



Regulation of Inhibitory Signaling at the Receptor and Cellular Level; Advances in Our Understanding of GABAergic Neurotransmission and the Mechanisms by Which It Is Disrupted in Epilepsy

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Inhibitory signaling in the brain organizes the neural circuits that orchestrate how living creatures interact with the world around them and how they build representations of objects and ideas. Without tight control at multiple points of cellular engagement, the brain's inhibitory systems would run down and the ability to extract meaningful information from excitatory events would be lost leaving behind a system vulnerable to seizures and to cognitive decline. In this review, we will cover many of the salient features that have emerged regarding the dynamic regulation of inhibitory signaling seen through the lens of cell biology with an emphasis on the major building blocks, the ligand-gated ion channel receptors that are the first transduction point when the neurotransmitter GABA is released into the synapse. Epilepsy association will be used to indicate importance of key proteins and their pathways to brain function and to introduce novel areas for therapeutic intervention.

Keywords: GABAergic, inhibition, epilepsy, GABR transcription, GABA-A receptor, GABAAR trafficking

INTRODUCTION

Epilepsy is a chronic neurological disorder characterized by repeated, unprovoked seizures. Seizure prevalence in those with epilepsy can range from one seizure every few months, to hundreds of seizures a day, the latter of which is usually seen in cases of genetic epilepsies (Epilepsy data and statistics CDC, 2020). Yet, even for those individuals who only experience seizures on rare occasions, the unpredictability of seizure occurrence often results in avoidance of social situations and a loss of independence that comes from a restriction in many of the abilities we take for granted, such as driving and even crossing the street alone.

Neurological disorders characterized as epilepsy can emerge throughout the lifespan, with cases of genetic epilepsy syndromes emerging in the first few years of life, childhood-restricted epilepsies, and new onset epilepsy in adulthood (Beghi, 2020). Frequently, adult-onset epilepsy stems from some type of insult to the brain, including traumatic brain injuries, strokes, or a single prolonged seizure [status epilepticus (SE)], and is commonly referred to as acquired epilepsy, of which

temporal lobe epilepsy (TLE) is the most common (Téllez-Zenteno and Hernández-Ronquillo, 2012; Asadi-Pooya et al., 2017). At any given time, more than 1% of Americans are living with active epilepsy, and worldwide more than 50 million individuals have epilepsy (Epilepsy, 2022).

Though numerous classes of medications have come into use for epilepsy in the past several decades, nearly all of them come with a heavy load of side effects that may exacerbate symptoms co-morbid with epilepsy such as mood disorders and learning and memory difficulties (Keezer et al., 2016). Additionally, while anti-seizure medications provide seizure control for many patients, breakthrough seizures still occur, often with devastating effects. In addition, nearly one in three individuals with TLE cannot achieve seizure freedom with existing medications and often elect to undergo invasive surgery to remove portions of their temporal lobe in the hopes of eliminating the seizure loci (Asadi-Pooya et al., 2017). Perhaps more important than the unwanted side effects and lack of efficacy for many patients with epilepsy, all existing “epilepsy” medications are more correctly classified as anti-seizure drugs, as they do nothing to modify the disease, only treat the symptoms (Rogawski et al., 2016; Sills and Rogawski, 2020; Löscher and Klein, 2021).

For a long time, epilepsy has been viewed, at a most basic level, as a disorder of disrupted inhibition/excitation balance, favoring the latter and leading to repeated, synchronous firing not adequately constrained by inhibitory signaling (Fritschy, 2008; Hines et al., 2012). Initial support for this theory came from pharmacological studies, in which glutamate receptor agonists, such as kainic acid, or the toxin domoic acid, were seen to be capable of inducing acute seizures (Ben-Ari et al., 1980; Sperk, 1994; Ramsdell, 2010). Additionally, early classes of anti-seizure drugs, and indeed most of those currently in use today, work by broadly decreasing excitation, or increasing inhibition, as is the case for barbiturates and benzodiazepines, both of which augment the function of type A γ -aminobutyric acid (GABA) receptors (GABA_ARs) (Griffin et al., 2013; Rogawski et al., 2016; Sills and Rogawski, 2020; Löscher and Klein, 2021). Further evidence in favor of a disruption in inhibitory/excitatory balance in epilepsy came from early studies using animal models of acquired epilepsy, and alterations in the levels of GABA_ARs seen in surgically resected or post-mortem tissue from patients with intractable epilepsy (Schwarzer et al., 1997; Tsunashima et al., 1997; Shumate et al., 1998; Houser and Esclapez, 2003; Pirker et al., 2003; Peng et al., 2004; Nishimura et al., 2005; Zhang et al., 2007).

Replication of these studies, along with more recent immunohistochemical datasets from surgically resected tissue of patients with intractable epilepsy, have produced inconsistent findings that often do not validate earlier reports (Schwarzer et al., 1997; Tsunashima et al., 1997; Shumate et al., 1998; Houser and Esclapez, 2003; Pirker et al., 2003; Peng et al., 2004; Nishimura et al., 2005; Zhang et al., 2007; Drexel et al., 2013; González et al., 2013, 2015; Stefanits et al., 2019; Sperk et al., 2021). Additionally, recent discoveries of mutations in epilepsy syndromes in ion channels that would be expected to promote increased inhibition, such as SCN1A and potassium channel gain of function mutations, along with upregulation of inhibition

in models of absence epilepsy, suggest that E/I imbalance as a general predisposing factor for epilepsy may not be a sufficient descriptor of the condition itself (Miri et al., 2018; Shao et al., 2019; Owen et al., 2021).

Over the past 20 years there has been an expanded exploration into the basic mechanisms of inhibitory plasticity. A complex and dynamic signaling landscape has emerged that contains unique cell populations which deliver GABA into the synapse and a tightly controlled feedback system that regulates its receptors in discrete cellular compartments of specialized neuronal populations. In this review, we will examine various mechanisms that have been shown to alter inhibitory signaling, with a special focus on GABA_ARs and their efficacy as the brain's major inhibitory sensor. We will explore regulation of (1) GABA receptor trafficking, stabilization, and localization, (2) GABA_AR subunit composition (with associated changes in channel properties), (3) inhibitory synapse formation and elimination, and identification of (4) vulnerable GABAergic neuronal populations. Additionally, we will provide examples where a particular nexus of control is known, or proposed, to be dysregulated with epilepsy, either in animal models or in tissue samples collected from human epilepsy patients. Finally, we will discuss how the proliferation of single cell technologies and genetic disease models may allow for a more refined understanding of inhibitory brain dynamics that impact seizure disorders and how this may inform the development of more targeted therapies.

GABA-A RECEPTOR TRAFFICKING, STABILIZATION, AND LOCALIZATION

For GABA_ARs to exert an inhibitory effect in neurotransmission, they must be located within the plasma membrane, either at the synapse (synaptic) or away from the synapse (extrasynaptic). Given the need for fine-tuned control of inhibitory signaling, neurons possess numerous mechanisms that they use to dynamically regulate GABA_ARs at the cell surface (Lüscher et al., 2011). This includes the initial formation of the pentameric receptor, that requires specific subsets of receptor subunits to participate in an assembly process within the endoplasmic reticulum (ER) or to be tagged for turnover in a ubiquitin-dependent manner. Once GABA_ARs are assembled, they next travel to the Golgi apparatus where specific subunits can undergo modifications, such as palmitoylation, before being packaged into vesicles for trafficking to the plasma membrane. Initially, GABA_ARs are inserted at extrasynaptic sites, from which they can laterally diffuse to the synapse (Jacob et al., 2005). Following insertion into the plasma membrane, they may be stabilized extrasynaptically and/or undergo dynamic lateral diffusion between synaptic and extrasynaptic compartments, influenced by interactions with other proteins, as well as posttranslational modifications (Vithlani et al., 2011). Additionally, GABA_ARs undergo dynamic endocytosis and endocytic cycling, with additional interacting proteins, such as huntingtin-associated protein 1 (HAP1), and the phosphorylation status of a particular subunit, determining whether they are recycled back to the cell

surface membrane, or targeted to the proteasome for degradation (Lorenz-Guertin et al., 2018).

