Early-life seizure sensitive neurones: Their characteristics and role in epilepsy pathology

By

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Abstract

There is a large body of literature exploring early-life-seizures (ELS), however, much of this research focuses on whole brain regions. This thesis aims to understand the characteristics of ELS-sensitive neurones and their role in future seizure pathology. Using c-Fos-GFP/c-Fos-tTA based transgenic mice, we found that at P10 ~18% of pyramidal neurones are activated in the CA1 pyramidal layer of the hippocampus in response to a kainic acid (KA) seizure. Electrophysiology recordings of ELS-sensitive neurones found a decrease in spontaneous GABA activity. Using c-Fos-GFP/c-Fos-tTA/TRE-hM3Dq mice, which flags ELS-sensitive neurones with the excitatory DREADD hM3Dq, we found that reactivating ELS-sensitive neurones is sufficient to evoke seizures. Finally, using c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mice with the inhibitory DREADD hM4Di, we found that suppressing ELS-sensitive neurones after a seizure recovers the increase in excitability. These results indicate that ELS-sensitive neurones play a crucial role in future seizure pathology and pose a unique target for further research.

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4′,6-diamidino-2-phenylindole	DAPI
adenosine deaminase acting on RNA 2	ADAR2
alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid	AMPA
alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor	AMPAR
bovine serum albumin	BSA
clozapine-N-oxide	CNO
deionized water	DI
dimethylsulfoxide	DMSO
doxycycline	dox
designer receptor exclusively activated by designer drugs	DREADD
early life seizure	ELS
enhanced green fluorescent protein	eGFP
electroencephalogram	EEG
G-protein-coupled receptor	GPCR
glutamic acid decarboxylase 67	GAD67
gamma-aminobutyric acid	GABA
gamma-aminobutyric acid receptor	GABAR
green florescent protein	GFP
intraperitoneal	i.p.
kainic acid	KA
long-term potentiation	LTP
N-methyl-D-aspartate	NMDA

N-methyl-D-aspartate receptor	NMDAR
paraformaldehyde	PFA
phosphate buffer	PB
phosphate buffered saline	PBS
post-natal day #	P#
potassium–chloride cotransporter 2	KCC2
sodium-potassium-chloride cotransporter 1	NKCC1
spontaneous excitatory postsynaptic currents	sEPSCs
spontaneous inhibitory postsynaptic currents	sIPSCs
subcutaneous	s.c.
subdermal wire electrodes	SWE
tet response element	TRE
tetracycline transactivator	tTA

1. Introduction

1.1 Overview of Epilepsy

Epilepsy is defined as an individual having recurrent unprovoked seizures and is one of the most common neurological disorders affecting almost 50 million individuals worldwide (Michael & Ellul, 2019). According to the most recent estimates from Statistics Canada, approximately 0.4% of the population is diagnosed with epilepsy; this makes it a particularly pressing issue within Canada today (Gilmour, Ramage-Morin & Wong, 2016; Statistics Canada, 2012). Typically, the brain maintains a balance of excitation and inhibition with the neurones firing in a partially synchronized and desynchronized fashion – when this normal balance is disrupted, one may experience a seizure (Smith, 2005). Seizures are defined as the synchronous firings of many neurones, although the exact manifestation of this activity in the brain can differ based on many factors such as seizure type and localization (Stafstrom & Carmant, 2015). Although the prevalence of epilepsy has been decreasing overall in recent years, it still affects a significant portion of the population, particularly in lower income households, where this trend of decreasing epilepsy is not present (Gilmour, Ramage-Morin & Wong, 2016; Michael & Ellul, 2019).

1.1.1 Epilepsy Diagnosis, Classification, and Treatment

Individuals must meet a defined set of criteria to be diagnosed with epilepsy. According to the International League Against Epilepsy, epilepsy is clinically defined as a condition where a patient has two or more unprovoked seizures over twenty-four hours apart, a seizure in a patient with a previous diagnosis of epilepsy syndrome, or a single seizure in a patient with certain criteria that raise the likelihood of more seizures (Fisher et al., 2014). Regarding practical epilepsy diagnosis, using an electroencephalogram (EEG) is the most common method once a

seizure is suspected. Particularly in recent years, computer learning algorithms have greatly advanced the field (Sinch & Malhotra, 2022; West, Woldman, Oak, McLean, & Shankar, 2022). These programs can not only successfully diagnose epilepsy but do it using shorter EEG recordings and can catch events that could be missed in a manual analysis. They can also be used in some cases to determine the type of seizure.

Seizure types are defined into three broad categories: unknown seizures, focal seizures, and generalized seizures (Berg et al., 2010). Unknown seizures are characterized by a brief synchronization of neurones called an epileptic spasm. Focal seizures are classified by seizure activity in a localized region of the brain. Finally, generalized seizures involve widespread seizure activity across many brain regions. Despite how common epilepsy is worldwide, there is still much unknown about the disease.

More than 50% of epilepsy cases globally have no known cause (Naghavi, 2019). This coupled with the present gaps in treatment mean there are many diagnosed with epilepsy that are without sufficient care. In fact, only two thirds of individuals diagnosed with epilepsy can have their condition managed with pharmacological intervention alone (Thijs, Surges, O'Brien & Sander., 2019). The remaining individuals may need to resort to more invasive interventions, such as resective surgery, severing the corpus collosum, or deep brain stimulation (Culler & Jobst, 2022; Foust & Wong, 2022). While these have been largely helpful in addressing those with drug resistant epilepsy, there are still those who have uncontrolled epilepsy after these interventions. Due to these gaps in both knowledge and treatment, we have realized understanding the diversity of epilepsy will help advance the field. Expanding our understanding of epilepsy's pathophysiology is critical to making progress, which is why future research should

not solely focus on the different causes of epilepsy but also how age and the development of the brain play a role in its etiology.

1.1.2 Early Life Epilepsy

Epilepsy is more common in infants and children than in adults; estimates vary, but it appears to affect between 0.5-3.3% of children before they reach the age of 18 (Aaberg et al., 2017; Gilmour et al., 2016; Russ, Larson, & Halfon, 2012). This increase in the incidence of ELS is thought to be, in part, due to the elevated ratio of excitatory to inhibitory synaptic strength and the highly plastic nature of the early life brain. There is a dramatic change in the first year of life as the brain matures, with a high level of synaptogenesis, neurone migration, and myelination driving this progression (Bakken, Miller, & Ding, 2016). In conjunction with the associated change in gene expression, the brain is vulnerable not only to the onset of epilepsy but also to increased associated adverse outcomes later in life.

ELS is associated with a wide variety of comorbid syndromes (Berg, Tarquinio, & Koh, 2017). Seizures are comorbid with many neurological disorders such as cerebrovascular disease, central nervous system neoplasm, and general brain malformation. Regarding somatic comorbidities ELS is associated with visual impairments, sleep disruptions, and metabolic disorders in addition to many more. However, some of the most common comorbidities with ELS are cognitive and psychiatric disorders (Berg, Tarquinio, & Koh, 2017; Pellock, 2004; Sillanpaa, 2004).

Children whose epilepsy onset is earlier show lower cognitive scores when compared to those where epilepsy onset was later in childhood (Cormack et al., 2007). Additionally, more than 85% of children that have epilepsy onset in the first year show intellectual dysfunction. Beyond intellectual ability, there is also an increase in behavioural disorders among those

diagnosed with epilepsy; this is particularly apparent in children. Aberrant behaviour in epileptic children can include being withdrawn, aggressive behaviour, anxiety, social interaction deficits, and depression (Choudhary, Gulati, Sagar, Kabra, & Sapra, 2014; Leniger, Driesch, Isbruch, Diener, & Hufnagel, 2003). In fact, some report that these comorbid syndromes are more debilitating than the seizures experienced (Hamiwka & Wirrell, 2009). It is not only during childhood that these cognitive and social deficits are present as the impact also extends far into adulthood too, regardless of if there is epilepsy remission (Pellock, 2004; Sillanpaa, 2004). This further exemplifies why research needs to focus on understanding the unique characteristics of ELS on the brain. We need to understand these critical periods and the interactions epilepsy has with development and comorbidities to develop novel treatments for those affected.

1.1.3 Sex Differences in Early Life Epilepsy

Population studies have revealed sex differences in the incidence and type of epilepsy. Overall, males are more predisposed to developing epilepsy (Annegers, Hauser, Lee, & Rocca, 1995; Hauser, Annegers, & Kurland, 1993; Kotsopoulos, VanMerode, Kessels, de Krom, & Knottnerus, 2002). However, when one looks further into the subset of types of epilepsy, an interesting pattern emerges. Females show a higher incidence of febrile status epilepticus, general onset epilepsy, childhood absence epilepsy and photosensitive epilepsy (Asadi-Pooya, Emami, & Sperling, 2012; Hauser et al., 1993; Nicolson, Chadwick, & Smith, 2004; Taylor, Berkovic, & Scheffer, 2013; Taylor, Marini, Johnson, Turner, Berkovic, & Scheffer, 2004). Males, in comparison, have a higher incidence of genetic syndromes such as Landau–Kleffner syndrome and West and Lennox Gastaut syndromes in addition to severe myoclonic epilepsy of infancy (Aicardi & Chevrie, 1970; Galanopoulou, Bojko, Lado, & Moshé, 2000; Tsai et al., 2013; Widdess-Walsh et al., 2013). It is interesting to note that despite this apparent difference in

the incidence and etiology of different early life epilepsies in males compared to females, few preclinical trials or animal-based experimental studies have focused on sex differences. Some studies have shown differences in gamma-aminobutyric acid (GABA) related systems and behavioural outcomes, but this is not a comprehensively studied field (Castelhano, Scorza, Teixeira, Arida, Cavalheiro, & Cysneiros, 2010; Galanopoulou, 2008).

1.1.4 Animal Models of Epilepsy

While population data and clinical studies give great insight into the onset and cause of epilepsy, it does come with limitations, especially regarding mechanistic causes of epilepsy. For this reason, animal models are valuable for gaining a greater experimental understanding of the cellular mechanism and consequences of epilepsy. This ability to use a more controlled model has allowed for significant advancements to be made in the field of both epilepsy and early life epilepsy.

In animal models of adult epilepsy, one of the primary outcomes observed is structural damage and structural alterations to the hippocampus and other temporal lobe structures. Particularly, we see atrophy of the CA1 of the hippocampus, excessive mossy fiber sprouting in the dentate gyrus of the hippocampus, and widespread cell death in the hippocampus, particularly in the CA3 (Ben-Ari & Lagowska, 1978; Buckmaster & Dudek, 1997; Jupp et al., 2012). Aside from the cognitive and memory deficits associated with this structural alteration, these changes also result in increased susceptibility to future seizures which creates a positive feedback loop. In early life models of epilepsy, we observe these same deficits, but these gross structural changes are not always seen.

When the same epilepsy models that produce widespread structural changes in adults are used in early life, similar states of spontaneous seizures can be found without any overt damage

(Bender & Baram, 2007; Raol, Budreck, & Brooks-Kayal., 2003). This indicates that similar states of the disease can be produced but with different mechanisms. Subsequent research has since revealed that these effects are primarily brought upon by altering neurogenesis, synaptogenesis, disrupting the excitation/inhibition balance, and altering intrahippocampal signalling pathways (Bender & Baram, 2007; Holmes, 2016).

