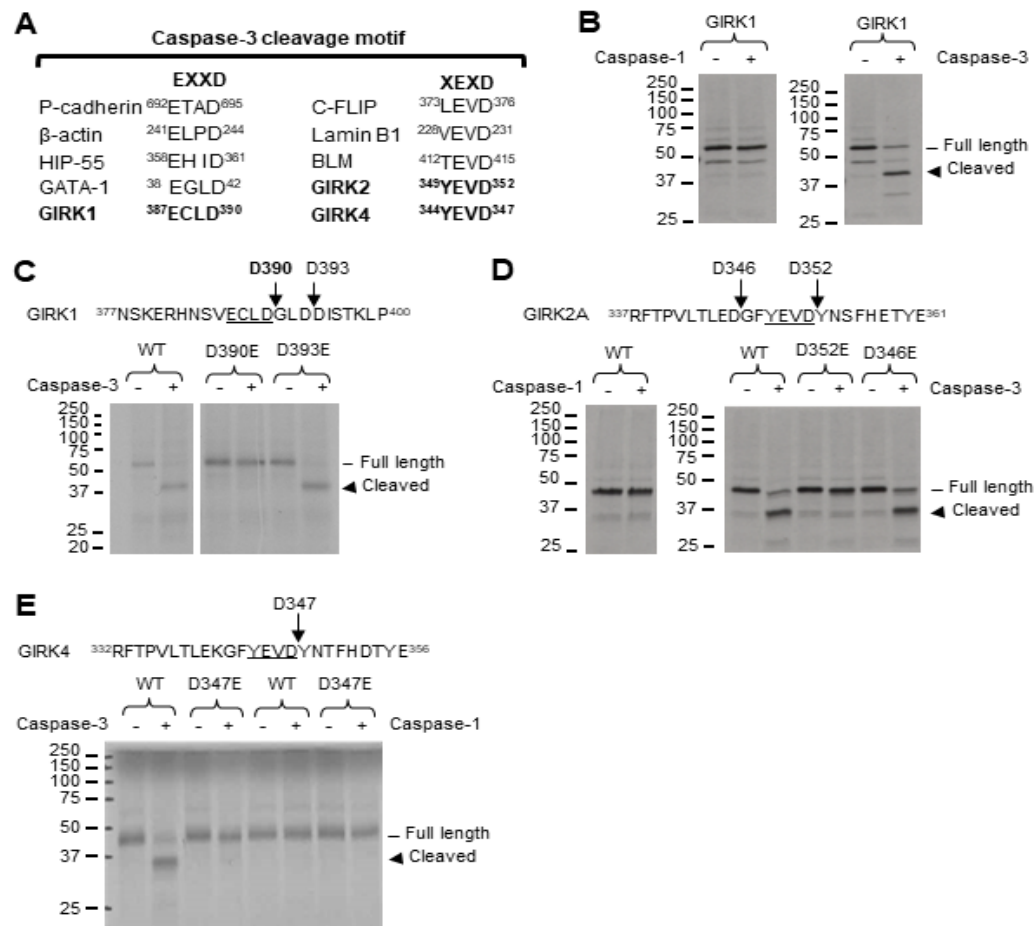


**Figure 2.3 Caspase-3 directly cleaves GIRK1, GIRK2, and GIRK4 in vitro.** (A) Sequence alignment of GIRK subunits with caspase-3 cleavage sites of known substrates that resemble consensus EXXD and XEXD motifs. (B-E) In vitro transcription and translation of GIRK subunits with <sup>35</sup>S-methionine followed by in vitro cleavage assay with purified caspase-1 or caspase-3. The cleavage reaction products were separated by SDS-PAGE gel and visualized by autoradiograph. (B) Caspase-3 but not caspase-1 cleaved wild type (WT) GIRK1. (C) Caspase-3 also cleaved mutant GIRK1-D393E but not mutant GIRK1-D390E, indicating that D390 within <sup>387</sup>ECLD<sup>390</sup> motif is the caspase-3 cleavage site. (D) Caspase-3 but not caspase-1 cleaved GIRK2 WT. Caspase-3 also cleaved mutant GIRK2-D346E but not mutant GIRK2-D352E, indicating that D352 within <sup>349</sup>YEVD<sup>352</sup> motif is the caspase-3 cleavage site. (E) While caspase-1 had no effect, caspase-3 cleaved GIRK4 WT but not mutant GIRK4-D347E, indicating that D347 within <sup>344</sup>YEVD<sup>347</sup> motif is the caspase-3 cleavage site.

**Figure 2.4**

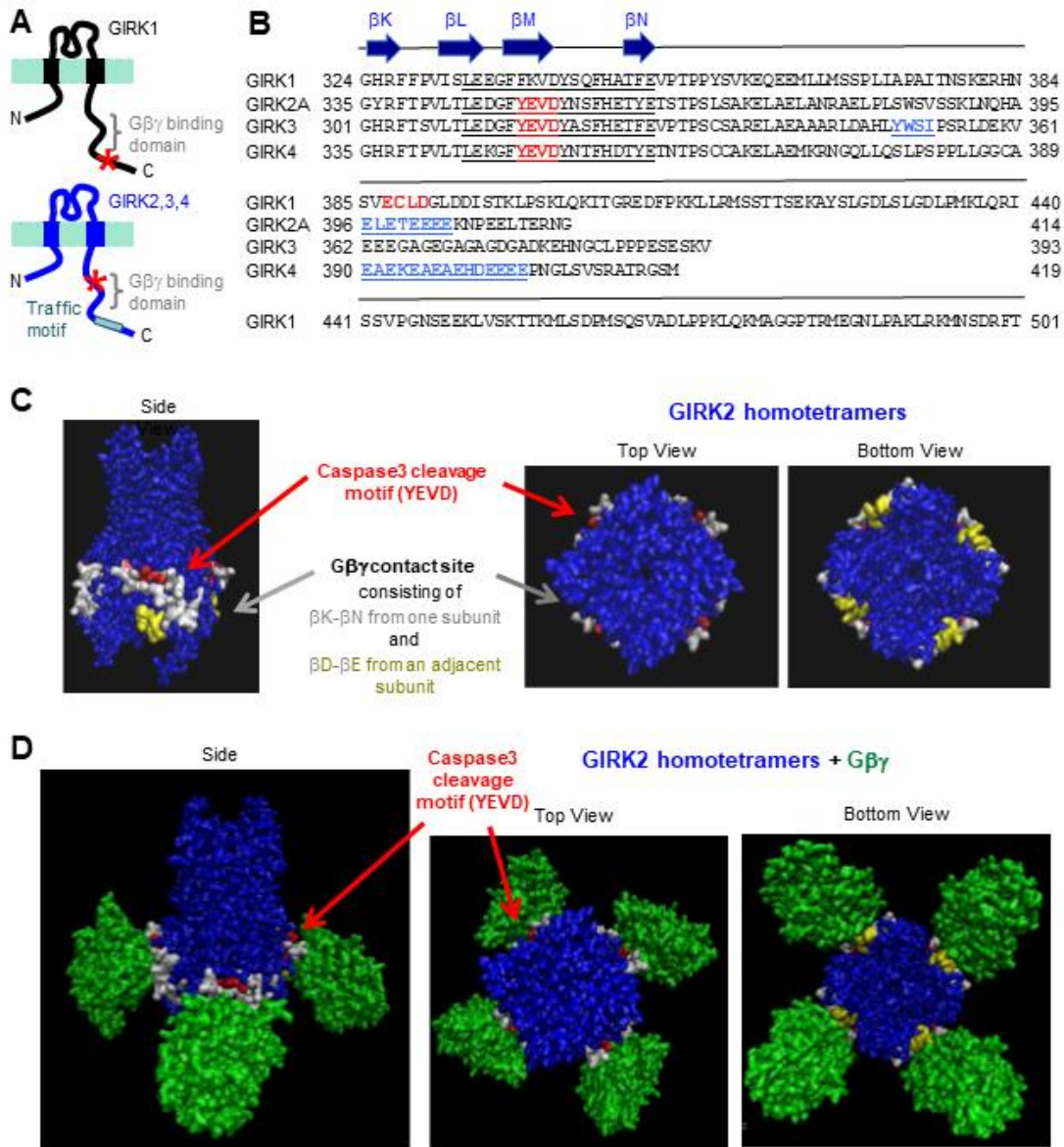


**Figure 2.4 Caspase-3 cleavage motif is located at the G $\beta\gamma$  contact site of GIRK2.** (A) Schematic cartoons (not to scale) of GIRK1 and GIRK2 subunit, highlighting the G $\beta\gamma$  binding site and an ER export motif in their cytoplasmic C-terminal domains. \* indicates caspase-3 cleavage motifs. (B) Amino acid sequence alignment of GIRK1 (NP\_113798.1), GIRK2A (NP\_001020755.1), GIRK3 (NP\_032455.2), and GIRK4 (NP\_058993.1) using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). Caspase-3 cleavage sites in GIRK1 (<sup>387</sup>ECLD<sup>390</sup>), GIRK2A (<sup>349</sup>YEVD<sup>352</sup>), GIRK3 (<sup>315</sup>YEVD<sup>318</sup>), and GIRK4 (<sup>344</sup>YEVD<sup>347</sup>) are shown in red. The traffic motifs are shown in light blue. The G $\beta\gamma$  contact sites are underlined. (C-D) Molecular surface representation of the crystal structure of GIRK2 homotetrameric channels alone (C, shown in blue) or complexed with G $\beta\gamma$  (D, shown in green) viewed from the side, top, and bottom (Protein Data Bank (PDB): 4kfm). Residues in red show caspase-3 cleavage site of GIRK2 (<sup>349</sup>YEVD<sup>352</sup>). Residues in yellow and white show the G $\beta\gamma$  contact site of GIRK2.

**Figure 2.5**

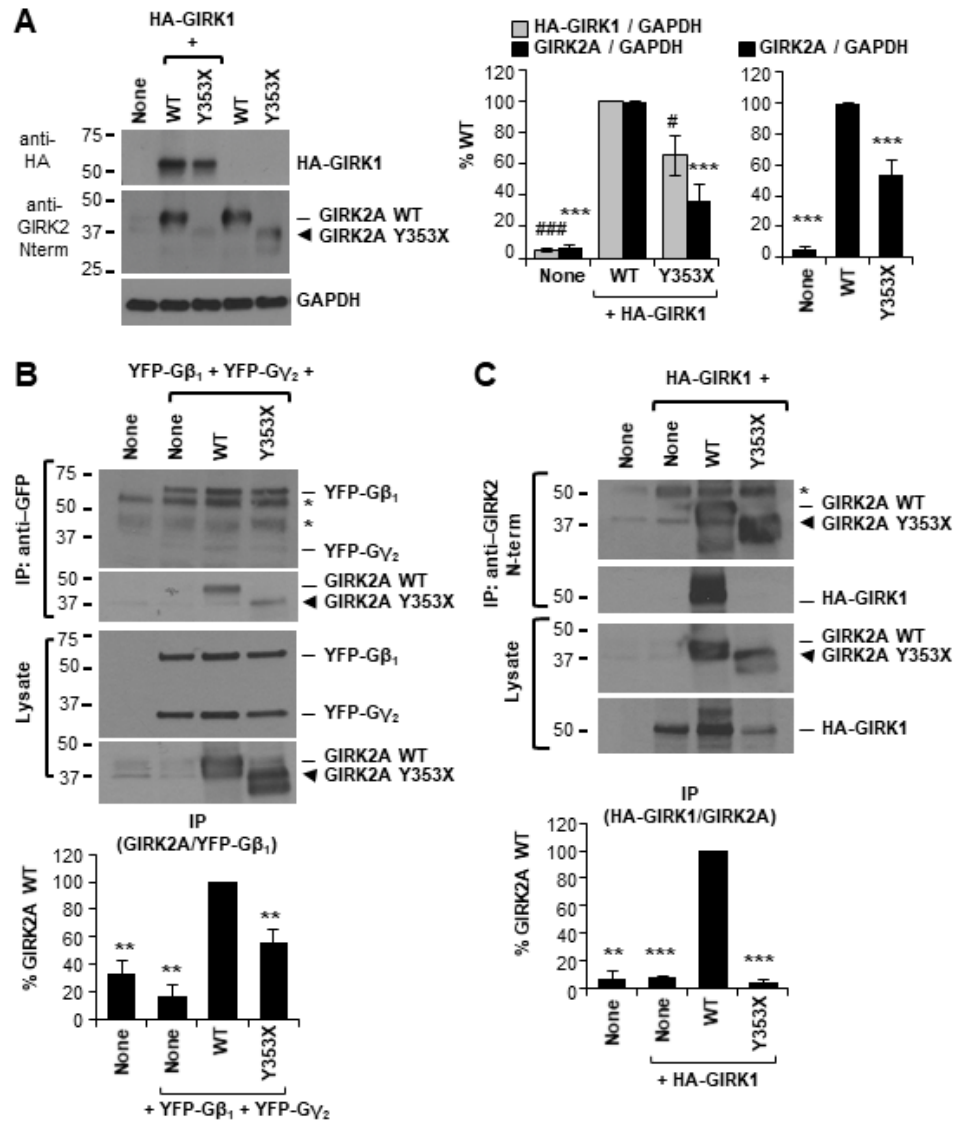


**Figure 2.5** GIRK2A truncated at <sup>349</sup>YEVD<sup>352</sup> motif shows decreased binding to Gβγ and GIRK1. (A) Quantitative immunoblot analyses of the HEK293T cells transfected with equal amounts of plasmids containing GIRK1 with extracellular HA tag (HA-GIRK1) and GIRK2A wild type (WT) or GIRK2A-Y353X (n = 7-8 each), GIRK2A WT alone, or GIRK2A-Y353X alone (n = 6-7 each). GAPDH served as a loading control. GIRK2A-Y353X expression was significantly lower than GIRK2A WT expression. Coexpression of GIRK2A-Y353X decreased HA-GIRK1 expression compared to coexpression of GIRK2A WT. (B) Immunoprecipitation (IP) with anti-GFP antibodies was performed from untransfected HEK293T cells (none, n = 4), or the cells transfected with YFP-Gβ<sub>1</sub> and YFP-Gγ<sub>2</sub> alone (n = 4) or together with GIRK2A WT (n = 6) or GIRK2A-Y353X (n = 6). To increase GIRK2A-Y353X expression to a similar extent as GIRK2A-WT expression, we doubled the amount of GIRK2A-Y353X plasmid for transfection compared to GIRK2A-WT plasmids. Coimmunoprecipitation of GIRK2-Y353X with Gβ<sub>1</sub>γ<sub>2</sub> was decreased compared to GIRK2A WT. (C) IP with anti-GIRK2 N-terminal antibodies was performed from untransfected HEK293T cells (none), or the cells expressing HA-GIRK1 alone or together with GIRK2A WT or GIRK2A-Y353X (n = 3 each). To increase GIRK2A-Y353X expression to a similar extent as GIRK2A-WT expression, we doubled the amount of GIRK2A-Y353X plasmid for transfection compared to GIRK2A-WT plasmids. Coimmunoprecipitation of HA-GIRK1 with GIRK2-Y353X was reduced compared to GIRK2 WT. In b-c, \* points at IgG bands. Data shown represent the mean ± SEM (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.005 against GIRK2 WT; #p < 0.05, ###p < 0.005 against HA-GIRK1 + GIRK2 WT). The cropped gray-scale blots are displayed. Full-length blots are included in the Supplementary Fig. S5.

**Figure 2.6**

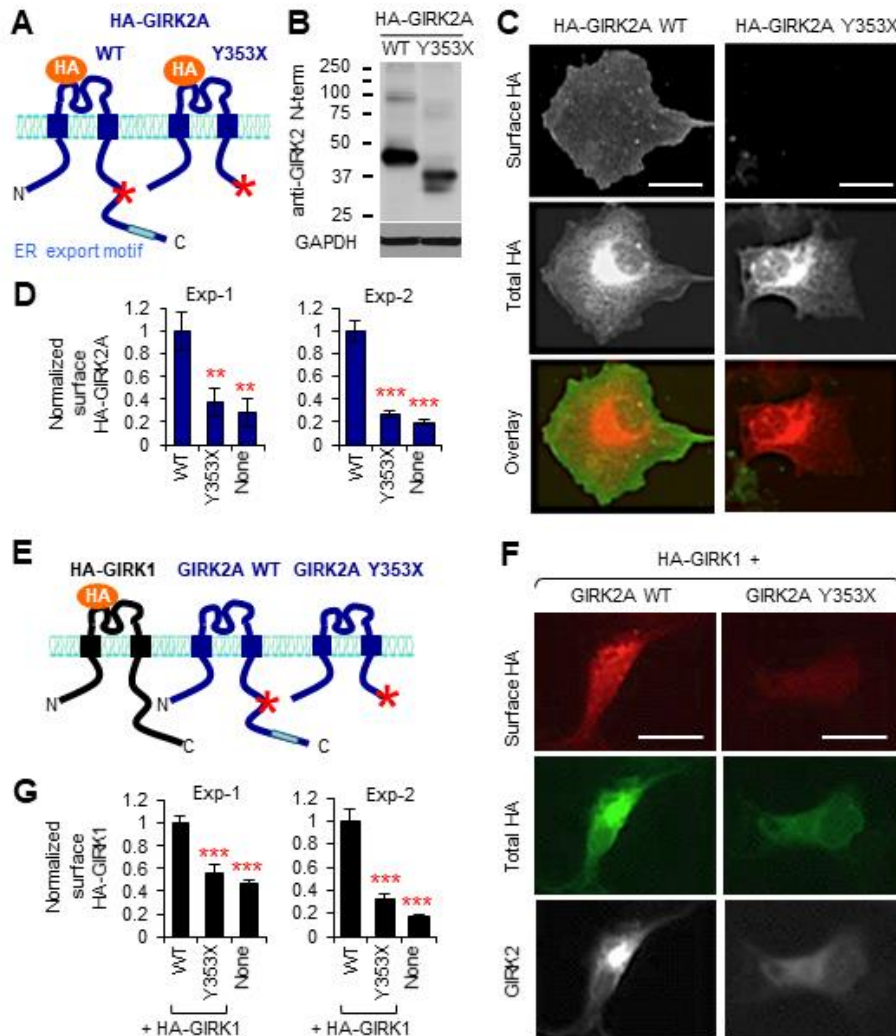


**Figure 2.6** GIRK2A truncated at <sup>349</sup>YEVD<sup>352</sup> motif does not express at the plasma membrane. **(A)** Schematic cartoon (not to scale) of GIRK2A WT or Y353X containing extracellular HA tag (HA-GIRK2A). \* indicates caspase-3 cleavage site of GIRK2A. **(B)** Immunoblot of HA-GIRK2A WT or Y353X in transfected COS7 cells. **(c-d)** Surface immunostaining of COS7 cells transfected with HA-GIRK2A WT or Y353X was performed without permeabilization using mouse anti-HA antibodies and Alexa488-conjugated secondary antibodies (Exp-1, n = 10 cells per transfection) or biotin-conjugated secondary antibodies followed by Alexa488-conjugated streptavidin (Exp-2, n = 12-15 cells per transfection). Following fixation and permeabilization, their total (surface and intracellular) expression was visualized with rabbit anti-HA antibodies and Alexa594-conjugated secondary antibodies. **(C)** Representative images showing low level of HA-GIRK2 Y353X at the plasma membrane compared to WT channels. Scale bars are 15  $\mu$ m. **(D)** Background subtracted, mean fluorescence intensities of surface HA in untransfected COS7 cells (none) or cells expressing HA-GIRK2A WT or Y353X. **(E)** Schematic cartoon (not to scale) of GIRK2A WT, Y353X, and GIRK1 containing extracellular HA tag (HA-GIRK1). \* indicates caspase-3 cleavage site of GIRK2A. **(F-G)** Surface immunostaining of COS7 cells transfected with HA-GIRK1 and GIRK2A WT or Y353X was performed without permeabilization using rabbit anti-HA antibodies and Alexa594-conjugated secondary antibodies (Exp-1, n = 24-26 cells per transfection) or biotin-conjugated secondary antibodies followed by Alexa594-conjugated streptavidin (Exp-2, n = 10 cells per transfection). Following fixation and permeabilization, total HA-GIRK1 and GIRK2 proteins were labeled with mouse anti-HA and rabbit anti-GIRK2 N-term antibodies, respectively, followed by Alexa488- and Alexa-680-conjugated secondary antibodies. **(F)** Representative images showing low level of HA-GIRK1/GIRK2 Y353X channels at the plasma membrane compared to WT channels. Scale bars are 15  $\mu$ m. **(G)** Background subtracted, mean fluorescence intensities of surface HA-GIRK1 in untransfected COS7 cells (none) or cells coexpressing GIRK2A WT, or Y353X. Data shown represent the mean  $\pm$  SEM. \*\* $p$  < 0.01, \*\*\* $p$  < 0.005.

