University of California

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Plasticity of GABAergic Inhibition

A dissertation submitted in partial satisfaction

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Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

by

Isabella Ferando

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Abstract of the Dissertation

Plasticity of GABAergic Inhibition

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Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

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Professor Istvan Mody, Chair

γ-aminobtutyric acid (GABA) is a transmitter molecule found in several organs and virtually all organisms. In the vertebrate central nervous system it is the major inhibitory neurotransmitter. Here GABA released by GABAergic neurons binds to GABA receptors and regulates neuronal excitability, network synchrony and neuronal development. $GABA_A$ receptors ($GABA_ARs$) are highly conserved ligand-gated ion channels permeable to Cl^- and HCO_3^-, and upon binding GABA they generate currents that can be functionally distinguished in phasic or tonic. Physiology of tonic GABAergic conductances is of particular interest as these exercise powerful constrain on neuronal excitability and gain. Tonic inhibition is mediated by $GABA_ARs$ containing δ or $\alpha5$ subunits localized outside of the synaptic cleft and activated by ambient GABA. Object of this dissertation is plasticity of δ-$GABA_ARs$, as their function and distinct pharmacology makes them relevant to many human diseases. δ-$GABA_ARs$ are naturally insensitive to benzodiazepines while uniquely sensitive to low concentrations of neurosteroids, which act on them as positive allosteric modulators. These endogenous steroids are the neuroactive form of progesterone, cortisol and testosterone. They are locally synthetized in both neurons and glia and their brain concentration varies in parallel with oscillations in plasma precursors. The present work shows how during times of altered neurosteroid production δ-$GABA_ARs$ expression homeostatically changes

with functional consequences on neuronal network functioning. In particular, plasticity of δ-GABA$_A$Rs during pregnancy and the postpartum affects hippocampal excitability and γ oscillations frequency *in vitro*. Moreover, over the ovarian cycle, plasticity of δ-GABA$_A$Rs on interneurons is necessary for fluctuations in γ oscillations amplitude *in vivo*. More and more evidence suggests an involvement of δ-GABA$_A$Rs in different kinds of neurological and psychiatric disorders, such as epilepsies, postpartum depression, pre-menstrual dysphoric disorder, and schizophrenia. Interestingly a convenient therapeutic strategy may be available, given the anatomical distribution of these receptors. In fact, δ-GABA$_A$Rs expressed on excitatory neurons of the cortex contain $\alpha 4$ subunits, whereas on interneurons they contain $\alpha 1$ subunits. This allows to pharmacologically differentiate δ-GABA$_A$Rs on specific neuron. Development of drugs optimized to selectively modify δ-GABA$_A$-mediated tonic inhibition of excitatory or inhibitory neurons may lead to critical clinical applications.

The dissertation of Isabella Ferando is approved.

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a Giacomo

CONTENTS

LIST OF FIGURES

LIST OF TABLES

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3. **Ferando I**, Mody I (2012) $GABA_A$ receptor modulation by neurosteroids in models of temporal lobe epilepsies. *Epilepsia* 53 Suppl 9:89101.
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1. Soss J, **Ferando I**, Engel J, Jr (2008) Diagnostic and Localizing Behavioral Features of Epileptic Seizures. In: *Epilepsy and Intensive Care Monitoring: Principles and Practice*, pp 191-200. Edited by Fisch B.J. Demos Medical Publishing

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1. **Ferando I**, Maguire J, Simonsen C, Mody I. Region-specific GABA$_A$R plasticity during pregnancy: Effect on neuronal excitability. 416.1/B105. *2009 Neuroscience Meeting Planner*. Chicago, IL: Society for Neuroscience, 2009. Online.

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CHAPTER 1

INTRODUCTION

The small molecule γ-aminobtutyric acid (GABA) is a widely used messenger, and can be found in virtually all living organisms. It is synthetized from glutamate and packaged in membranous vesicles by the releasing cell which then can use it to communicate over short distances in a paracrine way. In the central nervous system, where GABA is the major inhibitory neurotransmitter, GABAergic communication has evolved into a complex and sophisticated system comprising of a heterogeneous family of highly specialized neurons that release GABA, and an equally elaborate set of GABA binding proteins. GABAergic neurons can be local (interneurons) or long projecting neurons, they amount to about 20% of all mammalian neurons and are characterized by an incredible variety, to the point that consensus on their classification is still labile (DeFelipe et al., 2013). Through well-timed and tightly regulated GABA release, these neurons control neuronal excitation and can synchronize networks (Freund and Buzsaki, 1996; Klausberger and Somogyi, 2008).

Once GABA is released from the presynaptic neuron, it binds to specific transmembrane receptors both on the postsynaptic and on the presynaptic cell. Metabotropic $GABA_B$ receptors are G-protein coupled receptors that are linked to potassium channels and inhibit

transmitter release (Craig and Mcbain, 2014; Bettler et al., 2004). Ionotropic $GABA_A$ receptors ($GABA_ARs$) are heteropentameric $Cl^-/HCO3^-$ permeable channels that comprise three different subunits chosen from a pool of several (α1-6, β1-3, γ1-3, δ, ϵ, θ, π, and ρ1-2). Upon GABA binding and channel gating, an ionic conductance follows, which nature (hyperpolarizing, depolarizing or shunting) depends on the Cl^- reversal potential (Staley and Mody, 1992; Mitchell and Silver, 2003; Song et al., 2011). To this regard, GABA can be very a versatile transmitter molecule. While it is generally inhibitory on mature neuron, it is excitatory during development (Ben-Ari, 2002), and can act as a trophic factor (Represa and Ben-Ari, 2005).

Inhibition is functionally divided in phasic and tonic (Farrant and Nusser, 2005), and $GABA_ARs$ will mediate one or the other depending on their localization in subcellular compartments (synaptic vs. non-synaptic) and subunit composition. Tonic inhibition is of particular interest as it allows for a strong and long-lasting control of the gain of neuronal input-output functions (it is calculated that as much as three fourths of the total inhibitory charge received by the postsynaptic neuron can be ascribed to tonic inhibition) (Mody and Pearce, 2004). Tonic inhibition is mediated by $GABA_ARs$ localized peri- or extra-synaptically, in an ideal position to act as sensors of ambient GABA accumulated in the vicinity of inhibitory synapses following neurotransmitter spillover (Wei et al., 2003). In particular $GABA_ARs$ containing either δ or α5 subunits can mediate tonic conductances (Glykys et al., 2008; Saxena and Macdonald, 1994; Farrant and Nusser, 2005).

Focus of this dissertation will be the study of $GABA_ARs$ containing the δ subunit (δ-$GABA_ARs$), as their distinct pharmacology makes them relevant to a number of neurological and psychiatric disorders. δ-$GABA_ARs$ are expressed on both principal cells, where they comprise α4 subunit, and on several interneurons, where they contain α1 subunit (Glykys et al., 2007). All δ-$GABA_ARs$ are characterized by high affinity for GABA, modest desensitization, benzodiazepine insensitivity and low GABA efficacy, which can be greatly increased by some endogenous neuroactive steroids (3α-hydroxy ring A-reduced pregnane steroids) -

NS that act on them as positive allosteric modulators (Belelli and Lambert, 2005; Stell et al., 2003). NS are locally synthesized through the enzyme 5α-reductase in both neurons and glia, from precursor steroids (progesterone, cortisol/corticosterone, and testosterone). Although precursors are also synthesized locally starting from cholesterol (Rupprecht et al., 2009, 2010), brain NS concentrations will readily follow plasma precursor fluctuations. Therefore during conditions of altered steroid production, such as pregnancy, the ovarian cycle, stress and puberty, the mammalian brain will be exposed to largely variable levels of NS. The greatly potentiating effects NS have on δ-GABA$_A$Rs even at very low concentrations (Stell et al., 2003), expose δ-GABA$_A$Rs-expressing neurons to substantial fluctuations in their tonic inhibition. Previous work from this lab has shown how δ-GABA$_A$Rs in the hippocampus respond with a highly plastic behavior following NS fluctuations over the ovarian cycle, during pregnancy, and after acute stress (Maguire et al., 2005; Maguire and Mody, 2007, 2008).

Some neuromodulators that belong to the NS family don't have an effect on GABA$_A$Rs. Among these, for example, is estradiol, a NS that has been implicated with synaptic plasticity, and whose local concentrations greatly vary (Rune and Frotscher, 2005). Estradiol (17-β-estra-1,3,5(10)-triene-3,17-diol) belongs to the subfamily of androgen steroids and its local synthesis by the enzyme aromatase has been documented in both males and females in different brain areas, where it controls sexual behavior, modulates cognition and synaptic plasticity and can act as a neuroprotectant agent (Balthazart and Foidart, 1993; Rune and Frotscher, 2005; Garcia-Segura, 2008; Foy et al., 2008; Srivastava and Penzes, 2011). Local synthesis of estradiol can rapidly change during social interaction, independently of plasma fluctuations of its precursor testosterone, and in a Ca^{2+} dependent manner (Remage-Healey et al., 2008, 2011). This dissertation will focus on those NS that belong to the subfamily of progestogens, and in particular on how their fluctuations tightly correlate with plasticity of δ-GABA$_A$Rs during pregnancy and the ovarian cycle.

The first part of this dissertation presents two reviews that explore the current literature on tonic inhibition modulation in models of temporal lobe epilepsies, and the state of the art

of what is known about δ-GABA$_A$Rs-mediated tonic inhibition on different types of cortical interneurons. Then follows original work that explores three major questions.

First we investigated whether plasticity of δ-GABA$_A$Rs on hippocampal principal cells alters network excitability (Chapter 4). Secondly, we asked whether δ-GABA$_A$Rs expressed on interneurons also change during pregnancy and the ovarian cycle, and with what consequences on network synchrony (Chapter 5 and 6). Lastly, we investigated the possibility to pharmacologically selectively modulate a network behavior controlled by δ-GABA$_A$Rs on interneurons, rather than principal cells, in the perspective of possible therapeutic applications (Chapter 7). In fact, mounting evidence seems to suggest that alterations in the physiology of δ-GABA$_A$Rs may precipitate or predispose to neurological and psychiatric disorders, among which are different epilepsies, postpartum depression, premenstrual dysphoric disorder, and major depression (Lovick, 2006; Maguire and Mody, 2008; Peng et al., 2004; Dibbens, 2004; Macdonald et al., 2010; Feng et al., 2010; Ferando and Mody, 2012).

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CHAPTER 2

$GABA_A$ RECEPTOR MODULATION BY NEUROSTEROIDS IN MODELS OF TEMPORAL LOBE EPILEPSIES

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MOLECULAR PLASTICITY

$GABA_A$ receptor modulation by neurosteroids in models of temporal lobe epilepsies

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Summary

Epilepsies consist of a spectrum of neurologic disorders typically characterized by unpredictable and dysfunctional network behaviors in the central nervous system (CNS), which lead to discrete episodes of large bouts of uncontrolled neuronal synchrony that interfere with the normal functioning of the brain. Temporal lobe epilepsy (TLE) is accompanied by changes in interneuronal innervation and modifications in different γ-aminobutyric acid $(GABA)_A$ receptor subunits. Hormones play an important role in modulating the overall excitability of neurons, and at the same time hormonal pathways are frequently modified during epilepsy. This review focuses on TLE-correlated modifications of GABAergic transmission, and in particular on the implications of some of our own findings related to $GABA_A$Rs containing the δ subunits (δ-$GABA_A$Rs). These are extra- or perisynaptic $GABA_A$Rs that mediate tonic inhibition, a major component of the inhibitory mechanism in the brain. The most potent endogenous modulators of δ-$GABA_A$Rs are neurosteroids, which act as positive allosteric modulators. Plasticity of δ-$GABA_A$Rs during TLE consists of down-regulation of the subunit in the dentate gyrus granule cells (DGGCs), while being up-regulated in interneurons. Surprisingly, the level of tonic inhibition in DGGCs remains unchanged, consistent with the idea that it becomes mediated by $GABA_A$Rs containing other subunits. In parallel, tonic inhibition in a TLE model ceases to be sensitive to neurosteroid potentiation. In contrast, as predicted by the anatomic plasticity, interneuronal tonic current is increased, and remains sensitive to neurosteroids. These findings have important pharmacologic implications. Where neurosteroids normally have sedative and anticonvulsant effects, bimodal and cell-type specific modulations in their natural targets might weaken the inhibitory control on the dentate gate, under circumstances of altered neurosteroids levels (stress, ovarian cycle, or the postpartum period).

KEY WORDS: Temporal lobe epilepsy, Neurosteroids, $GABA_A$ receptors, Tonic current.

Hormones in the Brain

Network excitability may be viewed as the summation of the activity of discrete neuronal networks resulting from a balanced reciprocal control between inhibitory and excitatory components. Shifting the weight of one or the other neuronal population will determine the degree of excitability of a given brain area. This modulation can be achieved by regulating the expression and kinetics of ligand-gated or voltage-gated ion channels, by shifting the concentration gradient of the corresponding permeable ions, or by regulating neurotransmitter release probability and duration of action. Of the many possible ways of modulating network excitability, hormonal modulation stands out as a long-range and efficient mechanism for the task.

Hormones are classified into different chemical classes, and they act on diverse families of receptors. They serve as messengers for long-range, fast-acting, and long-lasting communication between organs and, as tuning molecules, they contribute to metabolic needs and functionality of the organism. Their ability to dynamically modulate neuronal outputs ultimately leads to their efficacy in influencing brain activity, behavior, and mood. Given these premises, it is not surprising how in pathologic states such as epilepsy, perturbation of the homeostatic hormonal effects on the brain can bring about an aggravation of the underlying disease, and reciprocally, physiologic hormone secretion

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can be disrupted by dysfunctional neuronal activity. Biochemically, hormones can be classified into lipid-derived (of which cholesterol-based steroids are the most abundant class), protein and peptide-derived, and catecholamines (adrenaline, noradrenaline, and dopamine) (Koeppen & Stanton, 2010).

Hormonal receptors can be found in different cellular compartments, depending on the nature of the ligand and the specific effect they trigger. For example, receptors for estrogen and progesterone (ER-α and ER-β and PR, respectively) are located in the cytoplasm or in the nucleus, in a strategic position for gene expression regulation. In this case, receptor activation normally leads to slow and long-lasting effects. Hormonal receptors can also be present in the plasma membrane, as is the case of the metabotropic receptors for dopamine and endocannabinoids, or the G-protein–coupled estrogen receptors (GPER1) that mediate nongenomic, fast effects of estrogen (Olde & Leeb-Lundberg, 2009). Their activation leads to cascades involving second messengers that can both act in a fast and transitory way by modulating the responsiveness of ion channels, or create long-lasting effects by enhancing or dampening the activity of transcription factors (Gómez-Ruiz et al., 2007; Mackie, 2008; Wegener & Koch, 2009; Beaulieu & Gainetdinov, 2011; Girault, 2012). Lastly, some hormones can bind directly to specific binding sites on ligand-gated and voltage-gated ion channels, resulting in fast and transitory effects on network excitability. Synthesis of neuroactive hormones can take place both peripherally (gonads, adrenal gland), and in the central nervous system (CNS). Basal levels of steroid hormone production (testosterone, estrogens) are always present in both the female and male brain, but in some cases, as for example following brain injury, their synthesis is tremendously increased (Mirzatoni et al., 2010). Locally synthetized steroids can act in a cell-autonomous way, or in a paracrine fashion on neighboring cells.