Kainic acid (KA) is one of the most common models for inducing a seizure in mice (Kandratavicius et al., 2014). KA is an analog for L-glutamate and binds readily to the primary glutamate receptors found in excitatory synapses in the brain: KA receptors, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, and N-methyl-D-aspartate (NMDA) receptors (Lévesque & Avoli, 2013). Initially created as an anti-parasitic treatment, observations of its ability to stimulate excitatory receptors in the brain shifted its use as a tool in epilepsy research. KA-induced seizures as a model of epilepsy became particularly common as it matched the structural damage seen in naturally occurring adult epilepsy (Ben-Ari & Lagowska, 1978). Its continued use has shown it to also be a good model for early-life epilepsy as kainic acid can produce status epilepticus and recurrent spontaneous seizures without overt structural damage, just like what is found in naturally occurring early-life epilepsy in humans (Holmes & Thompson, 1988).

1.2 Impaired Excitation Inhibition Balance in Epilepsy

As previously mentioned, epilepsy is defined as recurrent unprovoked seizures presenting in an individual. What will always predicate these seizures is a disruption in the excitation inhibition balance that underlies normal brain functioning (Stafstrom, 2006). Two significant mediators in this balance are excitatory Glutamate and inhibitory GABA receptors. Historically, much focus has been placed on understanding the changes in AMPARs and GABARs, but more

recently a focus on the age-dependent nature of this balance and its relation to epilepsy come into effect (Ben-Ari, Khalilov, Kahle, & Cherubini, 2012; Bradford, 1995)

1.2.1 AMPA Receptor Role, Composition and Location in the Brain

AMPA receptors, one of the aforementioned targets of KA in animal models, play a critical role in both the initial seizure and their continued pathophysiology. AMPA receptors are ionotropic glutamate receptors that are present at the majority of excitatory synapses within the brain (Rogawski et al., 2013). Thus, their composition and density in synapses have been implicated in a wide variety of fields ranging from normal functions such as learning and memory to abnormal pathology such as epilepsy. AMPA receptors are homodimers or heterodimers composed of a selection of four protein subunits: GluA1, GluA2, GluA3 and GluA4 (Lu et al., 2009). Of these four subunits, GluA2 is of particular note as it controls the calcium permeability of the receptor. In post-transcriptional editing of most GluA2 subunits found in the brain, ADAR2 (adenosine deaminase acting on RNA) acts on the intramembranous TMII region of the strand and leads to arginine instead of glutamine being coded. This change causes any receptors containing this modified subunit of GluA2 to be calcium impermeable. Detailed cell analysis has revealed that under typical conditions, over 80% of the AMPAR in the CA1 of the adult hippocampus are GluA1/2 dimers (Lu et al., 2009). Even further highlighting its role, over 99% of AMPAR contain this edited form of GluA2 in the adult brain. The blocking of calcium ion influx is often seen as protective, and it can reduce the likelihood of a cell undergoing exocytotic cell death (Henley et al., 2021). The non-edited version of GluA2 is implicated heavily in synaptic transmission plasticity and various neurological disorders (Szczurowska & Mareš, 2013).

1.2.2 AMPAR-lacking NMDAR only Silent Synapses

Silent synapses are a critical marker of maturation within the brain related to AMPARs. Briefly, silent synapses are new glutaminergic synapses that do not elicit an excitatory response. Their widespread presence was first confirmed when the number of functionally active synapses were significantly less when compared to the number of structurally identified synapses (Kullmann 1994). As previously mentioned, excitatory synapses contain three primary receptors: KA receptors, AMPARs, and NMDARs. The most widely accepted model of silent synapses suggests that the postsynaptic membrane of the synapse only contains NMDARs and does not contain a functional AMPA receptor (Hanse, Seth & Riebe., 2013). This would lead to the silencing of these synapses because at resting membrane potential, the synapse will not become active as partial depolarization is required for the NMDAR's voltage-sensitive Mg^{2+} ion to be removed from the channel and allow for receptor activation. Without functional AMPAR present to cause this membrane potential to change, it is extremely difficult for these synapses to show activity – hence the name silent synapses (Kerchner & Nicoll, 2008). The evidence supporting this theory includes morphological analysis of silent synapses showing a lack of AMPARs on the postsynaptic membrane (Nusser, Lujan, Laube, Roberts, Molnar, & Somogyi, 1998; Petralia et al. 1999) and that when exocytosis of receptors is inhibited, unsilencing and stabilization of these synapses cannot occur (Ward, McGuinness, Akerman, Fine, Bliss, & Emptage, 2006).

Throughout typical development, as critical periods pass, most of these synapses are eliminated, but some are unsilenced and stabilized with the introduction of functional AMPARs via exocytosis (Szczurowska & Mareš, 2013). Although this is still a developing field, overall, it is thought that consistently correlated pre-and post-synaptic activity, formation of perineuronal nets, and consistent inhibitory GABA activity leads to the stabilization of these synapses into

functional synapses and this lack of activity to their elimination (Hans et al., 2013). Thus, silent synapses are largely eliminated by the end of development, except for on GABAnergic inhibitory neurones. However, there is a critical period of vulnerability before this occurs where indiscriminate unsilencing of these silent synapses can lead to aberrant function. This period allows atypical factors to alter development and lead to long-term consequences for the individual—epilepsy is one such disorder. In particular, the hyperactivity seen in seizures may lead to this increased unsilencing of synapses (Zhou et al., 2011; Sun et al., 2018), and this increase in excitatory synapses between neurones may lead to increased susceptibility to future seizures, especially in the hippocampus (Hans et al., 2013).

1.2.3 AMPA Receptors as a Target in Epilepsy

One of the critical aspects of AMPARs is that their state in the synapse or on the cell membrane is constantly shifting. Under standard conditions, around 50% of AMPARs are contained within the cell and have no biological function (Greger, Khatri, Kong, & Ziff, 2003; Holman & Henley, 2007). Of the remaining AMPARs, between 50-80% are not located at the synapse. These receptors' location and composition are critical for normal brain functions such as synaptogenesis and long-term potentiation (LTP). The location and composition of surface AMPARs is also critical when examining the pathology of epilepsy.

Although determining if the AMPAR changes have a causal or resultant relationship with epilepsy is difficult, research has consistently shown an alteration in both the subunit composition of AMPARs and the number of synaptic and non-synaptic surface receptors in epilepsy. For example, an increase in the GluA1/GluA2 receptor ratio indicates that more calcium-permeable receptors are being created (Henley et al., 2021). Additionally, mutations in various genes related to AMPAR activity or expression have predisposed individuals toward

epilepsy, further indicating their importance (Salpietro et al., 2019; Tomita et al., 2005; Tomita et al., 2007).

Early in life, AMPARs show different expression compared to the adult brain. One of the most notable is the expression profile of the subunit receptor GluA2 (Szczurowska & Mareš, 2013). Early in development, there is a marked decrease in the expression of GluA2 and a relatively higher expression of GluA3 and GluA4. The increased amount of calcium-permeable AMPARs readily available is thought to contribute to the increased risk of epilepsy development in early life. However, within the AMPAR trafficking process may lie one of the reasons that cell death is less likely in early life due to a seizure. This is because, after the initial seizure, the composition for AMPAR subunits temporarily changes to decrease the number of GluA3/GluA4 subunits and increase the number of calcium impermeable GluA2 subunits (Szczurowska & Mareš, 2013). This is thought to allow for transient protection against exocytotic death seen after a seizure (Hu, Ondrejcak, & Rowan, 2012).

1.2.4 GABA Receptor Composition and Location in the Brain

GABA and GABARs compose one of the key inhibitory systems in the brain (Briggs & Galanopoulou, 2011; Treiman, 2001). There are three types of GABA receptors: $GABA_A$, $GABA_B$, and $GABA_C$. Both $GABA_A$ and $GABA_C$ are ionotropic receptors and allow chloride and bicarbonate to pass into the cell, causing hyperpolarization under normal adult functioning. $GABA_A$ receptors are composed of 5 subunits. Typically, you have two α subunits, two β subunits, and the fifth most commonly being a δ or γ subunit (Briggs & Galanopoulou, 2011). The various subunits contribute to different pharmokinetic properties and location on the cell membrane (Kasaragod & Schindelin, 2019). $GABA_C$ has a very similar structure and function to $GABA_A$, however, it differs in subunit composition. $GABA_C$ is composed of 5 identical ρ

subunits. Although most highly concentrated in the retina, $GABA_C$ is found in many other locations in the brain such as the limbic system (Calero et al., 2011; Cunha, Monfils, & Ledoux, 2010).

$GABA_B$ receptors are metabotropic receptors that have a slow inhibitory effect by decreasing calcium and subsequent signalling cascades (Treiman, 2001). Its structure is that of a G-coupled protein receptor and it is always found in a heterodimer of R1 and R2 subunits (Terunuma, 2018). $GABA_B$ enacts its effect by decreasing neurotransmitter release and altering transcription in a way that modifies the excitability of the neurone (Briggs & Galanopoulou, 2011; Terunuma, 2018). Together these three GABA receptor subtypes create both a short and a long-term inhibitory effect.

1.2.5 Age-Dependent Function of GABA and its Role in Epilepsy

The inhibitory effect that $GABA_A$ and $GABA_C$ receptors have on neurones is entirely dependent on the chloride concentration within the cell (Ben-Ari, Khalilov, Kahle, & Cherubini, 2012). In the typical adult brain, there will be higher concentrations of chloride outside of the cell, so when these passive channels open, there is an influx of negative ions leading to hyperpolarization. The maintenance of this balance is primarily determined by cation-chloride cotransporters. Although many cotransporters play a role in determining chloride concentration, two of the principal transporters within the brain are KCC2 and NKCC1 (Ben-Ari et al., 2012; Rivera, Voipio, & Kaila, 2005). KCC2 is a potassium-chloride symporter that removes potassium and chloride from the cell, and NKCC1 is a sodium-potassium-chloride symporter that increases cellular sodium, potassium, and chloride concentrations. In adulthood, there is a much higher concentration of KCC2, largely contributing to the hyperpolarization effects of GABA. However, in early life, it is an entirely different story as GABA during this period acts as an

excitatory neurotransmitter. A large concentration of NKCC1 and a low concentration of KCC2 contribute to this effect. This switch appears to be driven mainly by the KCC2 concentration reversal that begins at P6 (Riveral et al., 2005).