Broadly speaking, cellular processes that regulate the presence of GABA_ARs at the plasma membrane can be divided into GABA receptor trafficking, localization, and stabilization; each is discussed in turn in the following sections. [For an in-depth review of proteins and processes, see Moss et al. (2008), Luscher et al. (2011), Mele et al. (2019)].

Regulation of GABA-A Receptor Trafficking

Trafficking of Receptors to the Plasma Membrane

Trafficking of GABA_ARs begins with the assembly of the receptor subunits into a pentamer in the ER, followed by their movement to the Golgi apparatus, where the pentameric receptor undergoes additional modifications before arriving at the plasma membrane (**Figure 1E**). The $\gamma 2$ subunit, which is found in up to 90% of GABA_ARs in the brain, plays an important role in facilitating the movement of newly assembled GABA_ARs out of the ER, and varying mutations in this subunit have been discovered in cases of genetic epilepsies (Huang et al., 2014; Kang, 2017; Lorenz-Guertin et al., 2018). In fact, not only do mutations in GABA_AR subunits result in a receptor with altered functionality, but the mutant protein can suppress expression and trafficking of the wildtype protein leading to a failure of $\gamma 2$ -containing GABA_ARs to exit the endoplasmic reticulum (Kang et al., 2013; Kang, 2017). Interestingly, a recent paper by Yuan and colleagues identified Cleft lip and palate associated transmembrane protein 1 (CLPTM1) as a negative regulator of the movement of GABA_ARs to the membrane, instead trapping them in the ER and/or Golgi apparatus leading to decreased strength of the resultant inhibitory synapses (Ge et al., 2018). A tandem affinity purification assay was used to isolate YFP-tagged $\gamma 2$ from transgenic mice and mass spectrometry was used to identify proteins in complex with $\gamma 2$ -containing GABA_ARs. This assay identified 39 potential candidates, aside from those already known to interact with $\gamma 2$ that may play a role in modulating GABA_AR density at synapses. Three of these 39 interactions were validated via *in vitro* studies in transfected HEK293 cells, including CLPTM1, Integral membrane protein 2C (Itm2c), and Golgi glycoprotein 1 (Glg1). However, only the presence of CLPTM1 altered GABAergic currents, leading to decreased amplitude of GABA_AR mediated currents, but having no effect on the frequency of currents. Importantly, by preventing the progression of GABA_ARs out of the ER, and to the plasma membrane, CLPTM1 modulates both tonic and phasic inhibition.

Additional regulation of the trafficking of GABA_ARs to the plasma membrane comes from proteins that play a role in promoting re-insertion of receptors that have undergone endocytosis (**Figure 1D**). One such protein is HAP1 that, under normal conditions, promotes the trafficking of endocytosed GABA_ARs back to the plasma membrane rather than to the lysosome for endocytosis degradation (Kittler et al., 2004). Kittler and colleagues determined that direct interactions of HAP1 with endocytosed GABA_ARs protects them from degradation (Kittler et al., 2004). In fact, they demonstrate that HAP1 is a

label used by cells to prevent GABA_AR turnover and instead, promote receptor recycling back to the plasma membrane of synapses. Importantly, this leads to greater numbers of cell surface GABA_ARs without a compensatory increase in endocytosis, leading to an increased magnitude of miniature inhibitory postsynaptic currents (mIPSC's). This important study also highlighted the specificity of the HAP1- GABA_AR subunit interaction which occurs with $\beta 1$, $\beta 2$, or $\beta 3$ subunits. Given that all GABA_ARs will have one of these β subunit subtypes, HAP1 could have an important role in the receptor recycling of most GABA_ARs in the nervous system (Kittler et al., 2004).

Trafficking of Receptors Away From the Plasma Membrane (Endocytosis)

Research has demonstrated that there is a continuous cycling of GABA_ARs into and out of the plasma membrane, such that any one receptor does not remain at the cell surface for long (Thomas et al., 2005). However, under steady state conditions, there is an equal rate of insertion and endocytosis and thus no net change in receptor levels at the plasma membrane. Trafficking of receptors away from the membrane; however, can provide a means to dynamically modulate inhibitory signaling when needed by disturbing this equilibrium.

Interestingly, TNF-alpha causes increased association between $\beta 3$ and protein phosphatase 1 (PP1), leading to de-phosphorylation of key residues on the subunit. Due to this dephosphorylation event, the $\beta 3$ is no longer protected from association with endocytic machinery, mainly the adaptor protein AP2 (**Figure 1D**). Ultimately, this leads to increased endocytosis of $\beta 3$ -containing GABA_ARs and decreased inhibitory synaptic strength. In fact, Pribiag and colleagues found that TNF-alpha exposure impaired both the magnitude and frequency of inhibitory postsynaptic currents (IPSC's) (Pribiag and Stellwagen, 2013).

One important setting in which GABA_AR endocytosis plays a critical role is in activity dependent trafficking, both under physiologic and pathologic conditions (**Figure 1B**). Perhaps the most significant physiological condition under which GABA_ARs are trafficked away from the synaptic membrane is in response to excitatory signaling through N-methyl-D-aspartate receptors (NMDARs) (Muir et al., 2010; Eckel et al., 2015). Part of the role of this trafficking is to allow for excitatory synapses to be strengthened in the setting of learning, and its potential cellular correlate long-term potentiation, which would not be possible if a targeted increase in excitatory glutamatergic signaling was met with a reciprocal increase in GABAergic. And yet, despite these early findings of induction of GABA_AR dispersion due to NMDAR-mediated excitatory signaling, additional investigations have uncovered a potential role for NMDAR activation in inhibitory long-term potentiation (iLTP), and even inhibitory synaptogenesis (Marsden et al., 2007; Petrini et al., 2014; Gu et al., 2016; Chiu et al., 2018; Wu et al., 2021a).

An early paper demonstrated that the same mechanism that drives the NMDAR activation of AMPA receptor endocytosis, in the context of LTD, regulates the presence of GABA_ARs at the cell surface. This receptor-dependent potentiation of inhibitory synaptic plasticity relied on NMDAR-mediated calcium influx,

inhibitory currents. Interestingly, GluN2A-containing NMDA receptors are needed for tonic long-term potentiation in adaptation to prolonged alterations in neuronal activity. The impact of NMDAR subunit composition varied across development into adulthood. In immature neurons, signaling through GluN2B-containing NMDARs directly regulates the strength of tonic inhibition, whereas manipulation of those receptors with GluN2A was without effect. In contrast, in mature neurons, both GluN2B- and GluN2A containing-NMDARs serve to regulate the strength of tonic inhibition through promotion or blockade of endocytosis of $\alpha 5$ containing extrasynaptic GABA_ARs, respectively (Wu et al., 2021a).