Changes to several hormonal pathways have been implicated in the epileptogenic process and in the triggering of ictal periods, both in human and animal models of TLE. Herein we report a few examples of hormonal pathways that are directly involved in excitability modifications during epilepsy. Estradiol is an ovarian hormone, which in rats has been found to increase hippocampal seizure susceptibility while lowering their severity. The mechanism of action seems to be mediated by estrogen receptor-α (ER-α), and involves decrease in γ-aminobutyric acid (GABA) release accompanied by an augmented release of the anticonvulsant molecule neuropeptide Y (NPY) (Ledoux et al., 2009). There is evidence for a switch in the expression of ER-α and ER-β from principal cells to reactive astrocytes in the epileptic hippocampus of rats (Sakuma et al., 2009) and for a selective increase in ER-α expression in human hippocampi under certain antiepileptic drug (AED) treatments (Killer et al., 2009).

Another hormonal pathway that is profoundly altered in epilepsy is that of corticotrophin-releasing hormone (CRH). Numerous independent studies have reported increase in CRH synthesis in different brain areas, including the hippocampus, in epileptic humans and mice (Wang et al., 2001; Wu et al., 2012). Although it was long known that CRH can act as proconvulsant in the developing brain (Baram & Schultz, 1991), its role together with the hypothalamic pituitary adrenal (HPA) axis during epilepsy is currently under extensive investigation (Sarkar et al., 2011).

Neuregulin-1 (NRG-1) has recently been implicated in the regulation of excitability of a subclass of interneurons and seems to play a protective role in epilepsy (Li et al., 2012; Tan et al., 2012). Briefly NRG-1 is a peptide that acts as neurotrophic factor by activating the receptor tyrosine-protein kinase ErbB4. It increases inhibition by increasing the excitability of fast-spiking basket cells, and the downstream molecular pathway has been shown to be downregulated in human chronic epilepsy (Li et al., 2012). Further studies are needed to understand to what extent this hormonal pathway can be a potential target for treatment. Of interest, it seems that NRG-1 secretion is under progesterone control, via cytoplasmic progesterone receptor activation (Lacroix-Fralish et al., 2007). Progesterone is a well-established anticonvulsant and sedative (Majewska et al., 1986; Söderpalm et al., 2004), and these effects are mostly mediated by its neuroactive form allopregnanolone through an increase in $GABA_A$ receptor–mediated inhibition (Belelli & Lambert, 2005). Nonetheless, NRG-1 is a good candidate as an additional molecular pathway through which progesterone can decrease brain excitation.

Ongoing investigation of hormonal molecular pathways, and of their modulation during health and disease, constitutes an important basis for development of targeted pharmacologic approaches in order to lower frequency and magnitude of seizure activity in epileptic patients. This review will focus on 3α-hydroxy ring A–reduced pregnane steroids and their role during states of altered neuronal excitability such as epilepsy. Our main findings pertain to neurosteroid regulation of the inhibitory component of neuronal networks in health and disease. Neurosteroids are brain-derived metabolites of gonadal and adrenal gland steroids and are synthetized in both neurons and glia by the enzyme 5-α-reductase. In particular, our main topics will be the progesterone-derived allopregnanolone (ALLO) and the cortisol-derived tetrahydrodeoxycorticosterone (THDOC), which at physiologic concentrations preferentially act as positive allosteric modulators of extrasynaptic δ-containing $GABA_A$ receptors, and through this biochemical mechanism, fulfill their sedative, anticonvulsant, and anxiolytic effects (Belelli & Lambert, 2005).

Interneurons, GABA$_A$ Receptors and Tonic Inhibition

In the mammalian brain, the GABAergic system is in charge of the inhibitory control of neuronal output, and it plays a pivotal role in orchestrating synchronicity of local networks and functional coupling of different brain regions (Mann et al., 2005; Montgomery & Buzsáki, 2007; Buzsáki & Wang, 2012). Its fundamental components are highly specialized and heterogeneous families of neurons that synthesize and release GABA (Freund & Buzsaki, 1996; Klausberger & Somogyi, 2008), and an equally complex and diverse system of receptors that bind GABA (Mody & Pearce, 2004). Epilepsy comprises many syndromes, each of which is characterized by pathologic modifications of neuronal excitability and network synchrony (Engel, 2006; Engel et al., 2009). Hence it is not surprising that a number of brain region–specific changes in both GABA-releasing cells and GABA-binding receptors have been implicated in TLE, although the direct functional consequences of each modification are difficult to predict. Equally problematic is a clear-cut separation between compensatory and aggravating alterations (Mody, 1998; Zhang et al., 2007; Mann & Mody, 2008; Avoli & de Curtis, 2011). Understanding the many facets of GABAergic plasticity during epileptogenesis and during the chronic phase of TLE will be a fundamental step toward the development of specific drugs for the control and prevention of seizures. In order to investigate the functional consequences of GABAergic plasticity in TLE, in the following section we review anatomy, physiology, and the functional role of the GABAergic system in the healthy CNS.

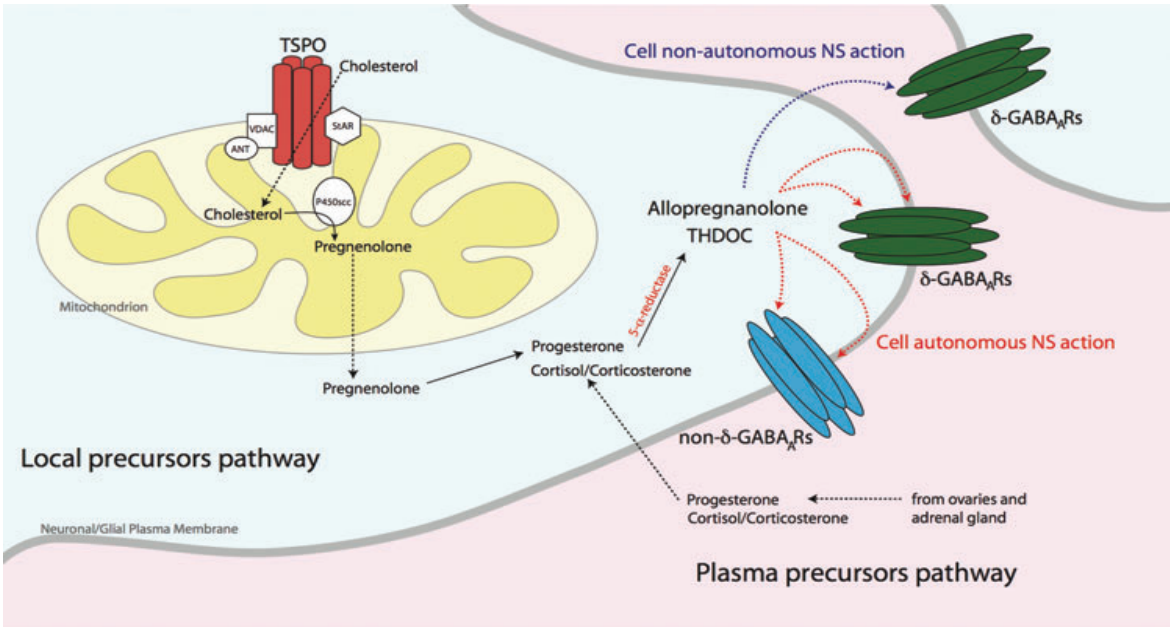

Figure 1.
Schematic representation of two pathways for neurosteroid synthesis and possible differential neurosteroid action on GABA$_A$Rs. Brain-derived precursors are synthetized from cholesterol both in neurons and glia. The rate-limiting step is the cholesterol transport into the mitochondrial matrix by the transporter protein TSPO (formerly known as peripheral benzodiazepine receptor, PBR). Cholesterol is converted to pregnenolone by the enzyme P450scc, located on the inner mitochondrial membrane. Pregnenolone is the precursor for ALLO and THDOC, which are synthetized in the smooth endoplasmic reticulum by the enzyme 5-α-reductase. Neurosteroidogenesis can also use plasma-derived precursors. Progesterone and cortisol/corticosterone synthesized in the ovaries and adrenal cortex, respectively, can diffuse into neurons and glia, where they are converted into ALLO and THDOC. The highly lipophilic neurosteroids accumulate in the plasma membrane and thus can modulate GABA$_A$Rs. Plasma membrane concentrations of neurosteroids might be sufficient to modulate both δ-GABA$_A$Rs and non-δ-GABA$_A$Rs in a cell-autonomous fashion. However, low concentrations of neurosteroids diffusing through the extracellular space from neighboring neurons and glia may only act on δ-GABA$_A$Rs, in a cell nonautonomous manner.

Among GABAergic neurons are the interneurons that coordinate the activity of local networks (Klausberger & Somogyi, 2008; Isaacson & Scanziani, 2011), but some GABAergic neurons can project long range, and synchronize far apart brain regions (Alonso & Köhler, 1982; Jinno et al., 2007; Melzer et al., 2012). Interneurons can be classified by their electrophysiologic properties, by certain exclusive proteins they express, by the neuronal compartments they innervate, or by areas they project to (Freund & Buzsaki, 1996). A powerful restraint on principal cell excitability is held by perisomatic inhibition. This type of GABAergic innervation is established by a subclass of interneurons which contacts on principal cells are localized on the soma and the axon initial segment, in a particularly advantageous position for the control of neuronal output (Freund & Katona, 2007). Both human and mouse-model TLE exhibit heterogeneous modifications of perisomatic GABAergic innervation of principal cells (Maglóczky, 2010; Wyeth et al., 2010; Li et al., 2012; Tan et al., 2012), suggesting the importance of this type of inhibitory innervation in maintaining adequate levels of neuronal excitability.

On the other side of the synaptic cleft, and its vicinity, are $GABA_A$Rs. These receptors are heteropentameric ligand-gated Cl^-/HCO_3^- permeable channels, which belong to the Cys-loop receptor family, and are usually made of three different proteins, chosen from an array of several subunits (α1-6, β1-3, γ1-3, δ, ϵ, θ, π, and ρ1-2) (Laurie et al., 1992; Wisden et al., 1992; Sieghart & Sperk, 2002; Sun et al., 2004). The subunit composition will determine the pharmacologic characteristics of the receptor. For example, γ-subunit–containing receptors mediate different benzodiazepine effects depending on what α subunit they are paired with (Rudolph et al., 1999). In addition, certain α subunits (α4 and α6) are naturally insensitive to benzodiazepines. Moreover, the presence of a δ subunit in the $GABA_A$R complex confers a high degree of neurosteroid-dependent modulation to the receptor (Belelli & Herd, 2003; Stell et al., 2003; Belelli & Lambert, 2005). Molecular and functional alterations of this subunit on different families of neurons during TLE are the main focus of this review. The varied pharmacologic properties of $GABA_A$Rs allow for the development of highly specific drugs for differential application in a wide range of clinical situations (epilepsy, depression, anxiety, amnesia, insomnia).

Anatomic localization of the different receptors is another variable that correlates with subunit composition. The same neuron (excitatory or inhibitory) can express assorted combinations of subunits in separate subcellular compartments, and some subunits are typical of different brain areas (Lüddens & Wisden, 1991; Hevers & Lüddens, 1998; Pirker et al., 2000). For example, γ and δ subunits are mutually exclusive (Shivers et al., 1989; Araujo et al., 1998), with δ subunits generally found outside the synapses (Nusser et al., 1998b; Wei et al., 2003). In addition, δ subunits classically pair up with α6 in the cerebellum, whereas in the forebrain, they pair up with α4 in principal cells and α1 in interneurons (Glykys et al., 2007). Likewise, various subunits can be differentially expressed during development and in the adult brain (Laurie et al., 1992).

In addition to inhibition mediated by postsynaptic $GABA_A$Rs, there is increasing evidence for the existence of axonal $GABA_A$Rs (Trigo et al., 2008). Their physiologic role is controversial: activation of presynaptic $GABA_A$Rs has been shown to both facilitate and inhibit transmitter release from the synapse, depending on the brain area, the developmental stage of the animal, and the neuron type. Moreover, presynaptic $GABA_A$R agonism might function in a bimodal way, acting as excitatory at states of low receptor activation and inhibitory at higher degrees of activation (Jang et al., 2005). Although little is known of the subunit composition and the pharmacologic properties of presynaptic $GABA_A$Rs on different kinds of cells, anatomic accessibility of mossy fiber boutons to patch clamp experiments has allowed for extensive functional investigation of presynaptic $GABA_A$Rs on these terminals (Alle & Geiger, 2007; Ruiz et al., 2010). Although anatomic evidence of axonal δ-$GABA_A$Rs presence is missing, the $GABA_A$Rs of mossy fiber boutons pharmacologically behave as δ-$GABA_A$Rs and their activation seems to facilitate synaptic transmission (Ruiz et al., 2010).

Lastly, subunit composition is responsible for the electrophysiologic behaviors of the $GABA_A$Rs (Farrant & Nusser, 2005). Upon agonist binding, an ionic conductance ensues, which correlates with the synaptic or peri/extrasynaptic localization of the receptor, and the nature of the conductance depends on the Cl^- reversal potential. In addition, the receiving pyramidal cell seems capable of controlling local Cl^- concentration depending on what type of interneuron synapses onto it, thanks to differential postsynaptic expression of ClC-2, a hyperpolarization-activated Cl^- channel (Armstrong & Soltesz, 2012). ClC-2 is also found in interneurons, suggesting that this mechanism that is actively controlled by the postsynaptic cell might also involve interneuron-to-interneuron synapses. Synaptic $GABA_A$Rs generate rapid postsynaptic currents known as inhibitory postsynaptic currents (IPSCs) and thus mediate phasic inhibition (Macdonald et al., 1989; Mody et al., 1994; Haas & Macdonald, 1999). Nonsynaptic $GABA_A$Rs are localized either far or in the proximity of GABAergic synapses and generate a more recently discovered, persistent ‘‘tonic’’ conductance and are implicated in the management and maintenance of tonic inhibition (Mody, 2001; Semyanov et al., 2004; Farrant & Nusser, 2005). Tonic inhibition accounts for three fourths of the inhibitory charge received by the postsynaptic cell (Mody & Pearce, 2004), and pathologic modifica-

tions that directly interfere with its functionality during epilepsy will greatly influence the overall gain of neuronal networks (Semyanov et al., 2004). Functional relevance of tonic inhibition has recently been demonstrated in vivo (Duguid et al., 2012), as modulation of tonic inhibition in cerebellar granule cells (CGCs) has been shown to be a regulator of sensory information processing. In particular, basal levels of tonic inhibition seem to be optimal for seamless and accurate sensory transmission: CGCs spontaneous activity is kept low, while they are still readily responsive to sensory stimuli. Blockade or enhancement of CGCs tonic inhibition shifts the network from this optimal zone where signal-to-noise ratio is at a maximum, toward areas where increase in spontaneous activity or decrease of the power of sensory-evoked responses, respectively, decrease the efficiency of sensory transmission.

As it is true for interneurons, GABA$_A$Rs can also be greatly altered in TLE, both in number and in subunit composition (Houser & Esclapez, 1996; Nusser et al., 1998a; Loup et al., 2000; Houser & Esclapez, 2003; Peng et al., 2004). In addition, some genetic epilepsies result from mutations that affect specific GABA$_A$R subunits (Macdonald et al., 2010). These receptor modifications alter the response of target cells to an already modified inhibitory network, most likely pushing baseline activity toward more excitable and synchronized states.