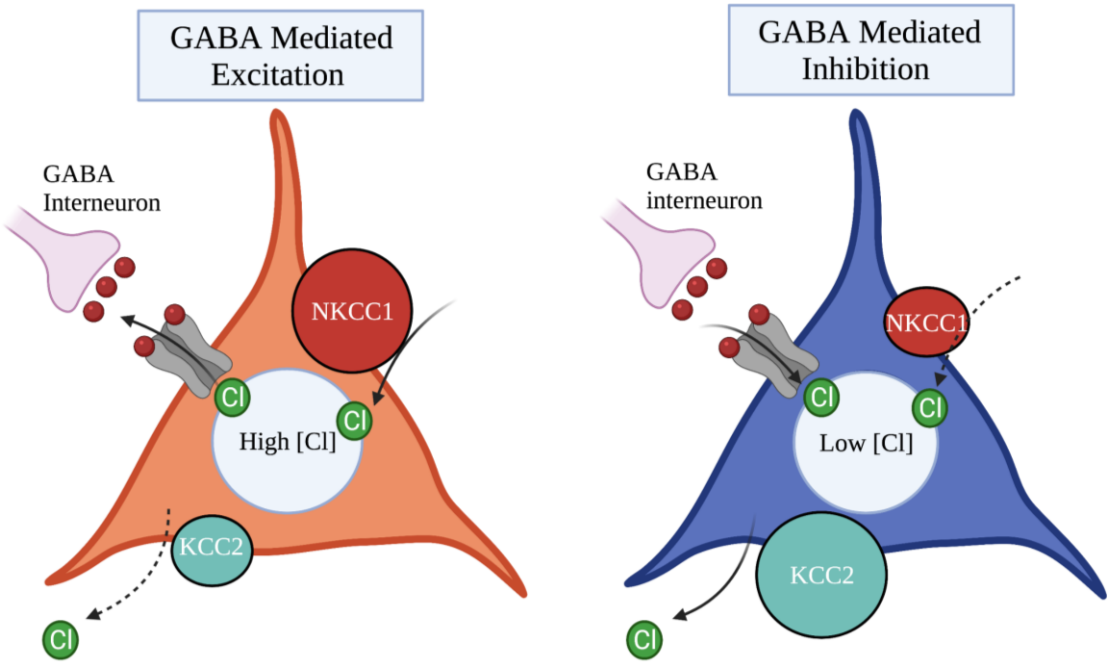

Figure 1: Role of NKCC1 and KCC2 in the excitation inhibition balance of CA1 pyramidal neurones. High levels of NKCC1 and low levels of KCC2 lead to GABA-mediated excitation. However later in development the amount of these cotransporters shifts towards low levels of NKCC1 and high levels of KCC2 leading to GABA-mediated inhibition.

This has implications for epilepsy treatments in humans as we typically find that traditional epilepsy treatments that include GABAergic inhibition are less effective or completely ineffective in the first year of life (Briggs & Galanopoulou, 2011). This is thought to be influenced by the excitatory role GABA plays at this time. Therefore, further understanding of what mediates this change, the timeline of such a change, and how we can use this information to treat the age group that experiences the most seizures is essential.

1.2.6 Sex Differences in AMPARs and GABARs

The glutamatergic and GABAergic systems, specifically AMPA and GABA receptors, show some distinct sex differences (Davis, Ward, Selmanoff, Herbison, & McCarthy, 1999; Wickens, Bangasser, & Briand, 2018). Female rodents tend to show a more significant AMPA receptor synaptic response in the hippocampus than male rodents (Monfort, Gomez-Gimenez, Llansola, & Felipo, 2015). This could be in part due to the increased AMPA/NMDA ratio seen in the CA1 of females compared to males or increased phosphorylation of the GluA2 subunit (Monfort, Gomez-Gimenez, Llansola, & Felipo, 2015). However, despite this increased response, females show decreased plasticity.

When discussing the sex differences in the silent synapses and epilepsy interaction, there is not a large body of literature. In fact, there is little research on the sex differences in silent synapses alone. Many studies use only male participants or do not differentiate data by sex (Ackman, Moshe & Galanopoulou, 2014). However, this is a crucial area of interest where research is beginning to expand because many areas of brain development where sex differences are seen, such as the GABAergic and glutaminergic systems, have been implicated in epilepsy. Overall, females tend to mature quicker hormonally and either quicker or at the same rate in brain development than males (Ackman et al., 2014).

Regarding the hippocampus, female rodents see an early shift towards a lower GluA1/GluA2 ratio (Bian, Zhu, Guo, Xiong, Cai, & Zhang, 2012). Behaviourally, it has been shown that males seem to show a greater social and spatial memory deficit compared to females (Castelhano, Scorza, Teixeira, Arida, Cavalheiro, & Cysneiros, 2010). However, much still needs to be understood about how seizures and sex interact regarding AMPA receptors, specifically silent synapses.

GABA shows distinct sex differences in early life, with there being a higher concentration of the neurotransmitter in the CA1 in males compared to females, however, this difference does disappear with age (Davis et al., 1999). GABA is mainly implicated in sexual differentiation in the early brain, with estradiol increasing the length of time that GABA acts as an excitatory neurotransmitter and increasing the calcium ion influx in response to GABA (McCarthy, Auger, & Perrot-Sinal, 2002). A proposed mechanism of this has been suggested that estradiol may interact with the cation-calcium cotransporters increasing intracellular chloride. Although not directly linked to estradiol, there have also been indications that the various subunits of GABA receptors have an age-by-sex interaction (Ethiraj et al., 2021). Overall, GABA shows several key sexual dimorphisms, further exploration of how this may interact with epilepsy in early life is required.

1.3 Heterogeneity of Hippocampal Neurones

Historically much research on the impact of epilepsy has been done on the brain and specific brain regions. For a considerable period, differences between brain regions in response to epilepsy were the only form of heterogeneity observed (Rempe, Mangan, & Lothman, 1995). While this has led to tremendous advancements in research, it does not fully explain the situation. Within a given brain region, it is possible that not every neuron is activated during a seizure, especially because during early postnatal development, neuronal networks at baseline are known to be both spatially and temporally heterogeneous. A better understanding of why this phenomenon occurs and why there is heterogeneity within a region in the response of cells to a seizure is a more recently proposed question. Several papers have commented on the heterogeneity of cells selected for electrophysiological recordings in epilepsy research (Feldt, Muldoon, Soltesz, & Cossart, 2013; Sparks, Liao, Li, Grosmark, Soltesz, & Losonczy, 2020).

However, little research on this difference has been conducted even with this observation. So far, most research has been focused on the difference between various interneuron morphologies and functions in epilepsy (Maglóczky & Freund, 2005). Regarding heterogeneity, it has also been observed that within active brain regions, active cells tend to form functional and spatial clusters (Szabo, Schneider, & Soltesz, 2015). However, there is still much unknown about the implications of this phenomenon and the specific role of these seizure-sensitive neurones.

Regarding early life, there is even less research on the heterogeneity of neurones within regions, especially in the CA1. We have observed in previous research that at P10, not every neurone is activated by KA in *ex vivo* hippocampal slices (Lippman-Bell, Zhou, Sun, Feske, & Jensen., 2016). It is also of interest to explore the different timelines that the GABA depolarization-to-hyperpolarization may have on each cell, as this has yet to be examined. This lack of research, in part, may have been due to the difficulty of identifying active cells and tracing their influence on subsequent pathology. However, the development of novel animal models allows us to observe this with a more detailed approach and increases our ability to further understand epilepsy. One novel model that will be utilized in this study is the use of an activity-dependent labelling and manipulating system with the designer receptors exclusively activated by designer drugs (DREADDs) technology to identify and control the activity of seizure-sensitive neurones in the developing brain.

1.3.1 TetTag Model

Our TetTag models, or specifically the c-Fos driven Tet-OFF model used in this study, is doxycycline-dependent. In this model, mice with three transgenes are used. The first gene uses a c-Fos promoter to cause the expression of green florescent protein (GFP). The second transgene is the tetracycline activator (tTA). The third transgene uses a tet response element (TRE) to drive

the expression of a DREADD G-coupled protein-coupled receptor (GPCR). The tTA transgene can only activate TRE when there is an absence of doxycycline – hence why it is called a Tet-OFF model. This model allows a specific window to be opened where the activity of cells leads to the permanent flagging of said cells with a GPCR.

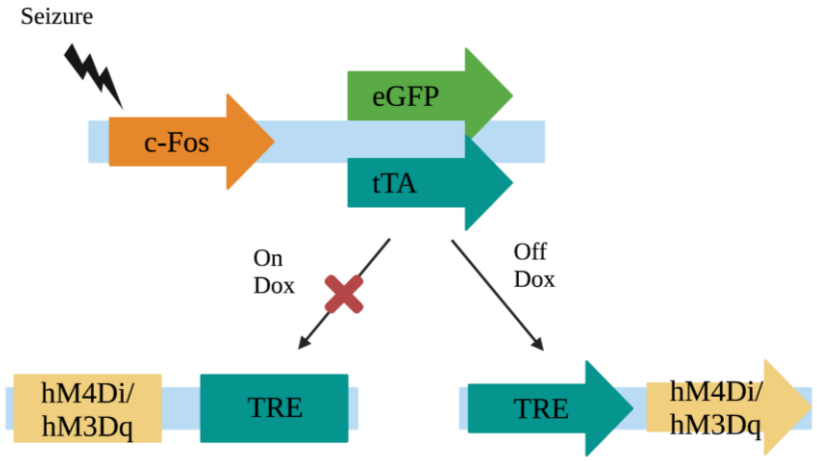

Figure 2: Schematic demonstrating the activation process of the triple transgenic model. Seizure-induced c-Fos production leads to the expression of eGFP and tTA. When the mouse is on a "dox diet," this will not cause a change, but off of a dox diet, tTA will activate TRE and lead to the production of a DREADD.

Broadly there are two categories of GPCR DREADDs used in transgenic research, Gq or excitatory GPCR DREADDs, or Gi or inhibitory GPCR DREADDs (Roth 2016). Of the excitatory GCPRs, the hM3Dq receptor is most commonly used, and of the inhibitory GPCR's hM4Di is most commonly used. These receptors are selectively activated by clozapine-N-oxide (CNO) – a drug that readily permeates the blood-brain-barrier. Overall, this model, combined with these receptors, creates an activity-dependent labelling system, and allows for selective activation or inhibition of neurones with high activity during a selected window of time – this makes it an ideal model for exploring the importance of seizure-sensitive neurones in subsequent seizure-related activity.

1.4 Rational and Experimental Aims

Epilepsy is a disorder that impacts millions worldwide, and despite many advances in recent years, over a third of patients are still treatment-resistant (Thijs, Surges, O'Brien &

Sander., 2019). Currently, this field of research is primarily focusing on improper ion channel function, the dysfunction of both excitatory and inhibitory outputs, and the influences leading to cell death (Blair, Sombati, Lawrence, McCay, & DeLorenzo, 2004; Löscher, Potschka, Sisodiya, & Vezzani, 2020; Murase, 2014; van Loo et al., 2019). Within this field, there is currently no focus on the role of the individual seizure-sensitive neuron in pathology. Therefore, the overarching purpose of this thesis is to identify what makes neurones activated by an early life seizure unique and their role in future pathology. The kainic acid model of epilepsy will be used to address this question as it has been shown to produce long term histological and behavioural deficits that are similar to those seen in human epilepsy (Koh, Chung, Xia, Mahadevia, & Song, 2005; Koh, Storey, Santos, Mian, & Cole, 1999). Considering these similarities, I hypothesize that ELS-sensitive neurones will show greater baseline activity and that their activity will be critical in the resulting seizure pathology.