In contrast, in epilepsy models where there is excessive excitation, activity-dependent trafficking of GABA_ARs can serve to make runaway excitation worse. Under normal conditions, increased neuronal activity driven by NMDA receptors leads to a calcium influx. This influx activates the phosphatase calcineurin, which then dephosphorylates Serine 327 on the GABA_AR $\gamma 2$ subunit, disrupting one of the stabilizing factors that normally keeps $\gamma 2$ -containing receptors at the synapse (Muir et al., 2010). In the absence of Serine 327 $\gamma 2$ phosphorylation, GABA_ARs are free to diffuse laterally, ultimately resulting in fewer GABA_ARs at synapses (Figure 1B). In fact, additional studies have demonstrated a role for calcineurin in the alteration of GABAergic signaling efficacy, specifically in the setting of seizures (Eckel et al., 2015). Eckel and colleagues, in an *in vitro* model of SE, found that SE resulted in the downregulation of GABA_ARs at 20 and 60 minutes after induction of SE, an effect brought about by calcineurin (which subsequent studies have demonstrated alters a key phosphorylation site on the GABA_AR subunit $\alpha 2$ so that it is no longer stabilized at the synapse) (Muir et al., 2010; Eckel et al., 2015; Nicholson et al., 2018).

Likewise, *in vivo* studies in the lithium chloride-pilocarpine SE model report that phosphatase inhibitors prevent the activity-dependent trafficking of $\gamma 2$ subunit-containing GABA_ARs away from the synapse, suggesting that indeed, calcineurin-dependent dephosphorylation of $\gamma 2$ promotes dispersal of $\gamma 2$ -containing GABA_ARs with increased neuronal activity (Joshi et al., 2015). Importantly, a similar mechanism involving calcineurin activation, downstream of a phospholipase C delta (PLC- δ)-mediated increase in intracellular calcium, has been shown to contribute to benzodiazepine pharmacoresistance in the treatment of SE in patients with long-term benzodiazepine treatment (Nicholson et al., 2018).

Regulation of GABA(A) Stabilization

While there is always an exchange of GABA_ARs into and out of the plasma membrane under baseline conditions, the interaction of specific GABA_AR subunits with selective proteins can influence the duration of time they spend at the membrane, and hence their stabilization.

The most important class of proteins responsible for the stabilization of GABA_AR populations at the membrane are scaffolding proteins which help tether receptors in specialized units (Figure 1A). Gephyrin is the most widely known scaffolding protein in association with inhibitory synapses, and loss or downregulation of gephyrin impairs clustering of GABA_ARs at the synapse (Essrich et al., 1998; Kneussel et al., 1999, 2001;

Lévi et al., 2004). Importantly, gephyrin can be regulated not just at the transcriptional level, but through its various post-translational modifications or its interacting partners that impact its association with GABA_ARs (Mukherjee et al., 2011, 2017; Tyagarajan et al., 2011, 2013; Wu et al., 2011; Kalbouneh et al., 2014; Flores et al., 2015; Ghosh et al., 2016). For example, phosphorylation of key residues on several of the receptor subunits, including Ser-359 of $\alpha 2$ and $\alpha 1$, impair the ability of these subunits and the receptors containing them to interact with gephyrin (Mukherjee et al., 2011; Nakamura et al., 2020). Gephyrin itself undergoes several key forms of post-translational modification, including phosphorylation, SUMOylation and acetylation (Tyagarajan et al., 2011, 2013; Ghosh et al., 2016). Interestingly, it seems that SUMOylation of residues within gephyrin impair its function as a scaffold for GABA_ARs and cause a resultant decrease in GABA_AR clustering and magnitude of IPSC's (Ghosh et al., 2016). Additionally, both acetylation and phosphorylation at key residues examined in this study resulted in differing impacts on the size and number of gephyrin clusters, with diminished gephyrin clustering resulting from both modifications. In fact, activity-dependent dispersion of GABA_ARs in part relies on phosphorylation of gephyrin and the resulting disruption of the clustering effects that the gephyrin scaffold provides (Flores et al., 2015).

Various intracellular pathways may alter the phosphorylation state of gephyrin, including mitogen-activated protein kinase (MAPK), glycogen synthase kinase 3 Beta (GSK3B), and cyclin-dependent kinase 5 (CDK5). Many of the exogenous factors that disrupt gephyrin clustering likely act through these pathways, including estradiol/estrogen and brain-derived neurotrophic factor (BDNF) (Tyagarajan et al., 2011, 2013; Wu et al., 2011; Kalbouneh et al., 2014; Flores et al., 2015; Mukherjee et al., 2017; Brady et al., 2018). Given the critical role of gephyrin in scaffolding at most inhibitory synapses, it is not unsurprising that disruptions to gephyrin function or structure are associated with epilepsy (Gonzalez, 2013; González et al., 2013, 2015; González, 2019; Garcia et al., 2021). For example, calpain, a key protease which gets activated during epileptogenesis, leads to subsequent cleavage of gephyrin, thus disrupting the clustering of GABA_ARs and the efficacy of inhibitory synapses (González, 2019). In a recent paper utilizing an oxygen and glucose deprivation model of excitotoxicity, the authors showed that at least two separate signaling pathways play a role in augmenting fast inhibitory signaling following an excitotoxic insult; one, which leads to disruption of GABA_AR clustering at synapses, and is dependent on calcineurin, and the second, a pathway that relies on calpain and disrupts gephyrin assembly (Garcia et al., 2021).

It is now known that gephyrin is not the only protein that scaffolds GABA_ARs at the synapse, as gephyrin-independent clustering of $\gamma 2$ -containing GABA_ARs has been identified. In a search for potential auxiliary subunits associated with GABA_ARs, like those previously seen and defined at excitatory synapses, lipoma HMGIC fusion partner-like 3 and 4 [referred to as the GABA_AR regulatory LFRPL (GARLH family proteins that contain LH3- and LH4-like protein)] were identified as putative auxiliary GABA_AR subunits that act as alternative scaffolding molecules at GABAergic synapses (Yamasaki et al., 2017). In the absence of LHFPL4, fewer inhibitory synapses develop

in a neuronal culture assay, and mice lacking LHFPL4 in cerebellar granule cells demonstrate enhanced susceptibility to seizures as well as disrupted motor behavior. Additionally, collybistin scaffolds GABA_ARs to the membrane through its binding to both GABA_AR subunits and gephyrin itself (Kins et al., 2000; Papadopoulos et al., 2008). Mutations in collybistin have been directly implicated in genetic causes of epilepsy (Shimojima et al., 2011).

Other classes of proteins are also essential to the tethering or maintenance of GABA_ARs at the plasma membrane. One such protein, neuroligin 2 (NLGN2), serves to stabilize GABA_ARs at synapses by interacting with the $\gamma 2$ subunit, in association with the GARLH protein LH4-like (see above) that helps to stabilize $\gamma 2$ -containing GABA_ARs at the synapse (Varoqueaux et al., 2006; Zhang et al., 2015; Yamasaki et al., 2017). In fact, LH4-like protein may even be important in the function of gephyrin with GABA_ARs, as its knockout (KO) leads to a decrease in gephyrin clustering (Davenport et al., 2017; Yamasaki et al., 2017). More functional diversity comes from the contribution of S-SCAM, a cell adhesion molecule that interacts with NLGN2, immunoglobulin superfamily member 9B (IGSF9B), and β -dystroglycan in its role as a GABA_AR scaffolding protein (Shin et al., 2020). Interestingly, researchers have demonstrated that the level of S-SCAM must be controlled within a tight range of expression, as both up- and downregulation attenuates the function of GABAergic synapses. Importantly, deletion of one copy of the S-SCAM gene leads to infantile spasms, one of the most severe forms of epileptic encephalopathy (Marshall et al., 2008; Kang, 2017; Shin et al., 2020).