Depending on the neuron type, tonic inhibition can be mediated solely by δ-containing GABA$_A$Rs (δ-GABA$_A$Rs), α5-containing GABA$_A$Rs, or a combination of both (Glykys et al., 2008). Interest in δ-GABA$_A$Rs increased once it was discovered that they are uniquely sensitive to low concentrations of neurosteroids, which act on them as potent positive allosteric modulators, thus increasing tonic inhibition in a fast and sustained way. The three main functional characteristics that distinguish phasic inhibition–mediating and tonic nhibition–mediating receptors are their differential affinity for GABA, desensitization rate, and agonist efficacy.

Typical of extrasynaptic GABA$_A$Rs is their high affinity for GABA (half maximal effective concentration [EC$_{50}$] 1 μM), which allows them to respond to very low GABA concentrations, and makes them an ideal sensor for ambient GABA, which oscillates in the near micromolar range (Nyitrai et al., 2006). In the DGGCs of the hippocampus, δ-GABA$_A$Rs are localized in proximity to GABAergic synapses, where they can readily detect GABA spilled over from nearby boutons (Wei et al., 2003). In other cases, δ-GABA$_A$Rs are found extrasynaptically scattered across the dendritic tree, as in CGCs (Nusser et al., 1998b), indicating the existence of ambient GABA fields larger than those found around inhibitory synapses. Cerebellar astrocytes were recently identified as a source for ambient GABA, and upon activation they can tonically release GABA through nonspecific anion channels (Best-1) (Lee et al., 2010). In addition, GABA can be released in the extracellular compartment by a particular type of cortical interneuron, the neurogliaform cell (NGFC), the release of which produces effects on δ-GABA$_A$Rs independently of synaptic transmission (Oláh et al., 2009). The axonal cloud of these cells can cover an area with a diameter of 200 μm, rendering this type of communication particularly fit to level and synchronize the amount of excitation of a network over broad distances. GABA spillover and volume transmission are the sources of extracellular GABA. However, the net ambient GABA concentration detected by extrasynaptic GABA$_A$Rs results from the summation of GABA release and GABA reuptake, which relies on an efficient class of transporters, localized both on neurons and glia (GAT-1 and GAT-3, respectively) (Walker & Semyanov, 2008). Dysfunction of the reuptake machinery will alter the level of tonic inhibition in receiving neurons, as shown in neocortex layer II–III pyramidal cells poststroke (Clarkson et al., 2010). After depolarization, GABA transporters may act in reverse and release GABA into the extracellular space. Of interest, there is evidence of decreased ambient GABA in epilepsy (Minamoto et al., 1992), whereas evidence of alteration in transporter expression and function during epilepsy is controversial. Altered distribution was reported in human in human postsurgical TLE hippocampi (Lee et al., 2006), where overall decrease in transporter expression was reported in several mouse model studies (During et al., 1995; Bouilleret et al., 2000; Silva et al., 2002). Nonetheless, the mechanism of action of some AEDs like tiagabine is mediated by selective blockade of GABA uptake (Madsen et al., 2010), or as in the case of vigabatrin, by a reduced GABA catabolism. In both cases, ambient GABA levels would be increased, and tonic currents would become potentiated. The mechanism of action of these AEDs, which are already in use in the treatment of chronic epilepsy, suggests that increasing the tonic current may be a good strategy for the development of novel drugs.

The second characteristic that makes δ-GABA$_A$Rs capable of sustaining a current that is always on in the continuous presence of GABA is their relatively modest desensitization to the agonist (Haas & Macdonald, 1999). Desensitization is a common property of ligand-gated ion channels, and describes a state of prolonged closures during which the agonist is still bound to the channel (Jones & Westbrook, 1996). In other words, rapidly desensitizing channels are designed to respond to fast, transitory, and large changes in agonist concentration, whereas nondesensitizing channels are capable of being functional for the entire time they are exposed to the agonist. Ambient GABA oscillations are slow and small compared to changes in synaptic GABA, which means that δ-GABA$_A$Rs need to be able to respond to extracellular GABA concentrations that vary little in the short run. δ-GABA$_A$Rs in the thalamus and cerebellum have been

shown to be unable to respond to GABA spilled over from the synapse, and this was mainly ascribed to their desensitization in the presence of ambient GABA. This would suggest that discrete populations of δ-$GABA_A$Rs, simultaneously activated, contribute to the generation of tonic conductance. Once a population becomes desensitized, tonic inhibition would be mediated by another population of extrasynaptic $GABA_A$Rs (Bright et al., 2011).

Thirdly, despite their high affinity for the agonist, GABA efficacy on δ-$GABA_A$Rs is relatively low compared to other $GABA_A$Rs (Meera et al., 2009). This results from a low-level coupling between channel gating and agonist binding. This property offers extensive modulatory potential of δ-$GABA_A$R-mediated tonic currents. In other words, their high affinity and low efficacy make these receptors very good listeners but very poor translators of extracellular GABA concentrations. Increasing their efficacy might be the major mechanism by which tonic inhibition is controlled in the CNS. Endogenously, neurosteroids promote tonic inhibition by increasing the efficacy of GABA on δ-$GABA_A$Rs, in response to physiologic homeostatic needs. Exogenously, this constitutes a pharmacologic mechanism that can be used to clinical advantage. The sedative effects of steroid metabolites have been described decades ago (Atkinson et al., 1965), and the molecular mechanisms involving the GABAergic system through which these effects are mediated have been thoroughly investigated (Majewska et al., 1986; Stell et al., 2003; Belelli & Lambert, 2005; Herd et al., 2007). Neurosteroids are the most potent endogenous modulators of GABA-mediated transmission, and at low concentrations (nanomolar range) they enhance the GABA efficacy of δ-$GABA_A$Rs. Their selectivity is likely to be ascribable to their kinetic effect rather than to a preferential interaction with δ-$GABA_A$Rs: the already high efficacy of GABA on non-δ-$GABA_A$Rs makes neurosteroid potentiation on them less likely.

Following $GABA_A$R gating, the effect of GABA transmission on the excitability of a network depends on the Cl^- reversal potential of the postsynaptic cell. In particular, the balanced expression of two Cl^- transporters will determine if the cell will be depolarized or hyperpolarized by the opening of its $GABA_A$Rs. NKCC1 and KCC2 are two symporters that, respectively, use the Na^+ or K^+ gradient generated by the Na-K pump to move Cl^- across the plasma membrane. In particular, NKCC1 activity increases intracellular Cl^-, whereas KCC2 decreases it. The developmental GABA switch from excitatory to inhibitory neurotransmitter is caused by molecular changes implemented in mature neurons, which increase KCC2 and lower NKCC1 expression (Ben-Ari, 2002). KCC2 expression has been shown to be impaired in the rat hippocampus following pilocarpine-induced status epilepticus and in the latent period, and chronically in sclerotic hippocampi of patients with TLE (Huberfeld et al., 2007; Pathak et al., 2007; Barmashenko et al., 2011). A reversed Cl^- gradient will further reduce the capability of the GABAergic network to maintain control of fleeting bursts of excitation. It follows that key structures to keep excitation in check, as for example the dentate gate, which is normally under stringent inhibitory control, start losing reliability in the way they process and pass on information.

Lastly, it is important to remember that a depolarizing GABA current will not necessarily excite a neuron. After all, inhibition can be achieved not only by hyperpolarization, but also by clamping the cell membrane at a certain potential, still hyperpolarized to the threshold of powerful voltage-gated conductances (Na^+ or Ca^{2+}). This phenomenon de facto dampens the neuron firing probability in what's known as shunting inhibition (Staley & Mody, 1992; Mitchell & Silver, 2003). The effectiveness of shunting inhibition is proportional to GABA conductance, and at least in some interneurons seems to come into play only at higher ambient GABA concentrations (Song et al., 2011). This means that certain cells naturally maintain a membrane potential so close to GABA reversal potential that they will respond with either an increase or a decrease in their gain depending on the extracellular GABA concentration: at low concentration, the depolarizing effect will prevail, whereas it will be overcome by shunting inhibition at higher concentrations. In conclusion, the final network effect of GABAergic transmission rests upon a dynamic and delicate equilibrium between GABA release, $GABA_A$Rs modulation, GABA transporters, and Cl^- gradient.

In the epileptic brain, this equilibrium is modified (Bernard et al., 1999; Cohen et al., 2002; Engel et al., 2009; Zhang et al., 2012), and although most of the time inhibition is capable of finely regulating excitation, changes in the many modulators of the GABAergic system, which would fall under normal homeostatic fluctuations in the healthy brain, may hence facilitate the triggering of epileptic discharges typical of clinical seizures.

Neurosteroids and Tonic Inhibition

Neurosteroids are synthetized in the cytoplasm of neurons and glia starting from steroid hormones released by the gonads and the adrenal gland, or produced locally in the cytoplasm of both neurons and glia (Belelli & Lambert, 2005). Plasma concentrations of progesterone and cortisol can greatly oscillate from virtually no detectable levels to hundreds of nM, depending on the physiologic state of the organism. In mammals during the ovarian cycle, progesterone levels vary between 2 nM in the follicular phase and 15 nM in the luteal phase. Physiologic stress response, both acute and chronic, modifies plasma levels of cortisol from a few nM to tens of nM (Reddy & Rogawski, 2002; Porcu et al., 2003). During the last third of pregnancy, circulating levels of progesterone are at

their highest, around 100 nM (Paul & Purdy, 1992). Modifications in neurosteroid levels are also typical of physiologic aging, when circulating steroid levels are reduced (Schumacher et al., 2003). In general, neurosteroids are readily synthetized in the brain, and their concentration correlates to that of their precursors: hence, oscillations in steroid levels are faithfully followed by similar oscillations in neurosteroids (Paul & Purdy, 1992). Local steroidogenesis can take place both in neurons and glia, and the rate-limiting enzyme is the cholesterol-translocator transporter protein TSPO localized on the mitochondrial membrane (Rupprecht et al., 2010). Neurosteroids synthetized in neurons or glia could potentially serve different purposes and overall have different effects on network excitability by acting in a cell-autonomous way as opposed to paracrine action influencing neurons of specific astrocytic microdomains (Fig.1).

Up to 100 nM, neurosteroids preferentially if not uniquely potentiate δ-GABAAR-mediated tonic current (Stell et al., 2003), by increasing GABA efficacy on these receptors. The functional effect is a fast and strong increase in tonic inhibition on the receiving cell. The amount of tonic current augmentation is neurosteroid concentration–dependent, and in DGGCs at 10 and 100 nM it ranges from 100% to >300% increase compared to control (Stell et al., 2003). Given the fact that δ-GABA$_A$Rs distribution is not homogenous throughout the brain, and that certain neurons express a weighted combination of δ-GABA$_A$Rs and α5-GABA$_A$Rs to sustain their tonic inhibition, neurosteroid effects will be stronger on those cells and in those brain areas, which preferentially utilize δ-GABA$_A$Rs for their tonic GABA conductance. In the hippocampus, δ-GABA$_A$Rs are heavily expressed on the dendrites of DGGCs, and on certain interneurons, whereas hippocampal pyramidal cells, exclusively (CA3) or preferentially (CA1) express α5-GABA$_A$Rs (Glykys et al., 2007). The hippocampal dentate gyrus has been extensively investigated in epilepsy, and malfunction of its inhibitory component has been postulated to play an important role in the initiation or propagation of seizures (Heinemann et al., 1992). This function of the dentate gyrus has been termed as a filter or gate, and this gate is under inhibitory control. Dentate excitability is naturally very low compared to other areas of the hippocampus: excitatory input onto the dentate from the entorhinal cortex through the perforant path excites both granule cells and local interneurons (feed-forward inhibition), an activity fundamental for the maintenance of the gate function (Coulter & Carlson, 2007). Typically in TLE, GABA$_A$R expression in both DGGCs and dentate interneurons changes, and this may contribute to a compromised efficiency of the gate.

At higher concentrations, neurosteroids become agonists on δ-GABA$_A$Rs, thus triggering channel gating independently of GABA presence (Callachan et al., 1987). Lastly, exogenous drugs, such as ethanol and certain antidepressants, can alter neurosteroid levels. For instance, there is increasing evidence that ethanol effects on GABA$_A$Rs might be mediated, at least in part, by neurosteroids, through an NMDA receptor–dependent increase in their synthesis (Sanna, 2004; Tokuda et al., 2011). Fluoxetine seems to normalize neurosteroid levels in major depression, posttraumatic stress syndrome (PTSD), and after social isolation, conditions characterized by reduced neurosteroid synthesis (Uzunova et al., 1998; Pinna et al., 2003; Pinna, 2004). This led to hypothesize that neurosteroids contribute to the antidepressant actions of this drug (Pinna et al., 2006).

The molecular mechanism of action of the allosteric modulation of GABA$_A$Rs by neurosteroids most likely involves direct interaction of neurosteroids with the receptor. Two sites on the α subunit have been identified as necessary for the potentiating and activating effects of neurosteroids (Hosie et al., 2006), although there is a debate on whether these sites physically bind neurosteroids (Li et al., 2009). Recently a new site on β3 subunit has been described to directly bind a neurosteroid analog, although its functional role is yet to be investigated (Chen et al., 2012). There is increasing evidence that neurosteroids might gain access to a membranous GABA$_A$R site of action through lateral diffusion into the plasma membrane. The interaction between neurosteroids and receptor would happen in the lipid phase, and would be dependent on the amount of neurosteroids that are accumulated into the lipid bilayer. This has several implications. First, the real neurosteroid concentration around the receptor might be hundreds of times larger than that calculated in the aqueous solution. Second, neurosteroid range of action will be limited to the synthetizing cell and immediate neighboring cells (autocrine and paracrine action). Third, this solubility-dependent, low-affinity and high-concentration interaction needs to be taken into account for the development of drugs that aim to mimic neurosteroid effect on GABA$_A$Rs (Chisari et al., 2010).

The possibility of mimicking neurosteroid effects in pathologic conditions characterized by excitation–inhibition dysfunctions has allowed for the development of drugs that can selectively and directly enhance δ-GABA$_A$R–mediated tonic inhibition. Clinical application of such pharmacologic agents has received increasing interest, as animal studies have shown intriguing potential. For example, direct enhancement of δ-GABA$_A$Rs with gaboxadol (4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol, or THIP) has been shown to lessen negative symptoms in a mouse model of postpartum depression (Maguire & Mody, 2008). At 500 nM, THIP acts as a δ-GABA$_A$R-selective agonist (Brown et al., 2002) and was previously proposed as a sleep-promoting drug (Wafford & Ebert, 2006), only to fail phase III clinical trials because of its side effects (Brickley & Mody, 2012). Safer δ-GABA$_A$R-specific agonists are currently under investigation (Wafford et al., 2009).

Ganaxolone is a synthetic neurosteroid currently under investigation as an AED for catamenial epilepsy, given the typical exacerbation pattern of this kind of epilepsy, which correlates with ovarian-cycle–linked steroid oscillations in the plasma of affected women. As it is true for other AED indications, not all kinds of epilepsies will benefit from drugs that increase tonic inhibition. In fact the effect of neurosteroid-like drugs on epileptic patients will depend on the anatomic and functional alterations typical of their specific syndrome. The clearest example is that of absence seizures. Of interest, THIP was used to trigger absence-like seizures in rats (Fariello & Golden, 1987), and in a case report, exogenous administration of progesterone increased seizure frequency in an absence epilepsy patient (Grünewald et al., 1992). These effects are most likely mediated by further potentiation of an already pathologically increased tonic inhibition on thalamic relay neurons described in animal models of absence seizures (Cope et al., 2009). In these cases, drugs with diametrically opposite effects might be of use. Unfortunately, to date no specific δ-GABA$_A$R antagonists have been developed.