1.4.1 Aim 1: To Characterize the Properties of KA-ELS Sensitive and KA-ELS Insensitive CA1 Pyramidal Layer Neurones in the Hippocampus.

Although there has been much research on the impact of ELS on the hippocampus and specifically the CA1, there has yet to be a focus on determining the difference between seizure sensitive and seizure insensitive neurones. Therefore, before exploring the role these neurones play in future seizure activity, I first want to explore how they differ from seizure insensitive cells.

Experiment 1: Does an early life seizure activate pyramidal neurones alone or both interneurones and pyramidal neurones in the CA1 pyramidal later of the hippocampus? To test this, I will use a GFP antibody to identify the activated neurones during the KA seizures. In addition, I will assess the phenotypes of the activated GFP+ neurones by using a standard

GABAergic neuronal marker, glutamic acid decarboxylase 67 (GAD67; Lariviere et al., 2002), to see if there is any colocalized staining to determine if ELS activates interneurons in addition to pyramidal cells.

Experiment 2: Do neurones activated by an ELS show any changes in the excitation-inhibition balance? To establish this, I will analyze spontaneous excitatory postsynaptic currents (sEPSCs) and spontaneous inhibitory postsynaptic currents (sIPSCs) in GFP positive CA1 pyramidal hippocampal neurones from the KA-ELS group compared to CA1 pyramidal hippocampal neurones in the control group.

1.4.2 Aim 2: To Determine Whether Activation of KA-ELS Sensitive Neurones could Evoke Seizures.

To test this, c-Fos-GFP/c-Fos-tTA/TRE-hM3Dq mice will be used. In the absence of doxycycline, KA-ELS will induce and tag the activated neurons with the excitatory receptor hM3Dq. Once the mice have been placed back on doxycycline, further hM3Dq tagging will be blocked. From P11-P17, CNO will be administered twice daily. An electroencephalograph will be used to monitor electrical seizure activity and a scoring system to monitor behavioural seizure activity during this period.

1.4.3 Aim 3: To Determine whether Inhibiting the Activity of KA-ELS Sensitive Neurones Rescues the Initial Seizure-Induced Changes.

In P10, c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mice without the doxycycline diet will experience a KA-induced seizure that will tag the inhibitory hM4Di receptor in all seizure-activated cells. Then, mice will be placed back on doxycycline and injected twice daily with the agonist for this receptor, CNO, to inhibit network alterations resulting from increased activity after a seizure. On P17, a second seizure will be induced with KA. The seizure latency, seizure

severity, and GFP activation will be compared to two control groups, one in c-Fos-GFP/c-Fos-tTA/TRE- hM4Di mice that received the same seizure and CNO treatment but do not have the hM4Di receptor embedded and one in c-Fos-GFP/tTA mice that only experience a seizure on P17.

2. Materials and Methods

2.1 Experimental Animals

P10 - P17 male and female c-Fos-GFP, c-Fos-GFP/c-Fos-tTA, c-Fos-GFP/c-Fos-tTA/TRE-hM3Dq, and c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mice with a C57BL/6 background were used for this study. All mice were kept on a 12h light/dark cycle in a room maintained at 22^{o}C. Dams were fed ad libitum. Standard diet was provided to dams with c-Fos-GFP and c-Fos-GFP/c-Fos-tTA litters. In addition, a special doxycycline (dox) diet and special control diet was provided to dams of c-Fos-GFP/c-Fos-tTA/TRE-hM3Dq and c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mice. Mice were kept on dox food except during P6-P10, which allowed for the removal of the dox from the brain and allowed hM3Dq or hM4Di expression to occur in response to seizure at P10. All experimental procedures were approved by and follow the guidelines of the Animal Care and Use Committee at Carleton University (protocols 106036 and 115763). All efforts were made to minimize animal suffering and the number of animals used.

2.2 Drug Preparation

Kainic Acid (KA; Tocris CAT# 0222) was prepared as a 1mg/mL solution in deionized water (DI) and kept at -20^{o}C. Before each use, an aliquot of KA was thawed, and a solution of either 0.1mg/mL or 0.45mg/mL was made by further diluting the liquid in DI. CNO (Tocris CAT# 4936) was prepared as a 5mg/mL solution by dissolving it in Dimethylsulfoxide (DMSO).

Immediately before use, an aliquot of this would be thawed, and a solution of 0.5mg/mL would be made by mixing the solution with 0.9% physiological saline.

2.3 ELS Model

Four experiments were conducted in this study with different time points for the induction of seizures. All seizures were elicited with an i.p. injection of KA. The dose of KA at P10 was 1mg/kg, and the dose at P17 was 4.5mg/kg. Dosage for P10 was determined by a preliminary study demonstrating that this dose would consistently induce a behavioral seizure without mouse death. Dosage for P17 was determined by establishing the minimum single dose that would consistently induce a behavioral seizure; this method was used to increase the sensitivity of experimental measures. Seizure latency, duration and severity of seizure were recorded. Severity was classified according to a modified Racine scale (Lüttjohann et al. 2009): 0 – no change in behaviour; 1 – sudden movement, arrest, freezing; 2 – head nodding; 3 – forelimb clonus, erect tail; 4 – forelimb clonus, rearing and falling movements; 5 – tonic-clonic activity with posture tone loss, jumping, and often results in death. After seizure induction, mice were monitored for 3 hours before being returned to the dam, or until euthanized for histology or electrophysiology experiments.

For aim 1, experiment 1, c-Fos-GFP/c-Fos-tTA mice were administered KA at P10. Mice were allowed to seize for 30-45 minutes before euthanization to obtain peak eGFP expression.

For aim 1, experiments 2, c-Fos-GFP mice were administered KA at P10. Mice were allowed to seize for 2.5 hours before cutting hippocampal slices for electrophysiology to ensure KA clearance from receptors.

For aim 2, c-Fos-GFP/c-Fos-tTA/TRE-hM3Dq mice were used. On P6, the dams were removed from the dox diet, and then on P10, a KA seizure was induced and continued until

cessation before the mice were placed back with their dam and placed back on the dox diet. The mice also received CNO (10mg/kg, i.p.) injections twice daily from P10-P17. The first dose was given concurrently with the P10 KA injection. Mice ages P12-P17, were selected to perform an EEG recording to detect the anticipated CNO induced seizures.

For aim 3, c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mice were used for the experimental group and c-Fos-GFP/c-Fos-tTA mice were used as a hM4Di negative control. On P6, the dams were removed from the dox diet, and then on P10, a KA seizure was induced and continued until cessation before the mice were returned to their dam and placed back on the dox diet. From P10-P17, the mice were administered 10mg/kg (s.c.) of CNO twice daily. The first dose was given concurrently with the P10 KA injection. Mice were allowed to seize for 30-45 minutes before perfusion for immunohistochemistry.

2.4 Genotyping

2.4.1 DNA Extraction

On P7, mice were ear punched, and samples were immediately placed in a sterile 1.5mL tube to prepare for extraction. Within the sample tube, 100μL of extraction solution (Sigma, CAT# E7526) and 25μL of tissue prep (Sigma, CAT# T3073) were added, and samples were vortexed and centrifuged. After a 10-minute waiting period, samples were heated at 95°C for 3 minutes before being allowed to cool for 5 minutes. Next, 100μL of neutralization buffer was added and samples were vortexed and centrifuged once more.

2.4.2 PCR

PCR primers (Invitrogen CAT# 10336022) for tTA (Primer #86112800), GFP (Primer #37792933), hM3Dq (Primer #10709436), hM4Di (Primer #10709436), and sex (Primer #86708376) were used to verify the genetic makeup of mice in this experiment. The PCR

solution for GFP and tTA was created using 10μL REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich CAT# R2523), 1μL each of forward and reverse primer, 6μL of UltraPure Sterile Water (Intermountain Life Sciences CAT# WPW-CMG-1X6) and 2μL of the extracted sample. The PCR solution for hM3Dq was created using 12.5μL KAPA2G Fast HotStart ReadyMix (Sigma-Aldrich CAT# KK5609), 1μL each of forward and reverse primer, 10.5μL of UltraPure Sterile Water and 1.5μL of the extracted sample. The PCR solution for hM4Di was created using 10μL KAPA2G Fast HotStart ReadyMix, 0.25μL each of forward and reverse primer, 7.5μL of UltraPure Sterile Water and 2μL of the extracted sample. The PCR solution for Sex was created using 10μL KAPA2G Fast HotStart ReadyMix, 0.19μL each of forward and reverse primer, 8.62μL of UltraPure Sterile Water and 1μL of the extracted sample. All heating and cooling procedures were completed using a T100™ Thermal Cycler (Bio-Rad) according to the manufacturing instructions unique to each primer. Only mice matching the genetic makeup required for their experimental group were included.

2.5 Electrophysiology

2.5.1 Hippocampal Slice Preparation

Hippocampal slices of 300μm thickness from P10 pups were prepared following the procedure previously described in Sun et al., 2018. Mice were rapidly dissected after decapitation. The brains were then placed in an oxygenated and chilled cutting solution containing (mM): 119 choline chloride, 5 KCl, 4 $MgSO_4$, 1.25 NaH_2PO_4, 0.8 $CaCl_2$, 26 $NaHCO_3$, 18 glucose, and 5 sucrose. The brain was then sectioned at 300μm using a vibrating microtome (Leica Microsystems VT1000S). Sections containing the middle third of the hippocampus were selected and transferred to a slice holding chamber containing artificial cerebral spinal fluid (ACSF) containing (mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 1.2 $MgSO_4$, 26

$NaHCO_3$, 2 $CaCl_2$, and 10 glucose, pH 7.4. The slice was kept at 35°C for 30 minutes and then kept at room temperature for at least one hour before electrophysiology recordings. The slices were then transferred to a 2.5mL recording chamber and placed in an upright Nikon FN1 microscope equipped with infrared and differential interference contrast imaging devices and constantly perfused with oxygenated ACSF at room temperature (22-24°C).

2.5.2 Whole-cell Patch-clamp Recording

Whole-cell patch-clamp recordings were made from pyramidal neurones of the CA1 pyramidal layer in the hippocampal brain slices using an Axopatch 700B amplifier (Axon Instruments, Molecular Devices), as previously described (Sun et al., 2013; Sun et al., 2018; Zhou et al., 2011). Electrodes filled with patch-pipette intracellular solution containing (mM): 0.3 GTP-Na3.110 Cs-methanesulfate, 10 TEA-Cl, 4 NaCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES, 4 ATP-Mg(Na2), and 0.3 GTP-Na3, with 5 QX-314 chloride and 7 Phosphocreatine-Na2, pH 7.25. with a resistance of 6-11MΩ were prepared from borosilicate glass capillaries with a Narishige micropipette puller (Model PC-100, Tokyo, Japan). $GABA_A$ receptor-mediated sIPSCs were recorded at 0mV holding potential. Spontaneous EPSCs (sEPSCs) were recorded at a -60mV holding potential to isolate the AMPAR mediated currents from the NMDAR mediated currents. Additionally, picrotoxin (PTX) (100 μM), which is a $GABA_A$ blocker, was applied to further pharmacologically isolate AMPAR mediated currents. Signals were filtered at 2kHZ, digitized at 10kHz by a Digidata 1550B interface, acquired by the pClamp 10.7 software, and analyzed with the Clampfit 10.7 program (Molecular Devices).