Mutations in protocadherin 19 (PCDH19), a protein involved in stabilization of extrasynaptic GABA_ARs that contribute to tonic inhibition, are also associated with another severe form of epilepsy: epileptic encephalopathy, early infantile 9 (Dibbens et al., 2008; Smith et al., 2018; Kolc et al., 2019; Serratto et al., 2020). PCDH19 binds directly to GABA_ARs and plays a role in GABA_AR recycling and miniature IPSC's. In a study by Serratto and colleagues, normal PCDH19 expression was found to be important for baseline tonic inhibition, while its downregulation leads to reduced GABA_AR mediated tonic currents (Serratto et al., 2020).

Regulation of GABA(A) Receptor Localization

The role played by GABA_ARs in brain inhibition is influenced not only by their presence at the plasma membrane, as opposed to the abundant intracellular pool of GABA_AR subunits, but critically whether they are located synaptically or extrasynaptically. Most importantly, there is a ready exchange between synaptic and extrasynaptic sites for receptors via lateral diffusion (Thomas et al., 2005; Bogdanov et al., 2006; Bannai et al., 2009; Hannan et al., 2020).

Localization of GABA_ARs is in part influenced by their subunit composition, with certain receptor subtypes primarily localizing in one compartment vs the other (Nusser et al., 1996; Caraiscos et al., 2004; Sun et al., 2004; Chandra et al., 2006; Glykys et al., 2008). For example, historical research has

documented the primary subunit composition of GABA_ARs in the hippocampus and shown that in the dentate gyrus, synaptic receptors tend to be $\alpha 1\beta\gamma 2$ or $\alpha 2\beta\gamma 2$ receptors, while extrasynaptic are $\alpha 4\beta\delta$ -containing receptors (Nusser et al., 1996; Sun et al., 2004; Chandra et al., 2006). In contrast, in the CA1 region of the hippocampus, extrasynaptic receptors are found to be primarily $\alpha 5\beta\gamma 2$ -containing receptors, illustrating heterogeneity of GABA_ARs even within a single region of the brain (Caraiscos et al., 2004; Glykys et al., 2008). Despite the existence of specific subunit assemblies for synaptic and extrasynaptic receptors, these are not hard and fast rules. A recent study from Saad et al. demonstrated that although GABA_ARs containing specific subunits are more likely to be found synaptically or extrasynaptically, all the receptor subunits under study (including $\alpha 2$, $\alpha 4$, $\alpha 5$, and δ) could be found at the synapse (as opposed to extrasynaptically). However, certain receptor subunit combinations were found more often at the synapse than others (Hannan et al., 2020).

Interestingly, the dwell time for $\alpha 2$ and $\alpha 4$ subunit-containing receptors at synapses was longer than those containing $\alpha 5$ or δ , which could help explain why δ and $\alpha 5$ are more likely to be found extrasynaptically. Under conditions with greater need for inhibitory signaling, receptor activation can result in increased lateral mobility of extrasynaptic versus synaptic receptors, such that extrasynaptic receptors can diffuse to the synapse and lead to increased hyperpolarization/amplitude of fast synaptic signaling (Hannan et al., 2020). However, instances have been reported where such recruitment decreases rather than increases brain inhibition, as extrasynaptic receptors have a higher affinity for GABA and can desensitize rapidly in this new environment (Lagrange et al., 2007).

In addition to subunit composition, specific interacting partners of GABA_ARs impact their localization. There are numerous proteins that play a role in the localization of GABA_ARs specifically to synaptic locations. Signaling via Noga-A, one of the most potent inhibitors of neurite outgrowth in the central nervous system, helps increase localization of GABA_ARs to the synapse by preventing the lateral diffusion of GABA_ARs in the compartment (Fricke et al., 2019). Also important for synaptic localization of GABA_ARs, is protrudin that was first associated with GABAergic signaling due to studies showing an association between a mutation in its gene and hereditary spastic paraplegia (Lu et al., 2019). In their 2019 paper, Lu et al. (2019) confirmed the relevance of protrudin to epilepsy by first examining its expression levels in tissue from human TLE and animal models where they found *downregulation* was *detrimental*. They then went on to overexpress protrudin in a mouse model and demonstrated that such overexpression provided a partial rescue of the seizure phenotype in both pentylenetetrazole (PTZ) and intra-hippocampal kainic acid injection (IHKA) SE mouse models.

Moreover, protrudin overexpression produced a significant decrease in spontaneous action potentials in pyramidal neurons, and a significant increase in the amplitude of mIPSCs and spontaneous inhibitory postsynaptic currents (sIPSCs). Using drugs that selectively block tonic inhibition, these same investigators demonstrated that protrudin overexpression

increased the amplitude of sIPSCs in both tonic and fast synaptic signaling. Additionally, they found that while protrudin did not impact overall levels of $\beta 2$ or $\beta 3$ -containing GABA_ARs, it resulted in greater localization of these receptors to the postsynaptic membrane. The functional response to altering protrudin levels likely stems from its role in exocytosis, potentially through its physical interactions with the GABA_AR and GABARAP (Lu et al., 2019). As discussed above, NLGN2, is located on the post-synaptic membrane and thus serves to stabilize GABA_ARs specifically at synapses (Varoqueaux et al., 2004, 2006; Pouloupoulos et al., 2009; Zhang et al., 2015). In contrast, there are also signaling pathways and interacting partners that promote dispersion of GABA_ARs from their synaptic localization. Excitatory signaling through NMDA receptors, which causes a calcium influx and activation of the phosphatase calcineurin, leads to the dispersion of GABA_ARs from the synapse via calcineurin's dephosphorylation of key residues on the $\gamma 2$ subunit of membrane GABA_ARs, promoting their lateral mobility (Muir et al., 2010).

While not as much is known about the proteins that promote extrasynaptic localization of GABA_ARs, some important progress has been made. In a study focusing on $\alpha 5$ -subunit containing GABA_ARs that are normally located extrasynaptically, Torben and colleagues describe an anchoring protein, radixin, that typically serves to keep $\alpha 5$ -containing GABA_ARs at the extrasynaptic membrane by preventing lateral diffusion. However, dephosphorylation of radixin, via Ras homologue family member A (RhoA)/Rho-associated protein kinase (ROCK)-mediated signaling disrupts the interaction between $\alpha 5$ and radixin, allowing $\alpha 5$ -containing receptors to diffuse to the synapse as needed (Figure 1B; Hausrat et al., 2015). Thus, radixin and its interaction with $\alpha 5$ allow for restriction and release of $\alpha 5$ subunit containing GABA_ARs from their extrasynaptic location (Hausrat et al., 2015). Another recently described regulator of extrasynaptic GABA_ARs, specifically $\alpha 5$ -subunit containing GABA_ARs, is the GABA_AR auxiliary subunit Shisa Family Member 7 (Shisa7). The role of Shisa7 at inhibitory synapses was discovered incidentally when authors were trying to study the role of Shisa6–9 at excitatory synapses (Han et al., 2019). Since this initial discovery, Shisa7 has been found to play a role in both tonic and synaptic inhibition. In tonic inhibition, Shisa7 plays a critical role in the strength of tonic inhibition currents, an effect that is mediated by Shisa7's influence on localization of $\alpha 5$ -subunit containing GABA_ARs to the cell surface (Wu et al., 2021b). Specifically, Shisa7 is needed for exocytosis of GABA_ARs. Shisa7 is also critical for the dynamic regulation of the strength of inhibitory signaling and plays a role in homeostatic plasticity at inhibitory synapses, which is significantly impaired in mice with neuronal Shisa7 KO (Wu et al., 2021b).