Lastly, ethanol is another molecule that can, at intoxicating concentrations (20–30 mM), increase δ-mediated tonic current in both interneurons and DGGCs (Glykys et al., 2007). Whether the effect is mediated by a direct interaction with the receptor or through the neurosteroid system via stimulation of neurosteroid synthesis is a topic of current controversy and fervent investigation.

Alterations of Neurosteroid-Sensitive GABA$_A$ Receptors in an Animal Model of TLE

GABA$_A$R plasticity in human TLE and animal models of the disease is complex and encompasses neuron-type and brain region–specific modifications in the expression of different subunits and will affect both types of inhibition, phasic and tonic (Schwarzer et al., 1997; Fritschy et al., 1999; Loup et al., 2000; Scimemi et al., 2005; Goodkin et al., 2008). For the extrasynaptic GABA$_A$Rs, changes in expression and modulation have been reported in different animal models, and consistently show decreased expression of δ-GABA$_A$Rs in the molecular layer in the DG starting 4 days after the initial epileptogenic insult, and progressively continuing until it stabilizes at constant low levels at about 12 weeks (Schwarzer et al., 1997; Peng et al., 2004; Zhang et al., 2007). In addition, the γ2 subunit begins to be ectopically expressed perisynaptically, where it most likely pairs up with α4 subunits, which also show an increased level of expression. Of interest, tonic current in these cells remains at control levels, and is largely mediated by α5-GABA$_A$Rs, and possibly by ectopic γ2-GABA$_A$Rs, in a molecular shift that will have important functional and pharmacologic consequences (Mtchedlishvili & Kapur, 2006; Zhang et al., 2007; Rajasekaran et al., 2010).

First of all, a neuron that changes its array of extrasynaptic receptors will respond to GABA very differently. Although there are no in vivo studies on how different GABA concentrations influence the activation of various GABA$_A$Rs, traditionally δ-GABA$_A$Rs are considered as having high affinity for GABA, whereas α5-GABA$_A$Rs seem to need concentrations 10 times higher to be activated. This implies that possibly a tonic current that shifts from being δ-GABA$_A$R mediated to being mostly α5-GABA$_A$R mediated will require higher ambient GABA levels in order to be as effective. This consideration, in addition to evidence of decreased overall GABA content in epileptic brains (Minamoto et al., 1992), corroborates the idea of a multilevel GABAergic inadequacy for the control of DGGC excitability.

Second, pharmacologic properties of α5-GABA$_A$Rs and δ-GABA$_A$Rs are very different. The low GABA efficacy on δ-GABA$_A$Rs, makes these receptors the preferential site of action of neurosteroids. In addition, δ-GABA$_A$Rs are naturally insensitive to benzodiazepines, because these drugs need γ subunit in order to bind to GABA$_A$Rs. Moreover, α5-GABA$_A$Rs are typically insensitive to zolpidem. In our own findings, diazepam-mediated increase in DGGC tonic current was unchanged after pilocarpine-induced SE, and there is little to no anatomic evidence of α5-GABA$_A$R up-regulation in the DG molecular layer, and on the contrary, α5 might even be slightly down-regulated (Zhang et al., 2007). Maintenance of DGGC tonic current at control levels in epileptic animals most likely relies on GABA$_A$Rs other than α5-GABA$_A$Rs, namely perisynaptic α4γ2-GABA$_A$Rs or receptors containing only α4 and β subunits. Pharmacology of the tonic current mediated by these receptors will also be different, given the low sensitivity of α4-GABA$_A$Rs to diazepam (Wisden et al., 1991; Benke et al., 1997). Perhaps the most important pharmacologic consequence of extrasynaptic GABA$_A$R plasticity in TLE is the loss of a major fraction of δ-GABA$_A$Rs. Functionally, DGGCs of epileptic animals do not respond to neurosteroid potentiation of tonic current, and in parallel, the ability of neurosteroids to lower dentate excitability turns into a net effect of increasing excitability (Zhang et al., 2007).

Before reaching final conclusions on δ-GABA$_A$R plasticity in TLE and its functional and practical pharmacologic consequences, we also need to consider δ-GABA$_A$R expression on interneurons. In the dentate, in molecular layer interneurons, the δ subunit is expressed paired with α1, rather than α4 subunits (Glykys et al., 2007), and the δ-GABA$_A$Rs mediate a neurosteroid sensitive tonic conductance. Of interest, in the same mouse model of TLE, δ expression on interneurons appears to be increased, contrary to what happens in granule cells (Zhang et al., 2007). Tonic current is also increased in molecular layer

interneurons cells, and neurosteroid sensitivity is maintained (Wei W, Mody I, unpublished data). Increasing tonic inhibition onto the inhibitory cells might have a dual effect. On one hand, slightly decreasing GABAergic drive onto DGGCs might help reduce their level of synchronization, since some interneurons are very efficient at synchronizing large populations of neurons (Cobb et al., 1995). More so if we keep in mind that a typical anatomic alteration in TLE is the formation of functional aberrant recurrent connections within DGGCs (Tauck & Nadler, 1985), which have been shown to facilitate the onset of DGGCs synchronization. On the other hand, some of the δ-GABA$_A$R-labeled interneurons in the hippocampus might be neurogliaform cells (NGFCs) and ivy cells (Armstrong et al., 2011, 2012). NGFCs abundantly express δ-GABA$_A$Rs (Oláh et al., 2009), and since they largely contribute to ambient GABA levels, decrease of their excitability would bring a net decrease in ambient GABA. Moreover, inhibiting the inhibitors can by itself lead to significant alterations in dentate excitability and bring basal excitability levels dangerously close to seizure threshold.

Most of the time the epileptic brain is capable of maintaining adequate and functional levels of excitation and inhibition, but compared to the healthy brain, it is a precarious equilibrium. Even small deviations can cause the inhibitory control to lose its grasp on the dentate gate. Typically TLE is sensitive to triggers, and there is much literature on the efficiency of stress as a trigger of seizures (Joëls, 2009). Catamenial epilepsy is a type of epilepsy during which seizures cluster around a specific period of the ovarian cycle (Herzog et al., 1997). In both these cases, neurosteroid fluctuations in the brain will be fast and large, and although they will fail to decrease DGGCs excitability, they will be able to inhibit interneurons by potentiating their already higher tonic current, possibly shifting the balance sufficiently to facilitate an epileptic event. Lastly, the role of ethanol as a precipitant of seizures might be played through its preferential δ-GABA$_A$Rs enhancement at low concentrations (Hauser et al., 1988; Ng et al., 1988).

However, during epilepsy a neurosteroid basal tone is necessary, because blocking neurosteroid synthesis with finasteride greatly increases seizure frequency, whereas it has no apparent effect on control mice (Lawrence et al., 2010). Finasteride is a 5-α-reductase blocker commonly used in the treatment of certain prostatic cancers, and at lower doses, as a remedy for androgenic alopecia (Mella et al., 2010; Nacusi & Tindall, 2011). In one case report, finasteride increased, whereas progesterone decreased, seizure frequency in a patient with catamenial epilepsy (Herzog & Frye, 2003).

One way to reconcile these results is to think of neurosteroid modulation of neuronal excitability as a heterogeneous phenomenon: compartmentalization of neurosteroid synthesis in neurons and astrocytes will dissociate their effects on different types of neurons and populations of receptors. Therefore, the fact that in vitro DGGCs do not show neurosteroid sensitivity could mean that part of the recorded tonic current was already potentiated by neurosteroid locally synthetized in the slice. If neurosteroid levels were sufficiently elevated to reach a ceiling effect on the few remaining δ-GABA$_A$Rs, then addition of neurosteroid will not cause a further increase in the tonic conductance. Therefore, in TLE, basal levels of neurosteroid are important to keep inhibitory–excitatory equilibrium, but at the same time the epileptic brain seems to be more sensitive to neurosteroid changes. Physiologic conditions of modified neurosteroid levels such as the ovarian cycle, pregnancy, and stress are accompanied by plastic homeostatic changes in δ-GABA$_A$Rs, but we do not yet know if similar changes are taking place in the epileptic hippocampus (Maguire et al., 2005; Maguire & Mody, 2007, 2008; Maguire et al., 2009). Nonetheless, in the face of the GABA$_A$R alterations in the dentate gyrus, rapid neurosteroid increases will likely favor more excitable states.

In conclusion, different hormonal pathways have the ability to regulate neuronal excitation. Neurosteroids are a potent class of hormones that enhance inhibition by positively modulating the efficacy of GABA at δ-GABA$_A$Rs. Plasticity of neurosteroid-sensitive GABA$_A$Rs has been consistently found in different models of TLE, and although neurosteroid effects on individual cell excitability have been tested, predicting the net effect of abrupt neurosteroid changes on the overall gain of a specific network in the epileptic brain is not straightforward. Stress and the ovarian cycle modify neurosteroid concentration in the brain and are also known seizure triggers in epileptic patients. Overall, neurosteroids appear capable of producing bimodal effects on excitability in the epileptic brain. Although basal neurosteroid synthesis seems to be necessary for control of seizure frequency, the epileptic brain is also more sensitive to neurosteroid changes, and inhibition is more likely to lose control over the dentate gate after substantial neurosteroid increase, since interneuronal sensitivity to neurosteroid is maintained while excitatory cells lose their ability to respond to neurosteroids. This bimodal behavior has to be taken into account when designing AEDs that act by potentiating the function of δ-GABA$_A$Rs, especially in patients exposed to abrupt changes in endogenous neurosteroids.

Disclosure

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

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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CHAPTER 3

INTERNEURONAL $GABA_A$ RECEPTORS INSIDE AND OUTSIDE OF SYNAPSES

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Interneuronal $GABA_A$ receptors inside and outside of synapses

Isabella Ferando[1,2,3] and Istvan Mody[1,2]

About 20% of the total number of neurons in the brain are interneurons (INs) that utilize GABA as their neurotransmitter. The receptors for GABA have been well studied in principal cells, but INs also express GABA receptors, in particular the $GABA_A$ type ($GABA_A$Rs), which may also be activated in an autocrine manner by the transmitter released by the INs themselves. As more and more neurological and psychiatric disorders are being discovered to be linked to malfunction or deficits of INs, this review will cover how INs communicate with each other through the activation of synaptic and extrasynaptic $GABA_A$Rs. The properties of $GABA_A$Rs specific to INs may differ significantly from those found on principal cells to open the prospect of developing IN-specific drugs.

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Introduction

In the mammalian brain inhibition and some forms of synchrony are generated by a complex and widely diversified network of interneurons (INs) [1,2] that release their transmitter (GABA) on an equally varied set of GABA receptors. Of these, the $GABA_A$ receptors ($GABA_A$Rs) are a class of heteropentameric ligand-gated ion channels permeable to Cl^- and HCO_3^- generally composed of three subunits out of an array of 19 (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π, ρ_{1-3}), with the clockwise $\alpha_1\beta_2\alpha_1\beta_2\gamma_2$ arrangement most commonly found in the brain [3–6]. Different subunits confer unique characteristics to the receptors including agonist affinity and efficacy, desensitization rate, and pharmacology [7,8]. Many widely used anxiolytic, hypnotic and anesthetic drugs act upon $GABA_A$Rs with specific subunits, for example, different α determine the sedative or anxiolytic effects of benzodiazepines [9,10•]. Some receptors are naturally insensitive to benzodiazepines (α4-, α6-, or δ-$GABA_A$Rs), while others confer a high degree of sensitivity to various exogenous or endogenous molecules, as is the case of ethanol and neurosteroids on δ-$GABA_A$Rs [11,12•]. Lastly, the localization of $GABA_A$Rs (synaptic or extrasynaptic) determines whether they mediate rapid synaptic (phasic) events activated by fast (<1 ms) and large (~1 mM) GABA transients in the synaptic cleft, or a persistent (tonic) conductance activated by low levels of GABA present in the extracellular space. Only a few subunit combinations (δ-$GABA_A$Rs or α5-$GABA_A$Rs) have a high affinity for and a marginal desensitization to GABA, or can open with a sufficiently large probability in the absence of agonists to mediate a tonic conductance [12•,13,14•].

The composition and assembly of $GABA_A$Rs on principal cells, their subcellular localization, plasticity in health and disease and specific pharmacology have been well studied in over three decades of research and clinical applications [10•,15]. These include stress, puberty, ovarian cycle, pregnancy, depression, the effects of ethanol, and of various anesthetics related to δ-$GABA_A$Rs [16–21], epilepsies for both δ-$GABA_A$Rs and α5-$GABA_A$Rs [22,23]; memory formation and consolidation, LTP induction, anesthesia-induced memory loss, functional plasticity and recovery after stroke, and power of γ oscillations for α5-$GABA_A$Rs [24–29]. In sharp contrast, research on $GABA_A$Rs of INs has progressed at a much slower pace. This can be ascribed to the inherent complexity of this diverse family of neurons that precludes consensus on a practical categorization of the INs themselves [30••]. This review will summarize the physiology of synaptic and extrasynaptic $GABA_A$Rs of INs, their plasticity, and the possibility of a correlation between IN development and their $GABA_A$Rs mediating tonic conductances.

$GABA_A$Rs and tonic/phasic conductances of INs

Compared to principal cells, INs in the forebrain seem to lack some specific $GABA_A$R subunits. The most striking difference is the absence of α4-$GABA_A$R and α5-$GABA_A$R, subunits that are involved in mediating tonic inhibition in glutamatergic neurons [12•], but some INs in the spinal cord may constitute an exception to this rule [31]. Below, without any presumption of completeness, we will summarize known properties of synaptic and extrasynaptic $GABA_A$Rs in a subset of INs.

Unidentified INs

Reports exist on GABAergic tonic conductances recorded in unidentified or poorly identified INs in many brain

areas, particularly in the molecular layer of the dentate gyrus (DGML), virtually all strata of CA1 and CA3 and various layers of the neocortex [22,27,32–36]. This conductance is largely mediated by a combination of subunits unique to INs (α1/δ-$GABA_A$Rs) and is highly sensitive to modulation by ethanol and neurosteroids [34]. In mice lacking the δ-$GABA_A$Rs (*Gabrd*$^{-/-}$), CA3 stratum pyramidale (SP)-INs show a complete loss of tonic conductances, whereas in wild type mice the same cells show a THIP-sensitive tonic current, which strongly argues for a δ-$GABA_A$R mediated tonic inhibition [33]. A recent study showed similar findings in DGML INs and CA1 stratum radiatum (SR) INs of a mouse with δ-$GABA_A$Rs selectively deleted from all GABAergic INs [37•]. Although the majority of tonic inhibition in INs seems to be mediated by α1/δ-$GABA_A$Rs, other subunits may also be involved, as CA1 SR INs show picrotoxin and zolpidem-sensitive tonic currents (the latter excludes the participation of δ-$GABA_A$Rs) [36], and DGML INs of *Gabrd*$^{-/-}$ mice, in which deletion of δ-$GABA_A$Rs was not restricted to INs, show a compensation in the tonic current, but receptor specificity was not determined [27]. These findings suggest that some INs may entirely or partially rely on δ-$GABA_A$Rs for generating a GABA-dependent tonic conductance.