**Figure 2.7**

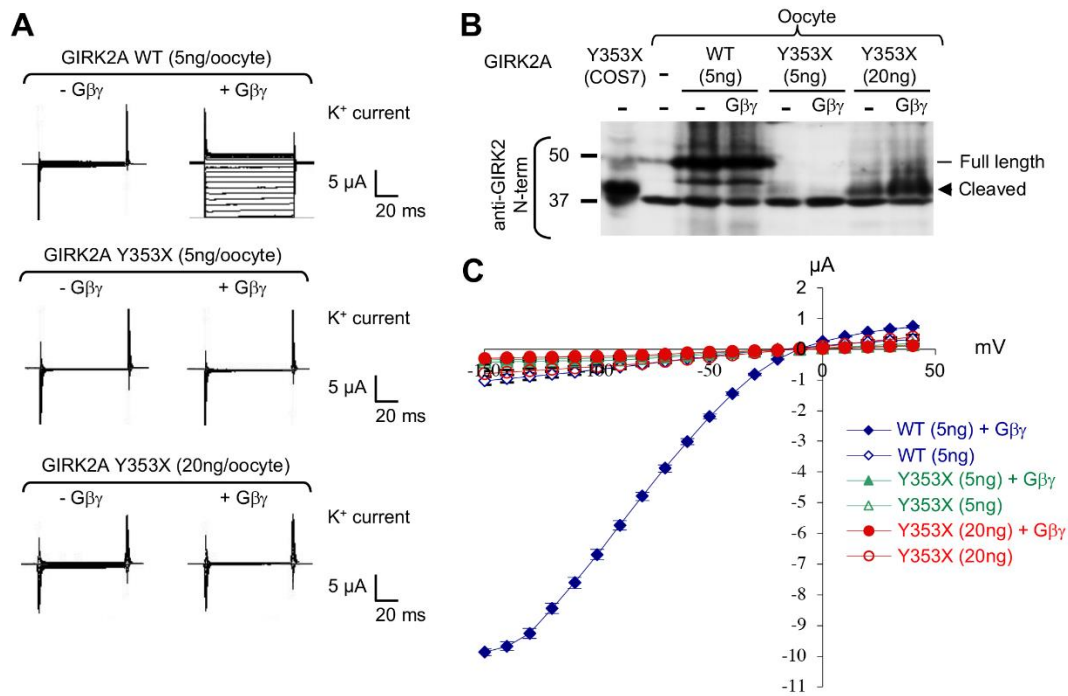
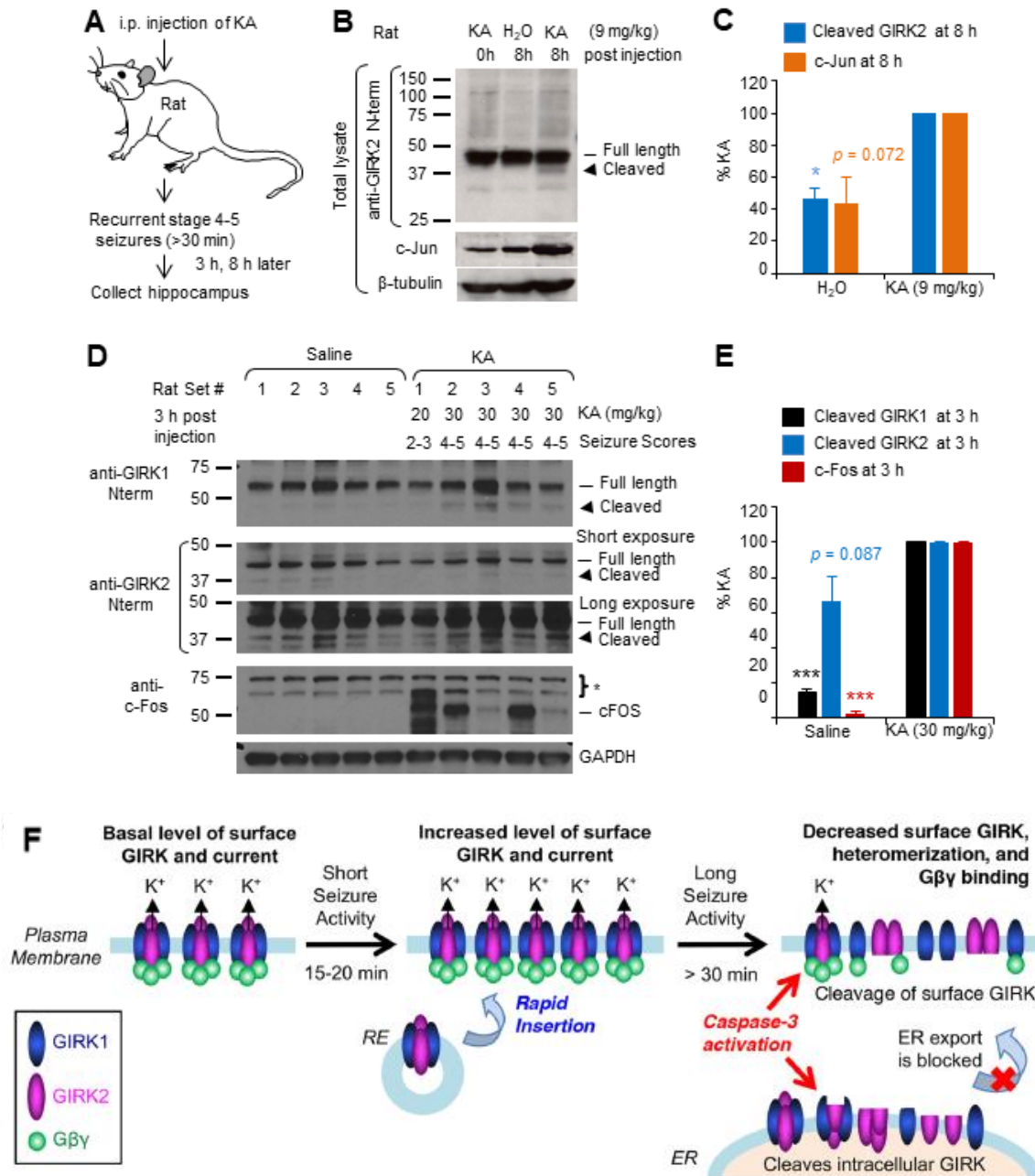


**Figure 2.7 GIRK2A channels truncated at <sup>349</sup>YEVD<sup>352</sup> motif do not express  $K^+$  current.**

Two-electrode voltage clamp recordings of  $K^+$  current from *Xenopus* oocytes expressing GIRK2A WT or Y353X were performed in 90 mM KCl external bath solution upon applying the voltage steps from -150 to 40 mV in 10 mV increments. **(A)** Representative current traces from oocytes expressing GIRK2A WT and Y353X bathed in 90 mM KCl. Truncated GIRK2-Y353X channels failed to carry  $K^+$  current in the presence or absence of  $G\beta_1\gamma_2$ . **(B)** Immunoblotting with anti-GIRK2 N-terminal antibodies on the lysate from COS7 cells expressing GIRK2A-Y353X and oocytes injected with cRNA of GIRK2A WT (5 ng) or Y353X (5 ng, 20 ng) with or without cRNA of  $G\beta_1$  and  $G\gamma_2$  (2 ng each). The cropped gray-scale blots are displayed. Full-length blots are included in the Supplementary Fig. S6. **(C)** Representative averaged current-voltage ( $I$ - $V$ ) relations obtained from *Xenopus* oocytes expressing GIRK2A WT ( $n = 5$ ), GIRK2A WT +  $G\beta_1\gamma_2$  ( $n = 8$ ), GIRK2A-Y353X ( $n = 8$ ), GIRK2A-Y353X +  $G\beta_1\gamma_2$  ( $n = 10$ ), 20 ng GIRK2A-Y353X ( $n = 4$ ), 20 ng GIRK2A-Y353X +  $G\beta_1\gamma_2$  ( $n = 5$ ).  $K^+$  current in oocytes expressing GIRK2A WT +  $G\beta_1\gamma_2$  were significantly larger than oocytes expressing GIRK2A WT alone or GIRK2A-Y353X +/-  $G\beta_1\gamma_2$  at all voltage steps except -20 to +20 mV. Data points in the  $I$ - $V$  curves represent the mean  $\pm$  SEM.

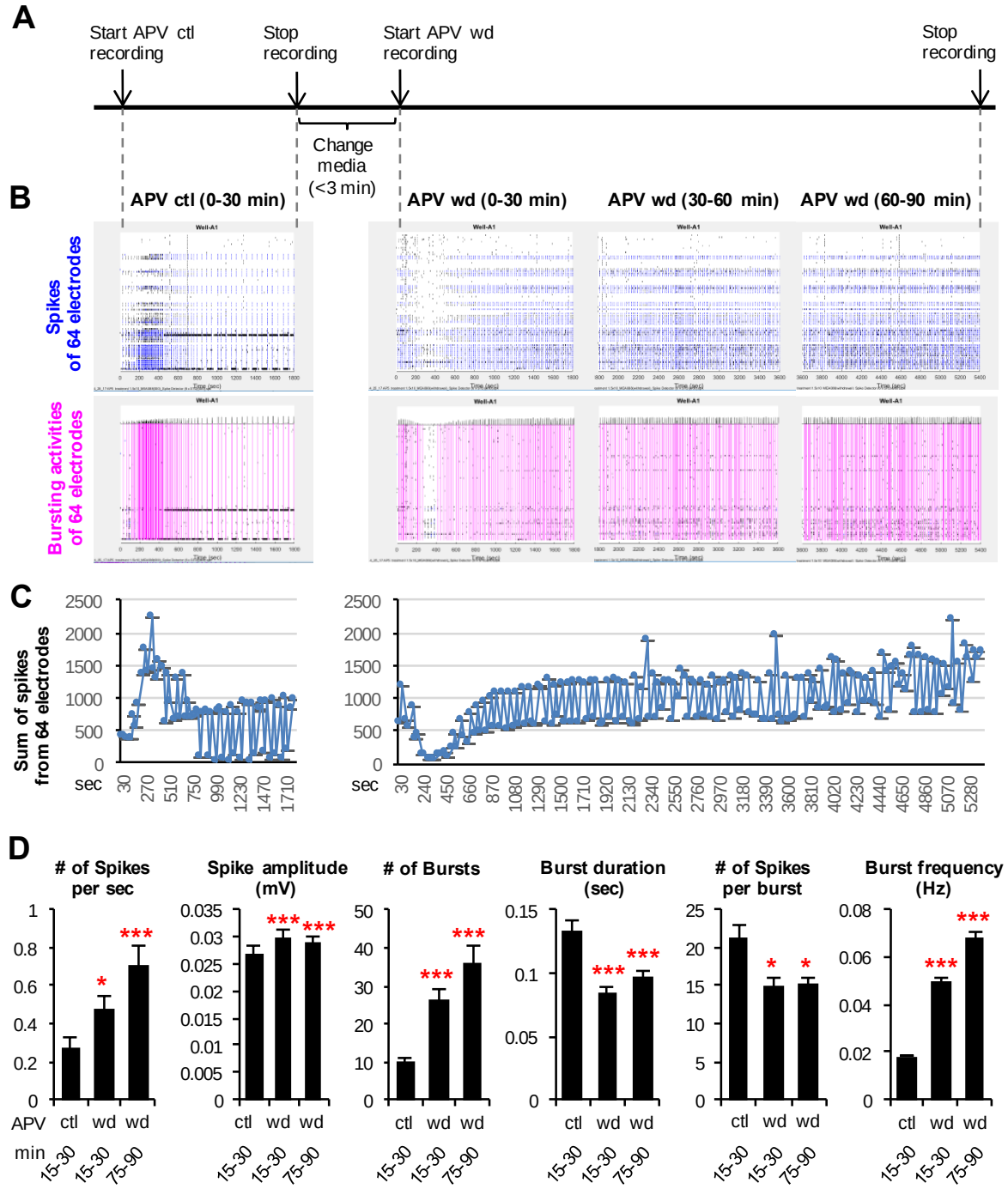
**Figure 2.8**



## Figure 2.8 continued

**Figure 2.8 Kainate-induced status epilepticus in rats induces C-terminal cleavage of GIRK1 and GIRK2 in the hippocampus.** (A) Schematic workflow of an experiment from kainate (KA)-mediated induction of status epilepticus in rats to collection of their hippocampi. (B-C) Immunoblot analysis of total hippocampal lysates from Sprague Dawley rats injected with kainate (9 mg/kg) and vehicle control (H<sub>2</sub>O) with antibodies against GIRK2 N-terminus, c-Jun (marker for seizure induction), and  $\beta$ -tubulin (loading control). Kainate-injected rats developed stage 4-5 seizures and status epilepticus (n = 3) whereas H<sub>2</sub>O-injected rats did not display seizures (n = 3). Representative immunoblots (B) and quantification (C) showed that status epilepticus induces C-terminal cleavage of GIRK2 in the rat hippocampi at 8 h post injection with 9 mg/kg kainate. (D-E) Immunoblot analysis of total hippocampal lysate from CD001 rats injected with kainate (20 and 30 mg/kg) or vehicle control (saline) with antibodies against GIRK1 and GIRK2 N-terminus, c-Fos (marker for seizure induction), and GAPDH (loading control). In one pair of CD001 rats (set #1), the rat injected with 20 mg/kg kainate developed a short duration of stage 2-3 seizures. In 4 pairs of rats (set #2-5), rats injected with 30 mg/kg kainate developed stage 4-5 seizures and status epilepticus. Saline-injected rats did not display seizures. Representative immunoblots (D) and quantification (E) showed that status epilepticus induced C-terminal cleavage of GIRK1 in the rat hippocampus within 3 h post injection with 30 mg/kg kainate (n = 4). The cropped gray-scale blots are displayed. Full-length blots are included in the Supplementary Fig. S7. Data shown represent the mean  $\pm$  SEM. \* $p$  < 0.05, \*\*\* $p$  < 0.005. (F) A working model for the regulation of GIRK channels by seizure activity. Induction of seizure activity increases surface expression and current of GIRK1/GIRK2 channels within 15-20 min by promoting their recycling possibly as a homeostatic defense to heightened excitability (Chung et al., 2009a; Chung et al., 2009b). In contrast, prolonged seizure activity (>30 min) leads to caspase-3 dependent cleavage of GIRK1/GIRK2 channels in the ER and plasma membrane, which may likely decrease their surface and current expression, heteromerization and/or interaction with G $\beta\gamma$ .

**Figure 2.9**



## Figure 2.9 continued

**Figure 2.9 Synaptic NMDAR activation upon APV withdrawal increased burst firing of action potentials that lasted up to 90 min. (A)** Flow chart of multi-electrode array field potential recordings under APV control (ctl) for 30 min and APV withdrawal (wd) for 90 min from the same MEA dish containing cultured hippocampal neurons (DIV 13) pretreated with DL-APV (200  $\mu$ M) for 2 days. **(B, C)** Representative traces of recordings from all 64 electrodes in one MEA plate. **(B)** Spikes per electrode from all 64 electrodes (top) and Raster plots showing bursting activities from 64 electrodes (bottom). **(C)** Sum of spikes from 64 electrodes. **(D)** AxIS data analyses of MEA recordings (n = 64 electrodes). Adaptive threshold crossing method was used for single spike detection settings to determine spike activity from noise. Inter-spike interval (Milh et al.) threshold algorithm was used for burst detection settings with maximum ISI of 100 ms and minimum 5 spikes per single burst. Because treatment and removal of medium as well as physical movement of the MEA plates before and after recording is shown to perturb network activity of neurons on the plate, the first 15 min of recording data in APV control and APV withdrawal were omitted from the statistical analyses. Data shown represent the mean  $\pm$  SEM. \* $p < 0.05$ , \*\*\* $p < 0.005$ .

**Figure 2.10**

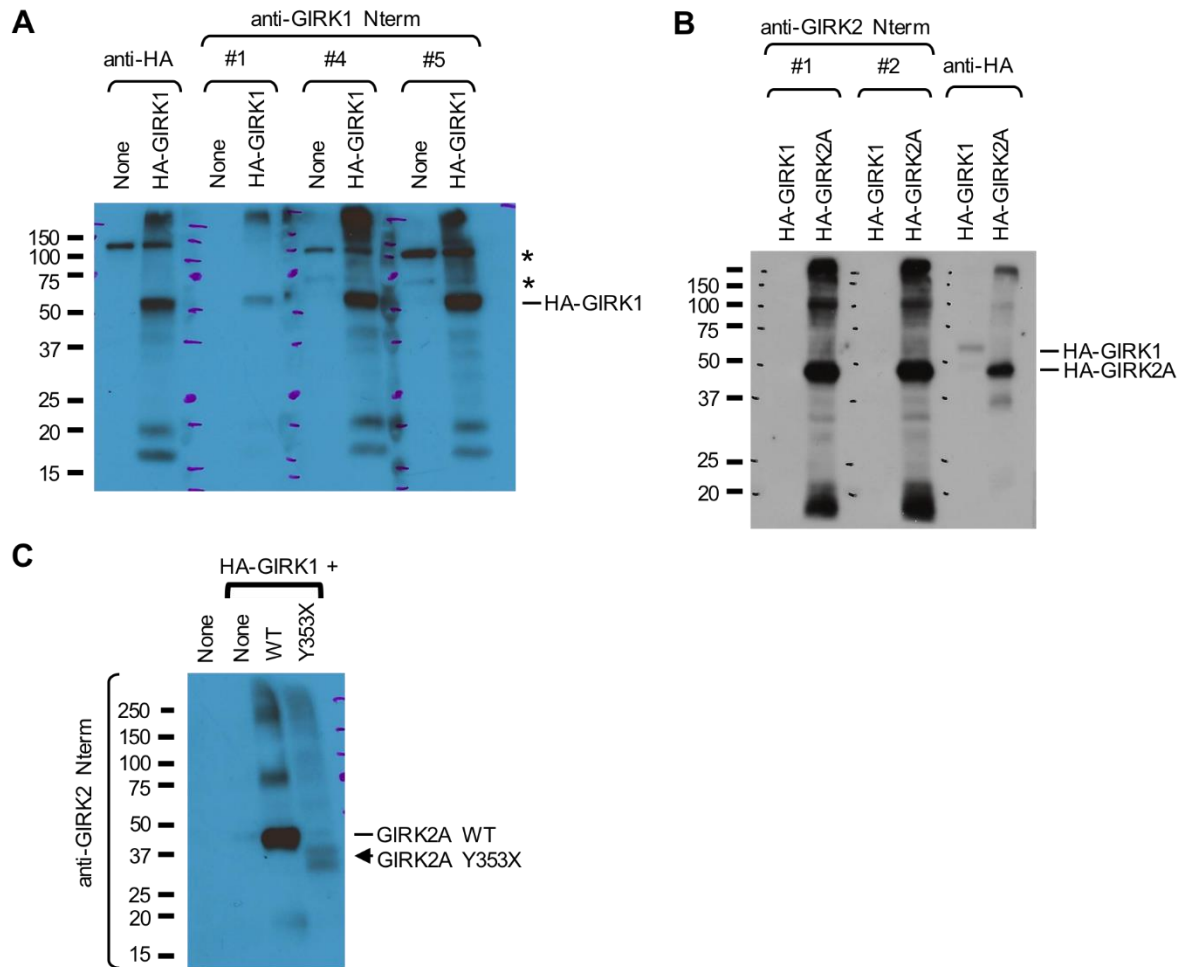


**Figure 2.10 Specificity of anti-GIRK1 and GIRK2 N-terminal antibodies used in Figures 1, 2, 7, and 8.** Rabbit polyclonal anti-GIRK1 and GIRK2 N-terminal antibodies were affinity-purified from sera by chromatography. **(A)** Characterization of anti-GIRK1 N-terminal antibodies in HEK293T cells that were untransfected (none) or transfected with HA-GIRK1. \* points at non-specific bands. **(B, C)** Characterization of anti-GIRK2 N-terminal antibodies in HEK293T cells that were untransfected (none) or transfected with HA-GIRK1 or HA-GIRK2A (B) or HA-GIRK1 and GIRK2A wild type (WT) or GIRK2A-Y353X in which Y353 residue right after <sup>349</sup>YEVD<sup>352</sup> motif was mutated to a stop codon. Expression of HA-GIRK1 and HA-GIRK2A were also examined by anti-HA antibodies **(A, B)**.

**Figure 2.11**

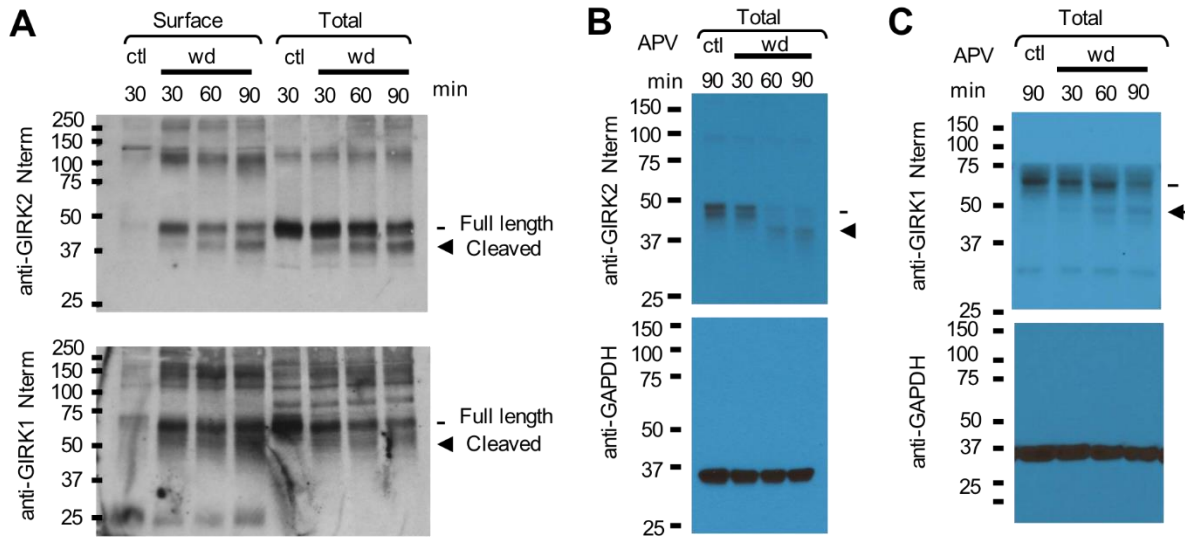


**Figure 2.11 Full-length immunoblots for cropped blots shown in Figure 1B-D.** (A) Surface biotinylation of cultured hippocampal neurons after APV control (ctl) or APV withdrawal (wd) was immunoblotted with anti-GIRK1 and GIRK2 N-terminal antibodies. (B, C) Total lysate of cultured hippocampal neurons after APV ctl or APV wd was immunoblotted with antibodies for GIRK2 N-terminus (B) and GIRK1 N-terminus (C).

**Figure 2.12**

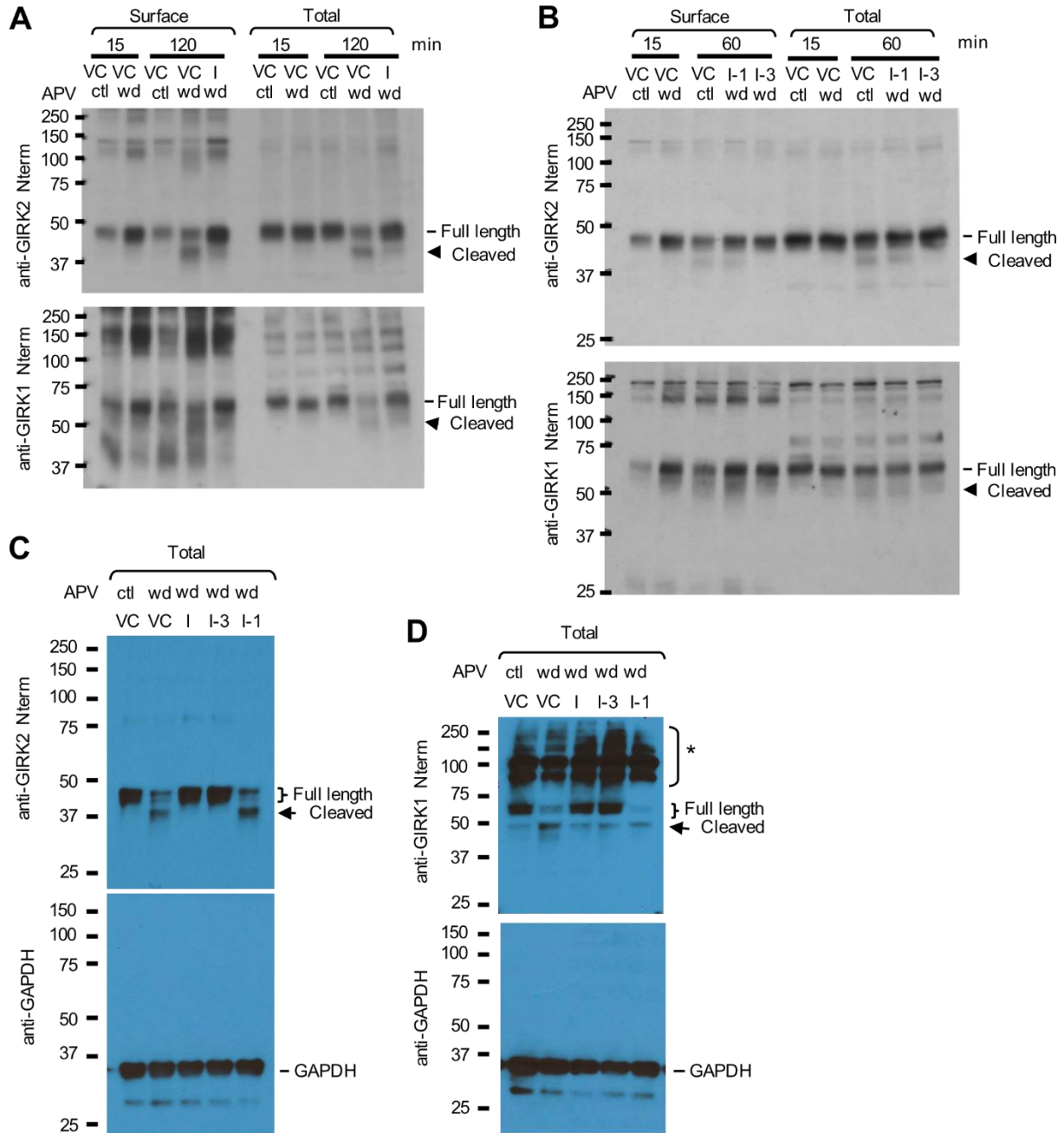


**Figure 2.12 Full-length immunoblots for cropped immunoblots shown in Figure 2. (A, B)** Surface biotinylation was performed after APV control (ctl) or APV withdrawal (wd) in cultured hippocampal neurons pretreated for 2 h with vehicle control (VC, 0.1% DMSO), pan caspase inhibitor ZVAD-Fmk (I, 100  $\mu$ M) (A), or caspase-1 inhibitor YVAD-cmk (I-1, 20  $\mu$ M), or caspase-3 inhibitor DEVD-fmk (I-3, 20  $\mu$ M) (B). (C, D) Immunoblot analyses were performed after APV ctl or APV wd for 120 min in cultured hippocampal neurons pretreated for 2 h with VC, ZVAD-Fmk (I), DEVD-fmk (I-3), or YVAD-cmk (I-1). \* points at non-specific bands.