MODULATION OF GABAR SUBUNIT COMPOSITION AND ASSOCIATED CHANNEL PROPERTIES

While the most dynamic regulation of inhibitory signaling in the brain may occur via a change in the trafficking,

stabilization, and/or localization of GABA_ARs, synaptically or extrasynaptically, a variation in the kind and/or levels of certain GABA_AR subunits may have a powerful impact on long-term changes in GABA_AR function that are associated with disease. The relative levels of various GABA_AR subunits impacts the formation of new GABA_ARs (or their failure to ever assemble and leave the ER), and the ultimate properties and localization of their resulting ligand-gated channels. However, even after the synthesis of new GABA_AR subunits, there are several additional layers of regulation at play that influence their ultimate functional contribution, and thus whether alterations in the transcript levels of unique GABA_AR subunits will ultimately impact inhibitory transmission of the nervous system is still an active area of investigation.

As briefly discussed above, the subunit makeup of GABA_ARs can play a critical role in the localization, and thus the resulting function of those receptors. Synaptic receptors are capable of mediating fast phasic synaptic transmission, whereas generation of additional receptors that localize to extrasynaptic spaces, especially $\alpha 5\gamma$ and $\alpha 4\delta$ -containing GABA_ARs, will alter tonic inhibition. On a more refined level, changes to the subunit makeup of GABA_ARs can influence what endogenous and exogenous modulators exert effects on the receptors, and even impact the dynamics of the resulting IPSC's produced with opening of the chloride channels. For detailed review of the major effects of subunit composition on GABA_AR function please see the earlier reviews of Rabow et al. and Fisher and Macdonald (Rabow et al., 1995; Fisher and Macdonald, 1997).

Alteration in the expression of GABA_AR subunit genes (GABRs) were some of the earliest transcriptomic datasets to be examined in epilepsy, both in resected patient tissue and animal models. Since the identification of these changes nearly 3 decades ago, significant work has illuminated the intracellular pathways that associate with epileptogenesis. To date, a range of molecular pathways have been implicated in epilepsy, from the Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) pathways to tropomyosin receptor kinase B (TrkB) and its neurotrophin brain derived neurotrophic factor (BDNF), REV-Erb alpha (Rev-Erb α), mammalian target of rapamycin (mTOR), among others (Kim et al., 1997, 2021; Tanaka et al., 1997; Brandon et al., 2000; Terunuma et al., 2008; Vaz et al., 2008; Weston et al., 2014; Zhang et al., 2021). Importantly, there is reason to believe that disruptions in many of these intracellular pathways are functionally relevant to the development or maintenance of epilepsy, as mutations in some of these pathways have been associated with epileptic syndromes (Kang, 2017).

Various studies conducted over the past two decades have provided us with a clearer understanding of how differential expression of heterogenous receptor subunits can define dynamic properties of GABA_ARs. In a 2014 study by Dixon et al. (2014), the authors used engineered synapses with specific subunit composition, as well as pharmacological compounds, to examine receptors containing $\alpha 1$, $\alpha 2$, $\gamma 1$, and $\gamma 2$ subunits in different combinations. Using macropatch currents and synaptic current measurements, they found that the identity of the α subunit of the receptors influenced the rate at which receptors deactivated after stimulation, with

$\alpha 2$ subunit-containing receptors displaying slower deactivation. Importantly, they demonstrated that this slower deactivation came about because $\alpha 2$ subunit-containing receptors had a greater affinity for GABA. This may have important physiologic implications, as with less GABA release, $\alpha 2$ subunit-containing receptors will be easier to activate and generate an inhibitory signal than $\alpha 1$ subunit-containing receptors (Dixon et al., 2014). Additionally, this study demonstrated $\gamma 1$ -containing receptors had a slower rise in the amplitude of inhibitory current, suggesting that the receptors were not as concentrated at synapses as $\gamma 2$ -containing receptors. The γ subunit composition of GABA_ARs also has important implications for the modulation of GABA_ARs by benzodiazepines, as $\gamma 1$ subunit-containing receptors are less responsive to benzodiazepines than $\gamma 2$ -containing receptors (Hanson and Czajkowski, 2008; Keramidis and Harrison, 2010).

The dynamics of fast synaptic inhibitory transmission are also influenced by the relative clustering or diffusivity of GABA_ARs at the postsynaptic membrane/in the synapse as well as the subunit composition of the receptors that lead to changes in kinetic properties of GABA_ARs and the IPSC's thus produced (Okada et al., 2000; Schofield and Huguenard, 2007; Eyre et al., 2012). Any alterations in subunit composition which may influence the clustering of GABA_ARs, therefore, can change the properties of the associated receptors and synapses. Interestingly, research has demonstrated that compared to other α subunits, the $\alpha 2$ subunit has lower affinity for gephyrin, which would impact its clustering properties (Maric et al., 2011).

Alterations in subunit composition of GABA_ARs is of direct relevance to epilepsy, as reflected by two lines of evidence. First, mutations in various subunit genes, including $\alpha 1$, $\beta 1$ - $\beta 3$, $\gamma 2$, and δ , are the primary ligand-gated ion channel genes found to be disrupted in cases of genetic epilepsy (Kang, 2017). More recent studies have found mutations in $\alpha 5$ specifically in cases of epileptic encephalopathy (Hernandez et al., 2019). Additionally, several decades of studies in animal models, and more recently in tissue resected from human cases of TLE, report alterations in the levels of varying receptor subunits, which may represent compensatory responses or alternatively, pathological changes that serve to contribute to epileptogenesis (González et al., 2013, 2015; Sperk et al., 2021). Mixed results, however, are reported in the literature that may reflect differences in the particular epilepsy model that was used and/or the time of data collection since the onset of SE.

Taken together, studies employing the pilocarpine (PILO) model of SE-induced TLE in mice, and rats, differ in which GABA_AR subunit, and subunit mRNAs, have alterations in expression. This lack of reproducibility may depend on the time elapsed since SE, and the region of brain examined. Overall, however, most studies examining levels of $\alpha 5$ and delta subunits, typically associated with tonic inhibition, report these subunits to be downregulated across most regions of the hippocampus and even in the entorhinal cortex (EC) and subiculum in the long-term after SE, once spontaneous seizures arise in these models (Tsunashima et al., 1997; Houser and Esclapez, 2003; Peng et al., 2004; Nishimura et al., 2005; Zhang et al., 2007; Drexel et al., 2013). Additionally, numerous studies examining

expression levels between 1-90 days post-SE have found increased levels of $\alpha 4$ and $\gamma 2$ subunits, and a decrease in $\alpha 1$ subunit expression, as well as an altered localization of $\gamma 2$ -containing receptors to perisynaptic locations (Peng et al., 2004; Zhang et al., 2007; González et al., 2015). In some studies, the increased levels of $\gamma 2$ and $\alpha 4$ transcripts and protein seem to be maintained in the chronic stages, but in others they do not (Tsunashima et al., 1997; Drexel et al., 2013; González et al., 2013). Alterations in $\beta 1$ - $\beta 3$ transcript and protein levels, as well as additional α subunits, have been described across numerous studies, but display less consistent patterns of change (Schwarzer et al., 1997; Tsunashima et al., 1997; Nishimura et al., 2005; Drexel et al., 2013; González et al., 2013, 2015). Results from studies conducted in tissue resected from human TLE patients undergoing surgical resection as a treatment for epilepsy, have also been varied and sometimes inconsistent with findings from animal models (Shumate et al., 1998; Pirker et al., 2003; Stefanits et al., 2019; Sperk et al., 2021). However, it may be important to keep in mind that in studies conducted using animal models, even with tissue harvested from animals with "chronic" epilepsy, the duration and severity of epilepsy, and the resultant remodeling of neural circuits, is likely less extensive and variable than the alterations present in resected human tissue. These cases represent only the most severe forms of human TLE that are insufficiently responsive to anti-seizure drug therapies.