Unequivocal anatomical and functional evidence from different sources shows heavy δ-$GABA_A$R presence on at least two kinds of INs of the neocortex and hippocampus [22,37•,38,39,40,41•,42]. These are the parvalbumin (PV) expressing INs (PV+INs) and neurogliaform cells (NGFCs).

Parvalbumin expressing INs (PV+INs)

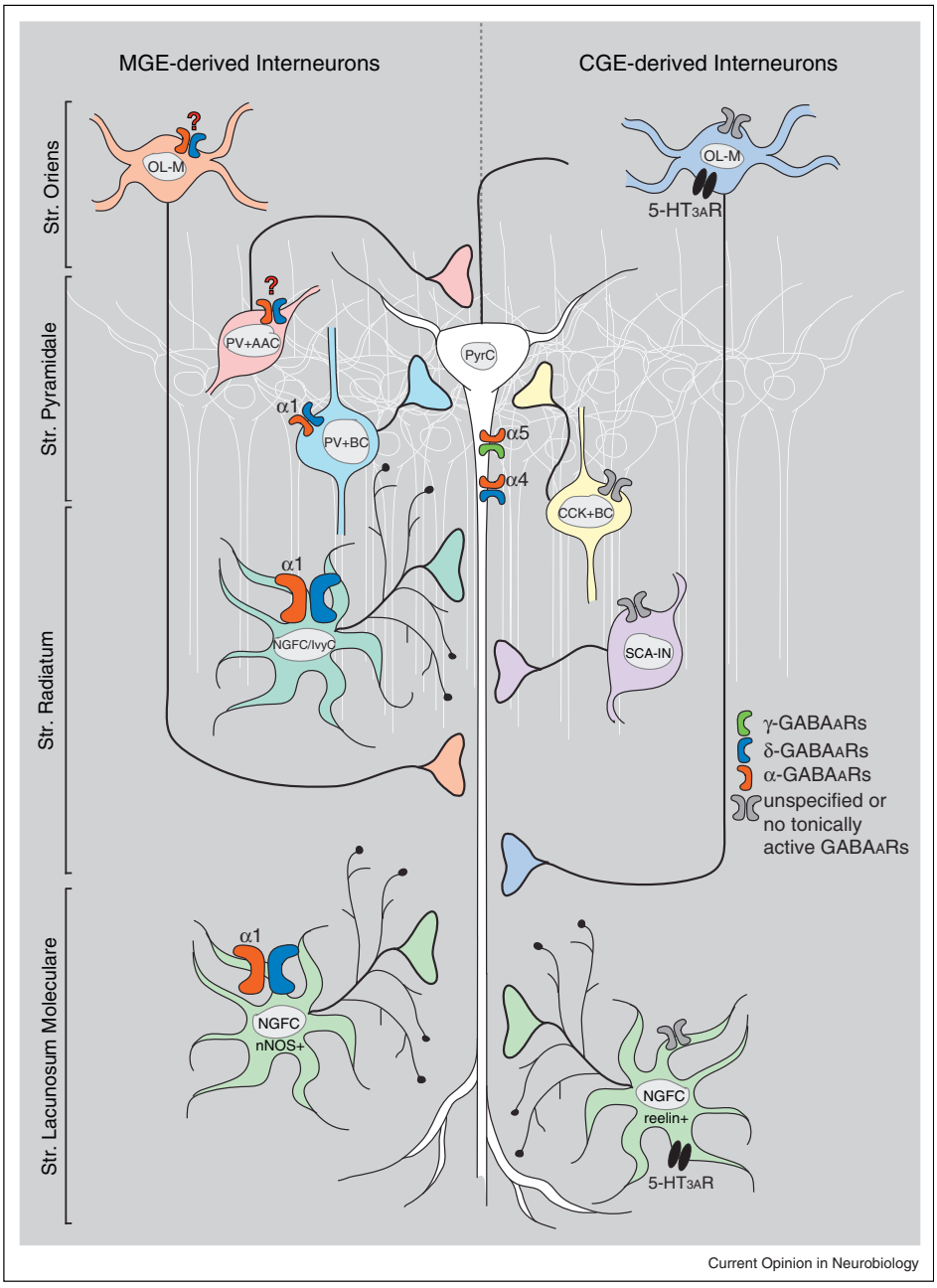
The vast majority of PV+INs in the brain are either basket cells (BCs) or axoaxonic cells (AACs), while a small fraction belong to other IN classes. These two predominant PV+INs target the somata and the axon initial segments of the target cell respectively, and thus are in an ideal position to strongly control neuronal output [1,2,43,44]. Most PV+INs are born in the median ganglionic eminence (MGE), and migrate to populate the hippocampus and the neocortex in late embryonic stages [45••,46••,47]. In the hippocampus, they most densely populate the area around the SP, and represent almost the entire population of δ-$GABA_A$R+INs in this area [40,41•,48•]. Deletion of this subunit and the consequent loss of tonic inhibition by these INs have major effects on the frequency of *in vitro* CA3 γ oscillations, a network behavior that is initiated and controlled by PV+BCs [33]. Moreover, selective potentiation of the δ-$GABA_A$R-mediated tonic conductance with neurosteroids, THIP or ethanol increases the power of γ oscillations (Ferando and Mody, unpublished). The δ-$GABA_A$Rs on PV+INs are modulated during physiological and pathological states, such as pregnancy and epilepsy [22,40,48•]. The GABAergic input onto these INs seems to be shunting in nature with kinetics that would help sustain γ oscillations [49], but after periods of intense activation, GABAergic transmission may become excitatory in an activity dependent switch [50]. Several studies have also investigated PV+BC to PV+BC synaptic connections. As expected from an IN type controlling fast network activity, PV+BCs have a high degree of reciprocal connectivity [51,52,53•]. Their synaptic $GABA_A$R-mediated events have faster kinetics, larger amplitudes and higher frequencies than IPSCs elicited in principal cells or in other INs. The PV+BC IPSCs are mediated by α1-$GABA_A$Rs, subunits heavily expressed in PV+BCs of the neocortex and hippocampus [54–58], as well as in PV+INs of the striatum [59]. The rapid synaptic $GABA_A$R signaling is responsible for the phase coupling of θ/γ oscillations, as demonstrated in mice where synaptic $GABA_A$Rs (γ2-$GABA_A$Rs) were selectively ablated from PV+INs; but the fundamental properties of γ oscillations were unaltered [60]. This is not necessarily surprising considering that of the large number of synapses (>16 000) received by PV+INs, only a small fraction (6.4%) are GABAergic [61]. This may also mean that the high density of α1-$GABA_A$Rs in PV+INs is not confined to synapses, but can be found, most likely paired with δ-$GABA_A$R, at extrasynaptic sites.

In contrast to most PV+INs found in the cortex and hippocampus, the PV+INs of the reticular nucleus of the thalamus (nRT) express no tonic $GABA_A$R-mediated conductance [62] and exhibit slowly decaying predominantly α3-$GABA_A$R-mediated synaptic events [63,64]. The PV+INs of the nRT are not generated in the MGE, but from prethalamic Olig2-lineage cells at day E 10.5 [65].

Neurogliaform cells (NGFCs) and Ivy cells (IvyCs)

NGFCs/IvyCs of the hippocampus and the cortex are the IN type probably expressing the highest levels of δ-$GABA_A$Rs located extrasynaptically [39,42]. Ontogenetically, NGFCs/IvyCs originate from two sources: the $5HT_{3A}$-R+, reelin+NGFCs/IvyCs derive from the caudal ganglionic eminence (CGE), whereas nNOS+NGFCs/IvyCs are born in the MGE [45••,66,67]. NGFCs/IvyCs release GABA slowly and diffusely; hence have been implicated in GABAergic volume transmission [42], but may also play a role in the dynamics of the θ rhythm [68–72]. In the hippocampus they are mostly localized in the str. lacunosum moleculare (NGFCs), in the DGML and in the CA1 SR (IvyCs). In the cortex, NGFCs are present in all layers [42], with reelin+CGE-derived NGFCs mostly inhabiting layer 1 [67]. We previously recorded the characteristics of tonic $GABA_A$R-mediated conductances from INs of the DGML [27,34] that, considering the abundance of NGFCs in this region [70], must have belonged to this group. It appears that these cells express the highly plastic α1/δ-$GABA_A$Rs and a corresponding ethanol-sensitive tonic current [34], and a low frequency

Figure 1



Schematic representation of δ-$GABA_A$R-mediated tonic conductances in major subclasses of hippocampal CA1 INs separated by their developmental profile. *Left panel*: Of the INs born in the medial ganglionic eminence (MGE) some have established α1/δ-$GABA_A$R-mediated tonic conductances while in others the presence of such a conductance is only hypothetical (marked by '?'). *Right panel*: According to evidence to date, INs born in the caudal ganglionic eminence (CGE) do not express δ-$GABA_A$Rs and maybe even no other $GABA_A$R would underlie a tonic conductance. For further explanation see text. *Abbreviations*: PyrC, pyramidal cell; OL-M, oriens-lacunosum moleculare IN; PV+AAC, parvalbumin + axo-axonic cell; PV+BC, parvalbumin + basket cell; NGFC, neurogliaform cell; IvyC, Ivy cell; CCK+BC, cholecystokinin + basket cell; SCA-IN, Schaffer collateral associated IN; nNOS, neuronal nitric oxide synthase; $5HT_{3A}$R, ionotropic serotonin receptor, type 3A.

of spontaneous $GABA_AR$-mediated events; but specific pharmacological manipulations have not been done to identify the receptors involved [70]. NGFCs of the neocortex also express high levels of δ-$GABA_ARs$ and clearly show a neurosteroid (THDOC)-sensitive tonic $GABA_AR$-mediated conductance [42]. Strong δ-$GABA_ARs$ labeling is found in the CA1 SLM where NGFCs from both embryological origin are localized [3,45••,66]. It remains to be determined whether NGFCs/IvyCs derived from the CGE and MGE express the same type of $GABA_ARs$.

Somatostatin expressing INs (SOM+INs)

One of the most typical members of the SOM+IN class is the hippocampal oriens-lacunosum moleculare (OL-M) IN, which has its soma in the str. oriens from where it targets the distal dendrites of principal cells in the LM. As most other SOM+INs, OL-M INs seemingly form a fairly homogenous population derived from the MGE [45••]. Surprisingly, they have been recently reported to have dual embryonic origins and distinct roles in network oscillations: the $5HT_3$-R negative OL-M INs originate from the MGE, while $5HT_3$-R positive OL-M INs are derived from the CGE [73••]. This bimodal ontogeny also appears to determine the mature subunit composition of AMPARs and NMDARs of INs [74•]. Perhaps analogous to how excitation is shaped by development, the specificity of $GABA_AR$ subunits may also be determined by embryonic origins. Interestingly, the presence of tonic inhibition on SOM+INs is yet to be fully investigated. Only a small fraction of dentate hilar SOM+INs express δ-$GABA_ARs$ [41•], but in the rest of the hippocampus they express α1-$GABA_AR$, the natural δ-$GABA_ARs$ partner in INs, in a diffuse histological distribution characteristic of extrasynaptic localization [57,58]. Moreover, hilar SOM+INs do not express the MGE-marker Nkx2.1 [75], which argues for their CGE origin. Consistent with the anatomical findings [41•], hilar SOM+INs identified in SOM-Cre mice also exhibit very little $GABA_AR$-mediated tonic conductance [76]. No specific characteristics are known about the synaptic $GABA_AR$-mediated events in SOM+INs, other than that they innervate other types of INs, but they generally do not inhibit each other [53•].

Cholecystokinin basket cells (CCK+BCs) and Schaffer collateral-associated INs (SCA-INs)

These two IN types represent a substantial group in the hippocampal formation with specific control of the pyramidal cells' somatic and dendritic compartments, respectively. Both derive from the CGE [45••], and are localized in the hippocampal SP (CCK+BCs) and the SR (SCA-INs). The CCK+BCs complement the PV+BCs in providing perisomatic inhibition to the pyramidal cells, but they express an entirely different set of synaptic inputs, receptors, voltage-gated Ca^{2+} channels, and a myriad of other properties that distinguish these two types of soma-targeting GABAergic neuron [43]. CCK+BCs do not express α1-$GABA_AR$ [57] and as a consequence they most likely do not express δ-$GABA_ARs$ either [40,41•,48•], although a complete coexpression study has yet to be performed. Compared to PV+BCs, CCK+BCs receive stronger GABAergic somatic synaptic input (83% of somatic synapses [77]). In spite of such abundant synaptic GABAergic innervation, some cortical regular spiking INs (likely CCK+BCs), lack a tonic $GABA_A$ conductance *in vitro* [78]. The SCA-INs make contacts with the apical dendrites of principal cells, and a fraction of them also expresses calbindin [79], which rarely co-localizes with δ-$GABA_ARs$ [41•,42]. The question remains open whether INs of the SR reported to have a zolpidem-sensitive GABAergic tonic conductance [35,36], incompatible with a δ-$GABA_ARs$ contribution, were genuine SCA-INs. The INs in the SR show faster IPSCs, have a more depolarized resting membrane potential compared to principal cells [80], with depolarizing or shunting GABAergic conductances depending on the ambient GABA concentration [35].

Conclusions and outlook

We have presented a brief overview of the $GABA_ARs$ and the corresponding phasic and tonic conductances present on a small sample of hippocampal and cortical INs. However blurred the picture may be, there are certain emerging trends when various properties of tonic inhibition in particular are put in the perspective of the embryonic origins of the INs. At this time, the GABAergic system of INs cannot be as clearly delineated on the basis of IN ontogeny as their glutamate receptors [74•]. Nevertheless, in Figure 1 we depicted a simplified scheme for the division of hippocampal INs, based on their CGE or MGE origins, into those that may possess δ-$GABA_AR$-mediated tonic conductances (MGE-derived) and those that do not (CGE-derived). A thorough investigation of phasic and tonic inhibition in these various INs in the future may answer significant questions about their function under normal and pathological conditions. Knowing the exact localization of δ-$GABA_ARs$ on different IN subtypes will offer a very powerful tool for differential potentiation or dampening of discrete networks, with interesting functional implications for generations of normal and altered brain rhythms in which INs are adamantly involved [2].

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CHAPTER 4

EXCITABILITY CHANGES RELATED TO $GABA_A$ RECEPTOR PLASTICITY DURING PREGNANCY

Excitability Changes Related to $GABA_A$ Receptor Plasticity during Pregnancy

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Alterations in $GABA_A$ receptor ($GABA_AR$) expression and function, similar to those we described previously during pregnancy in the mouse dentate gyrus, may also occur in other brain regions. Here we show, using immunohistochemical techniques, a decreased δ subunit-containing $GABA_AR$ ($\delta GABA_AR$) expression in the dentate gyrus, hippocampal CA1 region, thalamus, and striatum but not in the cerebral cortex. In the face of the highly elevated neurosteroid levels during pregnancy, which can act on $\delta GABA_ARs$, it may be beneficial to decrease the number of neurosteroid-sensitive receptors to maintain a steady-state level of neuronal excitability throughout pregnancy. Consistent with this hypothesis, the synaptic input/output (I/O) relationship in the dentate gyrus molecular layer in response to lateral perforant path stimulation was shifted to the left in hippocampal slices from pregnant compared with virgin mice. The addition of allopregnanolone, at levels comparable with those found during pregnancy (100 nM), shifted the I/O curves in pregnant mice back to virgin levels. There was a decreased threshold to induce epileptiform local field potentials in slices from pregnant mice compared with virgin, but allopregnanolone reverted the threshold for inducing epileptiform activity to virgin levels. According to these data, neuronal excitability is increased in pregnant mice in the absence of allopregnanolone attributable to brain region-specific downregulation of $\delta GABA_AR$ expression. In brain regions, such as the cortex, that do not exhibit alterations in $\delta GABA_AR$ expression, there were no changes in the I/O relationship during pregnancy. Similarly, no changes in network excitability were detected in pregnant *Gabrd*$^{-/-}$ mice that lack $\delta GABA_ARs$, suggesting that changes in neuronal excitability during pregnancy are attributable to alterations in the expression of these receptors. Our findings indicate that alterations in $\delta GABA_AR$ expression during pregnancy result in brain region-specific increases in neuronal excitability that are restored by the high levels of allopregnanolone under normal conditions but under pathological conditions may result in neurological and psychiatric disorders associated with pregnancy and postpartum.