2.5.3 AMPAR Mediated and $GABA_AR$ Mediated Spontaneous Postsynaptic Responses

AMPAR-mediated sEPSCs were recorded at a -60mV holding potential. GABA-mediated sIPSCs were recorded at 0mV holding potential. A 10-minute period was used to

record sEPSCs and sISPCs, and they were analyzed further using Clampfit 10.2. sEPSCs and sISPCs events were detected automatically, and frequency and amplitude histograms were constructed using Clampfit 10.2. The threshold for detecting sEPSCs and sISPCs events was set at 5-6pA, depending on the noise level in each recording. All events were confirmed visually by observing the rise and decay times. The cumulative distributions of the sEPSCs and sISPCs were constructed from at least 10 min of recording from each cell, using a bin width of 1 pA for amplitude.

2.6 Electroencephalography

Continuous subdermal EEG recording were acquired in unanesthetized mouse pups using hand-crafted thin Ag/Agcl Teflon coated subdermal wire electrodes (SWE). The mouse pups tolerated SWE implantation and removal with minimal discomfort. The hippocampal electrical signals were recorded using Axopatch 200B amplifier (gain, 100x, Axon Instruments, Molecular Devices). Signals were filtered at 1kHZ, digitized at 2kHz by a Digidata 1322A interface, acquired by the pClamp 8 software, and analyzed with the Clampfit 10.7 program (Molecular Devices).

2.7 Immunohistochemistry

2.7.1 Perfusion

Mice were anesthetized with 0.05– 0.1mL of sodium pentobarbital (200mg/Kg, Euthasol®, Virbac USA) and perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.08 M phosphate buffer (PB). The brains were extracted from the skull and placed in 4% PFA in 0.08% PB overnight at 4^{o}C. The next day the tissue was placed in 10% sucrose in 0.08PB for 24 hours, followed by a 20% sucrose for 24 hours, then a 30% sucrose solution for 48 hours.

2.7.2 Tissue Sectioning

After 48h in 30% sucrose, 0.08M PB brains were flash-frozen using Fisherbrand Super Friendly Freeze-it (Thermo Fisher Scientific CAT# 23022524) and sectioned in a cryostat (Thermo Fisher Scientific). The tissue was sectioned at 40µm thickness. Once sectioned, tissue was placed in 0.1% sodium azide in 0.08M PB.

2.7.3 Tissue Staining

Sections were rinsed 3 x 5 mins in 10mM phosphate buffered saline (PBS). The slices were then placed in block (5% normal goat serum (NGS, Sigma CAT# G9023) and 0.3% Triton-X in PBS) for 1 hour. Immediately after the slices were incubated in the primary antibody (GFP,1:5000-Aves Labs Inc. CAT# GFP-1020; GAD67 1:1000-EMD Millipore CAT# MAB5406) overnight at 4ºC in a primary solution (2% BSA, 5% NGS, 0.3% Triton-X in PBS). The following day the sections were rinsed 3 x 5 minutes in PBS and incubated in 1:1000 concentration fluorescent secondary antibody (488 goat-anti-chicken, Thermo Fisher Scientific CAT# A11039; 488 goat-anti-mouse, Thermo Fisher Scientific CAT# A11001; 647 goat-anti-chicken, Thermo Fisher Scientific CAT# A21449) dissolved in the secondary solution (2% bovine serum albumin (BSA), 5% NGS, 0.3% Triton-X in PBS) for 2 hours at room temperature. The slices were then rinsed 3 x 5 minutes in PBS followed by a 5-minute wash in 1:5000 concentration DAPI (4',6-diamidino-2-phenylindolel; Thermo Fisher Scientific CAT# D1306) in PBS. Following another 3 x 5 PBS wash, sections were then mounted onto positively charged slides (Thermo Fisher CAT# 12-550-15) and coverslipped (Thermo Fisher CAT# 12-545M) with Flouromount (Sigma-Aldrich CAT# F4680). Slices were left to air dry overnight and sealed with clear nail polish the next day. Slides were stored at -20ºC until imaging.

2.7.4 Imaging and Quantification

The middle CA1 Pyramidal cell layer's anatomical location was determined using the Allen mouse Brain Reference atlas (2008). All images were captured using the ZEISS AX10 microscope with the digital camera (HAMAMATSU C10600 attached; Zeiss Canada, ON). The number of GFP+ cells and GAD67+ cells colocalized with DAPI were manually counted in ImageJ. The percentage of GFP+ cells was calculated by the number of GFP+ neurones divided by the DAPI+ neurones. Three sections or six hemispheres were counted for each hippocampus.

2.8 Statistical Analysis

SPSS 28 was used to conduct all statistical analyses. The data was first tested for normality using the Shapiro-Wilk test. Normally distributed data was analyzed using a two-way ANOVA with a Fishers LSD Post-hoc procedure, a one-way ANOVA with a Fishers LSD Post-hoc procedure, or an unpaired student t-test. For data that failed normality, Kruskal Wallis test with Dunn's post hoc or a Mann Whitney U test was used. For weight comparison, an unpaired t-test was used. For aim 1 experiment 1, a two-way ANOVA (sex, experimental condition) was used to analyze P10 KA-ELS activated GFP+ neurones. For aim 1 experiment 2, analyzing the excitation-inhibition balance, GABAR sIPSC frequency, GABAR sIPSC amplitude, and AMPAR sEPSC amplitude were analyzed using an unpaired t-test. AMPAR sEPSC frequency was analyzed with Mann Whitney U test. For aim 2 seizure score comparison was analyzed with Mann Whitney U test. For the experiments under aim 3 all mice without a P17 seizure were excluded from analysis. Seizure score and latency were analyzed using Kruskal Wallis test. The percentage of GFP+ neurones was analyzed using a one-way ANOVA. All data is presented as mean ± SEM and significance will be set to $p<0.05$. All figure illustrations were created with BioRender.com.

3.0 Results

3.1 Characteristics of a KA Evoked ELS

Early life seizures were reliably induced by a single dose of KA (1mg/kg, i.p.) in P10 c-Fos-GFP/c-Fos-tTA based transgenic mice with an average latency of 200 ± 15s (n=9) and an Average accumulated duration of 2.11 ± 0.13h (n = 9). There was no significant difference found between the body weights of KA and control mice at P10 (KA-ELS mice, 4.57 ± 0.27 g, n = 10; control mice, 4.48 ± 0.22 g, n = 10, p = 0.79).

3.2 KA-ELS Selectively Activates a Subgroup of CA1 Pyramidal Layer Neurones

Due to the heterogeneity of hippocampal neurones in the developing brain, our first set of experiments were designed to determine if KA-induced ELS activates a selective group of neurones in hippocampal CA1. Using a GFP antibody to identify the activated neurones in c-Fos-GFP/c-Fos-tTA mice, we first verified that under baseline conditions there is very little activation as indicated by minimal number of GFP+ cells in control mouse pups (1.22±0.49%, n=10) (Figure 3B). Importantly, we found that KA-induced ELS significantly increased the number of GFP+ cells (18.04±3.75%, n=10) compared to control mice ($F(1,16) = 208.69$, n=20, $p<0.001$) (Figure 3B-C). When analyzed across sex, however, there was no difference in main effect or the condition-by-sex interaction ($F(1,16) = 1.20$, $p=0.29$; $F(1,16) = 1.80$, $p=0.20$) (Figure 3C). These results indicate that not every CA1 neuron is activated following KA-induced ELS, demonstrating an activation of a selective subgroup of CA1 neurones in KA-induced ELS (named ELS sensitive neurones).

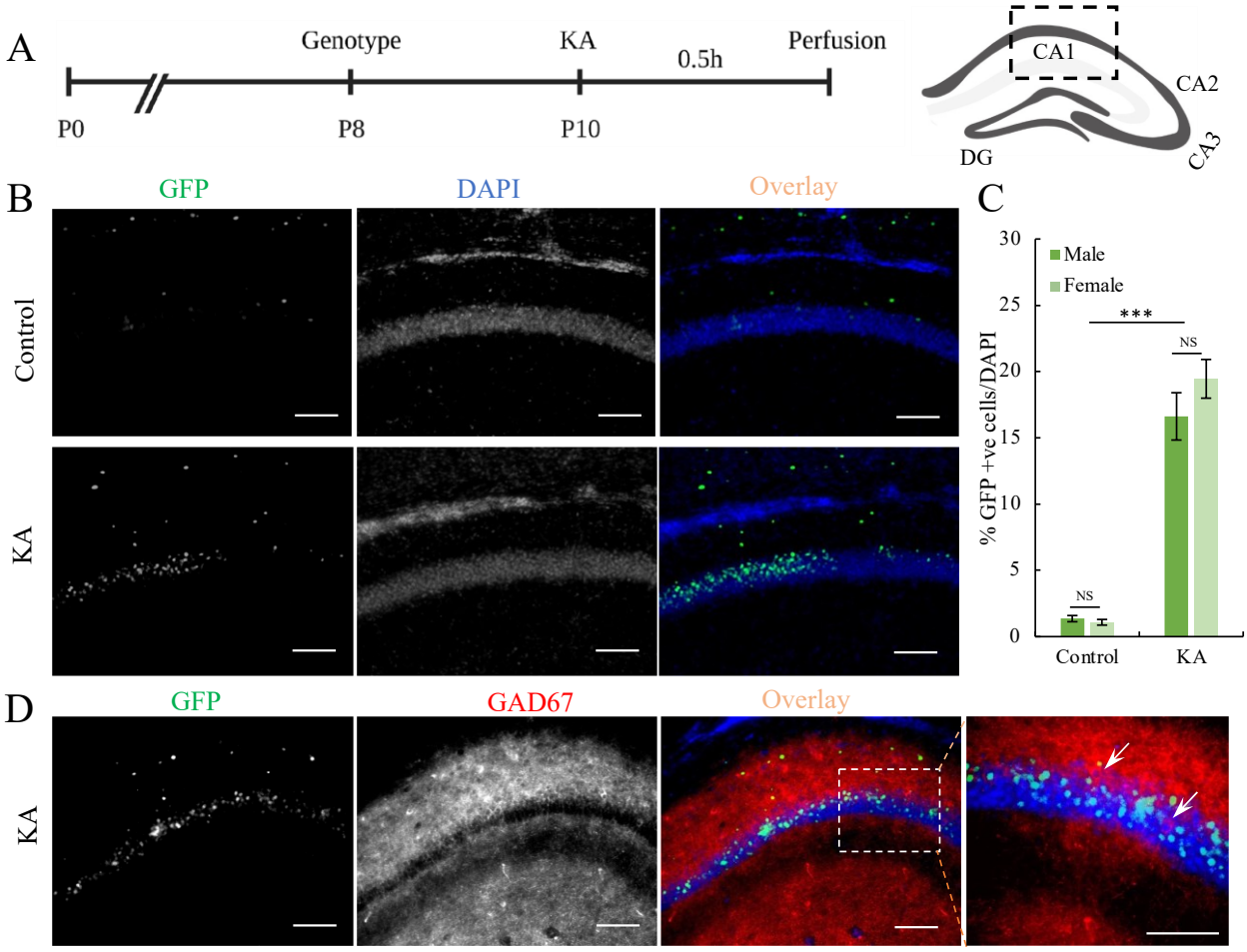

Figure 3: A KA-ELS seizure activated a selective group of CA1 pyramidal neurons. (A) The experimental timeline showing when KA administration occurred and a hippocampal schematic showing the area of CA1 imaged. (B) Images showing the GFP and DAPI separate and overlayed and that there is (C) significantly more GFP+ neurons in the KA group compared to the control. (D) GAD67+ and GFP+ staining showing that there is no colocalization found. Arrows are showing GAD67+ neurones that are GFP-. Bars represent mean, error bars SEM. ***p<0.001

To further characterize the KA-ELS activated CA1 neurones, we wanted to determine the types of neurones activated. We used a GAD67 antibody to determine if CA1 pyramidal layer interneurones are also activated by KA ELS. As shown in Figure 3D, no colocalizations of GFP+ and GAD67+ were identified in CA1 stratum pyramidal layer. This data indicates that at this age, KA-ELS selectively activates pyramidal neurones in the pyramidal later of the hippocampal CA1.