Figure 2.13

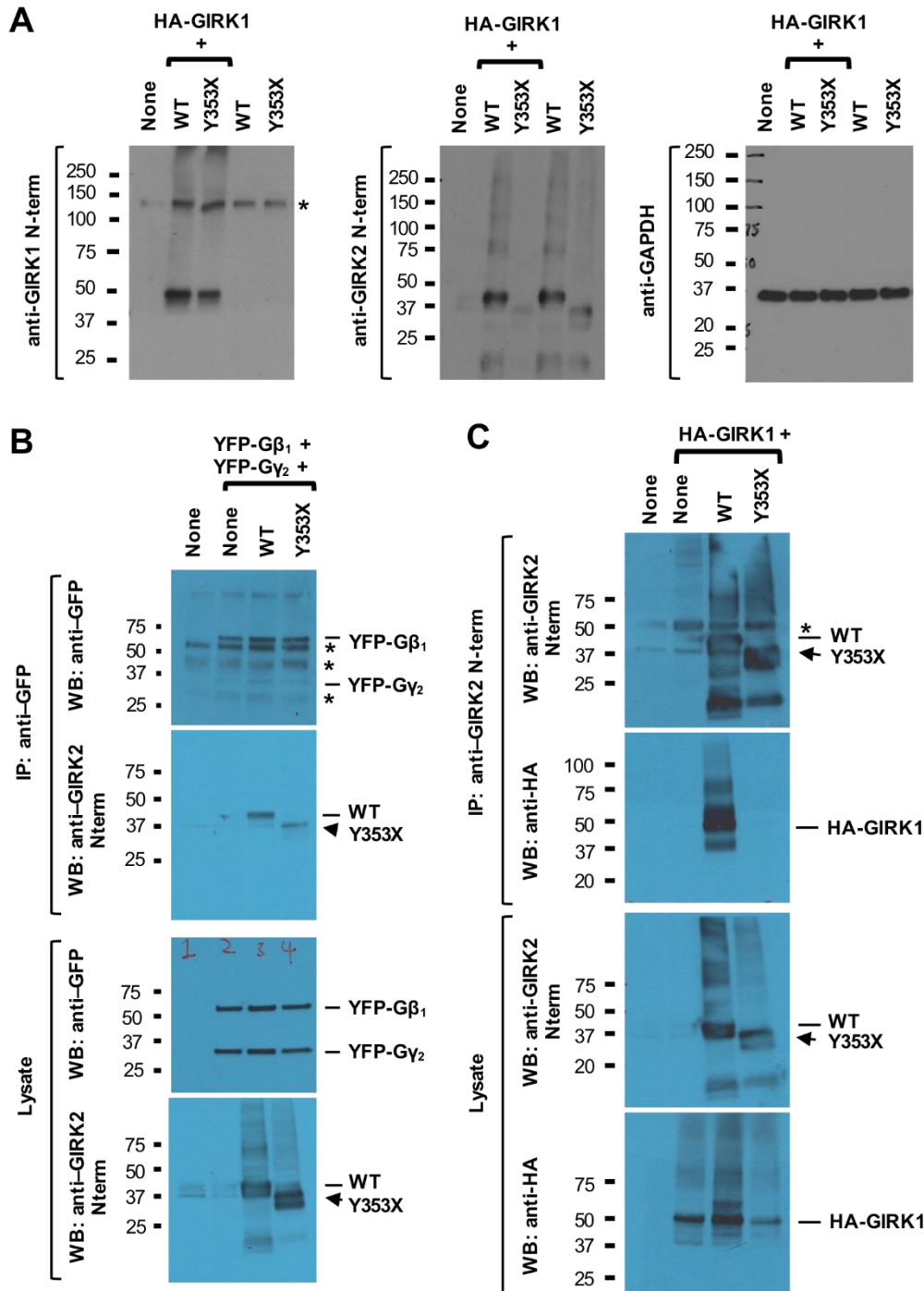


**Figure 2.13 Full-length immunoblots for cropped immunoblots shown in Figure 5. (A)** Immunoblot analyses of HEK293T cells expressing GIRK2A wild type (WT) or Y353X with or without HA-GIRK1. The cropped gray-scale blots are displayed in Figure 5A. GAPDH served as a loading control. \* points at non-specific bands. **(B, C)** Immunoprecipitation of YFP-G $\beta_1$  and YFP-G $\gamma_2$  **(B)** or HA-GIRK1 **(C)** from the HEK293T lysates co-expressing GIRK2A WT or Y353X. \* points at IgG bands. All antibodies used for immunoprecipitation and immunoblotting were from Cell Signaling.

**Figure 2.14**

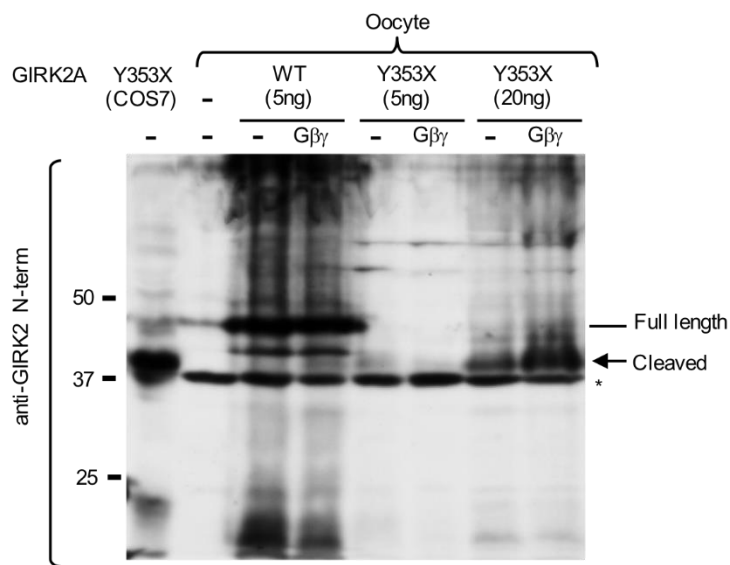


**Figure 2.14 Full-length immunoblot for a cropped blot in Figure 7B.** Immunoblotting of COS7 cells expressing GIRK2A-Y353X and the oocytes injected with GIRK2A wild type (WT) cRNA (5 ng) or Y353X cRNA (5 ng, 20 ng) with or without Gβ<sub>1</sub> (2 ng) and Gγ<sub>2</sub> (2 ng). \* points at non-specific bands.

**Figure 2.15**

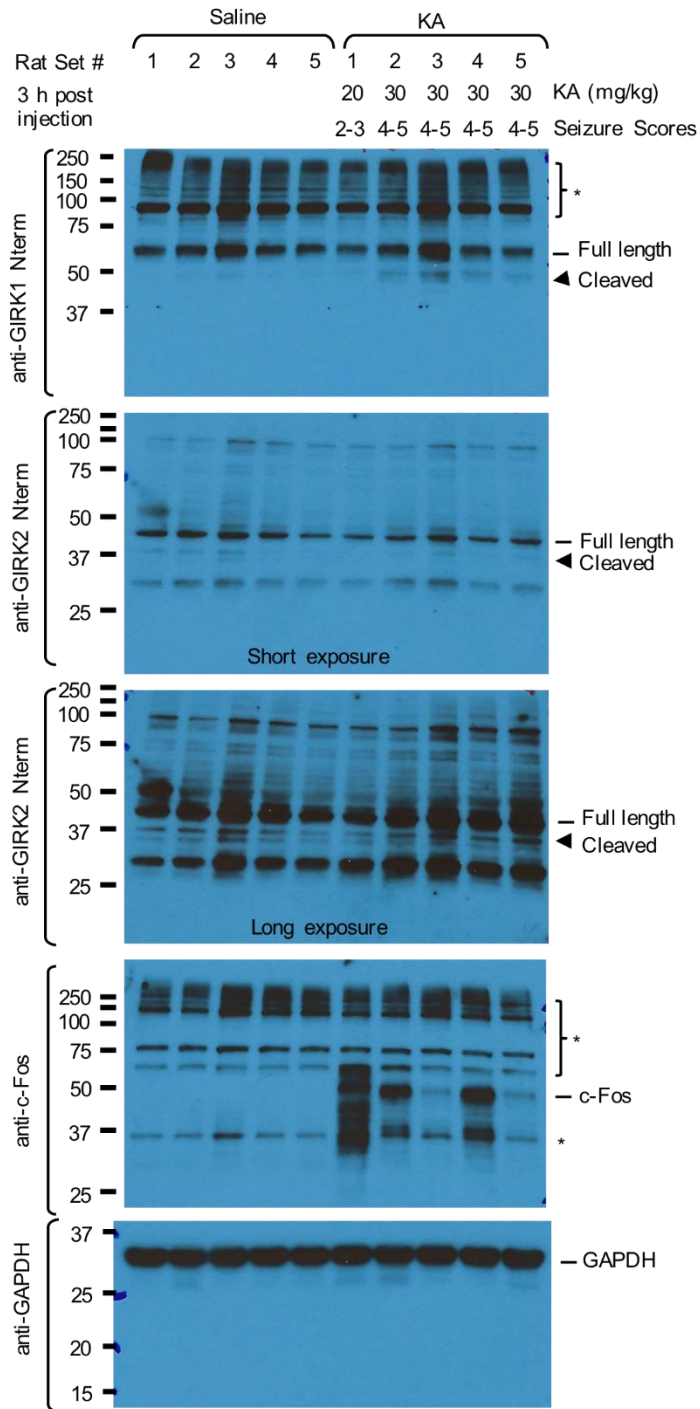


**Figure 2.15 Full-length immunoblots for cropped immunoblots shown in Figure 8D.** Immunoblotting of hippocampal homogenate from rats at 3 h after i.p. injection with kainate (15 and 30 mg/kg) or vehicle control (saline) using antibodies against GIRK1 and GIRK2 N-termini (described in Supplementary Fig. S2), c-FOS, and GAPDH (all from Cell Signaling). \* points at non-specific bands.

**Figure 2.16**

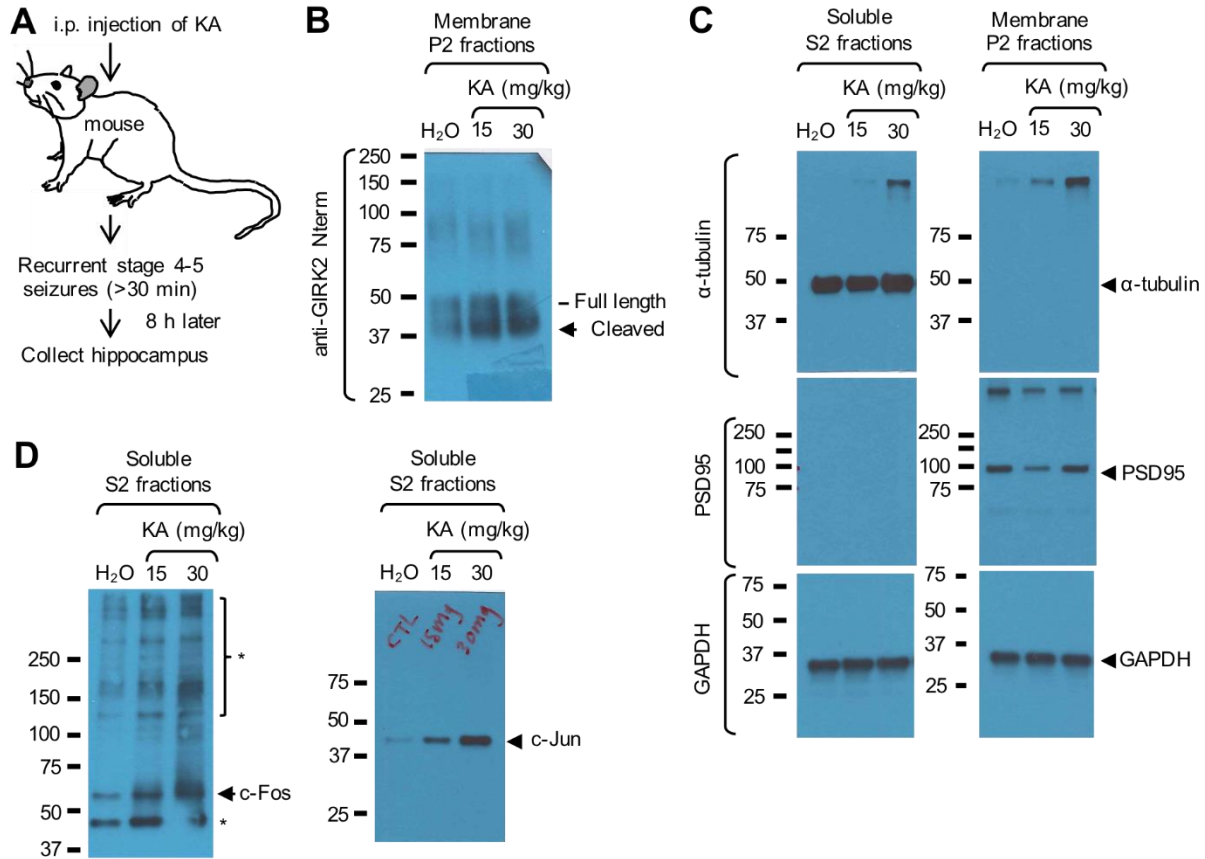


**Figure 2.16 Kainate-induced status epilepticus in mice induces C-terminal cleavage of GIRK2 in their hippocampi.** (A) Onset of behavioral hyperactivity within 20 min and Racine scale stage 3-4 seizures and 4-5 seizures in male mice was observed after i.p injection of kainate (15 and 30 mg/kg, respectively) but not vehicle control (saline). (B-D) Immunoblotting of hippocampal membrane and soluble fractions from male mice at 8 h after injection with kainate or vehicle control (H<sub>2</sub>O) using antibodies (all from Cell Signaling) against GIRK2 N-terminus (B), GAPDH (loading control) (C), c-Jun and c-Fos (positive control for seizures) (D). Immunoblot for PSD-95 and tubulin showed successful fractionations of soluble S2 and membrane P2 fractions. \* points at non-specific bands.

# CHAPTER 3: DOWNREGULATION OF ERK1/2 ACTIVITY AND AXONAL K<sub>v</sub>7.3 CHANNELS DURING HOMEOSTATIC SCALING OF INTRINSIC EXCITABILITY INDUCED BY PROLONGED BLOCKADE OF HIPPOCAMPAL NETWORK ACTIVITY <sup>3</sup>

## 3-1 Abstract

Voltage-gated K<sub>v</sub>7 potassium channels composed of K<sub>v</sub>7.2 and K<sub>v</sub>7.3 subunits are highly enriched at the axon initial segment (AIS) where they potently inhibit action potential firing. A reduction in their current expression has been observed in cultured hippocampal neurons during homeostatic scaling of action potential firing induced by prolonged activity blockade. Although homeostatic scaling of intrinsic excitability is mediated by reduced activation of brain-derived neurotrophic factor (BDNF) receptor TrkB, whether homeostatic scaling of intrinsic excitability involves a decrease in K<sub>v</sub>7 expression at the AIS via downregulation of TrkB signaling remains unknown. Here, we report that prolonged activity blockade in cultured hippocampal neurons decreases the activities of TrkB and its downstream extracellular signal-regulated kinase (ERK1/2). This concurrently occurs with significant reductions in the AIS levels of K<sub>v</sub>7.3 and ankyrin-G, which binds to and targets K<sub>v</sub>7.3 to the AIS. Similarly, prolonged pharmacological inhibition of ERK1/2 decreases not only *KCNQ3* and *BDNF* transcripts but also the density of K<sub>v</sub>7.3 and ankyrin-G at the AIS, implicating for the first time the downregulation of both ERK1/2 activity and the AIS expression of K<sub>v</sub>7.3 as a potential mechanism underlying homeostatic scaling of intrinsic excitability.

## 3-2 Introduction

Activity-dependent changes in synaptic strength and intrinsic excitability occur in many different types of neurons within the brain during development and throughout our lives. These changes in the brain can occur rapidly or over a prolonged period and could lead to extremely unstable, saturated neural networks. Homeostatic plasticity counteracts these destabilizing changes

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by adjusting their synaptic strength and intrinsic excitability within a physiological range (Turrigiano, 2012). For example, prolonged activity blockade using voltage-gated sodium channel blocker, tetrodotoxin (TTX) leads to homeostatic increase in action potential (AP) firing rate of hippocampal neurons in dissociated culture, slice culture, and *in vivo* (Aptowicz et al., 2004; Echegoyen et al., 2007; Lee et al., 2015). Homeostatic scaling of intrinsic excitability in cultured hippocampal neurons is associated with decreases in the transcripts and current densities of potassium ( $K^+$ ) channels critical for regulating intrinsic membrane properties including voltage-gated KCNQ/K<sub>v</sub>7 potassium channels (Lee et al., 2015).

Neuronal K<sub>v</sub>7 potassium channels are mainly composed of K<sub>v</sub>7.2 and K<sub>v</sub>7.3 (Delmas and Brown, 2005) which show overlapping expression in the hippocampus and cortex (Pan et al., 2006). They are active by membrane depolarization around -60 mV and produce slow activating and non-inactivating outward  $K^+$  current which potently suppresses repetitive and burst firing of APs (Brown and Passmore, 2009). In contrast, they are inhibited by membrane depletion of phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) upon activation of Gq/11-coupled receptors such as the M1 muscarinic acetylcholine receptor (Greene and Hoshi, 2017). K<sub>v</sub>7 channels also regulate AP threshold and contribute to resting membrane potential and resonance, intrinsic oscillations, medium and slow afterhyperpolarization currents, spike frequency adaptation, and suppression of temporal summation (Aiken et al., 1995; Yue and Yaari, 2004; Gu et al., 2005; Hu et al., 2007; Shah et al., 2008; Tzingounis and Nicoll, 2008; Hu et al., 2009). Subcellularly, surface expression of K<sub>v</sub>7 channels is higher in the axonal plasma membrane of hippocampal excitatory pyramidal and GABAergic inhibitory neurons compared to the somatodendritic surface with the greatest enrichment at the axon initial segment (AIS) (Chung et al., 2006; Pan et al., 2006). Their AIS localization is mediated by their interactions with ankyrin-G (Pan et al., 2006) and is critical for their ability to inhibit the repetitive firing of APs (Shah et al., 2008). However, whether a decrease in K<sub>v</sub>7 channel expression occurs at the AIS during homeostatic scaling of AP firing rate remains unknown.

Furthermore, the signaling pathway that mediates K<sub>v</sub>7 down-regulation during homeostatic scaling of intrinsic excitability is unclear. We have previously shown that homeostatic scaling of intrinsic excitability depends on a reduction in calcium ( $Ca^{2+}$ ) influx through N-methyl-D-aspartate-type glutamate receptors (NMDARs) (Lee et al., 2015). Interestingly, the application of brain-derived neurotrophic factor (BDNF) prevents, whereas the inhibition of its receptor TrkB

also produces homeostatic scaling of intrinsic excitability (Desai et al., 1999). There are multiple kinases downstream of NMDAR and TrkB involved in neuronal plasticity including MAPK, CaMKII, CaMKIV, and PKA (Fan et al., 2005; Lin et al., 2008; Rosenkranz et al., 2009). One potential signaling pathway downstream of NMDARs and TrkB involved in transcriptional regulation is extracellular signal-regulated kinase 1/2 (ERK1/2) (Thomas and Huganir, 2004; Kowianski et al., 2018). As one of the major components of the mitogen-activated protein kinase (MAPK) signaling pathway (Pearson et al., 2001), ERK1/2 contributes to the regulation of Hebbian synaptic plasticity including long-term potentiation (LTP) and plays a key role in learning and memory by regulating transcription of plasticity-related genes including *BDNF* (Adams and Sweatt, 2002; Thomas and Huganir, 2004). Importantly, ERK1/2 signaling is required for visual cortical plasticity during monocular deprivation (Di Cristo et al., 2001), which is a model of homeostatic plasticity of excitatory synaptic strength (Ranson et al., 2012; Zhou et al., 2017).

In this study, we investigated if homeostatic scaling of intrinsic excitability involves the downregulation of K<sub>v</sub>7 channels at the AIS via a reduction in ERK1/2 activity. We found that prolonged activity blockade in cultured hippocampal neurons decreased activities of TrkB and ERK1/2, *BDNF* and *KCNQ3* transcripts, and the AIS expression of K<sub>v</sub>7.3 and ankyrin-G. Prolonged pharmacological inhibition of ERK1/2 was sufficient to induce the same reductions, revealing ERK1/2 downregulation as a key signaling pathway that mediates homeostatic control of K<sub>v</sub>7 channels at the AIS.