Introduction

We recently demonstrated plasticity of $GABA_A$ receptors ($GABA_ARs$) during pregnancy and postpartum, in which the expression of the $GABA_AR$ δ and γ2 subunits in dentate gyrus granule cells are decreased during pregnancy, resulting in diminished tonic and phasic inhibitions mediated by these receptors (Maguire and Mody, 2008). Changes in GABAergic function during pregnancy have also been inferred from increased agonist binding during pregnancy (Majewska et al., 1989; Concas et al., 1999) and decreased expression of the mRNA encoding the $GABA_AR$ γ2 subunit during pregnancy (Concas et al., 1998; Follesa et al., 1998). $\delta GABA_ARs$ are the preferred, if not sole, site of action for neurosteroids in the physiological concentration range in the mammalian CNS (Stell et al., 2003; Belelli and Lambert, 2005). Therefore, it is reasonable to postulate that the GABAergic system and, in particular, the tonic inhibition mediated by $\delta GABA_ARs$ may play a role in steroid hormone-mediated changes in neuronal excitability.

Changes in neuronal excitability, e.g., changes in seizure frequency, associated with pregnancy are difficult to assess because of many factors, including but not limited to noncompliance with antiepileptic drug usage, alterations in the pharmacokinetics of antiepileptic drugs, hormonal changes, sleep deprivation, and stress. The available literature on seizures associated with pregnancy reveals that there is an increase in seizure frequency in 24% of the 2165 cases studied, a decrease in 23%, and no change in 53% of cases (Schmidt, 1982), demonstrating the complex relationship between pregnancy and epilepsy. More recent findings also support the idea that pregnancy has variable effects on seizure frequency (Sabers et al., 1998; Rück and Bauer, 2008).

Changes in seizure frequency during pregnancy are thought to result from hormonal changes; however, the mechanisms of steroid hormone-induced changes in neuronal excitability during pregnancy remain unclear. It is known that steroid hormone derivatives, or neurosteroids, are potent modulators of $GABA_ARs$; thus, it is likely that the production of neurosteroids throughout pregnancy may alter neuronal excitability via actions on $GABA_ARs$. In addition, steroid hormones have been shown to alter $GABA_AR$ subunit expression. Administration and with-

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drawal of exogenous progesterone alters $GABA_AR$ subunit expression (Concas et al., 1998; Smith et al., 1998; Follesa et al., 2000, 2001, 2002; Biggio et al., 2001; Smith, 2002). To complicate this picture, modulators acting on $\delta GABA_ARs$, such as allopregnanolone and ethanol, also alter $GABA_AR$ subunit expression (Follesa et al., 2004). The expression of the $GABA_AR$ $\alpha 1$, $\alpha 4$, and $\gamma 2$ subunits are altered by exposure to exogenous neurosteroids (Smith et al., 1998; Follesa et al., 2000, 2001, 2002; Smith, 2002). $GABA_AR$ $\gamma 2$ subunit mRNA levels are inversely correlated with neurosteroid levels, such that increased levels of allopregnanolone are associated with decreased $GABA_AR$ $\gamma 2$ subunit mRNA levels (Follesa et al., 2004). However, it is unclear how changes in $GABA_AR$ structure and function alter neuronal excitability in response to physiological conditions, such as pregnancy, which result in large elevations in progesterone and neurosteroid levels. Therefore, by examining synaptic input/output (I/O) function, we sought to determine whether the alterations in $GABA_ARs$ throughout pregnancy are associated with altered neuronal excitability.

The objective of this study was to determine possible changes in $\delta GABA_AR$ expression in several brain regions and to determine the effect of $GABA_AR$ plasticity on neuronal network excitability using extracellular field recordings. Here we demonstrate brain region-specific changes in $\delta GABA_AR$ expression, which increase network excitability in the absence of allopregnanolone. Physiological levels of allopregnanolone present in the intact brain of pregnant mammals dampen the increased neuronal excitability to pre-pregnancy levels, suggesting that the changes in $GABA_ARs$ constitute a homeostatic mechanism to ensure a normal level of inhibition throughout pregnancy.

Materials and Methods

Animals

Adult, ~3 months of age, C57BL/6 and *Gabrd*$^{-/-}$ mice (on C57BL/6 background) were housed and maintained by the University of California, Los Angeles (UCLA) Association for Assessment and Accreditation of Laboratory Animal Care accredited Division of Laboratory Medicine. All procedures used were Institutional Animal Care and Use Committee approved by the UCLA Chancellor's Animal Research Council. Animals were maintained on a normal light/dark cycle (12 h, lights on from 6:00 A.M. to 6:00 P.M.) and had *ad libitum* access to food and drinking water. All experiments were performed during the light cycle. Animals were killed between 10:00 A.M. and 12:00 P.M.. The virgin mice used in this study were anovulatory, noncycling mice. The pregnant mice used in this study were first-time pregnant mice at day 18 of pregnancy. The postpartum mice used were first-time dams at 48 h postpartum. All genotyping was performed by Transnetyx.

Immunohistochemistry

Age-matched adult virgin and day 18 pregnant C57BL/6 mice or *Gabrd*$^{-/-}$ mice were anesthetized and perfused intracardially with PBS, followed by perfusion with 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde overnight at 4°C. Brains were cryoprotected at 4°C in increasing concentrations (10–30%) of sucrose and then placed in a −80° freezer. Coronal sections (50 μm thick) prepared on a cryostat were placed in PBS until staining, which was performed within 48 h of sectioning. All staining procedures were done under nonpermeabilized conditions to ensure immunoreactivity of only surface, and therefore functionally relevant, proteins. Slices from virgin and pregnant mice were processed in parallel from the time of perfusion, throughout the processing, immunostaining, mounting, and analysis of the tissue. Endogenous peroxidase activity was quenched by incubating the sections in 3% H_2O_2 diluted in methanol for 1 h. Sections were washed with PBS incubated in 10% normal goat serum diluted in Tris-buffered saline (TBS). Sections were incubated overnight at 4°C with anti-GABA δ (1:1000, AB9752; Millipore Corporation) in TBS and 10% normal goat serum. After incubation with the primary antibody, the sections were washed three times in TBS before incubation with a biotinylated secondary anti-rabbit antibody (1:2000; Vector Laboratories) overnight at 4°C. The sections were washed three times in TBS, incubated with an HRP-conjugated avidin enzyme complex (ABC Elite; Vector Laboratories) for 1 h, washed again three times in TBS, and developed with diaminobenzidine (Vector Laboratories). The sections were mounted, coverslipped, and analyzed by light microscopy. Sections from paired animals were processed in parallel to ensure equivalent development of each experimental group for comparison, and pictures were taken in parallel to ensure equivalent exposure of each experimental group.

Optical density was determined using NIH ImageJ software. The optical density was measured in the region of interest, throughout serial sections in each animal. The region of interest in the dentate gyrus consisted of the entire molecular layer of the dentate gyrus. The optical density was measured in the dentate gyrus molecular layer in serial sections (~45 sections per animal). The region of interest in the cortex consisted of all six cortical layers (~25 sections per animal). The background staining was determined to be minimal, as assessed in *Gabrd*$^{-/-}$ mice, demonstrating the specificity of the antibody used for these experiments (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) (see Figs. 1–3). In addition, the background between experimental groups was low and not significantly different between experimental groups; thus, background subtraction was deemed unnecessary. The average optical density was determined for each animal, and the average across animals ($n = 5$ for each experimental group) was used for statistical tests.

Field potential recordings

Synaptic input/output curves. Coronal hippocampal slices (350 μm thick) were prepared from adult (~3 months old) wild-type virgin, day 18 pregnant, and 48 h postpartum mice. Hippocampal field recordings were performed at 32–34°C under standard conditions in an interface chamber perfused with normal artificial CSF (nACSF) solution containing [in mM: 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 1–2 $MgCl_2$, 1.25 $NaHPO_4$, 26 $NaHCO_3$, and 10 D-glucose, pH 7.3–7.4 (bubbled with 95% O_2 and 5% CO_2)]. Allopregnanolone at 100 nM (dissolved in 0.01% DMSO) was added, when indicated, to the nACSF in the incubation chamber and perfused into the recording chamber for at least 5 min before recording. Field potentials were evoked by lateral perforant path stimulation at 0.05 Hz, and responses were recorded in the dentate gyrus molecular layer. Bipolar electrodes were used to deliver a constant-current stimulus. The stimulus intensity was determined by recording a threshold response at a width (W) of 60 μs with no response at 20 μs. The W of the stimulus was increased stepwise by 20 μs to create I/O curves ranging from 20 to 240 μs. Four population field EPSPs (fEPSPs) were recorded at each W, and the maximum slope of the responses (volts per second) was measured over a 0.5–1 ms window of the fEPSP rising phase. The average slope was calculated at each W used.

Input/output curves were fit with a Boltzmann equation: $f(W) = (MAX/(1 + \exp((W - W_{50})/k)) + MAX)$, where W is stimulus width, MAX is the maximum response, k is a slope factor, and W_{50} is the stimulus width that elicits 50% of MAX. The fitted parameters were compared between experimental groups, and statistical significance was determined using a one-way ANOVA, followed by Bonferroni's corrected t tests.

Epileptiform activity induced by high KCl. Hippocampal slices were prepared and maintained as described above. Slices were tested before recordings to ensure their health and appropriate responses to electrical stimulation. Spontaneous extracellular field potentials were measured in the CA1 pyramidal cell layer of the hippocampus. Synchronous discharges were measured in increasing concentrations of extracellular KCl, normal ACSF (2.5), 5.0, 7.5, and 10 mM. The frequency and duration of discharges were measured. Only slices that exhibited synchronous discharges in 10 mM KCl were considered to have a sufficiently healthy local circuitry to be considered in the experimental results. Epileptiform discharges were considered to be population field potentials that had an increased amplitude of 2 SDs above the baseline activity recorded in nACSF. In addition, a discharge frequency of >1 Hz for at least 5 s was

required to constitute epileptiform discharges in this study. Allopregnanolone (100 nM) was added to the incubation chamber and also bath applied when indicated.

Results

Changes in δGABA$_A$R expression during pregnancy

We previously used Western blot analysis to demonstrate a downregulation in δGABA$_A$R expression in the hippocampus from pregnant mice (day 18) compared with virgin animals (Maguire and Mody, 2008). To determine whether there are changes in the expression levels of δGABA$_A$Rs in other brain regions during pregnancy, we performed immunohistochemical analysis throughout the brains of virgin and pregnant mice (day 18). A decrease in δGABA$_A$Rs was confirmed in the dentate gyrus of pregnant compared with virgin mice, similar to our previous results by Western blot analysis (Maguire and Mody, 2008). Representative bright-field and spectrum color-transformed δGABA$_A$R stains of the dentate gyrus in virgin and pregnant mice are shown in Figure 1*A*. The optical density of δGABA$_A$R immunoreactivity in the dentate gyrus molecular layer, in which this protein is predominantly localized (Peng et al., 2004), was measured throughout the hippocampus of each animal ($n = 3$ mice per experimental group, 46 sections per animal). There was a low variability in the δGABA$_A$R immunoreactivity between sections from the same animal (virgin SEM, 2.05; pregnant SEM, 1.84), demonstrating that there is little regional difference in δGABA$_A$R immunoreactivity between the rostral and caudal hippocampus. The optical densities in the 46 hippocampal sections were averaged for each mouse, and the average optical density from each individual animal was used to calculate the average optical density from all the animals in a given group. The data reveal a 41.9 ± 4.8% decrease in the optical density of δGABA$_A$R immunostaining in pregnant compared with virgin mice (Fig. 1*B*, Table 1). The lack of staining in the dentate gyrus molecular layer of *Gabrd*$^{-/-}$ mice confirms the specificity of δGABA$_A$R staining in the wild-type virgin and pregnant mice (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) (Fig. 1). In addition to changes in the dentate gyrus, there is a significant decrease in δGABA$_A$R in the striatum. Representative gray-scale and corresponding color-transformed images of δGABA$_A$R immunoreactivity in the striatum is shown in Figure 2*A* and Table 1. In each animal, the optical density was measured in 25 adjacent striatal sections, and the average from each individual animal was used to determine the average for each group. The optical density of striatal δGABA$_A$R immunoreactivity during pregnancy was decreased by 20.3 ± 4.0% compared with virgin levels (Fig. 2*B*, Table 1). Interestingly, there is no change in the expression of δGABA$_A$R in the cortex throughout pregnancy (Fig. 3, Table 1). Representative sections from virgin and pregnant mice reveal similar levels of δGABA$_A$R expression in the cortex (Fig. 3*A*, Table 1). Immunoreactivity was measured in 35 adjacent cortical sections per animal. There is no significant difference in the optical density measurements of δGABA$_A$R immunoreactivity in the cortex: pregnant mice were 107.8 ± 7.5% that of virgin mice (Fig. 3*B*, Table 1). These data demonstrate that the changes in δGABA$_A$R expression throughout pregnancy are brain region specific. A list of optical density measurements in different brain regions is given in Table 1. To note, there is a downregulation in δGABA$_A$R expression in the dentate gyrus, hippocampal CA1 region, striatum, and several nuclei of the thalamus but no change in the cortex. We did not observe an increase in δGABA$_A$R expression in any of the brain regions examined.