3.3 KA-ELS Activated CA1 Pyramidal Layer Neurones Show Enhanced Excitability

We next examined whether KA-ELS activated GFP+ CA1 pyramidal neurones show an elevated excitation/inhibition balance immediately following an ELS. To achieve this aim, we performed whole-cell patch clamp recordings in KA-ELS activated GFP+ cells from post KA-ELS mice and cells from littermate controls at 2.5h post ELS onset (Figure 4A). We first held the cell at the reversal potential of AMPAR and NMDAR mediated currents, 0 mV, to measure $GABA_A$ receptor mediated inhibitory sISPCs. The cell was then held at -60mV with $GABA_A$ receptor blocker PTX (0.1mM) to measure AMPAR mediated excitatory sESPCs. We found that KA-ELS activated GFP+ cells showed a significant decrease in frequency of $GABA_A$ receptor-mediated sIPSCs (0.057 ± 0.014 Hz, n=17, independent-samples t test, p=0.008) as compared to littermate controls (0.342 ± 0.088 Hz, n=15), while there is no significant difference in amplitude of $GABA_A$ receptor-mediated sIPSCs (GFP+ cells: 19.82 ± 2.36 pA, n=17; Control cells: 16.56 ± 1.47 pA, n=15, independent-samples t-test, p=0.249) (Figure 4B-D). In addition, there is no significant difference in both amplitude of glutamatergic AMPA receptor-mediated sEPSCs (GFP+ cells: 14.39 ± 1.52 pA, n=6; Control cells: 14.80 ± 1.29 pA, n=6, independent samples Mann-Whitney U, p=0.873) and frequencies of glutamatergic AMPA receptor-mediated sEPSCs (GFP+ cells: 0.024 ± 0.016 Hz, n=6; Control cells: 0.016 ± 0.010 Hz, n=6, independent samples

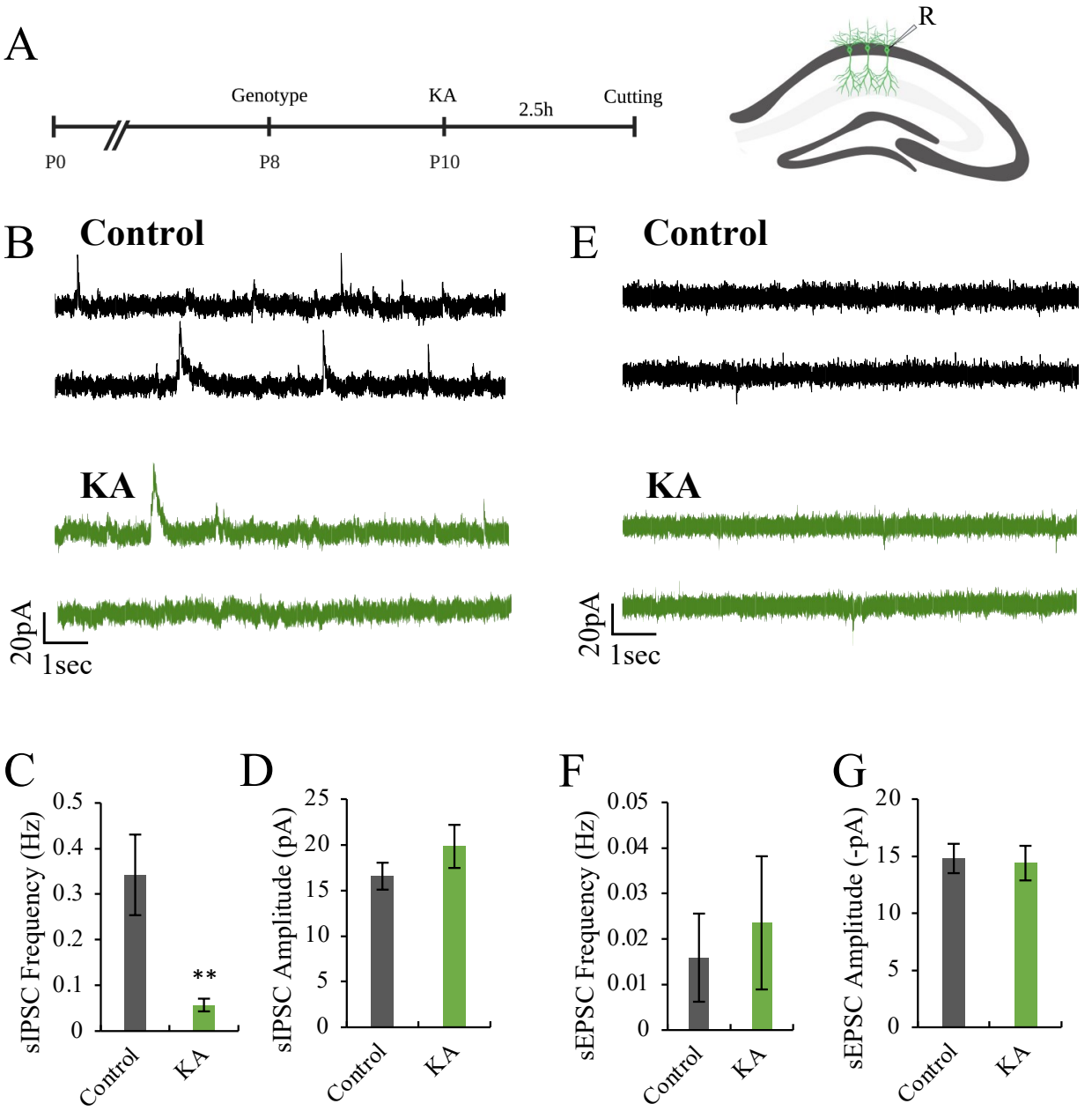

Figure 4: Electrophysiological recordings of $GABA_A$ receptor and AMPA receptor activity in GFP+ and control CA1 pyramidal layer neurones. (A) P10 mice had CA1 pyramidal neurones recorded to understand the excitation inhibition balance. (B) a comparison between $GABA_AR$ sIPSC (C) frequency and (D) amplitude found that there is a lower frequency of events in the KA group. (E) a comparison of AMPAR sEPSC activity found no difference in (F) frequency nor (G) amplitude. Bars represent mean, error bars SEM. **p<0.01

t-test, p=0.834) (Figure 4E-G). Therefore, this data supports an elevated excitation-inhibition balance in KA-ELS activated GFP+ cells, suggesting these selectively activated neurones might be a potential key player in ELS mediated long-term neural circuit changes.

3.4 Reactivation of KA-ELS Sensitive Neurones is Sufficient to Evoke Seizures

We next assessed whether precise activation of GFP+ cells would be sufficient to evoke seizures, we wanted to selectively activate these initial ELS-activated neurones and monitor behavioural seizures and electrical seizures using EEG recordings. To achieve this, a P10 seizure was induced with KA. From P11-P17 CNO was administered twice daily and behavioural seizures were observed (Figure 5B). We found that by P15, 62% of the hM3Dq mice experienced an obvious behavioural seizure of at least stage 2 and above (Lüttjohann et al. 2009). When comparing this hM3Dq mouse data to the controls where there was no hM3Dq receptor activation, we saw a significant increase in the maximum seizure score observed (hM3Dq: 2.75 ± 0.53, n=8, Con: 0 ± 0, n=6, independent samples Mann-Whitney U, $p<0.001$) (Figure 5F). It is known that behaviour does not always manifest itself in response to a seizure. To determine if electrical seizures below the behaviour threshold were occurring, we performed subdermal EEG recordings. Here, electrical seizure activities were observed in every animal, (n=4) regardless of age and previous visible seizure when CNO was administered, with no control mouse (n=2) showing epileptiform activity (Figure 5G).

3.5 Silencing of KA-ELS Sensitive Neurones Delays Seizure Onset

After establishing that we can evoke a seizure by selectively stimulating ELS neurones, we next wanted to determine if silencing their activity would prevent the network changes that would lead to an increased excitability in a second seizure. To determine this, we tagged the KA-