### **3-3 Materials and Methods**

#### ***Materials***

Sprague-Dawley rat ordered from Charles River. Culture dishes were purchased from Thermo Fisher Scientific. Additional 3 cm plastic dish for imaging (#81156) were purchased from ibidi. neurobasal medium (21103-049) was purchased from gibco. MEM media (15-010-CV) was purchased from corning. B27 supplement was purchased from Gibco. U0126 and TTX were purchased from Tocris. 20 mM stock U0126 was made by dissolving in dimethyl sulfoxide (DMSO) which was purchased from Sigma. TTX stock was made at 10 mM with double distilled deionized water. Paraformaldehyde (PFA, 32% v/v in water) was purchase from Electron Microscopy Sciences. Ankyrin-G antimouse antibody was purchased from Neuromab. *KCNQ3*

antirabbit antibody was purchased from abcam. Alexa Fluor 488/594 conjugated goat/donkey anti-mouse/rabbit antibodies, and goat/donkey serum were purchased from Thermo Fisher Scientific. Western blotting reagents including Tris, sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), Triton X-100, sodium dodecyl sulfate (SDS), deoxycholate, Halt Phosphatase inhibitor cocktail (78428), Halt Protease inhibitors (87786), Tween-20, glycerol, bromophenol blue, and tris(2- carboxyethyl)phosphine (TCEP) were purchased from various sources including Sigma, Thermo Fisher Scientific, and Bio-Rad. Horseradish peroxidase-conjugated antibodies against mouse and rabbit IgG were ordered from Jackson ImmunoResearch Laboratory. Multi-electrode arrays (M64-GL1-30Pt200) were purchased from Axion Biosystem. RNeasy kit purchased from Qiagen.

### ***Experimental animals***

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign in accordance with the guidelines of the U.S. National Institute of Health (NIH). KCNQ3<sup>-/-</sup> knock-out mice and wild-type littermates have been previously described (Tzingounis and Nicoll, 2008) and their fresh frozen brain tissues were kind gifts from Dr. Anastassios V. Tzingounis (University of Connecticut).

### ***Hippocampal neuronal culture***

Primary dissociated hippocampal neuronal cultures were prepared from Sprague-Dawley rat embryos at E18 as previously described (Brewer et al., 1993). Neurons were plated on Poly L-lysine (PLL) (0.1 mg/mL)-coated cell culture dishes at  $2.5 \times 10^5$  cells/cm<sup>2</sup> for QPCR and immunoblot analysis. For immunostaining, neurons were plated on PLL (0.1 mg/mL)-coated 12 mm glass coverslip ( $1.0 \times 10^5$  cells per coverslip) or 3 cm plastic bottom dishes (#81156, ibidi,  $5 \times 10^5$  cells per dish) which have polymer bottoms designed for better imaging results. These neurons were maintained in a neurobasal medium supplemented with B27 extract, 200 mM L-glutamine, and 100 U/mL penicillin and streptomycin in a cell culture incubator (37 °C, 5% CO<sub>2</sub>/O<sub>2</sub>). Neurons were fed twice a week by removing half of the medium and adding fresh medium. Neurons at 10 days *in vitro* (DIV) were treated with vehicle-control (0.1% H<sub>2</sub>O) or TTX (1 μM) for 24, 36, or 48 hours (h) to induce prolonged activity blockade by adding 1 μM TTX to culture media. Prolonged

pharmacological inhibition of ERK1/2 was achieved by 48 hour treatment with 20  $\mu$ M U0126 or vehicle-control (0.1% DMSO). For all drug treatments, drugs were refreshed after 24.

**Multi-electrode array (MEA) recordings.** Primary dissociated rat hippocampal neurons ( $1.5 \times 10^5$  cells) were plated onto poly D-lysine (0.1 mg/mL, Sigma)-coated MEA electrode area containing 64 electrodes per plate (M64-GL1-30Pt200, Axion Biosystem) inside a 5 % CO<sub>2</sub>, 37 °C recording incubator. Neurons were fed twice a week by removing half of the medium and adding fresh medium. All drug treatments and MEA recordings were performed in media to maintain proper neuronal health. At 9 days in vitro (DIV), field potentials at each MEA electrode relative to a ground electrode were recorded with a sampling rate of 25 kHz using Axion Muse 64 channel system inside a MEA recording incubator (5% CO<sub>2</sub>, 37°C) for baseline recording for 30 min. After 30 min of baseline recording, the MEA was returned to the 5 % CO<sub>2</sub>, 37 °C, humidified growing incubator. Due to changes in network activity caused by physical movement of the MEA, only the last 15 min of each recording were used in data analyses. Following baseline recording TTX (1  $\mu$ M), U0126 (20  $\mu$ m), (0.1%) DMSO, or (0.1%) H<sub>2</sub>O control was added to the original medium in the MEA plate in bio-safety cabinet and then put back in the recording incubator for acute 30 minute recording. AxIS software (Axion Biosystems) was used for the extraction of spikes (i.e. action potentials) from the raw electrical signal obtained from the Axion Muse system. After filtering, a threshold of  $\pm 6$  standard deviations were independently set for each channel; activity exceeding this threshold was counted as a spike. The settings for burst detection were a minimum of 5 spikes with a maximum inter-spike interval of 0.1 s. Synchrony index was computed through AxIS software by taking the cross-correlation between two spike trains, removing the portions of the cross-correlogram that are contributed by the auto-correlations of each spike train, and reducing the distribution to a single metric. A value of 0 corresponds to no synchrony and a value of 1 corresponds to perfect synchrony. Raster plots were generated using NeuralMetricTool software.

## **QPCR**

Following drug treatment, total RNA was isolated from hippocampal neuronal culture using a RNeasy kit (Qiagen) and stored at -80°C. To synthesize cDNA, reverse transcription was performed using total RNA, random nanomers, dNTPs, M-MuLV reverse transcriptase, and RNase

inhibitor. The resulting cDNA was subjected to qPCR using the StepOnePlus real-time PCR system and SYBRGreenI with primers homologous to cDNAs corresponding to *BDNF*, *KCNQ3*, *KCNA1*, *KCNA4*, and *CaMK4* genes as well as glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) control gene as previously described (Lee et al., 2015). Following normalization to *GAPDH* cDNA levels, the relative quantification of the fold change for each treatment compared to control was determined.

### ***Biochemical fractionation of mouse cortices***

The brain tissues of a *KCNQ3*<sup>-/-</sup> knock-out mouse and a wild-type mouse were collected at 1.5 months of age and fast frozen. Cortices were dissected out and were biochemically fractionated in ice-cold homogenization buffer (solution A) containing (in mM): 320 sucrose, 1 NaHCO<sub>3</sub>, 1 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 0.4 HEPES (pH 7.4) and Halt protease inhibitors (Thermo Fisher Scientific). The resulting cell lysate was subject to centrifuging 800g for 10 min at 4°C to separate soluble (S1) supernatant from insoluble (P1) pellet. S1 was then centrifuged at 13,800g for 10 min at 4°C separating soluble proteins (S2) from the pellet (P2) which has been shown to contain an enriched of membrane-bound proteins (Jang et al., 2017). P2 was then resuspended in ice-cold (solution B) containing (in mM): 160 sucrose, 6 Tris-HCl, 0.5% Triton-X (pH 8.0) and Halt protease inhibitors. BCA assay (Pierce) was then used to determine optimal protein concentrations. The P2 fractions were mixed with 5X SDS sample buffer and heated at 75°C for 15 min. Samples were run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinyl difluoride (PVDF) membrane (Millipore), immunoblot analysis was performed.

### ***Immunoblot Analysis***

Following drug treatment, neuronal lysates were collected in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with Halt protease and phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) as previously described (Lee et al., 2015). Following SDS-PAGE electrophoresis and transfer to a polyvinylidene difluoride membrane (Millipore), immunoblot analysis was performed as previously described (Lee et al., 2015). In brief, the membrane was blocked for 1 h in blocking buffer (1% milk and 0.1% Tween-20 in TBS) and incubated overnight at 4°C with primary antibodies for K<sub>v</sub>7.3 (Alomone APC-051, 1:500), ERK1/2-pTyr<sup>202/204</sup>, ERK1/2, TrkA-

pTyr<sup>674/675</sup>/TrkB-pTyr<sup>706/707</sup>, TrkB, and GAPDH (Cell Signaling #9106, #4695, #4621, #4603, #2118, respectively, all 1:1000). After the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, 1:200) in blocking buffer for 1 h and washed, they were exposed to enhanced chemifluorescence substrate (ECL, Thermo Fisher Scientific). Luminescent signals were detected using an autoradiography film on a Konica SRX-101A film processor or Life Technologies iBright CL1000 (Thermo Fisher Scientific) imaging system and quantified by ImageJ software (NIH). The background-subtracted band intensity of the protein of interest was divided by that of GAPDH, and this ratio was normalized to the vehicle control.

### ***Immunocytochemistry***

Following drug treatment, neurons were washed once with artificial cerebral spinal fluid (ACSF) containing drugs and then immediately fixed for 10 min with 4% PFA and 4% sucrose in PBS. For K<sub>v</sub>7.3 immunostaining in a 3 cm plastic dish (#81156, ibidi), neurons were fixed for 3 min with 1% PFA and 4% sucrose in PBS. After washing with PBS, the neurons were blocked for 1 h in a blocking buffer containing 10% normal donkey serum (NDS) and 0.2% Triton X-100 in PBS. Neurons were then incubated overnight with antibodies for ankyrin-G (NeuroMab AB\_10673030, 1:500), K<sub>v</sub>7.3 (Alomone APC-051, 1:500), or MAP2 (Cell Signaling 4542, 1:500) in 3% NDS in PBS at 4°C. After the neurons were washed and incubated for 2 h with secondary antibodies conjugated to Alexa Fluor 488, 594, or 647 (Thermo Fisher Scientific A32766, A32754, A31571, 1:200), neurons were thoroughly washed with PBS and mounted in Fluoro-Gel (with Tris Buffer) antifade mounting medium (Electron Microscopy Sciences).

### ***Imaging and Image analysis***

High-resolution grayscale fluorescence images were acquired using a Zeiss Axio Observer inverted microscope with a Zeiss AxioCam 702 mono Camera using a 20x objective with appropriate excitation and emission filters. Each fluorescent wavelength was taken separately moving from the longest excitation wavelength to shortest. ZEN Blue 2.6 software was used to save images of 1920 x 1216 pixels as 16-bit CZI and TIFF files. To compare the fluorescence intensity of the neurons with different drug treatments, the images were acquired using the same exposure time within one experiment and each independent experiment was normalized to its own

control. Ankyrin-G-positive segment was defined as the enrichment of Ankyrin-G staining coming out from the cell body. We visual determination of the start of the AIS by eye. This was evident from a 200-400% increase in artificial unit intensity at this site. For example, the beginning of the AIS was determined in one sample to start when 1000 artificial units background suddenly jumps to 2000 artificial units within a few pixels measured. The end of the AIS was determined when the artificial unit intensity dropped back to the same as the beginning of the AIS which was also close to background intensity. These values were observed using ImageJ software (NIH) plot profile live view. We drew a line included 3-pixel on each side, down the center of the AIS from the start of the Ankyrin-G stained segment closest to the soma down the axon until stain reduced to background level. After drawing the ankyrin-G segment we would record the total intensity and length of Ankyrin-G. We would then change fluorescence channels to measure K<sub>v</sub>7 immunostaining using the same line to make sure we measure K<sub>v</sub>7 staining only within this Ankyrin-G-positive segment that defines the AIS. ImageJ was used to analyze the raw integrated fluorescence intensity and length of the ankyrin-G-positive segment at the AIS as well as K<sub>v</sub>7.3 intensity within the ankyrin-G-positive segment from the healthy neurons. The neurons with broken neurites or soma as well as regions containing fasciculation or overlapping neurites were excluded from the analyses. Background intensity was measured for each image as an area with no cells.

### ***Statistical analysis***

Data are reported as mean  $\pm$  SEM with individual data points. Statistical analysis was performed in either excel or OriginPro 2019. The Student's two-tailed *t*-test was used to compare between 2 groups. One-way ANOVA and posthoc Tukey were performed to compare groups  $\geq 3$ . The priori value (*p*) <0.05 was considered statistically significant.

## **3-4 Results**

### **Prolonged activity blockade decreases activities of TrkB and its downstream ERK1/2**

A prolonged reduction in BDNF activation of TrkB is previously shown to increase AP firing rate similar to that induced by prolonged activity blockade using TTX (Desai et al., 1999), suggesting that homeostatic scaling of intrinsic excitability may be mediated by a reduction in BDNF-TrkB signaling. To test if prolonged activity blockade decreases TrkB activation, we treated rat hippocampal neuronal culture with either vehicle control (0.1% H<sub>2</sub>O) or TTX (1  $\mu$ M)

for 48 h at DIV 10–12. Multi-electrode array recording of hippocampal neuronal network confirms the activity blockade by TTX application (Figure 1A-C), interestingly U0126 treatment also led to a decrease in activity similar to that found with TTX (Figure 1D-F). 48 h TTX treatment was previously shown to induce homeostatic scaling of AP firing rate in cultured hippocampal neurons (Lee et al., 2015). Quantitative immunoblot analysis with phosphorylation site-specific antibodies was performed to examine autophosphorylation of TrkB at Tyr<sup>706/707</sup>, which corresponds to TrkB activation upon BDNF binding and receptor dimerization (Huang and Reichardt, 2003). We found that 48 h TTX application significantly reduced the level of phosphorylated TrkA and TrkB at Tyr<sup>674/675</sup> and Tyr<sup>706/707</sup>, respectively, by  $49.9 \pm 6.5$  % compared to control treatment ( $p < 0.01$ ), with no change in the total TrkB expression (Figure 1G-H). Since TrkB and TrkC are the primary Trk receptors present in the hippocampus (Muragaki et al., 1995), the observed changes most likely represent a reduction in Tyr<sup>706/707</sup>-phosphorylation of TrkB indicative of decreased TrkB activity.

To investigate whether prolonged activity blockade decreases ERK1/2 activity downstream of TrkB receptor (Pearson et al., 2001), we treated hippocampal neuronal culture with TTX (1  $\mu$ M) for 24-48 h and performed immunoblot analysis for phosphorylation of ERK1 at Thr<sup>202</sup>/Tyr<sup>204</sup> and ERK2 at Thr<sup>185</sup>/Tyr<sup>187</sup> (Figure 1I-J), which leads to activation of ERK1/2 (Robbins et al., 1993; Zhou and Zhang, 2002). We found that TTX treatment for 24 h and 36 h significantly decreased the levels of phosphorylated ERK1/2 by  $70.5 \pm 5.3$  % ( $p < 0.01$ ) and  $55 \pm 3.5$  % ( $p < 0.01$ ), respectively, compared to vehicle treatment (Figure 1I-J). However, 48 h application of TTX did not affect the level of phosphorylated ERK1/2, and the total ERK1/2 level was unaffected by TTX treatment (Figure 1I-J). These findings suggest that prolonged activity blockade leads to a transient decrease in ERK1/2 activity.

### **Prolonged ERK1/2 inhibition decreases mRNA and protein expression of K<sub>v</sub>7.3.**

We have previously shown that prolonged activity blockade with 48 h TTX treatment leads to homeostatic scaling of excitability and concurrently down-regulates the expression of multiple K<sup>+</sup> channel genes including K<sub>v</sub>1 channels (*KCNA1*, *KCNA4*), K<sub>v</sub>7 channels (*KCNQ3*), and activated small conductance Ca<sup>2+</sup> activated SK channels (*KCNN1*) (Lee et al., 2015). Since TTX application for 48 h decreased TrkB and ERK1/2 activity (Figure 1G-J), we next tested if prolonged inhibition of ERK1/2 activity results in a similar decrease in *KCNQ3* transcript.

Hippocampal neuronal cultures were treated with vehicle control (0.1% H<sub>2</sub>O or 0.1% DMSO), TTX (1  $\mu$ M), or a MEK1/2 specific inhibitor U0126 (20  $\mu$ M) for 48 h. MEK1/2 specifically phosphorylates and activates ERK1/2 therefore MEK1/2 inhibition by U0126 leads to specific inhibition of ERK1/2 (Favata et al., 1998), indicated by a significant reduction in the levels of phosphorylated ERK1/2 upon 48 h U0126 application (Figure 2A-B).

Real-time qPCR analysis revealed that U0126 treatment for 48 h decreased the transcripts of *BDNF* by  $42.6 \pm 3.8$  %, *KCNQ3* by  $60.1 \pm 12.2$  %, *KCNA1* by  $53.9 \pm 5.0$ %, and *KCNA4* by  $43.8 \pm 6.7$  % compared to vehicle treatment ( $p < 0.01$ ) (Figure 2C). The extent of U0126-induced reductions in the expression of *BDNF* and axonal K<sup>+</sup> channel genes was comparable to that of TTX-induced reductions (Lee et al., 2015) (Figure 2C). In contrast, *CAMK4* expression was reduced only by 48 h application of TTX but not U0126 ( $p > 0.05$ ) (Figure 2C). This suggest that *CAMK4* expression is not being affected by ERK inhibition in contrast to prolonged activity blockade. *CAMK4* and has been implicated in regulation of homeostatic scaling (Joseph and Turrigiano, 2017). However, ERK inhibition does not appear to be working through regulation of *CAMK4* expression. Immunoblot analyses with the verified antibodies for K<sub>v</sub>7.3 (Figure 2D) showed that 48 h U0126 treatment decreased K<sub>v</sub>7.3 expression by  $15.6 \pm 3.3$  % compared to DMSO treatment ( $p < 0.05$ ) (Figure 2E-F) to a similar extent as 48 h TTX treatment (Lee et al., 2015). These findings indicate that prolonged ERK1/2 inhibition reduces K<sub>v</sub>7.3 mRNA and protein expression.

### **Prolonged blockade of neuronal activity or ERK1/2 decreases Ankyrin-G and K<sub>v</sub>7.3 expression at the axon initial segment**

K<sub>v</sub>7 channels composed of K<sub>v</sub>7.2 and K<sub>v</sub>7.3 are highly enriched at the AIS through the interaction between their C-termini and ankyrin-G (Chung et al., 2006; Pan et al., 2006), which is a scaffolding protein at the AIS and maintains neuronal polarity (Pan et al., 2006; Leterrier, 2018). Therefore, we next examined if prolonged activity blockade reduces K<sub>v</sub>7.3 expression at the AIS by immunostaining cultured hippocampal neurons after 48 h treatment with either vehicle control (0.1% H<sub>2</sub>O) or TTX (1  $\mu$ M). Immunostaining for ankyrin-G revealed the AIS, which does not overlap with the somatodendritic marker microtubule-associated protein 2 (MAP2) (Dehmelt and Halpain, 2005), demonstrating neuronal polarity and health regardless of the treatments (Figure

3A). However, 48 h TTX application induced a small but statistically significant reduction in K<sub>v</sub>7.3 immunodensity at the ankyrin-G positive AIS by  $12.7 \pm 3.5\%$  compared to vehicle treatment ( $p < 0.05$ ) (Figure 3B, D). Ankyrin-G immunodensity was also reduced by  $10.2 \pm 2.9\%$  ( $p < 0.05$ ) in TTX-treated neurons compared to H<sub>2</sub>O-treated neurons without altering the Ankyrin-G-positive AIS length (Figure 3B-C). These findings indicate that prolonged activity blockade leads to a decrease in both K<sub>v</sub>7.3 and ankyrin-G expression at the AIS.

We next tested if pharmacological ERK1/2 inhibition alters K<sub>v</sub>7.3 and ankyrin-G at the AIS. Cultured hippocampal neurons were treated for 48 h with either vehicle control (0.1% DMSO) or U0126 (20  $\mu$ M). Immunostaining for ankyrin-G and MAP2 showed normal neuronal polarity and health regardless of the treatments (Figure 4A). However, U0126 application decreased ankyrin-G immunodensity by  $21.2 \pm 1.9\%$  and ankyrin-G-positive AIS length by  $8.0\% \pm 5.5$  compared to DMSO control ( $p < 0.01$ ) (Figure 4B-C). K<sub>v</sub>7.3 immunodensity was also decreased by  $16.8\% \pm 1.8$  in U0126-treated neurons ( $p < 0.05$ ) (Figure 4D), indicating that prolonged ERK1/2 inhibition leads to reductions in not only ankyrin-G and K<sub>v</sub>7.3 expression at the AIS, but also the AIS length.

### 3-5 Discussion

Functional stability of the neuronal network is maintained by homeostatic plasticity. Homeostatic scaling of intrinsic excitability induced by prolonged activity blockade is shown to involve a reduction in K<sub>v</sub>7.3 transcript and K<sub>v</sub>7 current (Lee et al., 2015). However, the molecular mechanism underlying the homeostatic regulation of K<sub>v</sub>7 channels remains largely unknown. In this study, we provide evidence that prolonged activity blockade decreases the activation of TrkB and its downstream ERK1/2 (Figure 1), and that prolonged pharmacological inhibition of ERK1/2 is sufficient to decrease K<sub>v</sub>7.3 transcripts and expression at the AIS (Figures 2-3), the key site at which K<sub>v</sub>7 channels suppress AP firing in hippocampal neurons (Shah et al., 2008). Our study has therefore revealed downregulation of K<sub>v</sub>7.3 density at the AIS via a reduction in ERK1/2 activity, offering new mechanistic insights into homeostatic control of intrinsic excitability in response to prolonged activity blockade.

### **Decrease in ERK1/2 activity as a mechanism for homeostatic downregulation of K<sub>v</sub>7.3.**

ERK1/2 can activate or stabilize gene transcription (Whitmarsh, 2007) by directly phosphorylating transcription factors (Hollenhorst, 2012) or influencing ERK1/2 substrates by altering the stability of these proteins (Whitmarsh and Davis, 1996). In long-term memory formation and persistence, ERK1/2 is shown to link multiple cell surface receptors such as NMDAR and TrkB with transcriptional regulation of key synaptic plasticity-associated proteins including Arc/Arg3.1 and BDNF (Medina and Viola, 2018). Hippocampus-dependent learning and memory not only increases but also depends on ERK1/2 activation (Atkins et al., 1998; Blum et al., 1999; Walz et al., 1999; Schafe et al., 2000; Kelleher et al., 2004). ERK1/2 is also necessary for induction of NMDAR-dependent LTP at the CA3-CA1 synapse of the hippocampus (English and Sweatt, 1997; Rosenblum et al., 2002). Consistently, NMDAR stimulation increases ERK1/2 activation and dendritic spine density, whereas its inhibition prevents new spine formation (Wu et al., 2001) and BDNF-induced increase in spine density in hippocampal neurons (Alonso et al., 2004).

Importantly, ERK1/2 is shown to facilitate protein synthesis required for homeostatic scaling of excitatory synaptic strength (Rutherford et al., 1998; Wang et al., 2013a), which is mediated by reduced BDNF signaling in excitatory pyramidal neurons (Rutherford et al., 1998; Turrigiano, 2008). However, much less is known for the role of ERK1/2 in homeostatic scaling of intrinsic excitability. We show that prolonged activity blockade decreased TrkB and ERK1/2 activation (Figure 1) and *BDNF* transcripts (Figure 2) (Lee et al., 2015). Since homeostatic scaling of intrinsic excitability also involves reduced BDNF-TrkB signaling (Desai et al., 1999) and reduced NMDAR activity (Lee et al., 2015), our findings suggest that reduction in ERK1/2 activity downstream of TrkB and NMDAR may mediate homeostatic scaling of intrinsic excitability.