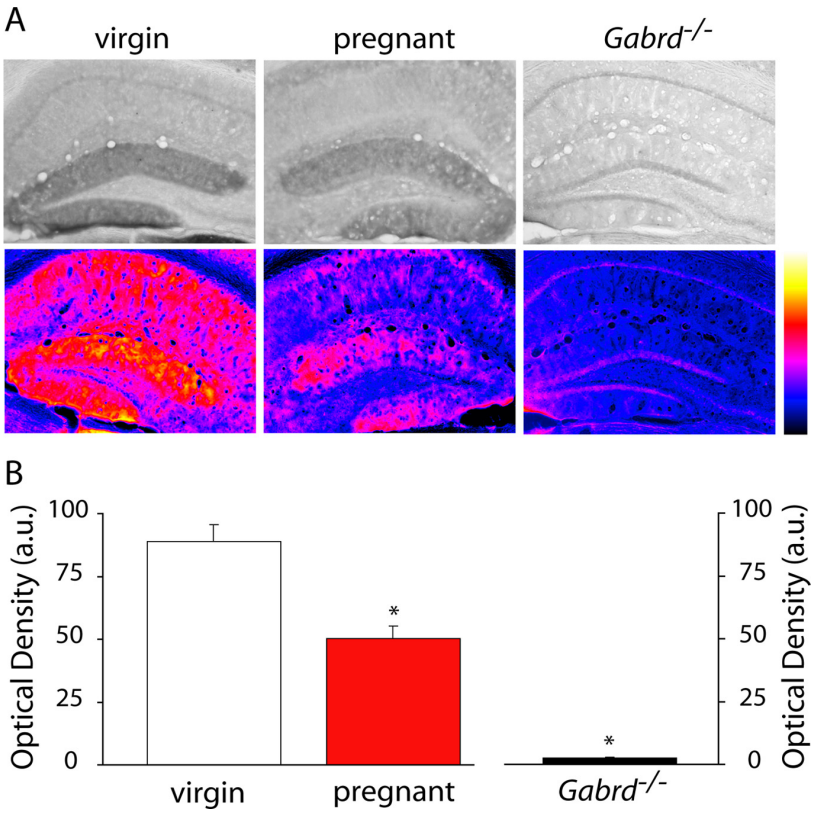


Figure 1. Decreased GABA$_A$ receptor δ subunit expression in the dentate gyrus in pregnant animals. ***A***, Representative bright-field and spectrum color-transformed images of the dentate gyrus from wild-type virgin, pregnant, and *Gabrd*$^{-/-}$ virgin mice stained with an antibody specific for the GABA$_A$R δ subunit. ***B***, Optical density measurements show a significant decrease in GABA$_A$R δ subunit in wild-type pregnant mice compared with virgin mice. The lack of staining in *Gabrd*$^{-/-}$ virgin mice confirm the specificity of the antibody. *$p < 0.05$ using the Student's *t* test compared with virgin wild type. a.u., Arbitrary units.

Changes in synaptic I/O function during pregnancy

The effect of decreased δGABA$_A$R expression in the molecular layer of the dentate gyrus (Fig. 1) on synaptic excitability was investigated by analyzing the synaptic I/O relationship of the dentate gyrus of virgin and pregnant mice. The fEPSP was measured in the molecular layer in response to lateral perforant path stimulation of varying stimulus widths (see Materials and Methods). There was no significant difference in the stimulus intensity between experimental groups in wild-type mice (Table 2). However, the stimulus intensities required to evoke fEPSPs in *Gabrd*$^{-/-}$ were lower compared with those used in wild-type mice (Table 2). Representative fEPSP raw traces and I/O curves from individual wild-type virgin and pregnant mice in the pres-

Table 1. Optical density measurements in multiple brain regions in virgin and pregnant mice

Hippocampal formation	Virgin	Pregnant	Two-way ANOVA		
			Gestational stage	Animal	Interaction
Dentate gyrus	**69.8 ± 2.0**	**40.6 ± 1.8**			
n (sections)	138	138			
n (mice)	3	3	$F_{(330.3, 1, 270)}$, **9.30×10^{-49}**	$F_{(7.337, 2, 270)}$, **7.89×10^{-4}**	$F_{(4.566, 2, 270)}$, **1.12×10^{-2}**
CA1	**25.2 ± 2.2**	**16.3 ± 2.0**			
n (sections)	64	61			
n (mice)	3	3	$F_{(25.59, 1, 119)}$, **1.55×10^{-6}**	$F_{(10.55, 2, 119)}$, **6.05×10^{-5}**	$F_{(3.234, 2, 119)}$, **4.29×10^{-2}**
Cortex	**65.1 ± 2.1**	**69.1 ± 2.0**			
n (sections)	105	105			
n (mice)	3	3	$F_{(2.614, 1, 204)}$, 1.07×10^{-1}	$F_{(16.73, 2, 204)}$, **1.87×10^{-7}**	$F_{(6.865, 2, 204)}$, **1.30×10^{-3}**
Striatum	**168.7 ± 3.9**	**135.1 ± 3.7**			
n (sections)	74	84			
n (mice)	3	3	$F_{(115.6, 1, 152)}$, **2.09×10^{-20}**	$F_{(45.71, 2, 152)}$, **2.86×10^{-16}**	$F_{(2.915, 2, 152)}$, **5.72×10^{-2}**
Thalamus					
VL	**162.2 ± 5.6**	**145.0 ± 5.0**	$F_{(14.41, 1, 73)}$, **3.01×10^{-4}**	$F_{(5.346, 2, 73)}$, **6.81×10^{-3}**	$F_{(0.3652, 2, 73)}$, 6.95×10^{-1}
VA	**126.3 ± 7.2**	**108.2 ± 6.1**	$F_{(10.19, 1, 73)}$, **2.08×10^{-3}**	$F_{(1.981, 2, 73)}$, 1.45×10^{-1}	$F_{(0.1531, 2, 73)}$, 8.58×10^{-1}
VPL	**159.7 ± 4.6**	**129.1 ± 5.7**	$F_{(35.48, 1, 73)}$, **8.34×10^{-8}**	$F_{(2.301, 2, 73)}$, 1.07×10^{-1}	$F_{(1.887, 2, 73)}$, 1.59×10^{-1}
n (sections)	37	42			
n (mice)	3	3			

The average optical density was determined for each individual animal, and the average optical density from each animal was used for statistical analysis. Statistical significance was determined using a two-way ANOVA for gestational stage and individual variability. The *F* values are given as $F_{(\text{sum of squares, degrees of freedom, mean square})}$. Bold *F* values denote significance at $p < 0.05$.

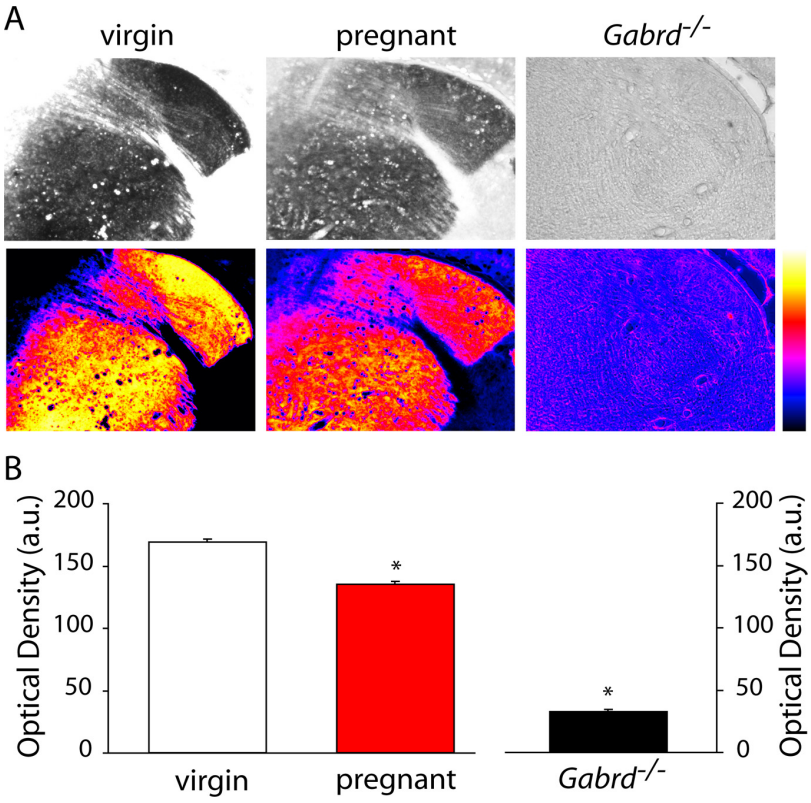


Figure 2. Decreased $GABA_A$ receptor δ subunit expression in the striatum in pregnant animals. ***A***, Representative bright-field and spectrum color-transformed images of the striatum from wild-type virgin, pregnant, and *Gabrd*$^{-/-}$ virgin mice stained with an antibody specific for the $GABA_AR$ δ subunit. ***B***, Optical density measurements show a significant decrease in $GABA_AR$ δ subunit in wild-type pregnant mice compared with virgin mice. The lack of staining in *Gabrd*$^{-/-}$ virgin mice confirm the specificity of the antibody. *$p < 0.05$ using the Student's *t* test compared with virgin wild type. a.u., Arbitrary units.

ence or absence of 100 nM allopregnanolone (equivalent to physiological levels during pregnancy) (Paul and Purdy, 1992) are shown in Figure 4*A*. The symbols represent the percentage of averaged fEPSP slope relative to the slope of the largest fEPSP in four stimulation trials in a representative experiment from virgin and pregnant mice. The average I/O relationships for these representative experiments were fit with a Boltzmann function, where W_{50} is the stimulus width that elicits the half-maximal response and the *k* value is the slope factor, and the Boltzmann curve from representative experiments are represented as a solid line in Figure 4*B*. These representative experiments demonstrate a shift to the left in the I/O relationship in slices from pregnant mice compared with virgin, suggesting an increase in neuronal population excitability. The addition of allopregnanolone (100 nM) shifted the I/O curve in slices from pregnant mice back toward virgin levels.

To obtain the grand average I/O relationship from all experiments, the fitted parameters of the individually normalized (MAX = 1) Boltzmann functions were averaged from virgin and pregnant slices in the presence or absence of allopregnanolone (Fig. 5*A*). The I/O relationships were compared between experimental groups, and the W_{50} and *k* values are given in Table 2. The W_{50} in pregnant mice was decreased to 75% that of the W_{50} in virgin mice, consistent with an increase in network excitability in slices from pregnant mice. The addition of physiological levels of allopregnanolone (100 nM) restored the W_{50} to122% of virgin levels (Fig. 5*A*). These data demonstrate an increase in synaptic excitability in the dentate gyrus during pregnancy in the absence of allopregnanolone. However, when levels of allopregnanolone found in pregnancy are restored in the slices, the synaptic I/O function is comparable with that found in virgin animals. Thus, the downregulation of $\delta GABA_ARs$ appears to be a homeostatic mechanism to maintain a

constant level of synaptic I/O function in the face of elevated allopregnanolone levels throughout pregnancy (see Discussion).

Consistent with the proposed importance of δGABA$_A$R changes on neuronal excitability during pregnancy, changes in synaptic I/O curves were only present in brain regions that exhibited changes in δGABA$_A$R expression. Brain regions with no changes in δGABA$_A$R expression during pregnancy, such as the cortex, do not display changes in network excitability associated with pregnancy. The I/O curves generated from the averages of the fitted parameters (MAX = 1) from wild-type virgin and pregnant mice in the presence or absence of allopregnanolone are similar in the cortex (Fig. 5*B*, Table 2). There is no difference in the W_{50} in pregnant mice, with the W_{50} values being 111% that of virgin mice. Interestingly, the addition of physiological levels of allopregnanolone (100 nM) did not significantly alter the W_{50} in virgin mice (98%) or pregnant mice (88%) (n = 3–5 mice, 15–18 slices per experimental group) (Fig. 5*B*). These data suggest that changes in neuronal excitability throughout pregnancy are brain region specific and restricted to areas that exhibit alterations in δGABA$_A$R expression.

These changes in network excitability in the dentate gyrus during pregnancy can be attributed to the changes in δGABA$_A$R expression, because there is no significant difference in the I/O relationship between *Gabrd*$^{-/-}$ virgin and *Gabrd*$^{-/-}$ pregnant mice (Fig. 5*C*, Table 2). The average Boltzmann functions (MAX = 1) generated using the average fitted parameters are shown in Figure 5*C*. Lateral perforant path stimulation produces a similar half-maximal width (W_{50}) in virgin *Gabrd*$^{-/-}$ mice that is 115% that of pregnant *Gabrd*$^{-/-}$ mice (Fig. 5*C*), suggesting that pregnancy does not alter neuronal excitability in *Gabrd*$^{-/-}$ mice. In addition, allopregnanolone does not have an effect on neuronal excitability in virgin *Gabrd*$^{-/-}$ mice, with W_{50} values increased by 115% in virgin *Gabrd*$^{-/-}$ mice and 109% in pregnant *Gabrd*$^{-/-}$ mice (n = 3–4 mice, 12–13 slices per experimental group) (Fig. 5*C*), likely attributable to the fact that the main site of action of allopregnanolone, the δGABA$_A$R, is absent in these mice. These data highlight the importance of specific δGABA$_A$R changes in mediating in the altered neuronal excitability throughout pregnancy.

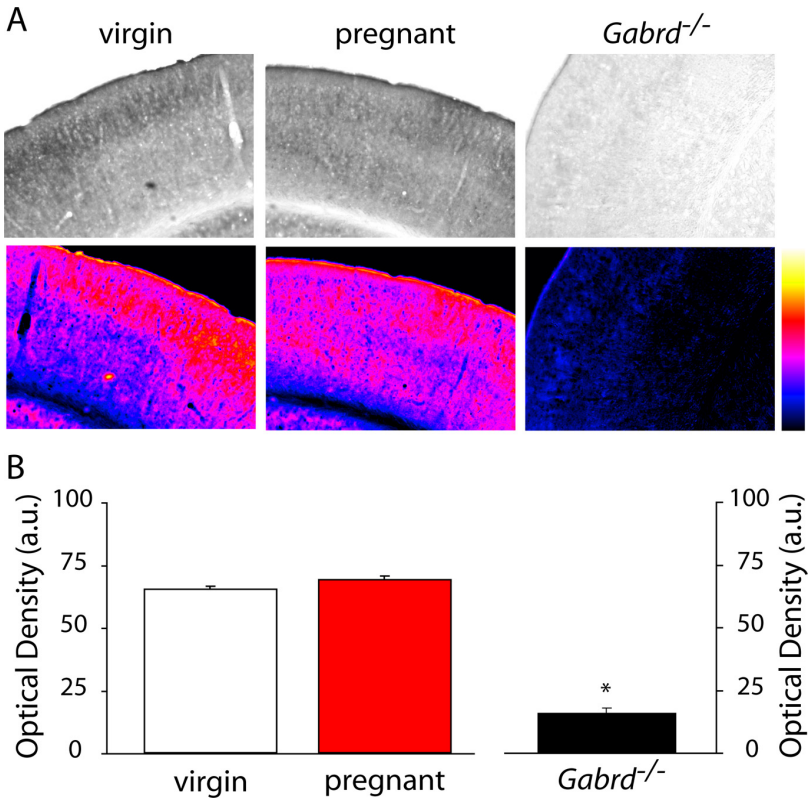


Figure 3. No change in GABA$_A$ receptor δ subunit expression in the cortex of pregnant mice. ***A***, Representative bright-field and spectrum color-transformed images of the somatosensory cortex from wild-type virgin, pregnant, and *Gabrd*$^{-/-}$ virgin mice stained with an antibody specific for the GABA$_A$R δ subunit. ***B***, Optical density measurements show no significant change in the expression of GABA$_A$R δ subunits in wild-type pregnant mice compared with virgin mice. The lack of staining in *Gabrd*$^{-/-}$ virgin mice confirm the specificity of the antibody. No significance detected using the Student's *t* test. a.u., Arbitrary units.