Given the significant implication of subunit composition to the functional properties of GABA_ARs, along with clear evidence demonstrating alterations in mRNA levels and protein expression of numerous receptor subunits in animal and human TLE cases, an important area of past and current research exists for the development of new epilepsy therapeutics based on identifying the intracellular pathways that contribute to the genome expression of GABRs. Work from our own lab, in collaboration with our colleagues in the Brooks-Kayal lab, has helped to identify the intracellular signaling that leads to the transcriptional alterations in $\alpha 1$ and $\alpha 4$ gene expression in the context of epilepsy (Brünig et al., 2001; Hu et al., 2008; Lund et al., 2008). We were the first to show that these genes are differentially regulated by BDNF and that their levels are altered in the PILO model of TLE in response to increased BDNF release that is the immediate aftermath of SE (Roberts et al., 2006; Lund et al., 2008). Functionally, this leads to an increased presence of $\alpha 4\gamma 2$ containing receptors in hippocampal neurons that replaces $\alpha 1\gamma 2$ (Lund et al., 2008).

A unique property of GABA_ARs is the negative feedback regulation of their levels that occurs upon chronic exposure to GABA, which also produces an uncoupling of the receptor response to positive modulation by benzodiazepines, first described by Farb and colleagues in 1990 (Roca et al., 1990). While downregulation of GABA_AR levels in response to GABA can be the product of complex intracellular pathways for endocytosis and degradation, as discussed above, it can also occur directly at the level of gene promoter engagement as was shown for *GABRB1* through a binding activity at the initiator sequence InR that contains the transcriptional start site (Russek et al., 2000). Recognition of this site by the polycomblike protein PHF1b and its association with Suz12 suggests that GABRs

may be epigenetically regulated via InRs that act as sensors for overactive GABA signaling (Saha et al., 2013). Such chromatin regulation in additional subunit genes and its relationship to disorders such as epilepsy remain to be described.

In contrast, the core promoter of *GABRA1* (*GABRAp*) through sequences upstream of the InR can be upregulated via the binding of the c-AMP response element binding protein (CREB) in response to protein kinase C signaling that solidifies its role in learning and memory, as well as in synaptic plasticity (Hu et al., 2008). However, even when CREB is bound to *GABRA1p*, expression of the inducible c-AMP early repressor (ICER) can convert the “On” response to “Off”. Interestingly, this can occur in response to c-AMP signaling or in the case of BDNF, through a specific activation of the Janus Kinase (JAK)/signal transducer and activator of transcription (STAT) pathway that is dependent on TrkB (Figure 2; Raol et al., 2006; Lund et al., 2008). In parallel, BDNF signaling upregulates *GABRA4p* and GABAR subunit $\alpha 4$ transcript and protein levels through induction of early growth response factor 3 (EGR3) via MAPK signaling (Roberts et al., 2005, 2006). Increases in both $\alpha 4$ and Egr3 have been reported during spontaneous seizures weeks after SE and accompany increases in BDNF (Grabenstatter et al., 2014). While much is known about the JAK/STAT pathway in glia, its novel role in neurons remains to be determined (Nicolas et al., 2013). Current work in our labs is focused on this mystery using mouse genetics to unravel the relationship of JAK/STATs to other signaling pathways that control inhibitory neurotransmission via expression of the mammalian genome.

INHIBITORY SYNAPSE FORMATION AND ELIMINATION

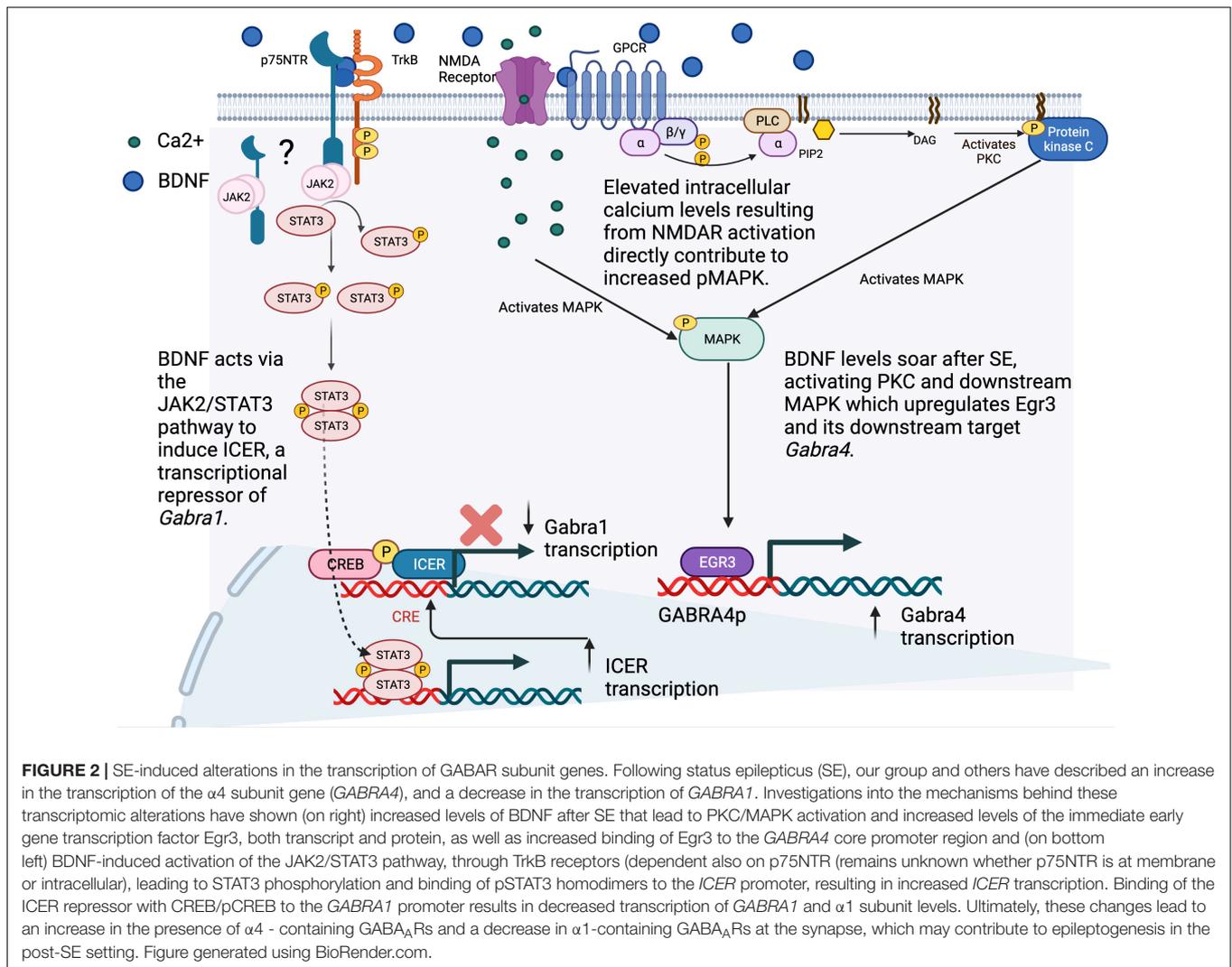
Another key regulator in the control of inhibitory signaling in the CNS is modulation of the formation, number/efficacy, and elimination of inhibitory synapses. The efficacy and number of inhibitory synapses plays an important role in how effective inhibitory signaling in the brain is at restraining runaway excitation. In fact, many of the genetic epilepsies that have been identified involve mutations that impact the function of proteins or channels that play an important role at inhibitory synapses. *SCN1A*, a sodium channel present in both excitatory and inhibitory neurons, is of particular relevance to epilepsy given that 80% of patients with Dravet syndrome, which presents with severe epilepsy in children, have loss of function *SCN1A* mutations (Marini et al., 2011). In mutant mice engineered with the human *SCN1A* mutation, heterozygous mice develop fewer inhibitory synapses than WT, and display diminished inhibitory signaling, as evidenced by smaller IPSCs at the few inhibitory synapses that do form (Marini et al., 2011; Uchino et al., 2021). Some genetic epilepsies alternatively result from mutations in genes whose proteins impact the efficacy of inhibitory synapses through presynaptic mechanisms (Figure 1C). Most of these proteins, including syntaxin binding protein 1 (STXB1), proline rich transmembrane protein 2 (PRRT2), and dynamin 1 (DNM1), are part of the machinery responsible for exocytosis of neurotransmitters, including GABA. By altering the size of