### **Significance of ERK1/2-dependent homeostatic downregulation of K<sub>v</sub>7.3 at the AIS.**

The computational modeling work has shown that activity-dependent regulation of ion channel transcripts can serve as one mechanism for neuronal homeostasis (O'Leary et al., 2013). Indeed, one way to express homeostatic scaling of intrinsic excitability is to down-regulate

transcripts of multiple K<sup>+</sup> channels including axonally localized K<sub>v</sub>1 and K<sub>v</sub>7 channels (Lee et al., 2015). The axonal expression of K<sub>v</sub>1.1 was recently reported to decrease in hippocampal CA3 pyramidal neurons following activity blockade in organotypic hippocampal slices (Zbili et al., 2021). In this study, we found that both prolonged activity blockade and prolonged pharmacological inhibition of ERK1/2 decreased gene expression of axonal potassium channel subunits including K<sub>v</sub>1.1, K<sub>v</sub>1.4, and K<sub>v</sub>7.3 (Figures 2C). This is in line with a previous report showing that conditional deletion of MEK1/2, the kinase directly upstream of ERK1/2, results in decreased expression of many ion channels and neurotransmitter receptors that regulate neuronal excitability (Xing et al., 2016). Importantly, K<sub>v</sub>7.3 protein expression was reduced by prolonged activity blockade (Lee et al., 2015) and prolonged ERK1/2 inhibition (Figure 2).

Reduction of K<sub>v</sub>7.3 density at the AIS upon prolonged activity blockade or ERK1/2 inhibition (Figure 3) is critically important due to several reasons. First, the AIS is the key site at which K<sub>v</sub>7.2/K<sub>v</sub>7.3 heterotetrameric channels suppress AP firing in hippocampal neurons (Shah et al., 2008) and chronic pharmacological inhibition of K<sub>v</sub>7 channels themselves can also induce a homeostatic regulation of the firing rate (Lezmy et al., 2017). Second, compared to K<sub>v</sub>7.2, K<sub>v</sub>7.3 has a stronger interaction with ankyrin-G (Xu and Cooper, 2015), which targets and concentrates K<sub>v</sub>7.2/K<sub>v</sub>7.3 channels at the AIS (Pan et al., 2006), whereas multiple epilepsy mutations decrease their enrichment at the AIS (Chung et al., 2006; Cavaretta et al., 2014; Kim et al., 2018; Zhang et al., 2020). Third, K<sub>v</sub>7.3 has a higher binding affinity to PIP<sub>2</sub> than K<sub>v</sub>7.2, allowing for a greater maximal channel open probability and resistance to PIP<sub>2</sub> depletion (Li et al., 2005). This is important since K<sub>v</sub>7.2/ K<sub>v</sub>7.3 channels conduct significantly larger currents than homomeric K<sub>v</sub>7.2 channels (Schwake et al., 2000), and homomeric K<sub>v</sub>7.3 channels are non-functional due to an ER retention motif (Nakajo and Kubo, 2008). Therefore, reduction in K<sub>v</sub>7.3 expression would decrease not only the AIS localization of K<sub>v</sub>7.2/K<sub>v</sub>7.3 channels but also their current, thereby hindering their ability to suppress AP firing and leading to increased AP firing.

### **Mechanism underlying homeostatic downregulation of K<sub>v</sub>7.3 at the AIS.**

We found that prolonged activity blockade and prolonged ERK inhibition decreased the AIS expression of not only K<sub>v</sub>7.3 but also ankyrin-G, whereas only prolonged ERK inhibition decreased the length of ankyrin-G-positive AIS (Figure 3). Our findings are different from the

reported effects on the AIS of an avian brainstem auditory neuron upon prolonged auditory deprivation (Kuba et al., 2010; Kuba et al., 2015). Prolonged 7-day deprivation of auditory input increases the AIS length,  $\text{Na}_v$  current, neuronal excitability (Kuba et al., 2010), and the AIS expression of  $\text{K}_v7.2$  while decreasing that of  $\text{K}_v1.1$  (Kuba et al., 2015). We speculate that different experimental conditions (*in vivo* sensory deprivation versus pharmacological activity blockade in neuronal culture) and different neurons (auditory vs. hippocampal) could contribute to the differences in activity-dependent regulation of the AIS length, its starting location, and the AIS density of ion channels and ankyrin-G (Yamada and Kuba, 2016). For example, different plating densities were found to regulate AIS position in cultured hippocampal neurons by altering the extent of BDNF activation (Guo et al., 2017).

How prolonged activity blockade or ERK1/2 inhibition decreases the AIS expression of  $\text{K}_v7.3$  and ankyrin-G is unclear. Previous reports state that the strength of ankyrin-G binding is the greatest for  $\text{Na}_v1.2$  followed by  $\text{K}_v7.3$  and the weakest for  $\text{K}_v7.2$  (Xu and Cooper, 2015). Therefore, decreases in  $\text{K}_v7.3$  mRNA and protein levels may reduce ankyrin-G binding to both  $\text{K}_v7.3$  and  $\text{K}_v7.2$ , but may retain its binding to  $\text{Na}_v1.2$ , thereby contributing to the overall scaling of intrinsic excitability upon prolonged activity blockade or ERK1/2 inhibition. It is also possible that reduced ERK1/2 activity may also regulate targeting of  $\text{K}_v7.2/\text{K}_v7.3$  channels to the AIS by indirectly influencing phosphorylation of ankyrin-G critical for its clustering at the AIS via recruitment of  $\beta 4$ -Spectrin (Yang et al., 2019) or regulating phosphorylation of  $\text{K}_v7.2$  and  $\text{K}_v7.3$  by casein kinase 2 which facilitates ankyrin-G binding (Xu and Cooper, 2015). The mechanism underlying the downregulation of  $\text{K}_v7.3$  and ankyrin-G at the AIS during homeostatic scaling of intrinsic excitability warrants future studies.

### **3-6 Acknowledgements**

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## **Contribution to the Field Statement**

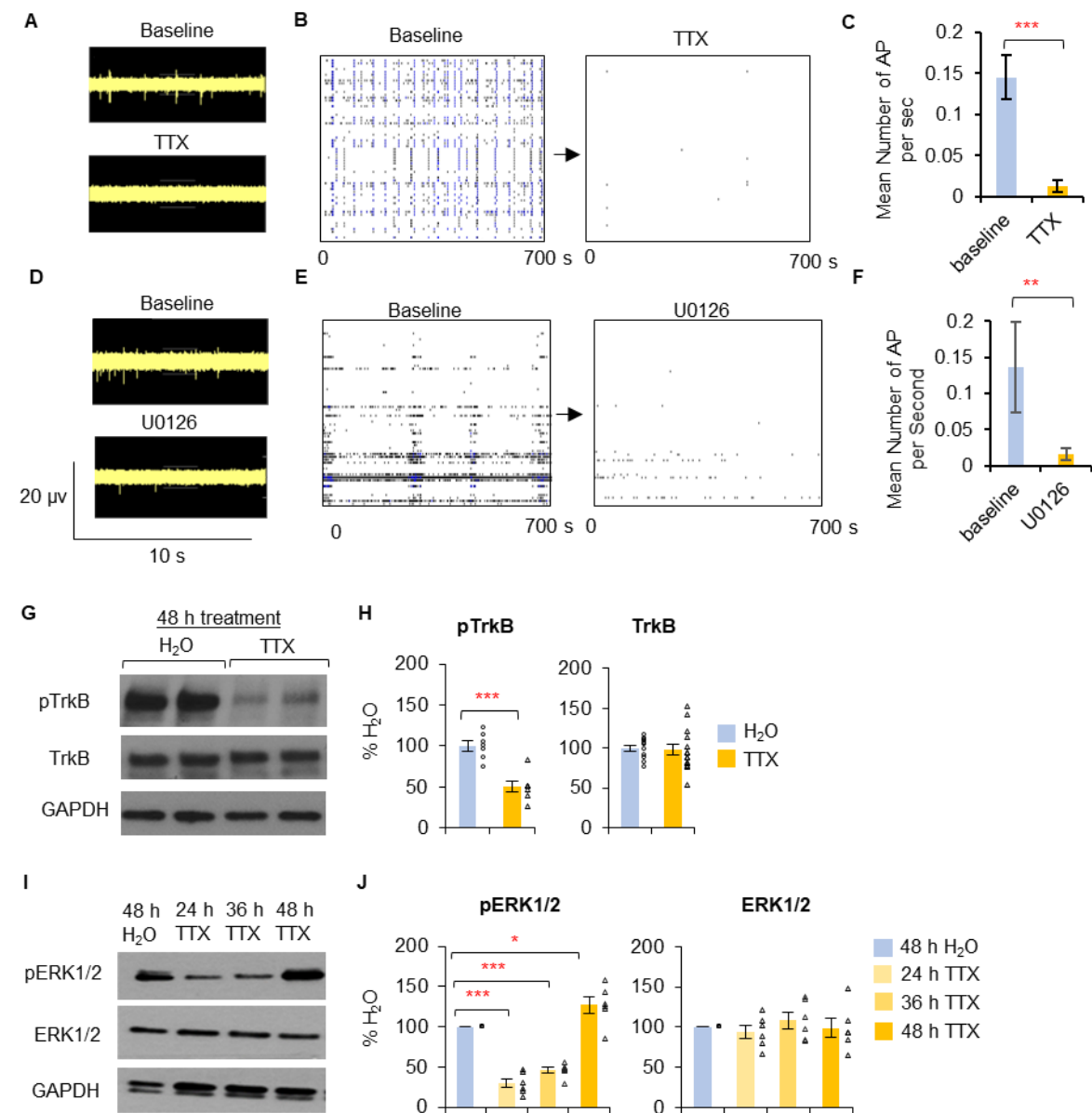
Functional stability of the neuronal network is maintained by homeostatic plasticity. Homeostatic scaling of intrinsic excitability induced by prolonged activity blockade involves a reduction in brain-derived neurotrophic factor (BDNF) receptor TrkB and currents through voltage-gated K<sub>v</sub>7 potassium channels which are critical negative regulators of neuronal excitability. However, the molecular mechanism underlying homeostatic regulation of K<sub>v</sub>7 channels remains largely unknown. This study presents a novel discovery that prolonged activity blockade of cultured hippocampal neurons decreases the activities of TrkB and its downstream kinase ERK1/2 involved in transcriptional regulation, and concurrently, reduces K<sub>v</sub>7.3 transcripts and protein expression at the axon initial segment (AIS), the key site for action potential generation in hippocampal neurons. This study further shows that prolonged pharmacological inhibition of ERK1/2 is sufficient to decrease K<sub>v</sub>7.3 transcripts and expression at the AIS, suggesting the downregulation of ERK1/2 activity and the AIS expression of K<sub>v</sub>7.3 as a potential mechanism underlying homeostatic scaling of intrinsic excitability.

## **Conflict of Interest**

None of the authors have any conflict of interest to disclose.

3-7 Figures

Figure 3.1



## Figure 3.1 continued

**Figure 3.1 Prolonged activity blockade decreases activities of TrkB and its downstream ERK1/2.** (A-C) AxIS software (Axion Biosystems) was used for the extraction of spikes (i.e. action potentials) from the raw electrical signal obtained from the Axion Muse system. Adaptive threshold crossing method was used for single spike detection settings to determine spike activity from noise. Inter-spike interval (ISI) threshold algorithm was used for bursting detection settings with maximum ISI of 100 ms and minimum 5 spikes per single burst. MEA recordings were recorded at baseline DIV 9 and 15 min following the addition of (A-C) TTX (1  $\mu$ M) or (D-F) U0126 (20  $\mu$ M) to media. (A and D) Sample traces. (B and E) Rosta plot of spikes. (C and F) Quantification of the mean number of APs per sec. Recordings consisted of 30 min. The last 15 min were analyzed following baseline (before drug treatment) and after drug application. Data shown represent the mean  $\pm$  SEM from 64 electrodes. The Student's t-test was used (\*\*\*p < 0.005). (G-H) Cultured hippocampal neurons were treated for 48 h with vehicle control (0.1% H<sub>2</sub>O) and TTX (1  $\mu$ M) at DIV 11-12. Immunoblot analysis was performed with antibody for pTrkB (pTrkA<sup>Tyr674/675</sup> / pTrkB<sup>Tyr706/707</sup>), TrkB, and GAPDH. (G) Representative immunoblots. (H) Quantification of pTrkB and total TrkB immunodensities. The immunodensity ratios (pTrkB/GAPDH and TrkB/GAPDH) were normalized to CTL (pTrkB: n = 7 per treatment, TrkB: n = 11 for H<sub>2</sub>O, n = 11 for TTX). The Student's t-test was used (\*\*\*p < 0.005). (I-J) Cultured hippocampal neurons were treated for 24 h, 36 h, and 48 h with vehicle control (0.1% H<sub>2</sub>O) or 1  $\mu$ M TTX at DIV 11-12. Immunoblot analysis was performed with antibodies for pERK1/2 (pERK1<sup>Thr202/Tyr204</sup> / pERK2<sup>Thr185/Tyr187</sup>), ERK1/2, and GAPDH (n = 6 per treatment). (I) Representative immunoblots. (J) Quantification of pERK1/2 and total ERK1/2 immunodensities. The immunodensity ratios (pERK/GAPDH and ERK/GAPDH) were normalized to CTL (n = 6 per treatment). One-way ANOVA and posthoc Tukey were performed (\*p < 0.05, \*\*\*p < 0.005). Data shown represent the mean  $\pm$  SEM with individual data points.

**Figure 3.2**

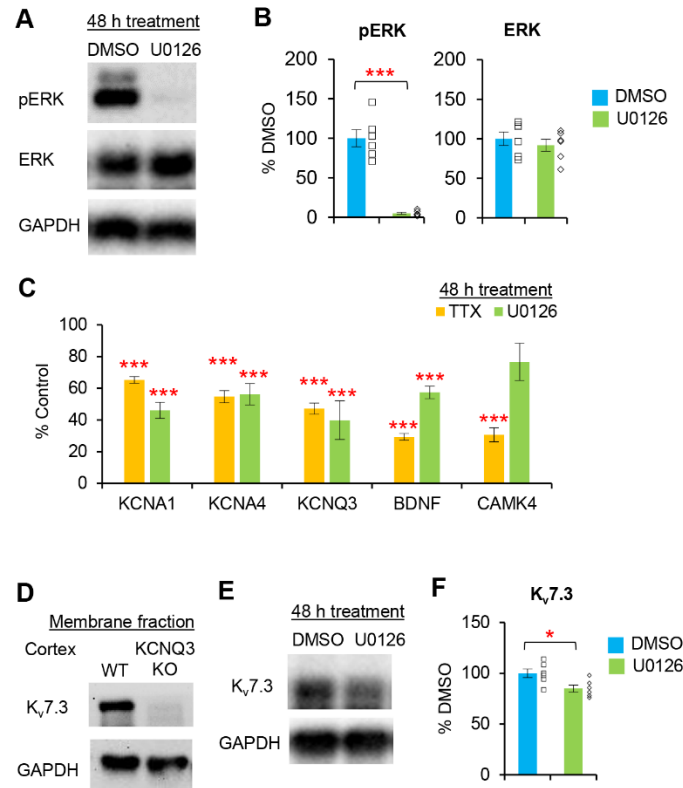


**Figure 3.2 Prolonged pharmacological inhibition of ERK1/2 decreases mRNA and protein expression of Kv7.3.** (A-B) Cultured hippocampal neurons were treated for 48 h with vehicle control (0.1% DMSO) or 20  $\mu$ M U0126 at DIV11-12. Immunoblot analysis was performed with antibodies for pERK1/2 (pERK1<sup>Thr202/Tyr204</sup> / pERK2<sup>Thr185/Tyr187</sup>), ERK1/2, and GAPDH (n = 6 per treatment). We normalize to GAPDH and then normalized to H<sub>2</sub>O control. (A) Representative immunoblots. (B) Quantification of pERK1/2 and total ERK1/2 immunodensities. (C) Cultured hippocampal neurons were treated for 48 h with vehicle control (0.1% DMSO or 0.1% H<sub>2</sub>O), 1  $\mu$ M TTX, and 20  $\mu$ M U0126 at DIV 11-12. Quantitative PCR analysis was performed using the cDNA which was prepared from 2  $\mu$ g of total RNA isolated from cultured hippocampal neurons and the validated primers for *Kcna1*, *Kcna4*, *Kcnq3*, *Bdnf*, *Camk4*, and *Gapdh* (n=5 per treatment). Data were analyzed using the comparative threshold cycle (Ct) method and *Gapdh* control gene. One-way Anova with Tukey post hoc was used \*\*\*(p < 0.005). (D) Immunoblot analysis of hippocampal tissues of Kv7.3 knock-out (cKO) mice and wild-type (WT) mice to verify the specificity of anti-Kv7.3 antibody. Hippocampal tissues were fractionated into membrane (P2) and soluble (S2) lysate fractions, and the membrane fractions were then subjected to immunoblotting with anti-Kv7.3 and anti-GAPDH antibodies. (E-F) Hippocampal cultured neurons were treated for 48 h with vehicle control (0.1% DMSO) or 20  $\mu$ M U0126 at DIV 11-12. Immunoblot analysis was performed with antibodies for Kv7 and GAPDH (n = 6 per treatment). (E) Representative Immunoblot. (F) Quantification of Kv7.3 immunodensities. Data shown represent the mean  $\pm$  SEM with individual data points. The Student's t-test was used (\*p < 0.05).

**Figure 3.3**

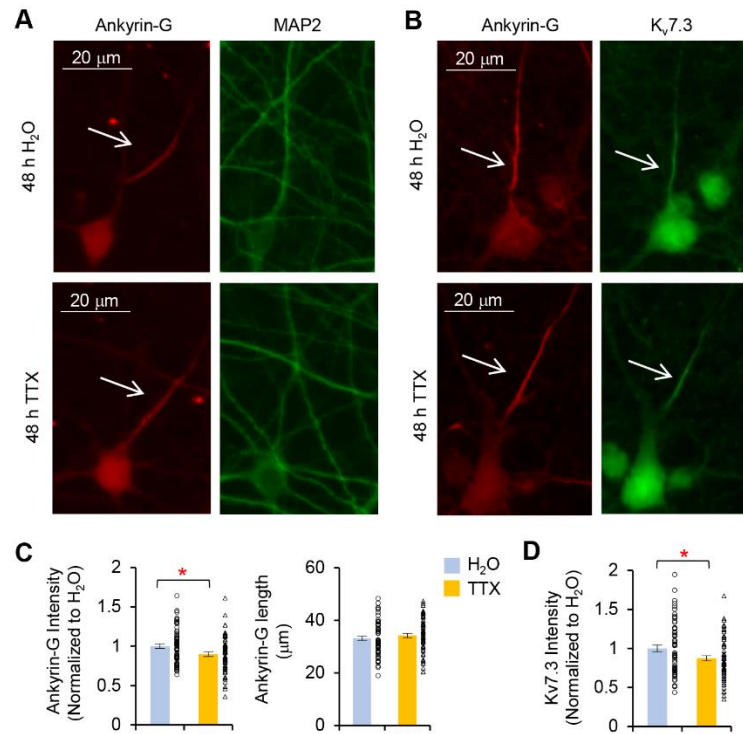


**Figure 3.3 Prolonged activity blockade decreases Kv7.3 and ankyrin-G expression at the AIS.**

Cultured hippocampal neurons were treated for 48 h with vehicle control (0.1% H<sub>2</sub>O) or 1  $\mu$ M TTX at DIV 11-12, fixed, permeabilized, and subjected to immunocytochemistry with antibodies for the AIS marker ankyrin-G, somatodendritic marker MAP2, and Kv7.3. **(A-B)** Representative fluorescent images of immunostained ankyrin-G and MAP2 (A) or Kv7.3 (B). **(C)** Quantification of the background-subtracted raw integrated fluorescent intensity of ankyrin-G at the AIS, which was normalized to vehicle control and the length of the ankyrin-G-positive AIS segment. **(D)** Quantification of the raw integrated fluorescent intensity of Kv7.3 within the ankyrin-G-positive AIS segment. Data shown represent the mean  $\pm$  SEM with individual data points (H<sub>2</sub>O = 62 neurons from 46 images, TTX = 61 neurons from 43 images). Images were collected from 2 independent experiments). The Student's t-test was used (\*p < 0.05). Scale bar = 20  $\mu$ m.

**Figure 3.4**

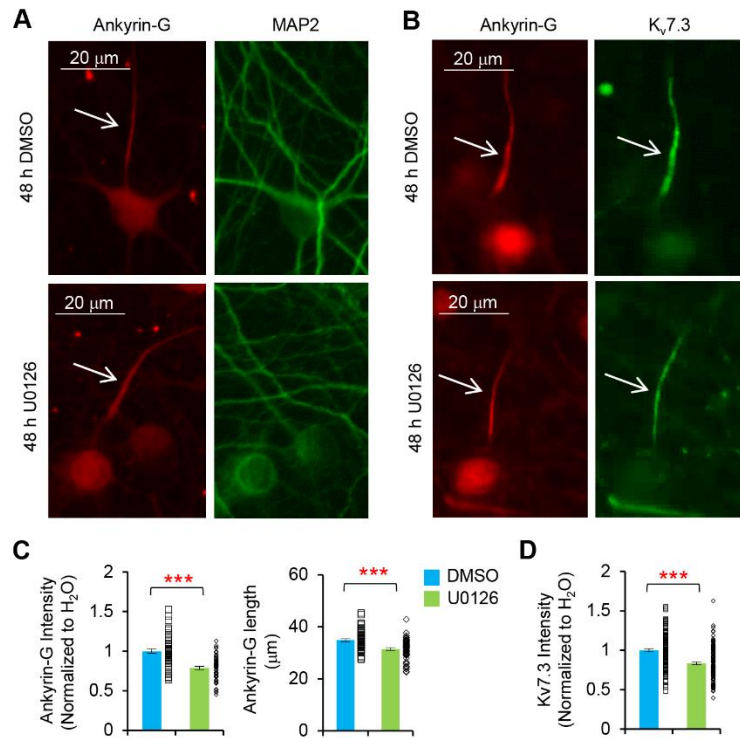


**Figure 3.4 Prolonged pharmacological inhibition of ERK1/2 decreases Kv7.3 and ankyrin-G expression at the AIS and the AIS length.** Cultured hippocampal cultured neurons were treated for 48 h with vehicle control (0.1% DMSO) or 20 μM U0126 at DIV 11-12, fixed, permeabilized, and subjected to immunocytochemistry with antibodies for the AIS marker ankyrin-G, and somatodendritic marker MAP2, or Kv7.3. **(A-B)** Representative fluorescent images of immunostained ankyrin-G and MAP2 (A) or Kv7.3 (B). **(C)** Quantification of the raw integrated fluorescent intensity of ankyrin-G at the AIS, which was normalized to vehicle control, and the length of the ankyrin-G-positive AIS segment. **(D)** Quantification of the background-subtracted raw integrated fluorescent intensity of Kv7.3 within the ankyrin-G-positive AIS segment. Data shown represent the mean ± SEM with individual data points (DMSO = 132 neurons from 69 images, U0126 = 126 neurons from 53 images). Images were collected from 2 independent experiments. The Student's t-test was used (\*\*\*p < 0.005). Scale bar = 20 μm.