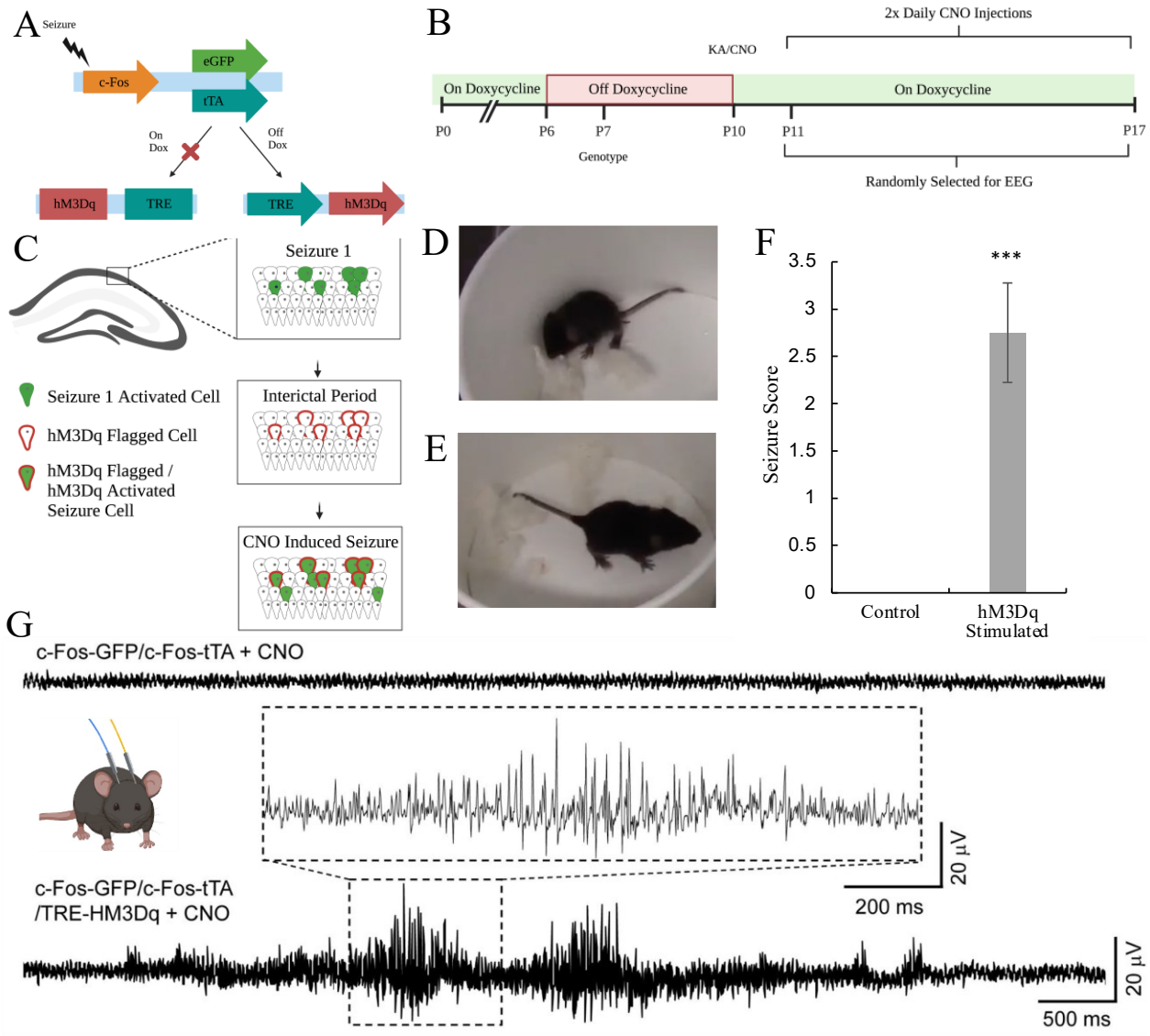

Figure 5: Reactivation of ELS sensitive neurones evoke a seizure at a later date. (A) Schematic demonstration of how being off a dox diet led to the embedding of hM3Dq receptors. (B) Timeline showing periods the dams were on a doxycycline diet compared to a control diet, in addition to when there were KA and CNO injections. Also demonstrates that EEG recordings were taking from P12-P17 when mice were selected (C) A seizure leads to embedding of hM3Dq receptors in those active cells. Mice that experienced CNO in the control group (D) did not experience any seizures whereas those in the CNO mediated hM3Dq activation (E) experienced seizures demonstrating (F) a significant difference in seizure score. (G) EEG recordings of mice that experienced CNO mediated hM3Dq activation also showed epileptiform activity compared to the control group without the hM3Dq receptors. Bars represent mean, error bars SEM. ***$p<0.001$

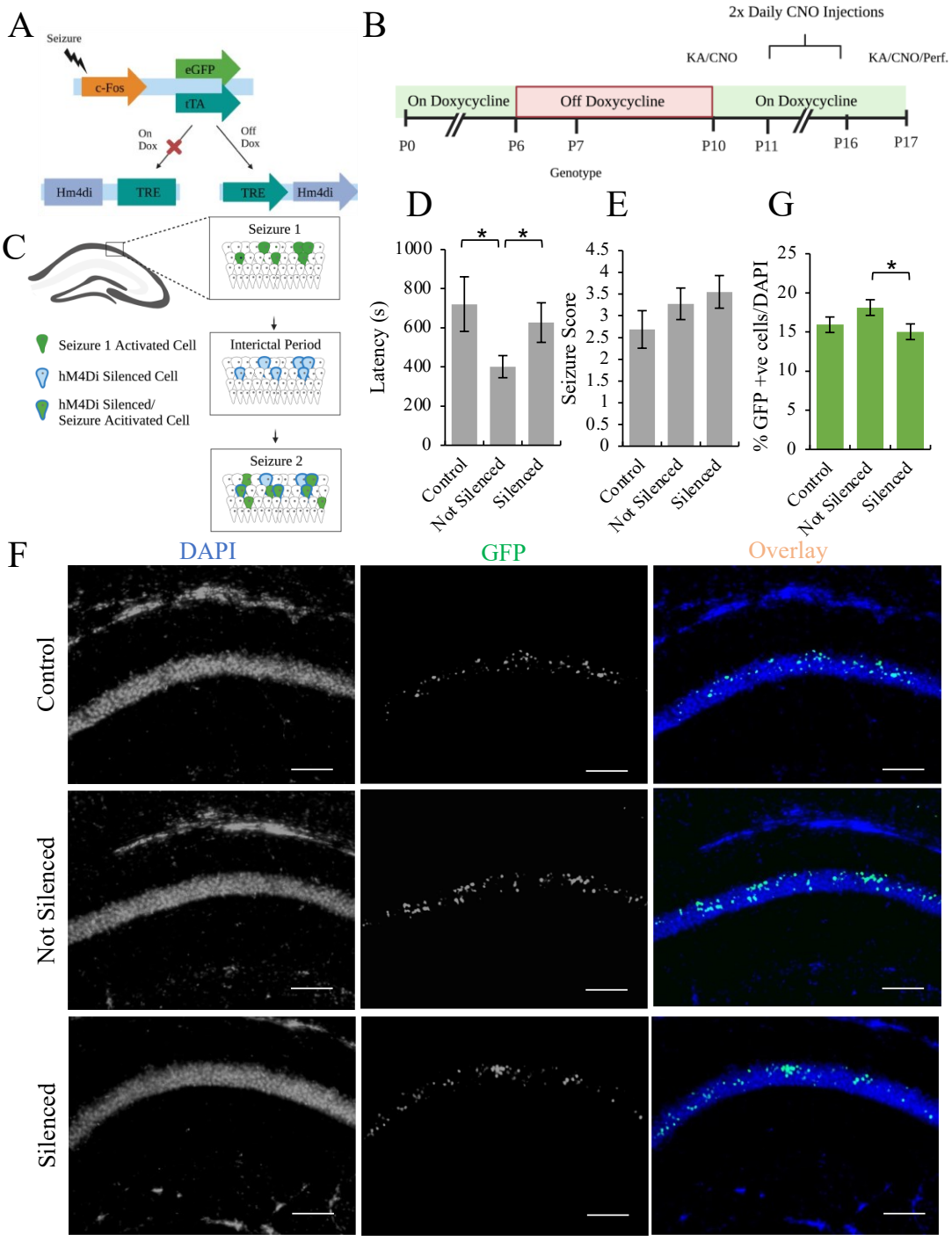

Figure 6: Silencing of ELS sensitive neurones inhibits a seizure induced changes in excitability. (A) Transgenic model showing activity dependent labelling system. (B) Timeline demonstrating periods the dams were on a dox diet compared to a control diet, and when their KA and CNO injections were. (C) A seizure embeds hM4Di receptors into the cell membrane of seizure activated neurones. (D) When stimulated there was a decrease in latency in the not silenced group compared to silenced and control. However, there was no difference in (E) seizure score (G) There was a decrease in GFP expression in the silenced group compared to the not silenced group, but not to control. (F) Shown in images. Bars represent mean, error bars SEM. *p<0.05

ELS activated neurones with inhibitory DREADD hM4Di at P10 in our unique c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mice and suppressed the activity of these neurones by daily CNO administration (Figure 6A-B). We compared the seizure latency, seizure severity score, and the percentage of GFP+ cells in response to a 2nd KA seizure challenge at P17. We found that KA-ELS at P10 induced significant reduction of latency to a 2nd KA seizure at P17 (722 ± 100 s, n=8 vs 402 ±56.4 s, n=9,), which was alleviated by suppression of KA-ELS-activated neurones with CNO (627 ± 160 s, n=10, p=0.038) (Figure 6D). While we detected significant effects of CNO treatment on seizure latency in response to a 2nd KA seizure, KA-ELS at P10 did not induce significant increases in the percentage of GFP+ pyramidal layer neurones in response to a 2nd KA seizure at P17 (15.93 ± 0.62%, n=8 vs 17.79 ± 0.99%, n=9). However, suppression of KA-ELS-activated neurones with CNO did significantly decrease the percent of GFP+ cells (15.26 ± 0.77%, n=10, p=0.047) (Figure 6F-G). In addition, no significant difference was found when examining the seizure score (F (2,24) =1.09, p=0.353) (Figure 6E). This data supports that KA-ELS activated neurones play a critical role in the early seizure-induced chronic changes in neural networks.

4.0 Discussion

Using a KA-ELS model, we report that a KA-ELS seizure activated a selective group of hippocampal pyramidal neurones in this study. These KA-ELS activated neurones show elevated excitation/inhibition balance. Interestingly, we demonstrated that selective re-activation of KA-ELS activated neurones is sufficient to evoke seizures. More importantly, we showed that selective suppression of KA-ELS activated neurones can alleviate KA-ELS induced increases in

susceptibility to additional seizures. Our data strongly support that KA-ELS activated neurones could play a critical role in ELS-induced long-term neural network changes.

4.1 Heterogeneity of Hippocampal CA1 Pyramidal Layer Neurones

During early postnatal development, neuronal networks at baseline are known to be both spatially and temporally heterogeneous. Here, we found that ~18% of CA1 pyramidal layer neurones were selectively activated in P10 pups (Figure 3C). Our further breakdown of the type of neurone using GAD67 staining led us to identify that only pyramidal neurones, and not interneurons, were activated in the CA1 pyramidal layer of the hippocampus (Figure 3D). This data supports our previous research that there is a heterogeneous response to KA in the CA1 pyramidal neurons in *ex vivo* hippocampal slices (Lippman-Bell et al., 2016).

Regarding the ~18% activation in the pyramidal layer, our next goal was to understand what characteristics made these neurones different. P10 occurs during a critical period where there is great plasticity within the brain (Szczurowska & Mareš, 2013). There is neuronal migration, receptor trafficking, and many other processes that affect activation and change the susceptibility of neurones to a seizure. Importantly, there is increased excitation/inhibition balance in the immature brain which makes the brain more excitable at baseline. Electrophysical recordings of the cells activated by an ELS indicate that there is a change in the excitation-inhibition balance (Figure 4). Interestingly this appeared to be driven by $GABA_AR$ changes, where we saw fewer spontaneous events in the ELS-activated neurons compared to the control (Figure 4B-C).

P10 is an interesting age point when it comes to GABA activity. Previous research has identified P10 as the timepoint where the hippocampus, particularly CA1, changes its response to GABA; GABA becomes inhibitory at this age (Nuñez & McCarthy, 2007). It is particularly

difficult at this age to determine what a change in GABA could mean. We could infer that since we observed a decrease in GABA, which was associated with increased neuronal activity, GABA is playing an inhibitory role at this time. However, further experiments would be required to determine if this is in fact the case. Additionally, it must be noted that these results were only obtained using male mice; it is yet unclear if this effect would be present in females. As was previously introduced, GABA activity is sexually dimorphic, with females seeming to have a delayed transition from excitation to inhibition due to estradiol (McCarthy et al., 2002). It would be of great interest to see if GABA plays a similar role at this age in females.