endocytic vesicles, or the release probability of these vesicles, mutations in these proteins can exert profound effects on the size and frequency of ISPCs.

Formation of inhibitory synapses, including clustering of GABA_ARs in a restricted space, is influenced by numerous factors, including essential interactions between GABA_AR subunit protein domains and scaffolding machinery within the cells. For instance, recent work by Nathanson et al. demonstrated the essential role of a particular sequence of amino acids (a motif) within the intracellular domain of the $\alpha 2$ subunit of GABA_ARs that promotes interactions between the receptors and their binding partners, collybistin and gephyrin, that helps cluster GABA_ARs to produce effective inhibitory synapses (Nathanson et al., 2019). Their evidence came from mutations to this key AA motif that prevent $\alpha 2$ from binding with collybistin, and ultimately impair GABA_AR localization to inhibitory synapses, especially axo-axonic. Previously these investigators demonstrated the relevance of $\alpha 2$ subunit interactions with collybistin by showing that disruption of $\alpha 2$ /collybistin interactions made mice susceptible to seizures and early death (Hines et al., 2018). In recent years, the importance of collybistin, encoded by *Cdc42* Guanine Nucleotide Exchange Factor 9 (ARHGEF9), to functional inhibitory synapses has become clear with the discovery of multiple patients with ARHGEF9 mutations that present with disease syndromes including epilepsy (Shimajima et al., 2011; George et al., 2021; Hines et al., 2022). Additionally, a recent study in mice demonstrated that increased expression of collybistin results in elevated GABAergic signaling and mitigates the development of seizures in an SE model (George et al., 2021).

NLGN2, in interaction with various other molecules, is also a key player in the formation of inhibitory synapses. Early research demonstrated impaired inhibitory synapse formation in mice lacking the cell adhesion molecule, NLGN2, yet only recently did researchers begin further defining which interacting partners of NLGN2 contribute to its role in synaptogenesis (Lee et al., 2013; Li et al., 2017; Wu et al., 2018).

In a study using mass spectrometry to identify binding partners of NLGN2 that may be necessary to promote development of inhibitory synapses, Wu, Tian et al showed the functional relevance of LHFPL4 to inhibitory synapse formation *in vitro* by demonstrating fewer inhibitory synapses formed in cultured neurons with LHFPL4 KO (Wu et al., 2018). Additionally, they demonstrated the functional consequences of LHFPL4 KO in cerebellar neurons as mice with this deletion display impaired motor behavior and were more susceptible to seizures than WT. In a separate study examining the interaction of Neuroligin 2 and Slitrk3 in the formation of inhibitory synapses, both Neuroligin 2 and Slitrk3 (ST3) participated in the development of functional GABAergic synapses, such that mice lacking either of these cell adhesion molecules had increased seizure susceptibility and altered network activity (Li et al., 2017). Through use of a combination of genetic KO models and RNAi-mediated knockdown (KD), Li et al. demonstrated an essential role for NLGN2, specifically in the *development* of inhibitory synapses, but after neurons mature, there is some recovery in inhibitory neurotransmission even in



NLGN2 KO mice. shRNA-KD of NLGN2 at 2 days *in vitro* (DIV2) significantly decreased GABAergic neurotransmission at DIV8, whereas shRNA-KD of ST3 at DIV2 did not, suggesting that NLGN2 is essential at that time but ST3 is not. Later in development, elimination of either ST3 or NLGN2 at DIV10 significantly decreased mIPSCs at DIV18, representative of more mature neurons, and the effects of KO were additive. MAM Domain Containing Glycosylphosphatidylinositol Anchor proteins (MDGAs), on the other hand, inhibit the role of NLGN2 in forming inhibitory synapses, as KD of these molecules in the adult brain led to increased inhibitory synapse formation and upregulation decreased the number of inhibitory synapses (Lee et al., 2013). MDGAs that interact with NGLN2 interact with NGLN2 in a cis formation (are on the same side of the synapse, postsynaptic).

Generation of inhibitory synapses is also regulated at the level of transcription, with neuronal PAS domain protein 4 (NPAS4) serving as a key transcription factor that is responsive to local neuron activity levels and plays a role in determining the number of functional inhibitory synapses that are formed

(Lin et al., 2008). Another recent study by Gu and colleagues illustrated the relevance of neuronal activity in promoting the development of inhibitory synapses, and provided one potential mechanism for the maintenance of excitatory/inhibitory balance in development. Using a mouse model with sparsely expressed, Cre-dependent KO of GLUN1 and GLUA1-3 in select neurons (totaling less than 1% of total neurons), they were able to track Cre-expressing neurons and study, at a single neuron level, the impact of AMPA and GLUN1 KO on the ability of select neurons to form inhibitory synapses (Gu et al., 2016). When neurons harvested from E18 embryos, and grown *in vitro* for 3–7 days, were recorded from, those lacking GLUN1 displayed decreased amplitude and frequency in synaptic mIPSCs. Additionally, acute hippocampal slices from these conditional KO mice demonstrated impaired GABAergic transmission, and immunostaining revealed reduced VGAT, gephyrin, and NLGN2 in Cre⁺ neurons.

When it comes to the elimination of inhibitory synapses, microglia are a key cell type involved in the process. During development, microglia play an essential role in synaptic

pruning, disruptions of which have been implicated in various neuropsychiatric disorders, including autism and schizophrenia. In addition to this developmental function, microglia continue to play a role in refinement of synaptic connections into adulthood (Andoh et al., 2019; Park et al., 2021). In order for microglia to know which synapses to phagocytose, they look for the presence of particular “tags” on them, which in the case of inhibitory synapses, seems to be stable presentation of phosphatidylserine (PTD) on the outer surface of the plasma membrane. Normally, the phospholipid-flipase chaperone protein Cdc50a regulates Ptd exposure, and the deletion of Cdc50a was found to lead to stable Ptd expression and subsequent elimination of inhibitory synapses by MER proto-oncogene tyrosine kinase (MERTK) microglial signaling (Park et al., 2021). Importantly, the role of microglia in synaptic pruning may be particularly relevant to epilepsy, as a recent review by Andoh et al. suggests that increased and dysregulated synaptic pruning in the epileptic brain, leading to the inappropriate elimination of inhibitory synapses by microglial phagocytosis, could contribute to an emergence of E/I imbalance (Andoh et al., 2019).