## CHAPTER 4: CONCLUSION

GIRK and K<sub>v</sub>7 channels regulate neuronal activity, while their dysregulation can result in hyperexcitability and neurodegeneration (Signorini et al., 1997; Watanabe et al., 2000). How does homeostatic plasticity and epilepsy regulate K<sub>v</sub>7 and GIRK channels?

In chapter 2, we examined the effect of prolonged high-frequency epileptiform activity on GIRK channels in cultured hippocampal neurons. GIRK1 and GIRK2 are the major subunits that mediate membrane hyperpolarization and synaptic inhibition (Liao et al., 1996). It is known that recurrent high-frequency epileptic seizures cause progressive hippocampal sclerosis, and this is associated with caspase-3 activation (Henshall, 2007; Thom, 2014). The goal was to investigate how this prolonged hyperactivity regulated GIRK1 and GIRK2 channels. In the process, we identified caspase-3 substrates that contribute to seizure-induced hippocampal atrophy. We found that  $\geq 30$  minutes of hyperexcitability lead to caspase-dependent cleavage of GIRK1 (<sup>387</sup>ECLD<sup>390</sup>) and GIRK2 (<sup>349</sup>YEVD<sup>352</sup>). The YEVD motif is highly conserved in GIRK2-4, and located within their C-terminal sites which include their G $\beta$  $\alpha$  protein binding site and is necessary to mediate GIRK channel activation. Indeed, the cleaved GIRK2 displays reduced binding to G $\beta$  $\alpha$  and cannot coassemble with GIRK1. Caspase cleavage of GIRK2 at the site (<sup>349</sup>YEVD<sup>352</sup>) also results in the loss of an ER export motif. When truncated GIRK2 with (<sup>349</sup>YEVD<sup>352</sup>) motif are expressed in HEK cells and oocytes this results in a loss of surface and current expression of GIRK2 homotetrameric channels. Kainate-induced status epilepticus causes GIRK1 and GIRK2 cleavage in hippocampal neurons *in vivo*. This demonstrates the role of caspase-3 mediated down-regulation of GIRK channels in hippocampal neuronal injury during prolonged epileptic seizures. Our findings show how GIRK1 and GIRK2 are regulated by high-frequency epileptiform activity and suggest the role of caspase-3 in mediated down-regulation of GIRK channel function and expression in hippocampal neurons following an injury during prolonged epileptic seizures.

In chapter 3, we examined the effects of prolonged activity blockade and ERK1/2 inhibition on K<sub>v</sub>7 channels in cultured hippocampal neurons. Voltage-gated K<sub>v</sub>7 potassium channels composed of K<sub>v</sub>7.2 and K<sub>v</sub>7.3 subunits are highly enriched at the AIS where they potentially inhibit action potential firing (Delmas and Brown, 2005). A reduction in K<sub>v</sub>7 current expression

has been observed in cultured hippocampal neurons during homeostatic scaling by prolonged activity blockade (Lee et al., 2015). The goal was to investigate the signaling pathway involved in homeostatic scaling of intrinsic excitability and whether it involves a decrease in  $K_v7$  expression at the AIS by downregulation of TrkB signaling. Prolonged activity blockade in cultured hippocampal neurons decreases the activities of TrkB as well as its downstream ERK1/2. There was a significant reduction in the AIS levels of  $K_v7.3$  and ankyrin-G following prolonged activity blockade. Similarly, prolonged pharmacological inhibition of ERK1/2 decreases the density of  $K_v7.3$  and ankyrin-G at the AIS. This implicates for the first time the downregulation of both ERK1/2 activity and the AIS expression of  $K_v7.3$  as a potential mechanism underlying homeostatic scaling of intrinsic excitability. When prolonged activity blockade is applied to cultured hippocampal neurons, this decrease in the activity of TrkB and its downstream kinase ERK1/2 was revealed to correlated change in transcriptional regulation, and concurrently, reduced  $K_v7.3$  transcripts and protein expression at the AIS which would result in a homeostatic increase in intrinsic excitability.

The studies discussed in this thesis support the emerging concept that GIRK and  $K_v7$  channels contribute to neural plasticity, memory, and behavior. However, there is a significant knowledge gap in our understanding of the underlying molecular and cellular mechanisms. The development of a more selective agonist/antagonist for GIRK,  $K_v7$ , and their numerous subunits could offer additional insights into their complex contribution to behavior. Determining whether the prevention of caspase-mediated cleavage and down-regulation of neuronal GIRK channels during seizures in human TLE patients may dampen their excitability and prevent part of the hippocampal sclerosis remains to be explored fully. Further investigation into the precise molecular mechanism underlying ERK1/2 regulation of  $K_v7.3$  at the AIS during homeostatic scaling of intrinsic excitability warrants future studies. Potassium channels are a diverse group of ion channels offering many novel possibilities in precisely controlling neuronal activity with many therapeutic possibilities.

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## **APPENDIX A**

### **Single-cell epidermal growth factor binding correlates with single-cell receptor translocation.**

**Author Contribution:** B.C.B performed western blot and analysis. P.L treated triple-negative breast cancer cells (MDA-MB-231) with QD-EGF and gefitinib and performed 3-D deconvolution microscopy and analysis. S.J.L. synthesized QDs and performed optical characterization.

#### **A.1. Introduction**

This work was performed in collaboration with Phuong le in Dr. Andrew Smith's laboratory in the Department of Bioengineering at the University of Illinois at Urbana-Champaign.

Fluorescent quantum dots (QDs) are tiny particles of semiconducting materials with diameters of 2-10 nm that can be engineered to display different fluorescent wavelengths. Traditional antibodies can be 10-15 nm and when combined with secondary fluorophores can reach excesses of 30 nm. This size limitation can be significant in areas where proteins are highly enriched and limit traditional antibody penetration, for instance, between the pre-and post-synaptic site which is only about 20 nm would not allow for the complete accessibility to traditional antibody staining. This drives the need to make new fluorophores that are smaller and can be introduced into live cells.

QDs have allowed for the counting of individual growth factors within a single cell (Lim et al., 2015). QDs could be used to observe receptor internalization/insertion into the cell membrane due to external stimuli and has the potential to be applied to any receptor-ligand interaction that causes a phenotypic response (Bowsher and Swain, 2014; Levchenko and Nemenman, 2014). How individual cells decide to respond to the same stimuli with such a heterogenous phenotype has been not well studied due to technological limitations of measuring the phenotype and the signal strength simultaneously (Kolitz and Lauffenburger, 2012).

However, QDs allow for the investigation of single molecules within individual cells and compares this to the other cells in a population. QDs achieve this due to their small size, fluorescence properties, and the ability to add binding motifs (Lim et al., 2015; Ma et al., 2016). The number of QDs that bind to receptors within a live cell and the phenotypic response can be

visualized. QDs provide superior spatial and temporal resolution of individual receptors within a cell, as well as, examining the substantial cell-to-cell variability between cells due to their small size and high fluorescent intensity. QDs can therefore be used for the investigation of cell heterogeneity and intrinsic variability and better understand cellular decision-making.

Dr. Andrew Smith's lab has developed novel technology using fluorescent QDs and calibrated 3-D deconvolution microscopy (QDC-3DM) technique to quantify biochemical stimulation and the resulting individual cell phenotype. To demonstrate this technology, QDC-3DM was applied to epidermal growth factor (EGF) signaling in triple-negative breast cancer (MDA-MB-231) cells grown on micropatterned islands and stimulated with EGF conjugated to QDs (QD-EGF) (Figure A1.1). Epidermal growth factor (EGF) binding to EGF receptors (EGFR) in MDA-MB-231 cells display a heterogeneous disease with a small portion of patients benefiting from EGFR inhibitors. This demonstrates the need to better understand the effects of EGFR inhibitors on individual MDA-MB-231 cells (Davidson et al., 1987; Nakai et al., 2016). EGFR was bound extracellularly with the fluorescent QDs fused to EGF. QDs emitting in the infrared spectrum were used to avoid interference from autofluorescence from other cellular components. Using the QDC-3DM technique Andrew Smith's lab observed proportionality between stimulation, receptor internalization, and inhibitor response.

We next examined whether the QDC-3DM technology could replicate physiological results similar to those found using previously validated techniques. To validate this novel technology, Dr. Andrew Smith has requested a collaboration for us to perform a traditional western blotting analysis. In this Appendix A-1, I present my immunoblot analysis findings that indicate reduced phosphorylation with increased EGFR inhibitor concentrations. EGF ligand bounding to EGFR results in autophosphorylation at multiple tyrosine residues leading to internalizing (Nakai et al., 2016). My results suggest that EGFR is internalized following EGF treatment, and this internalization was prevented with its inhibitor gefitinib.

This data was included in a publication "Counting growth factors in single cells with infrared quantum dots to measure discrete stimulation distributions" and is attached as a PDF.

This Appendix is part of the following manuscript that is published in Nature Communications.

Le P, Lim SJ, **Baculis BC**, **Chung HJ**, Kilian KA, Smith AM. Counting growth factors in single cells with infrared quantum dots to measure discrete stimulation distributions. *Nat Commun*. 2019 Feb 22;10(1):909. doi: 10.1038/s41467-019-08754-5. PMID: 30796217; PMCID: PMC6385258.

## **A.2. Results**

To test if EGF stimulation impacted EGFR internalization in a dose-dependent manner, I treated MDA-MB-231 cells with pharmacological EGFR inhibitor gefitinib. Gefitinib was used to prevent phosphorylation of EGFR, and therefore preventing receptor internalization (Nakai et al., 2016). Gefitinib was applied at 0, 0.51, 5.1, 51, 510, 5100 nM concentrations to MDA-MB-231 cells for 40 min before stimulation with QD-EGF. After 5 min stimulation with QD-EGF, the cells were lysed in RIPA buffer, and the cell lysate was subjected to western blot analysis for phosphorylation of EGFR at Tyrosine residue 1068 (Y1068) and total EGFR. Dose-dependent phosphorylation of EGFR at Y1068 correlates with the extent of receptor internalization (Figure A1.2A). My western blot results revealed that 510 nM gefitinib significantly prevented receptor phosphorylation. There is also a dose-dependent decrease in phosphorylation with increasing gefitinib drug concentration (Figure A1.2B).

## **A.3. Discussion**

Dr. Andrew Smith's lab used QDC-3DM to find an increase in EGFR inhibitor gefitinib concentration led to a decrease in EGFR internalization (A1.1D). Importantly, the Smith lab was able to observe the variability of individual cellular responses to gefitinib which increased with the drug concentration. My western blot findings indicate the same decreasing trend in phosphorylation of EGFR seen with increasing gefitinib concentrations (Figure A1.2A). These findings were consistent with QD-3DM results with a mean average (Figure A1.1) very similar to those found using western blotting analysis (Figure A1.2A). Increasing EGF inhibitor concentration reduces EGFR phosphorylation and therefore reduced internalization. However, increasing the concentration also resulted in greater cell to cell variability in their response to this inhibitor.

## **A.4. Methods**

### **Cell Culture**

MDA-MB-231 cells (300,000 cells) were seeded in each well of a 6- well plate for 72 h in DMEM supplemented with 10% FBS. Cells were then starved in serum-free DMEM supplemented with 0.8% BSA for 5 h. Serum-starved cells were then treated with gefitinib at the indicated concentrations for 40 min in serum-free DMEM containing 0.8% BSA. Cells were then stimulated with QD744-EGF (1 nM) in the presence of different concentrations of gefitinib for 5 min and washed three times with ice-cold Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5).

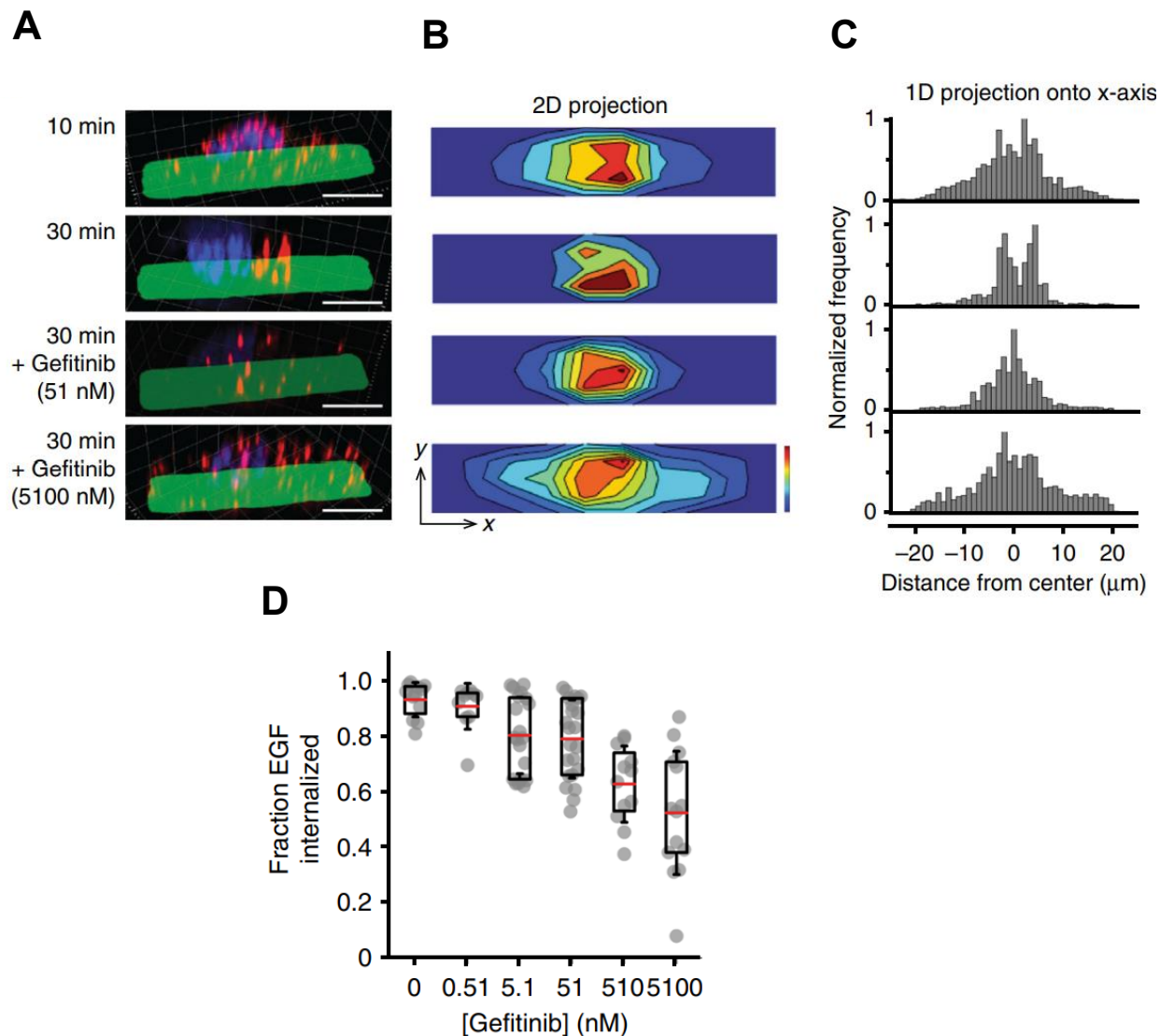
### **Western blot**

Cells were lysed by treatment with radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and phosphatase inhibitors (50 mM NaF, 1 mM NaVO<sub>3</sub>) on ice for 15 min. Cell lysates were collected after centrifugation for 15 min at 14,000g at 4 °C; a small fraction was aliquoted for protein concentration measurement using the bicinchoninic acid assay. Protein concentrations for each sample were adjusted to ~0.9 mg mL<sup>-1</sup>. Cell lysates were then mixed with 5× sample buffer (1 M Tris, pH 9, 10 g SDS, 12.5 mL glycerol, 100 μL 0.5 M EDTA, 50 mg bromophenol blue, 100 mM TCEP) to a final concentration of 1×, heated at 75 °C for 20 min, aliquoted, and stored at -80 °C until use. Samples were loaded into wells of an SDS-polyacrylamide gel; electrophoresis was performed, and gels were transferred to a polyvinylidene difluoride membrane (Immubilon-P membrane, Millipore). The membrane was washed three times with deionized water followed by Tween-20 (0.1%) in TBS for 5 min each. The membrane was then blocked with 5% milk and 0.1% Tween-20 in TBS for 1 h. The membrane was treated overnight at 4 °C with a solution of primary antibodies in 1% milk and 0.1% Tween-20 in TBS. Primary antibodies used were rabbit anti-EGFR (1:500 dilution), mouse anti-human pEGFR<sup>Tyr1068</sup> (1:250 dilution), and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000 dilution; Cell Signaling). Membranes were washed with 1% milk and 0.1% Tween-20 in TBS five times before incubation with horseradish peroxidase-conjugated secondary antibodies (anti-mouse or anti-rabbit, 1:5000 dilution) for 1 h. Membranes were again washed five times with 1% milk and 0.1% Tween-20 in TBS, and one time with 0.1%

Tween-20 in TBS before bands were developed by enhanced chemifluorescence substrate (ECL, Thermo Fisher Scientific) and imaged on autoradiography film (Denville Scientific). Images were analyzed using ImageJ software (National Institutes of Health). The band intensities for pEGFR and EGFR were divided by that of GAPDH; then, the band intensity of pEGFR/GAPDH was divided by EGFR/GAPDH. The intensities were normalized to sample treated with 1 nM QD-EGF without gefitinib to calculate the ratio of pEGFR to total EGFR under the different experimental conditions.

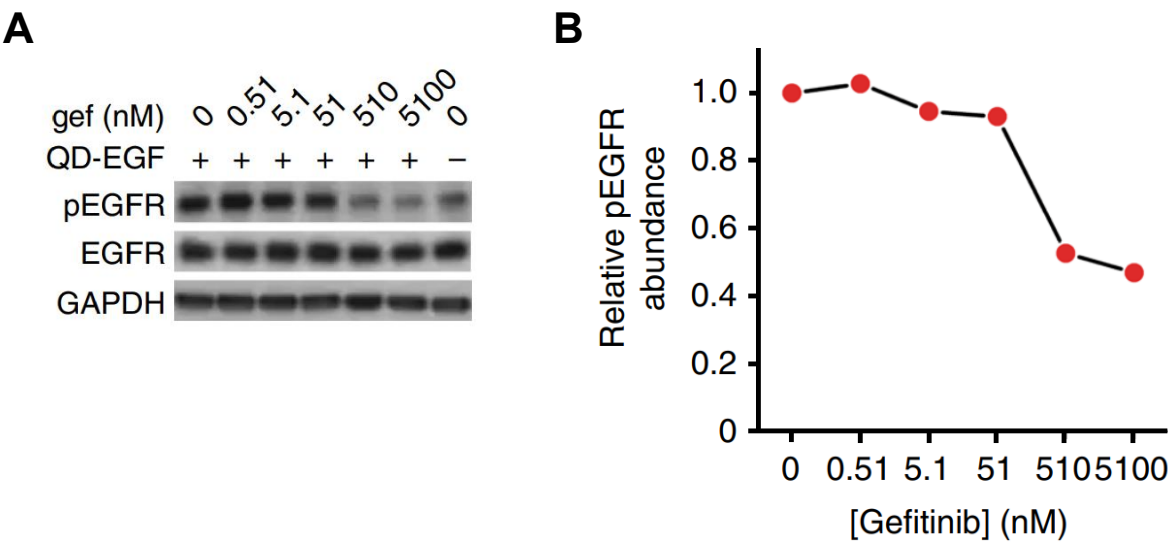
## A.5. Figures

### Appendix Figure A.1.



**Appendix Figure A.1. Fluorescent quantum dots and calibrated three-dimensional deconvolution microscopy of QD-EGF receptor binding spatial distribution and translocation in a single-cell and drug response.** (A) Representative three-dimensional images of triple-negative breast cancer (MDA-MB-231) cells after 10 or 30 minutes of stimulation with quantum dot-EGF in the absence or presence of EGFR inhibitor gefitinib. QDs are shown in red, nuclei are blue, and Alexa Fluor 488-conjugated fibronectin micropatterns, indicating the cell body, are shown in green. (B) Two-dimensional (2D) z-projections on x, y fibronectin micropattern planes and (C) one-dimensional (1D) projections on x-axes indicate the localization of single EGF averaged across cells. (D) Fraction of EGF internalized in single cells at different gefitinib concentrations at 30 min after the start of a QD-EGF stimulation. The box indicates 25/75th percentile; red lines are means; whiskers are s.d. Figure A1.1 was performed by Phuong Le in Dr. Andrew Smith's lab.

Appendix Figure A.2.



**Appendix Figure A.2. Western blot analysis of EGFR phosphorylation response to EFG stimulation and drug response.** (A) Representative image of western blot. The dose of gefitinib is indicated above QD-EGF stimulation or no stimulation indicated by + and – accordingly. (B) mean pEGFR abundance in MDA-MB-231 whole-cell lysates immediately after stimulation with QD-EGF in the presence of indicated gefitinib concentrations. Figure A1.2 was performed by Brian Baculis in Hee Jung Chung’s lab.

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## APPENDIX B

### TC-2153 inhibition of STriatal-Enriched protein tyrosine phosphatase decreases HCN1 in hippocampal neuronal culture

**Author Contribution:** B.C.B performed western blot of HCN and analysis. J.W. did seizure monitoring,  $\text{Ca}^{2+}$  imaging in acute slice, and E.C.K did electrophysiology in hippocampal neurons of  $I_h$ .

#### B.1. Introduction

This work was performed in collaboration with Jennifer Walters in my lab (Dr. Hee Jung Chung's lab) in the Department of Molecular and Integrative Physiology at the University of Illinois at Urbana-Champaign.

STriatal Enriched protein tyrosine Phosphatase (STEP) is a brain-specific tyrosine (Tyr) phosphatase (Goebel-Goody et al., 2012). STEP61 is expressed in the hippocampus and neocortex (Boulanger et al., 1995) where it has the ability to dephosphorylate AMPAR and NMDAR resulting in receptor internalization (Zhang et al., 2008; Kurup et al., 2010). Activity-dependent regulation of STEP61 has been shown to dephosphorylate GluN2B and GluA2 weakening synaptic strength (Jang et al., 2015). Interestingly, deletion of STEP gene resulted in resistance to pilocarpine-induced seizures (Briggs et al., 2011), suggesting STEP may be a target for seizure treatment.

TC-2153 is a selective STEP inhibitor for STEP and has been shown to reverse cognitive deficits in 3xTg mouse model of Alzheimer's Disease (Xu et al., 2014) which also display hippocampal hyperexcitability and seizures (Davis et al., 2014). It was also found that TC-2153 can block pentylenetetrazol-induced convulsions (Khomenko et al. 2009). However, it is unclear what is the molecular target of TC-2153 which mediates STEP inhibitions anticonvulsant properties.