Another interesting result of our research is that we did not find a change in AMPAR mediated sEPSCs following KA-ELS at P10 (Figure 4E-G). As was established earlier in this paper, KA targets AMPAR, and it is the activation of these receptors that mediates the seizure response. Given that we see such a robust response in cellular activation and behavioural seizures were always observed, one would anticipate an equally robust change in the receptor responsible for such events. However, this was not the case.

Now, this does not mean that AMPAR is not affected in an ELS, just that we failed to detect it under these conditions using spontaneous event recording. Determining if there is a change in silent synapses or the AMPAR response to stimulation should also be examined. This would allow us to assess if there is a difference that may not have been apparent with spontaneous recordings alone. Another potential reason could be that changes in AMPAR function are not yet present 2.5h after a seizure. If the change is mediated by gene expression and not in the trafficking of already existing receptors, the 2.5h may not have provided enough time for the change to be observed. As our research aimed to monitor the neurones specifically activated by a KA-ELS, we were limited to a timeframe where GFP could be detected and

wanted to ensure a strong signal. However, the cFos/GFP strain of mice can show GFP expression up to 8h after a seizure. Therefore, it may be possible to observe the signal from the seizure at this time and could pose an interesting time to observe a change in future research.

Overall, what this first aim demonstrated is that there is significant heterogeneity in the neurones activated by a KA-ELS. Within the CA1, 18% of the pyramidal neurones are seizure sensitive. Historically the pyramidal neurones within the CA1 have been regarded as a homogenous group. However, recently researchers have begun recognizing that it is a diverse, heterogeneous population that varies with experience and age (McKiernan & Marrone, 2017). Based on this relatively new understanding, one underlying theory that could explain what is unique about these neurones is that they are at a different stage in development when compared to the non-KA-ELS sensitive neurones. Our KA-ELS model could activate those neurones as they are at the stage where cells may be most vulnerable to KA. However, what would make these neurones uniquely vulnerable is unknown. This study aimed to determine how this population is different post-seizure. A future study could observe the baseline characteristics of a CA1 pyramidal neurone, then administer KA on the slice and see if it reacts. This could allow one to determine the fundamental differences in these neurones that would predispose them to be KA-ELS sensitive.

4.2 Reactivating KA-ELS Neurones

We have long known that one seizure increases one's susceptibility to a second seizure – this is something that is found in both early life and adulthood (Holmes & Thompson, 1988). However, the exact role that the neurones activated in the first seizure play in this phenomenon has yet to be established. Our experiments have demonstrated how these ELS neurones in a KA model play a critical role in subsequent seizure activity. We found that simply activating seizure-

sensitive neurones was sufficient to evoke a seizure up to seven days after the initial event (Figure 5). This study has a relatively short timeframe as the focus was on understanding these neurones in early life. Determining if these consequences extend into adulthood could be an exciting direction to move forward. Future research within the constraints of this model should explore if reactivating these neurones would evoke a seizure in an adult mouse.

It should also be noted that we did not always see a robust behavioural seizure even though electrical seizure activities were consistently identified in all mice, as evidenced by EEG recordings (Figure 5G). This indicates that more sub-behaviour seizures may have been occurring and could not be detected unless there was an EEG recording. As we were using a high dose of CNO (10mg/kg) to evoke the seizure to obtain that behavioural result, a low dose of CNO could provide a novel kindling model. The current kindling models in common use do not have large specificity and can require invasive procedures, such as in amygdala kindling, that may cloud the impact of kindling alone (Wang, Wei, Yan, Luo, & Zhao, 2022). This model could provide a way to activate a specific subset of neurones at a sub-threshold dose over a long period of time to fully understand how impacting these neurones without a seizure may affect future behaviour.

One key limitation to discuss regarding this model of how we tag neurones is that we cannot make it specific to only one region. Any neurone activated in the first seizure, regardless of region, will be reactivated when CNO is administered in our hM3Dq model. We focused on the changes observed in one region – the hippocampal CA1 pyramidal layer – but without examining every region, we cannot tell the whole story of how KA-ELS neurones, dependent on their location, affect future pathology. Nevertheless, this study still shows that KA-ELS neurones will play a role in future pathology. To gain greater specificity, an injectable virus version of the

transgenic model could be used. Early in life, an injection can be given in a specific brain region, and then the role of that brain region can be elucidated. Regardless of our focus on a specific region, what is undeniable is that the ELS neurones clearly play a crucial role in future pathology.

4.3 Impact of Inhibiting KA-ELS Neurones

Further evidence that the KA-ELS neurones play a crucial role in future seizure pathology is that when their activity is inhibited, we see that seizure activity is also affected. When measuring the latency of the seizure groups, we found that an initial P10 seizure led to a decreased latency of a second seizure compared to the control group that only had the later seizure induced (Figure 6D). Furthermore, we found that when we silenced the activity of these KA-ELS sensitive neurones, we were able to recover this decreased latency. This provides strong evidence that the activity of these neurones is critical in the network and receptor changes that lead to an increased susceptibility to future seizures.

Interestingly, we did not see any significant change in seizure severity score between the control, not silenced, and the silenced group (Figure 6E). It is possible that the dose we selected was high enough to obfuscate any difference. The KA, once it started the seizure, was at a high enough dose that a severe seizure was the highly likely outcome. A future study with a slightly lower dose or multiple subthreshold doses could be interesting to investigate if a true effect may exist.

An analysis of GFP activation after the seizure also found a significant difference between the silenced and not silenced group (Figure 6 F-G). Although this indicates that a post-seizure measure of GFP may work, it may not be the most sensitive result since we found no difference in seizure severity between the pure control and the not silenced group. This may be another

situation where different dosing could show a more precise effect, but there may be another solution. An interesting measure to determine baseline activity would be to assess GFP activation between our three groups, control, silenced ELS, and non-silenced ELS, which could be measured at P17 without any seizure activity. This would give increased insight into baseline activity changes that could reflect the change in latency we did observe.

4.4 Future Directions

This project has demonstrated that ELS neurones play a critical role in future seizure pathology. There are many directions that can be taken to further expand on what has been seen. Future research could focus further on the heterogeneity and differences between ELS and control neurones by doing cell sorting. This way, the RNA profile and any changes could be examined to find future targets. This would open the door to developing knockout or knockdown mice to assess any critical gene changes, and if inhibiting this change would provide a more specific target for epilepsy treatment in early life.

Yet to be established in any research is if these ELS-sensitive neurones continue to play a critical role beyond early life, as marked by P10 and P17 in this study. Examining any deficits or changes at P60 would also be interesting to examine the longevity of these effects. This age would also allow for a new way of measuring the difference between groups: behaviour. As the middle region of hippocampal CA1 pyramidal layer was the focus of our study, no behaviour tests could be used to examine if spatial memory, the behaviour associated with this region, was affected at this age (Alberini & Travaglia, 2017). In adult mice, using various spatial memory tests could be a way to tie this research to tangible behavioural deficits. Alongside behaviour, examining if a seizure can be evoked and if increased seizure sensitivity could be inhibited using

the hM3Dq and hM4Di models respectively, would be important to determine how long ELS neurones play an effect.

4.5 Conclusion

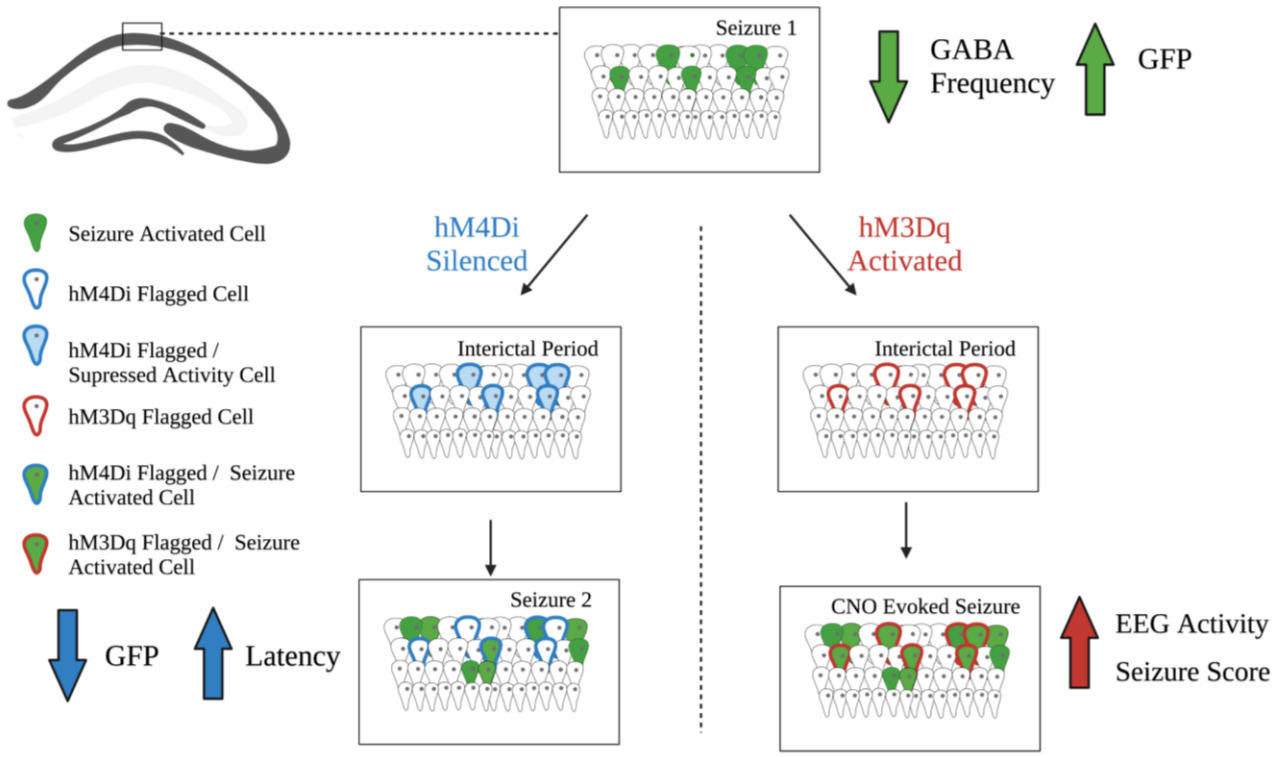

Figure 7: Summary schematic demonstrating how KA-ELS affects seizure sensitive neurones initially, and with hM3Dq or hM4Di manipulation.

Overall, we found that KA-ELS activates ~18% of the pyramidal layer neurones of the hippocampal CA1. Moreover, KA-ELS sensitive neurones show a distinct decrease in spontaneous GABA activity after a seizure. Stimulating these neurones is sufficient to evoke a seizure and inhibiting them decreases hyperactivity as measured by the increased latency and decreases GFP expression indicating a less severe seizure. This provides the critical information that KA-ELS sensitive neurones are a novel target for epilepsy research and could hold the key to a new age of targeted epilepsy treatments.

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