VULNERABLE GABAergic NEURONAL POPULATIONS

Early research into the molecular and cellular underpinnings of epilepsy focused on the potential selective loss of inhibitory neurons after insults in models of TLE. Indeed, excessive loss of inhibitory neurons compared to excitatory neurons could provide a reasonable explanation for the E/I imbalance that defines epilepsy. Early studies confirmed inhibitory neuron death in many epilepsy models, including SE models with kindling and pilocarpine (Dinocourt et al., 2003; Kobayashi and Buckmaster, 2003; Sayin et al., 2003; Dudek, 2020). However, broad inhibitory neuronal loss may cause varying phenotypes depending on the subclass of inhibitory neurons that is impacted. Early studies demonstrated that PV⁺ interneurons in the hippocampus are particularly vulnerable to insults (Bouilleret et al., 2000; Kuruba et al., 2011). Given that these PV⁺ neurons normally serve as the breaks for the key tri-synaptic circuit that projects information within and subsequently out of the hippocampus, their loss could have a profound effect on the ability of the hippocampus to dampen hyperactivity at a time when it could be managed.

One factor that is hypothesized to contribute to the differential vulnerability of specific classes of interneurons is the population of calcium binding proteins that they contain. Calcium binding proteins, including PV, calbindin (CB), and calretinin (CR), serve an important role as a buffering system for transient dysregulation in intracellular calcium levels. Thus, in the context of excessive excitatory signaling, calcium binding proteins that can buffer more calcium may provide added resilience against such insults when compared to the neuronal subtypes that primarily contain calcium binding proteins with lower buffering capacity. Of the 3 primary calcium binding proteins that are known to define distinct sets of inhibitory neurons in the hippocampus, PV has the lowest buffering capacity, while CR has the highest, which may contribute to the

almost invariant vulnerability of PV⁺ interneurons observed in animal models of TLE (Dinocourt et al., 2003; Kobayashi and Buckmaster, 2003; Marx et al., 2013). And yet, even CR-expressing interneurons in the hippocampus, which should be afforded the greatest protection against calcium influxes, appear vulnerable to degeneration in both animals and humans with TLE (Tóth and Maglóczy, 2014). While CB⁺ inhibitory neurons are largely protected against degeneration in epilepsy models, whether it is because of the calcium buffering capacity of CB, or perhaps other features of the neurons expressing CB, remains to be determined (Fairless et al., 2019). In addition to these studies, research has focused on other interneuron subsets that include those defined by the expression of specific markers, including SST, neuropeptide Y (NPY), or cholecystokinin (CCK). In an IHKA mouse model of TLE, PV⁺ interneurons displayed selective vulnerability compared to NPY⁺ neurons at 2 days and 3 weeks post-SE. This effect was seen most clearly in regions of the hippocampus closest to the injection insult, including the septal and dorsal hippocampus, but not in the temporal or ventral intermediate hippocampus in these early time points (Marx et al., 2013). In contrast, a separate study conducted using the same IHKA mouse model with KA injection into the dorsal hippocampus focused specifically on CCK⁺ interneurons and found that CCK⁺ basket cells were susceptible along the entire dorsal to ventral axis, not just in the regions immediately neighboring the insult (Kang et al., 2021). Taken together, the cellular responses of certain classes of neurons to a change in circuit behavior leaves them susceptible to irreparable damage and we need a better means to identify their nodes of engagement where balance can be restored.

With the advent and large-scale application of single cell technologies, future research will be poised to identify selectively vulnerable classes of inhibitory neurons, whose protection may offer a new therapeutic avenue in epilepsy (Bruzelius et al., 2021; Waloschková et al., 2021). Additionally, identification of classes of inhibitory neurons that are resilient to epileptic insults could provide insights into methods of promoting resilience in typically vulnerable inhibitory neurons, a technique which is currently being explored in the Alzheimer's field (Pfisterer et al., 2020; Roussarie et al., 2020; Leng et al., 2021). In the first single nuclei RNA sequencing (snRNAseq) study of surgically resected tissue from the temporal lobe of patients with TLE, researchers distinguished 23 different subclusters of GABAergic neurons, and even more refined characterizations will be possible through the acquisition of larger datasets (Pfisterer et al., 2020). To begin to identify especially susceptible neurons, the authors compared the relative abundance of each of the many GABAergic subclasses that exist between human TLE and control autopsy cases, as well as determined which subtypes display the greatest shift in their gene expression profile. In agreement with much of the existing research on broad classes of GABAergic neurons, the investigators found the greatest reduction in parvalbumin, sulfatase1 (SULF1)-expressing GABAergic neurons in tissue from cases compared to controls, pointing to a specific subset of PV⁺ neurons that may be particularly susceptible to degeneration in TLE. As for transcriptomic alterations, subsets of many broad classes of inhibitory neurons, defined by the authors

using known subtype-specific expression markers derived from literature, including PV⁺ neurons expressing SULF1, PV⁺ neurons expressing nitric oxide synthase 1 (NOS1), vasoactive intestinal peptide positive neurons expressing cerebellin 1 precursor, SST⁺ neurons expressing tachykinin precursor 1, and inhibitor of DNA binding 2 positive neurons expressing lysosomal associated membrane protein family member 5 and NOS1, displayed dramatic shifts between control and TLE cases, providing more detailed information than was previously possible regarding GABAergic neurons and their signaling in the setting of human chronic TLE.

In parallel with technological advances in single cell RNA-sequencing, recent advances in spatial transcriptomics are allowing researchers to capture spatially resolved single cell or even subcellular level transcriptomic and proteomic data. These spatially resolved techniques are a particularly valuable resource in epilepsy, as they will allow for an in-depth investigation of whole circuits that are disrupted and detail how alterations in one brain region may relate to those in another on an epigenetic, transcriptomic, and proteomic level. Spatial approaches that give context to transcriptomic findings may indeed provide important insights into the progression of epilepsy from the hyperactivity of neuronal subsets to a circuit level disorder in which epigenetic or genomic changes may drive epileptogenesis, providing new strategies for therapeutic intervention.

SUMMARY/FUTURE DIRECTIONS

Significant progress has been made in the past three decades to elucidate the various cellular mechanisms that allow for fine grained control over inhibitory signaling within the CNS. Great strides have been made in our understanding of factors regulating the formation, properties, stability, and localization of GABA_ARs, the primary receptors responsible for synaptic and extrasynaptic neurotransmission, as well as the formation, maintenance, and elimination of inhibitory synapses in both health and disease. In part, this progress has been informed by the genomic revolution, which in the past two decades has allowed for identification of

specific gene mutations in an increased number of individuals with genetic epilepsies, the exploration of which have propelled our understanding of GABAergic signaling forward. In the same vein, the single cell and spatial transcriptomic revolutions of the past five years have already begun to illuminate advancing complexity in the identity and function of cell types throughout diverse organs, including the brain, and offer a rich opportunity for enhancing our understanding of GABAergic neurons in the context of neural circuits that are dysregulated in a plethora of brain disorders, including epilepsy. The promise of multiomics to bring together the regulation of the genome with the functional proteomic landscape that is carved into the brain's connectome will open new opportunities to rethink the development of curative therapies to restore the capacity of the brain for rebalance using its dynamic and complex inhibitory signaling properties.

AUTHOR CONTRIBUTIONS

AT generated the original text for the document which was edited in collaboration with SR. AT generated the figures for general information to synthesize key concepts. Both authors contributed to the article and approved the submitted version.

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