To test whether TC-2153 also has anticonvulsive properties, Jennifer in our lab has investigated if TC-2153 reduces seizure severity in C57BL/6J mice. To induce acute seizures, she used a single systemic injection of kainate, which is shown to stimulate the hippocampus and induce status epilepticus at 3 hours after intraperitoneal (i.p.) injection with DMSO vehicle control

or TC-2153 (10 mg/kg, diluted in saline). She found that TC-2153 pre-treatment resulted in a reduced seizure severity in C57BL/6J mice and increased their survival. Using GCaMP6s mice which have a genetically encoded calcium indicator GCaMP6s, Jennifer also found that 1 hour treatment with 10  $\mu$ M TC-2153 (reduced KCl-evoked calcium signals in the acute hippocampal slices).

To better understand the mechanisms by which TC-2153 reduces the excitability of the acute hippocampal slices, Dr. Eung Chang Kim in our lab performed whole-cell patch-clamp recording in hippocampal neurons in dissociated culture. He found that 1 hour pretreatment with 10  $\mu$ M TC-2153 resulted in a hyperpolarized resting membrane potential, a reduction in instantaneous firing rate, voltage sag, rebound potential, and non-selective inward cationic current called  $I_h$  (Figure A2.1).

Since  $I_h$  mediates voltage sag, rebound potentials, and regulates resting membrane potential (RMP) in hippocampal pyramidal neurons (Biel et al., 2009), these recordings demonstrate the potential of STEP inhibitor TC-2153 in regulating hyperpolarization-activated cyclic nucleotide-gated ion (HCN) channels, which produce  $I_h$ .

My role in this project was to confirm the expression of HCN1 and HCN2 subunits in hippocampal neuronal culture at the same DIV when the recordings were performed. In this Appendix A-1, I present my immunoblot analysis findings that show the expression of both HCN1 and HCN2 in the hippocampal neuronal culture. My immunoblot analysis also revealed that TC-2153 reduces HCN1 expression, suggesting that TC-2153 may reduce neuronal excitability in part by decreasing HCN1 channel expression.

This Appendix is part of the following manuscript that has been submitted to *Epilepsia*.

Walters JM, Kim EC, Zhang J, Jeong HG, Bajaj A, **Baculis BC**, Tracy G, Ibrahim B, Christian-Hinman CA, Llano DA, Huesmann GR, **Chung HJ**. Pharmacological inhibition of STriatal-Enriched protein tyrosine Phosphatase by TC-2153 reduces hippocampal excitability and seizure propensity. *Epilepsia*. Submitted Nov 2021, and *Under minor revision*.

## **B.2. Results**

Dr. Eung Chang Kim had found TC-2153 treatment decreases  $I_h$  in culture neurons.  $I_h$  is mediated through HCN channels. To confirm that hippocampal neurons in dissociated culture

indeed express HCN1 and HCN2, the principal subunits of HCN channels that mediate  $I_h$ , neurons were lysed at 10 days in vitro (DIV) (Figure A2.2) when TC-2153-induced reduction in  $I_h$  current was observed. Before lysing, neurons were pre-treated for 1 h with either vehicle control (0.14% DMSO) or TC-2153 (10  $\mu$ M) to inhibit STEP. My western blotting analysis confirmed that neurons at DIV 10 expressed both HCN1 and HCN2 subunits. Interestingly, there was a reduction in HCN1 subunits following TC-2153 treatment (Figure A2.2).

### **B.3. Discussion**

Dr. Eung Chang Kim's recordings showed that  $I_h$  current was reduced following TC-2153 treatment in hippocampal dissociated culture. My western blots revealed that HCN1 and HCN2 expression are found in DIV 10 dissociated hippocampal neurons. Interestingly, the same TC-2153 treatment also revealed that TC-2153 reduces HCN1 expression suggesting that TC-2153 may reduce neuronal excitability in part by decreasing HCN1 channel expression. STEP has been shown to directly dephosphorylate NMDAR leading to their internalization (Jang et al., 2015). Whether STEP directly dephosphorylates HCN1 and HCN2 remains to be determined.

### **B.4. Methods**

#### **Hippocampal Neuronal Culture**

Primary dissociated hippocampal cultures were prepared from E18 Sprague-Dawley rat embryos as described (Jang et al., 2015) and plated at high density (330 cells/mm<sup>2</sup>) onto poly L-lysine-coated (0.1 mg/mL) 30 mm cell culture dishes. Neurons were treated at 9-10 days in vitro (DIV) for 1 h with vehicle control (0.14% DMSO) or TC 2153 (10  $\mu$ M) to inhibition of STEP.

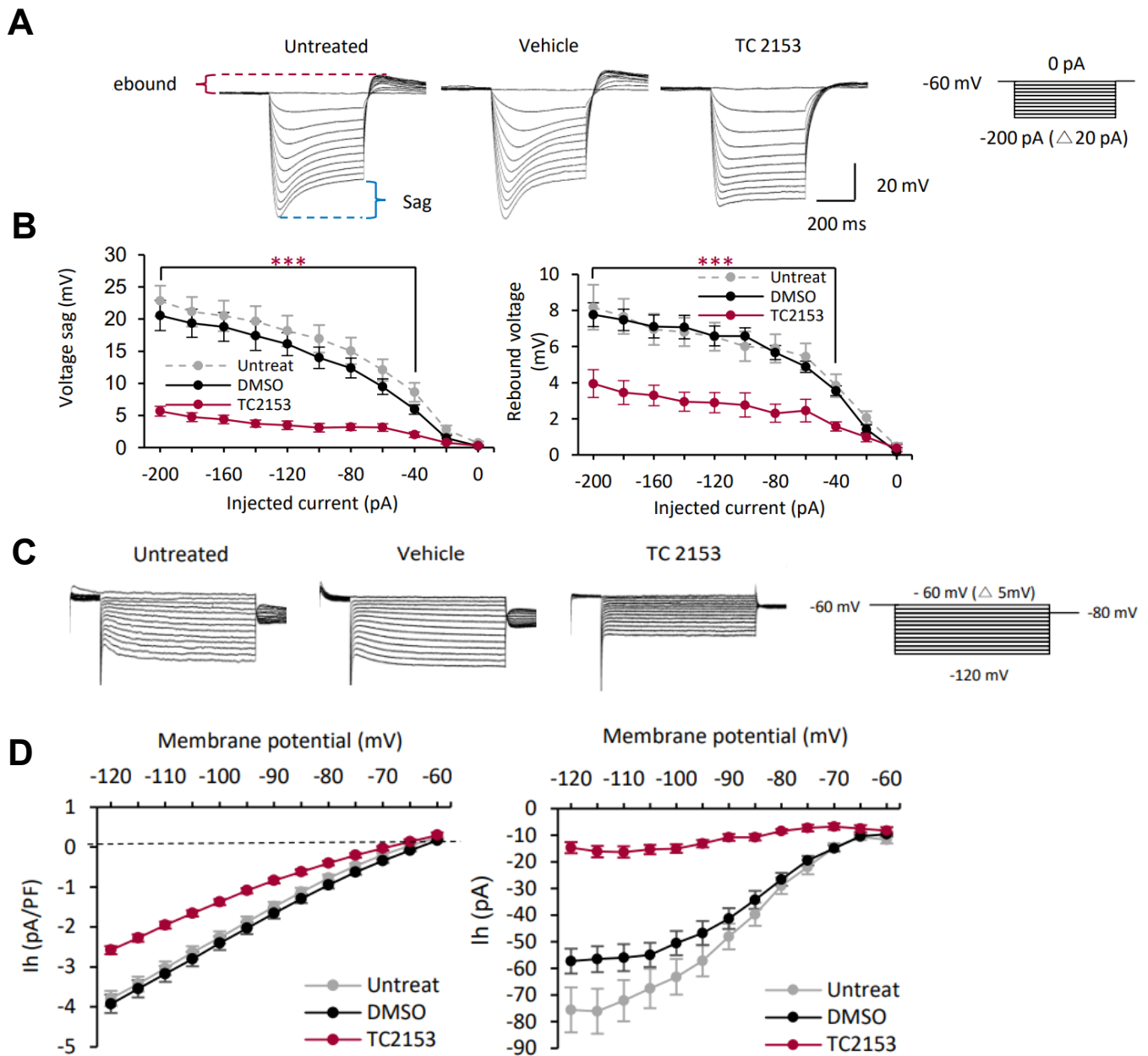
#### **Western blotting**

Following pharmacological treatment, hippocampal cultured neurons were lysed in ice-cold RIPA buffer (200  $\mu$ L per dish) which contained protease inhibitor cocktail and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The resulting lysate samples were subjected to immunoblot analysis. Cell lysates were collected after centrifugation for 15 min at 14,000g at 4  $^{\circ}$ C. Cell lysates were then mixed with 5 $\times$  sample buffer (1 M Tris, pH 9, 10 g SDS, 12.5 mL

glycerol, 100  $\mu$ L 0.5 M EDTA, 50 mg bromophenol blue, 100 mM TCEP) to a final concentration of 1 $\times$ , heated at 75  $^{\circ}$ C for 20 min, aliquoted, and stored at  $-80^{\circ}$ C until use. Samples were loaded into wells of an SDS-polyacrylamide gel; electrophoresis was performed, and gels were transferred to a polyvinylidene difluoride membrane (Immubilon-P membrane, Millipore). The membrane was washed three times with deionized water followed by Tween-20 (0.1%) in TBS for 5 min each. The membrane was then blocked with 5% milk and 0.1% Tween-20 in TBS for 1 h. The membrane was treated overnight at 4  $^{\circ}$ C with a solution of primary antibodies in 1% milk and 0.1% Tween-20 in TBS. Primary antibodies including HCN1 (#APC-056, Alomone, 1:1000), HCN2 (#75-111, Neuromab, 1:1000), and GAPDH (#2118, Cell Signaling, 1:1000). Densitometric quantification, following normalization to GAPDH was performed with ImageJ software (National Institutes of Health).

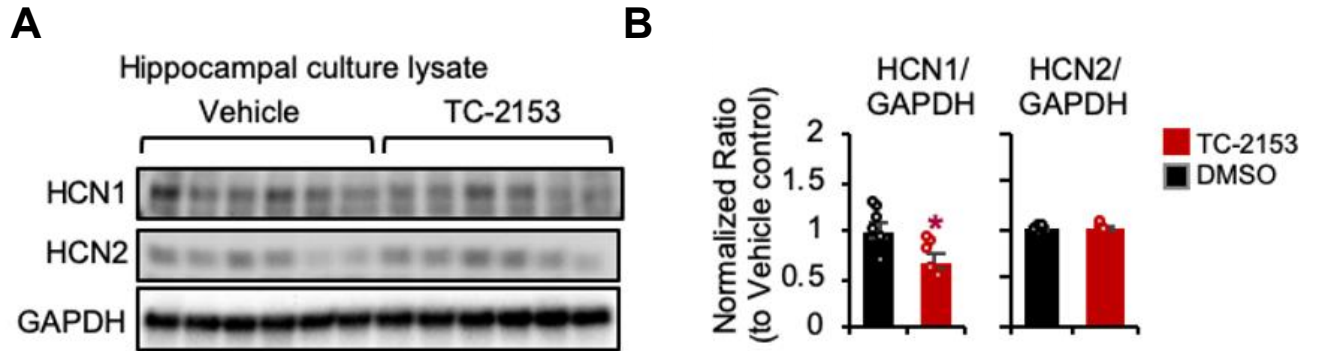
## B.5. Figures

### Appendix Figure B.1.



**Appendix Figure B.1. TC-2153 treatment reduces  $I_h$  in cultured hippocampal neurons.** Whole-cell current-clamp recording of cultured hippocampal neurons (DIV 8–10) was performed after 1 h treatment with TC 2153 (10  $\mu$ M) or DMSO (0.14%). **(A–B)** TC-2153 reduces voltage sag and rebound voltage in cultured hippocampal neurons. **(A)** Representative responses to hyperpolarizing current steps from -200 to 0 pA in 20 pA increments in current-clamp mode. The amount of voltage sag (blue lines) and rebound potential (red lines) was determined as the difference between the maximum and steady-state voltage during the hyperpolarizing current injection. **(B)** Average sag voltage. **(C)** Average rebound voltage. **(C–D)** In voltage-clamp mode,  $I_h$  was evoked by applying voltage steps from the holding potential of -60 mV to -120 mV in 5 mV decrements. **(C)** Representative traces of  $I_h$ . **(D)**  $I_h$  density at all voltage steps. Data shown as mean  $\pm$  STDEV. Post-hoc Tukey test results are shown for TC-2153 vs. untreated or DMSO (\*\*\* $p$  < 0.005). Figure A2.1 was performed by Dr. Eung Chang Kim in Dr. Hee Jung Chung's lab.

## Appendix Figure B.2.



**Appendix Figure B.2. TC-2153 application decreases HCN1 in hippocampal neuronal culture.** Dissociated rat primary hippocampal neuronal culture that were treated for 1 h with either DMSO as a vehicle control, or TC-2153 (10  $\mu$ M) at DIV (days in vitro) 9-10. **(A)** Representative immunoblots. **(B)** Quantification of immunoblots. Data shown represent the mean  $\pm$  SEM. The Student's t-test was used (\* $p < 0.05$ ). Figure A2.2 was performed by Brian Baculis in Hee Jung Chung's lab.

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## **APPENDIX C**

### **Generation and characterization of a genetic mouse model of in vivo activity blockade**

**Author Contribution:** (B.C.B) performed and analyzed all experiments.

#### **C.1. Introduction**

Epilepsy manifests as uncontrolled aberrant electrical hyperactivity in the brain and these seizures are recurrent and unpredictable. Temporal lobe epilepsy (TLE) is the most common form of focal epilepsy. One of the known causes of TLE is traumatic brain injury (TBI). Patients with TBI often develop seizures one day to one week following injury (Kandratavicius et al., 2014). TLE is commonly associated with neuronal death, network reorganization, and synaptic sprouting (Sharma et al., 2007). It has also been found that surviving neurons develop aberrant connections and find new synaptic targets (Sharma et al., 2007). These aberrant connections may contribute to hyperexcitability and seizure onset. Furthermore, loss of input to the surviving neurons could lead to homeostatic scaling of intrinsic firing. A study has shown that infusion of tetrodotoxin (TTX) into the hippocampus causes activity blockade inducing seizures (Galvan et al., 2000) and can increase intrinsic excitability as well as synaptic strength after 2 days of treatment (Echegoyen et al., 2007).

Homeostatic plasticity acts to stabilize neuronal activity, by altering synaptic strength and adjusting intrinsic excitability (Turrigiano, 2011). One powerful control over neuronal excitability is through the precise clustering and functional regulation of voltage-gated ion channels at subcellular domains (Lai and Jan, 2006; Beck and Yaari, 2008; Trimmer, 2015). A disruption in homeostatic plasticity is associated with multiple diseases, including Alzheimer's disease and epilepsy (Beck and Yaari, 2008).

However, whether prolonged activity blockade alone is enough to induce epileptogenesis is unknown. Kir2.1 is a potassium channel that hyperpolarizes neurons (Cui et al., 2021). Overexpression of Kir2.1 has been previously shown to induced prolonged activity blockade in vitro and in vivo (Paradis et al., 2001; Burrone et al., 2002; Yu et al., 2004). Since intracranial injection of TTX into the hippocampus causes an increase in intrinsic excitability, I hypothesize prolonged activity blockade through genetic overexpression of Kir2.1 will cause homeostatic scaling of intrinsic excitability and induce epileptogenesis.

To test this hypothesis, in this chapter (Appendix A-3.), I developed a genetic mouse model by crossing Tg(Camk2a-tTA)1Mmay/DboJ with Tg(tetO-Kcnj2,LacZ)1Gogo mice in which conditional hippocampal expression of *KCNJ2*/Kir2.1 channels and the reporter gene *LacZ* which encodes  $\beta$ -galactosidase ( $\beta$ -gal) are under the control of tetracycline operator. The resulting double mutant offspring displayed normal appearance and weights compared to single mutant control. Importantly, the double mutant mice displayed  $\beta$ -gal indicating the presence of Kir2.1 in hippocampal neurons including dentate gyrus granular neurons and CA1 and CA3 pyramidal neurons in P30 and P60 when doxycycline (Dox) was removed from gestation, and an increase in Kir2.1 when Dox was removed 10-20 days.

## C.2. Results

### **Generation of in vivo activity blockade mice to examine hippocampal homeostatic scaling and epileptogenesis.**

In order to investigate prolonged activity blockade in the hippocampus in vivo, we generated a transgenic mouse model by crossing Tg(Camk2a-tTA)1Mmay/DboJ with Tg(tetO-Kcnj2,LacZ)1Gogo mice on a C57BL/6J background. Tg(tetO-Kcnj2,LacZ)1Gogo mouse line contains tetO promoter which is activated by the tetracycline-controlled transactivator protein (tTa) allowing control over the expression of *Kcnj2*/Kir2.1 and *LacZ* genes (Yu et al., 2004). Tg(Camk2a-tTA)1Mmay/DboJ mouse line expresses tTa only in Camk2a containing neurons (Mayford et al., 1996). In this double mutant mouse, removal of Dox-containing food should allow the tetracycline-controlled tTa to bind to the tet-O promoter and give rise to the expression of *Kcnj2*/Kir2.1 and *LacZ*/ $\beta$ -galactosidase ( $\beta$ -Gal) in Camk2a- positive excitatory neurons in the hippocampal formation and forebrain. (Figure A3.1B-C). Utilizing this tTa system, I have spatial and temporal control over *LacZ*/ $\beta$ -gal and *Kcnj2*/Kir2.1 which are linked via IRES sequence. *LacZ* encodes  $\beta$ -Gal which is a commonly used reporter protein, which creates a blue stain upon undergoing an enzymatic reaction with X-gal. Kir2.1 overexpression has been previously shown to cause hyperpolarization-induced activity blockade (Burrone et al., 2002). This genetic control over the expression of *Kcnj2*/Kir2.1 should then allow us to silence spontaneous activity by hyperpolarizing these neurons for a specific amount of time and then allow for activity to return by feeding back Dox to stop Kir2.1 overexpression (Yu et al., 2004).

The resulting double mutants appeared normal in coat color and general features compared to C57BL/6J control. We observed no significant difference between the weights of male or female double mutants compared to their age-matched single mutant control when Dox was removed from gestation. On postnatal day 30 male double mutant mice weighed 13.3  $\pm$  0.35 g and single mutant mice weighed 12 g  $\pm$  0.33 (p=0.21). Female double mutant mice weighed 14.3  $\pm$  0.35 g and single mutant weighed 12.8  $\pm$  0.11 g (p=0.12). On postnatal day 50-55 male double mutant mice weighed 20.3  $\pm$  0.25 g and single mutant weighed 21.8  $\pm$  0.15 grams (p=0.08). Female double mutant mice weighed 17.6  $\pm$  0.25 g and single mutant weighed 16  $\pm$  0.22 g (p=0.06) (Figure A3.1D).

### **Characterization of in vivo activity blockade mice**

Dox removal was performed from gestation (E0) or weaning (P21) until postnatal day (P30) and (P60). Brain tissue was then collected for subsequent experiments. To examine  $\beta$ -Gal staining animals were transcardially perfused and fixed with 2% PFA. Brains were then removed and cryosectioned in 30  $\mu$ m coronal sections. We then performed  $\beta$ -Gal staining according to the manufacturer's instructions (Invitrogen Cat# K146501). I found that  $\beta$ -Gal staining was evident in the olfactory area, hippocampal formation, midbrain, cortical regions, cerebellum, cerebellar, and lateral forebrain fiber tracts, as well as lining the lateral and third ventricles and cerebral aqueduct when Dox removal was from E0-P30 and E0-P60 (Figure A3.2B). In the hippocampus, Dox removal from E0 to P30 or P60 leads to robust  $\beta$ -Gal staining in CA1, CA3, and Dentate gyrus of the hippocampus (Figure A3.2B). The degree of  $\beta$ -Gal staining increased from P30 to P60.

It was found that Dox removal from P25-P30 was enough time to induce  $\beta$ -Gal staining in only the olfactory bulb but no other regions. However, Dox removal from P25-60 was enough time to produce robust  $\beta$ -Gal staining in many brain regions including the hippocampal formation of CA1, CA3, and Dentate gyrus. Dox removal from P25-60 showed similar results as Dox removal was from E0-P30 and E0-P60. My preliminary data shows successful induction of  $\beta$ -Gal in the hippocampal excitatory neurons at P30 and P60 after Dox removal from weaning (Figure A3.2A) and gestation (Figure A3.2B). Since the *Kcnj2/Kir2.1* cDNA is linked to LacZ/ $\beta$ -Gal cDNA via IRES sequence and the transcription of *Kcnj2-IRES-LacZ* is under the control of the tet-

O promoter, my preliminary data (Figure A3.2A-B) suggest that Kir2.1 proteins are also expressed in the same regions of the brain.

To test if Kir2.1 expression was increased following Dox removal, I next performed western blot. Dox removal was begun from weaning and samples were collected at P31-41. For western blots hippocampal, cortex, and cerebellum tissue was rapidly dissected and flash-frozen before storage at -80C until used. We found double mutants seem to have an increased Kir2.1 expression following 10-20 days of Dox removal (Figure A3.3). A larger sample size and examining Kir2.1 expression at E60 after Dox removal from gestation could help confirm these preliminary findings.

To test if activity blockade was achieved in this mouse, we next examined neurons within the hippocampus that expressed  $\beta$ -Gal. We decided to start by recording activity from dentate gyrus granular neurons at P30 when Dox was removed from gestation. At this time, we wanted to see if Kir2.1 overexpression was silencing these neurons. Through whole-cell patch current-clamp recording from acute slice from double mutant or since mutant mice, I recorded spontaneous activity of dentate gyrus granule cells without any synaptic inhibitors (Figure A3.4). I also evoked action potential firing from dentate gyrus granule cells by using a current injection from -90 pA to +50 pA without any synaptic inhibitors and found no significant difference between Camk2a-tTa x tetO-Kir2.1 mice compared to controls (Figure A3.4). Examining intrinsic AP firing correctly by adding synaptic blockers and increasing the sample size would help determine if Kir2.1 overexpression indeed silenced these neurons.

### **C.3. Future Directions**

#### **Future Aim 1. Validation of our novel in vivo mouse model of activity blockade**

I will do this by performing whole-cell patch-clamp recordings of spontaneous action potential firing in acute slice preparation. To begin I will target dentate granule neurons because this area shows the most robust expression of  $\beta$ -Gal. I will then look at CA1 and CA3 pyramidal neurons to examine if they have reduced or blocked activity.

**Method:** Whole-cell patch current-clamp recording will be performed to examine spontaneous AP firing upon Dox removal from gestation. All recordings were carried out in an external solution

containing the antagonists for the fast excitatory and inhibitory synaptic transmission, CNQX (20  $\mu$ M) and APV (100  $\mu$ M), and Bicuculline (20  $\mu$ M).

**Expected Results and Interpretation:** Kir2.1 overexpression should silence neuronal activity in the hippocampus. Double mutant mice should display significantly less AP firing in the hippocampus compared to single mutant control.

## **Future Aim 2. Examine homeostatic scaling of intrinsic excitability utilizing in vivo model of activity blockade**

**Method:** Upon confirmation of activity blockade, I will examine intrinsic excitability and look for AIS plasticity. To examine homeostatic scaling of intrinsic excitability, I will record evoked action potential firing from acute hippocampal slices from double transgenic mice in the presence of Kir2.1 inhibitor (ML133) upon Dox removal. I will first examine granule cells where activity has been blocked at P30.

Immunostaining will be performed on fixed brain slices, followed by confocal imaging and analysis of the maximally projected z-stacked images. It would be interesting to compare how this activity blockade affects synaptic properties of these excitatory hippocampal neurons as well as other neurons where activity was not impaired, such as inhibitory neurons where Kir2.1 should not be expressed.

**Expected Results and Interpretation:** Prolonged Dox removal should cause a homeostatic increase in excitability. I expect to see Na<sub>v</sub>1 density and spread at the AIS to stay relatively normal while seeing a decrease in K<sub>v</sub>7 density and spread at the AIS. I also expect to see a change in synaptic properties of both excitatory and inhibitory neurons.

## **Future Aim 3. Examine seizure propensity after homeostatic scaling**

**Method:** To test if seizure propensity is altered due to homeostatic scaling, I will perform kainite injection of P90 double mutant mice following Dox removal and refeeding. Dox will be removed from E0 until P60 to induce homeostatic scaling. Activity blockade will be removed by refeeding Dox, which should remove Kir2.1 overexpression. Status epilepticus will be induced by injection

of kainite (30 mg/kg). The behavioral response will be recorded over a 2-hour period following injection following the Racine scale.

**Expected Results and Interpretation:** I expect mice that have undergone homeostatic scaling due to activity blockade will show increased seizure propensity due to heightened excitability. If this length of refeeding Dox is too long and allows homeostatic scaling to return to normal, I will test alternative periods of Dox refeeding on seizure propensity.

#### **C.4. Methods**

##### ***Mouse model of activity blockade***

I generated double transgenic mice in which removal of Dox will induce tTA-dependent induction of Kir2.1 expression in hippocampal excitatory neurons. I have crossed B6.Cg-Tg(Camk2a-tTA)1Mmay/DboJ mice with B6.Cg-Tg(tetO)-Kcnj2,lacZ)1Gogo/J (double mutant) mice to spatially and temporally control excitability in Camk2a containing neurons. The Kcnj2 gene is under the regulation of the tetracycline-responsive promoter element (tetO) promoter, which is activated by the removal of Dox that is given in food pellets. Breeders were given 625mg/kg Dox during gestation and development. Upon weaning, animals were genotyped and placed in 1 of 4 groups, double mutant with Dox feed, double mutant Dox removal, single or wild-type Dox feed, or single or wild-type Dox removal. Animals used for immunohistochemistry were anesthetized and transcardially perfused with 1X PBS for 5 minutes followed by perfused with 2% PFA for 5 minutes. Brains were cryoprotected in 30% sucrose for 24 hours and then frozen in cryomold using O.C.T. and stored at -80 Celsius.

At weaning, genotyping of these mice was determined by Polymerase Chain Reaction (PCR) on their tail genomic DNA. The primers used in genotyping were the B6.Cg-Tg(Camk2a-tTA)1Mmay/DboJ (Jax lab # 007004) forward primer (5'→3' GAC CTG GAT GCT GAC GAA GG) and reverse primer (5'→3' GCA GCT CTA ATG CGC TGT). B6.Cg-Tg(tetO-Kcnj2,lacZ)1Gogo/J (Jax Lab # 009136) forward primer (5'→3' AGG GCC AGC TGT TGG GGT GAG TAC T and reverse primer (5'→3' TAA CCA GCA TCC ACC TCC AGC G).

### ***β-gal/X-gal staining***

Brains were collected from P30 and P60 mice and underwent coronal brain cryosectioning into 30-micrometer sections. Brains fixed in 2% PFA were coronally cryosectioned and then stained with β-gal staining kit (Invitrogen Cat# K146501) according to manufactures instructions. β-gal staining contains a working concentration of 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 1 mM MgCl<sub>2</sub>, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in 92% 1x PBS. The staining solution was incubated on fixed tissues at 37 degrees Celsius for 24 hours. β-galactosidase (LacZ reporter) undergoes an enzymatic reaction with X-gal in the presence of potassium ferricyanide, potassium ferrocyanide, and magnesium chloride that produce blue staining. Brain sections stained with β -Gal were imaged at IGB using NanoZoomer Digital Pathology System (Hamamatsu).

### ***Fractionation and Western blotting***

Following genotyping and Doxycycline removal mice were euthanized and brains were removed, dissected for hippocampi, cortex, and cerebellum before storing at −80°C. The hippocampi of C57BL/6J mice were biochemically fractionated to the supernatant (S2) and the membrane (P2) fractions (0.5 mg/mL) as described (Jang et al., 2016a). Hippocampi were homogenized in ice-cold homogenization buffer (solution A) containing (in mM): 320 sucrose, 1 NaHCO<sub>3</sub>, 1 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 0.4 HEPES (pH 7.4) and Halt protease inhibitors (Thermo Fisher Scientific). After centrifuging 1,400g for 10 min at 4°C, the homogenate supernatant (S1) was separated from insoluble tissue and nuclear pellet (P1). The S1 fraction was then centrifuged at 13,800g for 10 min at 4°C. The supernatant (S2) was removed, and the remaining pellet (P2 membrane fraction) was resuspended in ice-cold solution B containing (in mM): 160 sucrose, 6 Tris-HCl, 0.5% Triton-X (pH 8.0) and Halt protease inhibitors. The S2 fraction is enriched with cytosolic soluble proteins. The P2 fraction is enriched with transmembrane proteins and membrane-bound proteins. BCA assay (Pierce) analysis was performed to determine protein concentrations across samples, which were subsequently normalized to 1 mg/mL. The S2 and P2 fractions were stored at −80°C until use. The resulting lysate samples were subjected to immunoblot analysis. Cell lysates were collected after centrifugation for 15 min at 14,000g at 4 °C. Cell lysates were then mixed with 5× sample buffer (1 M Tris, pH 9, 10 g SDS, 12.5 mL glycerol, 100 μL 0.5 M EDTA, 50 mg bromophenol blue, 100 mM TCEP) to a final concentration

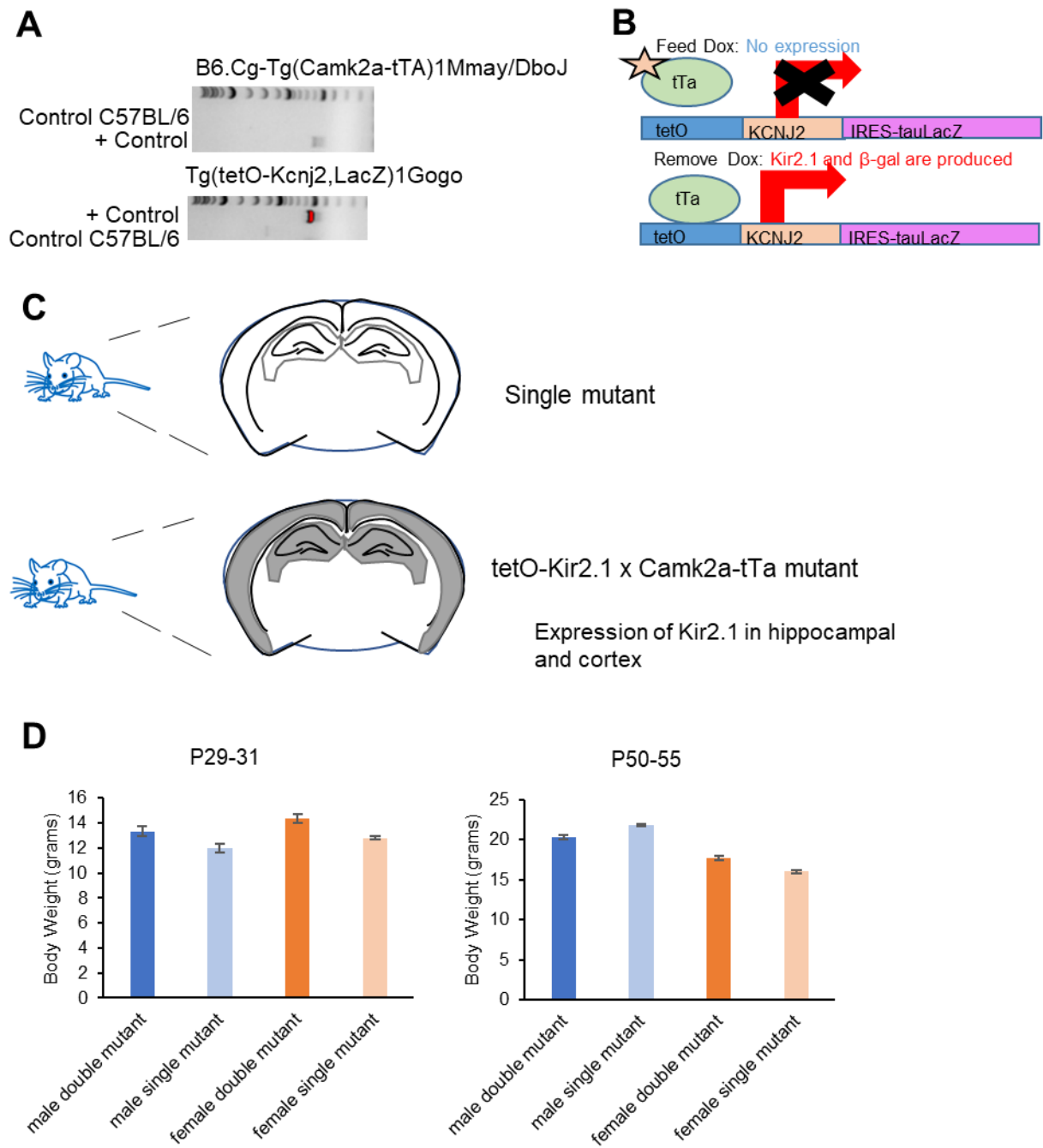
of 1×, heated at 75 °C for 20 min, aliquoted, and stored at −80 °C until use. Samples were loaded into wells of an SDS-polyacrylamide gel; electrophoresis was performed, and gels were transferred to a polyvinylidene difluoride membrane (Immubilon-P membrane, Millipore). The membrane was washed three times with deionized water followed by Tween-20 (0.1%) in TBS for 5 min each. The membrane was then blocked with 5% milk and 0.1% Tween-20 in TBS for 1 h. The membrane was treated overnight at 4 °C with a solution of primary antibodies in 1% milk and 0.1% Tween-20 in TBS. Primary antibodies including Kir2.1 (AB\_11000720, Neuromab, 1:1000), β-Gal (ab9361, abcam, 1:1000), and GAPDH (#2118, Cell Signaling, 1:1000). Densitometric quantification, following normalization to GAPDH was performed with ImageJ software (National Institutes of Health).

### ***Electrophysiology***

Whole-cell patch current-clamp recordings were performed to examine spontaneous AP firing upon Dox removal to confirm activity blockade. Using whole-cell patch-clamp recording chamber in external solution containing (in mM): 126 NaCl, 3 KCl, 2 CaCl<sub>2</sub>, 2 MgSO<sub>4</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub> and 14 Dextrose, bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4, 305–315 mOsm). Recording pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on micropipette puller (P-97; Sutter Instruments, Novato, CA, USA), and had a resistance of 3–5 MX when filled with an internal solution containing (in mM): 130 KmeSO<sub>4</sub>, 10 KCl, 10 HEPES/K-HEPES, 2 MgSO<sub>4</sub>, 0.5 EGTA and 3 ATP (pH 7.3, 285–295 mOsm). Current-clamp recordings were performed as previously (Lee and Chung, 2014) with the following modifications. Neurons were held at -60 mV. The AP firing rates (Hz) were measured upon delivering constant current pulses of 500 ms in the range 0–200 pA, and were averaged from 3 to 5 individual sweeps per current injection. Neurons were omitted if the resting membrane potential was >-50 mV, or if no Aps were discharged. The instantaneous AP firing rate at each current pulse was calculated as a reciprocal of the interspike intervals, measured as the time between the first and second AP peaks (i.e. 1/interspike interval) as (Lee and Chung, 2014). To examine homeostatic scaling, evoked AP firing from drug-treated slice culture and acute hippocampal slices from double transgenic mice in the presence of Kir2.1 inhibitor (ML133) upon Dox removal.

C.5. Figures

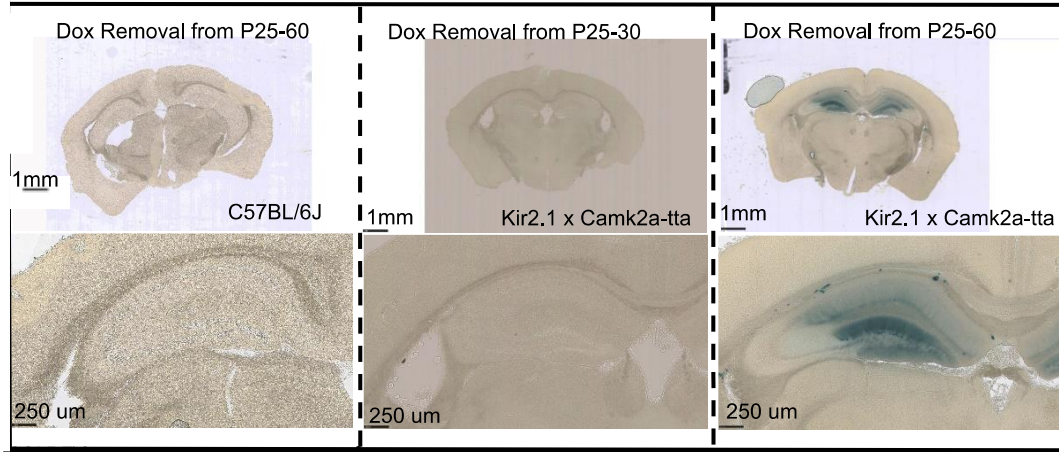
Appendix Figure C.1.



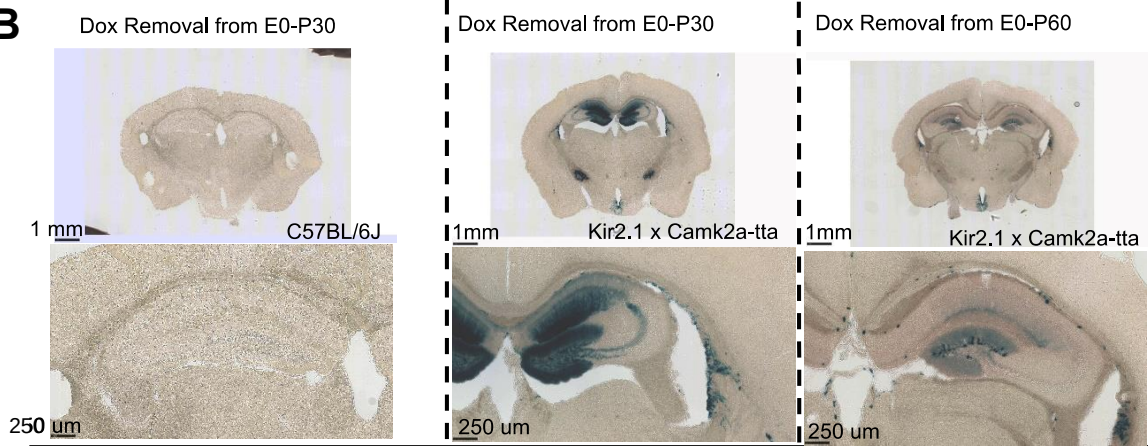
**Appendix Figure C.1. Generation of In vivo activity blockade transgenic mice** (A) Schematic representation of coronal brain sections of the progenies that result from breeding Tg(Camk2a-tTA)1Mmay/DboJ with Tg(tetO-Kcnj2,LacZ)1Gogo mice. (B) Representative example of a genotyping PCR using mouse tail genomic DNA as template. (C) Weights of single and double mutants collected at P30 and P60.

## Appendix Figure C.2.

**A**

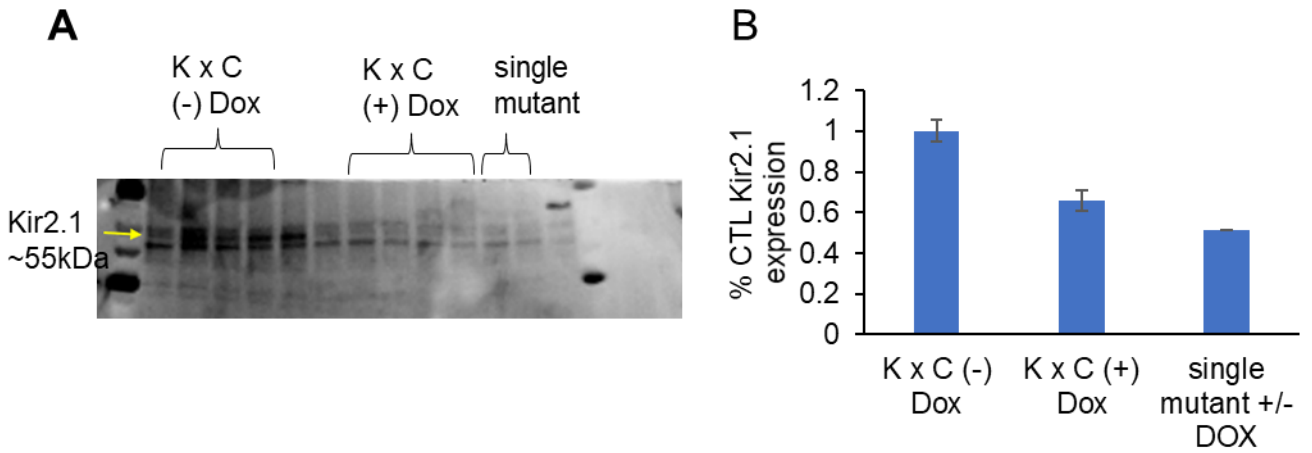


**B**



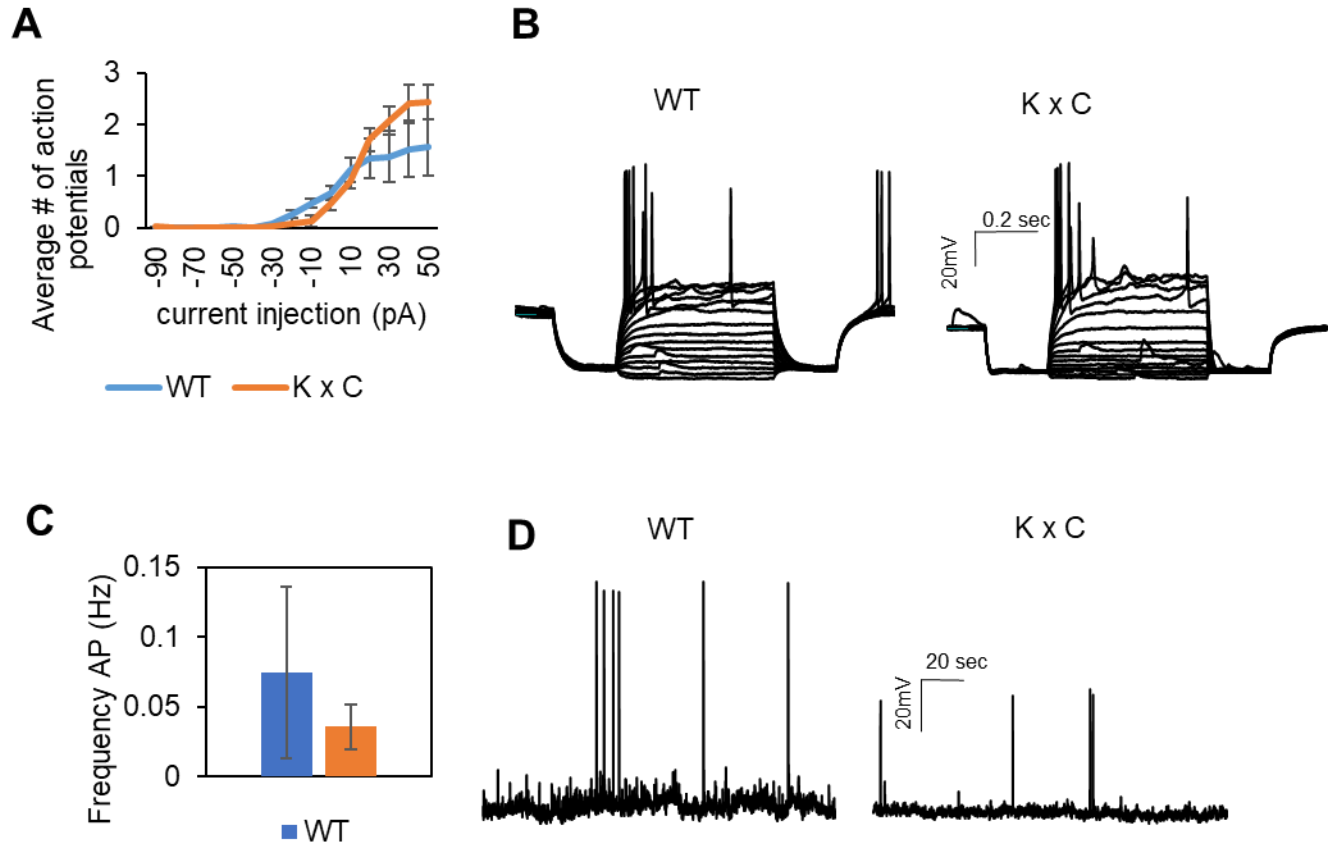
**Appendix Figure C.2. Characterization of Camk2a-tta age-dependent expression and length of Doxycycline removal on  $\beta$ -Gal expression in our transgenic mice model of in vivo activity blockade. (A) X-gal staining of  $\beta$ -Gal in our transgenic mice after removal of doxycycline from weaning until P30 and P60. (B) X-Gal staining of  $\beta$ -Gal in our transgenic mice after removal of doxycycline from gestation until P30 and P60.**

**Appendix Figure C.3.**



**Appendix Figure C.3. Expression of Kir2.1 in hippocampal lysate from double and single mutant mice.** Western blot of (A) Kir2.1 and (B) Quantification of Kir2.1 expression from tetO-Kir2.1 x Camk2a-tTa double mutant mice and single mutant mice  $\pm$  10-20 days after Doxycycline removal from P21-40 single and double mutant mice. Mean  $\pm$  SEM. (n=4 K x C (-) Dox, n=4 K x C (+) Dox, n=2 single mutant  $\pm$  Dox).

**Appendix Figure C.4.**



**Appendix Figure C.4. Electrophysiological activity in dentate gyrus granule cells in acute slice preparation from our transgenic mice model of *in vivo* activity blockade.** (A) Whole-cell patch current clamp recording of evoked action potential firing from -90 pA to +50 pA current injection steps. Mean  $\pm$  SEM (\*\*p < 0.01). (n: CTL= 18, K x C= 13). (B) Representative image of evoked action potential firing. Whole-cell patch current clamp recording of sporadic depolarizing synaptic potentials. Mean  $\pm$  SEM. (n: CTL= 4, K x C= 3 ). #) Representative image of sporadic depolarizing synaptic potentials. Tg(Camk2a-tTA)1Mmay/DboJ= C Tg(tetO-Kcnj2,LacZ)1Gogo= K. CTL= single or Wild type (WT) mice.

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