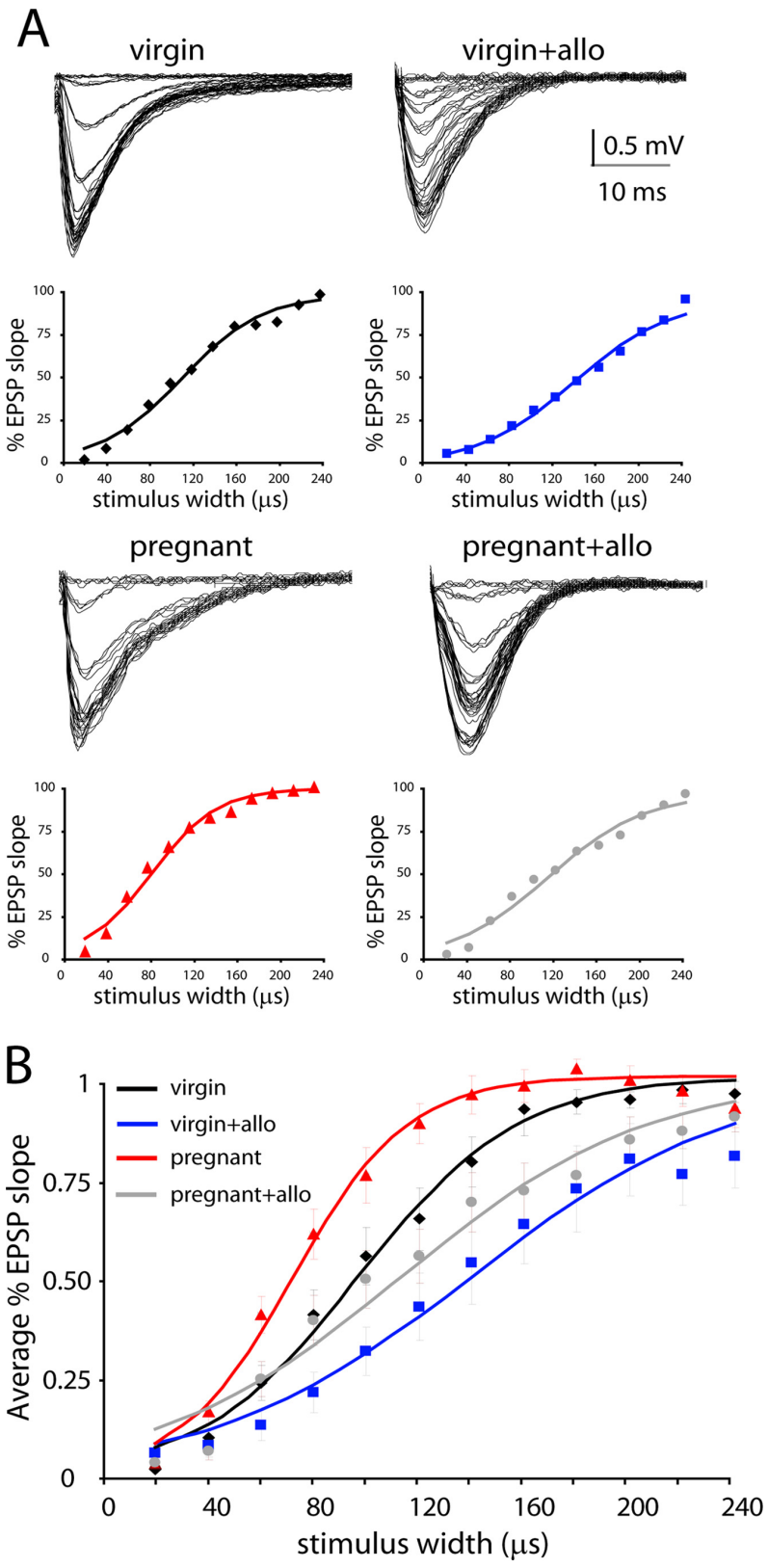
Table 2. Brain region-specific changes in fEPSP input/output relationships during pregnancy

	W_{50} (μs)	*k* values	Intensity (μA)	*n*
Wild-type dentate gyrus				
Virgin	101.2 ± 7.9	26.4 ± 3.7	554.2 ± 44.6	13
Virgin + Allo	158.4 ± 21.3*	42.9 ± 9.5*	557.7 ± 50.3	13
Pregnant	75.9 ± 5.5*	19.9 ± 2.6*	540.5 ± 46.9	13
Pregnant + Allo	123.7 ± 13.3	35.8 ± 4.3	669.4 ± 45.6	18
Wild-type cortex				
Virgin	108.9 ± 7.1	32.3 ± 2.9	500.0 ± 14.5	15
Virgin + Allo	106.2 ± 8.4	37.0 ± 3.8	482.1 ± 31.3	16
Pregnant	120.4 ± 10.2	38.7 ± 3.9	500.0 ± 0.0	18
Pregnant + Allo	106.5 ± 7.4	33.5 ± 3.9	500.0 ± 47.2	16
Gabrd$^{-/-}$ dentate gyrus				
Virgin	134.1 ± 11.8	38.0 ± 4.4	384.8 ± 27.1	13
Virgin + Allo	153.9 ± 14.6	39.5 ± 4.7	415.4 ± 36.9	13
Pregnant	116.8 ± 6.5	33.2 ± 3.0	454.5 ± 30.5	13
Pregnant + Allo	127.5 ± 13.7	37.8 ± 3.7	409.1 ± 38.0	12

* denotes significance compared with virgin animals in each group. Input/output relationships were constructed for virgin and pregnant mice in the wild-type dentate gyrus, in the wild-type cortex, and in the dentate gyrus of *Gabrd*$^{-/-}$ mice. The effect of 100 nM allopregnanolone (Allo) was determined for each condition. The input/output curves were fit with a Boltzmann function, and the W_{50} and *k* values are given. Statistical significance was determined using a one-way ANOVA, followed by Bonferroni's corrected *t* test.

Changes in epileptiform network excitability during pregnancy

To further investigate the effect of GABA$_A$R changes during pregnancy on network function, we examined the excitability of the network in response to elevated levels of extracellular KCl. The threshold to induce epileptiform activity in the CA1 region of the hippocampus was decreased in slices from pregnant mice compared with slices from virgin mice (Fig. 6). Although the CA1 region is not particularly rich in δGABA$_A$R, the decrease observed during pregnancy (Table 1) may result in an enhanced excitability of the interconnected CA1 network. Representative recordings from virgin, pregnant, and postpartum mice in the absence or presence of allopregnanolone are shown in Figure 6*A*. The percentage of slices discharging at 5 mM extracellular KCl was increased during pregnancy (61.5 ± 2.2%) compared with virgin



(0.0 ± 0.0%) and postpartum (18.8 ± 1.1%) mice. Similarly, the percentage of slices exhibiting epileptiform activity in 7.5 mM KCl was increased in slices from pregnant mice (92.9 ± 2.8%) compared with slices from virgin (25.0 ± 1.8%) and postpartum mice (43.8 ± 1.7%). The addition of physiological levels of allopregnanolone (100 nM) decreased the excitability in slices from pregnant mice back to virgin and postpartum levels at 5 mM KCl (9.1 ± 0.9%) and 7.5 mM KCl (54.5 ± 2.2%) (n = 3–5 mice, 7–18 slices per experimental group; significance determined using a one-way ANOVA at each concentration of KCl) (Fig. 6*B*). These data further suggest that, in the absence of allopregnanolone, the decrease in $\delta GABA_AR$ in the CA1 region combined with that in the dentate gyrus during pregnancy results in hippocampal hyperexcitability; however, in the presence of physiological levels of allopregnanolone, which would be present in the intact brain, there is no difference in excitability during pregnancy compared with virgin.

Mice deficient in $\delta GABA_AR$ regulation throughout pregnancy, such as *Gabrd*$^{-/-}$ mice, exhibit increased network excitability (Fig. 7) compared with wild-type mice (Fig. 6). At 5 mM KCl, 0.0 ± 0.0% of wild-type virgin slices exhibit epileptiform discharges compared with 71.4 ± 2.3% in *Gabrd*$^{-/-}$ virgin mice. At 7.5 mM KCl, 25.0 ± 1.8% of wild-type virgin slices exhibit epileptiform discharges compared with 100 ± 0.0% in *Gabrd*$^{-/-}$ virgin mice. However, *Gabrd*$^{-/-}$ mice do not exhibit changes in network excitability during pregnancy or postpartum. *Gabrd*$^{-/-}$ mice, either virgin or pregnant, exhibit epileptiform activity in the presence of increased extracellular KCl. Control and epileptiform activity in *Gabrd*$^{-/-}$ mice is shown in Figure 7*A*. Slices from *Gabrd*$^{-/-}$ mice are hyperexcitable and exhibit large depolarizing shifts with epileptiform discharges (Fig. 7*A*), although no significant difference is found between virgin and pregnant mice in the presence or absence

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Figure 4. Changes in network excitability during pregnancy reversed by allopregnanolone. ***A***, Representative traces and I/O curves from individual experiments in the dentate gyrus from wild-type virgin and pregnant mice in the presence or absence of allopregnanolone (allo). ***B***, The average I/O curves for virgin (◆), virgin plus 100 nM allopregnanolone (■), pregnant (▲), and pregnant plus 100 nM allopregnanolone (●) are indicated. The average I/O curves were fit with a Boltzmann function for virgin (black line), virgin plus 100 nM allopregnanolone (blue line), pregnant (red line), and pregnant plus 100 nM allopregnanolone (gray line).

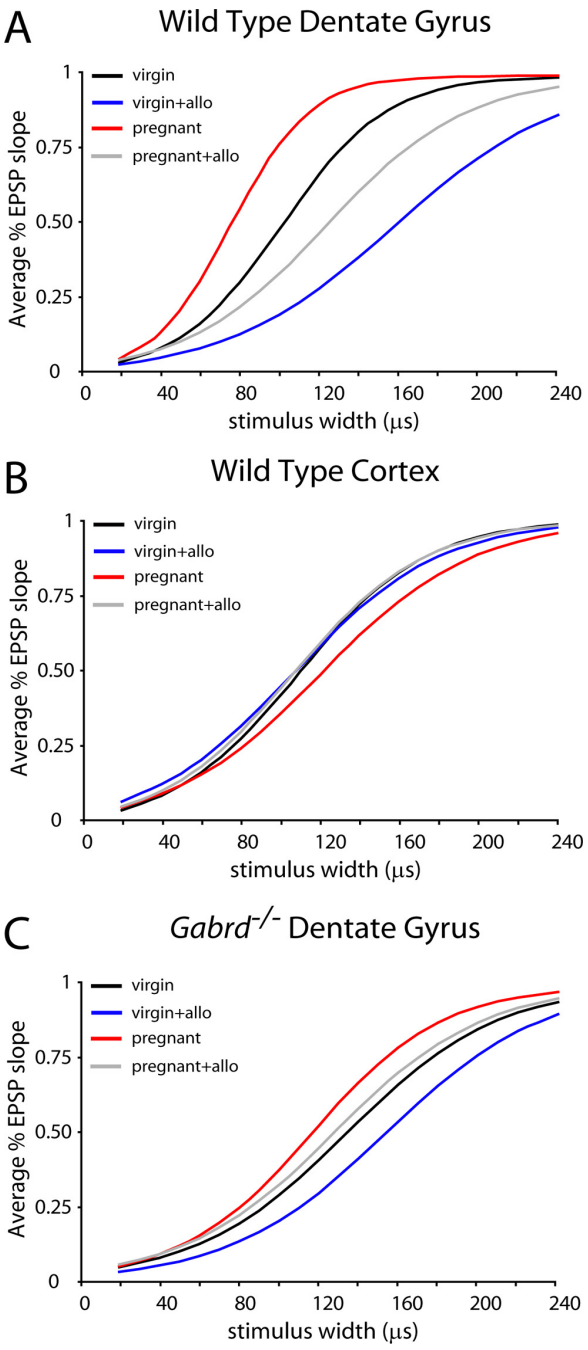

Figure 5. Changes in network excitability during pregnancy related to $GABA_AR$ δ subunit expression. ***A***, The average synaptic I/O function from the dentate gyri of wild-type virgin, pregnant, and postpartum mice is shown as a Boltzmann function generated by the averages of the fitted parameters. There is a shift toward increased excitability in the fEPSP slope I/O curves from wild-type pregnant mice (red line) compared with wild-type virgin mice (black line). This increased excitability is restored by 100 nM allopregnanolone (allo) (gray line). ***B***, The average I/O curves of fEPSP slopes from the cortex of wild-type virgin (black line) or pregnant (red line) with or without allopregnanolone (blue and gray lines) demonstrate no significant difference in the W_{50} between groups, suggesting that the changes in excitability related to pregnancy are brain region specific. ***C***, The average I/O curves of fEPSP slopes from *Gabrd*$^{-/-}$ virgin (black line), *Gabrd*$^{-/-}$ virgin plus 100 nM allopregnanolone (blue line), *Gabrd*$^{-/-}$ pregnant (red line), and *Gabrd*$^{-/-}$ pregnant plus 100 nM allopregnanolone (gray line). There is no significant difference in the W_{50} between *Gabrd*$^{-/-}$ groups. Statistical significance was determined using a one-way ANOVA, followed by Bonferroni's corrected *t* tests.

of allopregnanolone. The percentage of slices discharging at 5 mM extracellular KCl was similar in *Gabrd*$^{-/-}$ virgin mice (71.4 ± 2.3%), *Gabrd*$^{-/-}$ pregnant mice (66.7 ± 2.1%), and *Gabrd*$^{-/-}$ postpartum mice (73.3 ± 2.2%). Similarly, there was no difference in the percentage of slices exhibiting epileptiform activity in 7.5 mM KCl from virgin mice (100 ± 0.0%) compared with slices from pregnant mice (100 ± 0.0%) and postpartum mice (100 ± 0.0%) (n = 3–5 mice, 8–15 slices per experimental group; significance determined using a one-way ANOVA at each concentration of KCl) (Fig. 7*B*). In addition, the neuronal excitability in *Gabrd*$^{-/-}$ mice is unaffected by addition of 100 nM allopregnanolone (5 mM KCl, 62.5 ± 2.8%; 7.5 mM KCl, 100 ± 0.0%), likely attributable to the absence of $\delta GABA_ARs$ for allopregnanolone to act on. Thus, the inability to regulate $\delta GABA_AR$ expression may prevent changes in network excitability during pregnancy, rendering the network hyperexcitable.

Discussion

In this report, we demonstrate brain region-specific changes in $GABA_AR$ subunit expression during pregnancy. There is a decrease in the expression of $\delta GABA_ARs$ during late pregnancy, which may constitute a homeostatic mechanism to maintain a steady level of inhibition throughout pregnancy (Fig. 8). In this model, we propose that the expression of $\delta GABA_ARs$ must become downregulated in the face of extremely high levels of neurosteroids during pregnancy, which can act as positive allosteric modulators of these receptors, to maintain a level of inhibition throughout pregnancy equivalent to that before this stage (Fig. 8). At the time of parturition, neurosteroid levels abruptly decline and $\delta GABA_ARs$ must be recovered, again to maintain a constant level of inhibition throughout the postpartum period (Fig. 8). Failure to properly regulate these receptors throughout pregnancy and postpartum may play a role in neurological and neuropsychiatric disorders associated with this vulnerable period.

Changes in various $GABA_AR$ subunits have been observed during pregnancy; however, the contribution of these changes to neuronal excitability during pregnancy remained elusive. Changes in $GABA_ARs$ during pregnancy has been inferred from decreased agonist binding (Majewska et al., 1989; Concas et al., 1999) and decreased sensitivity to benzodiazepines (Concas et al., 1998; Follesa et al., 1998). Previous studies have demonstrated decreased expression of γ2 in the hippocampus (Concas et al., 1998; Follesa et al., 1998) and decreased expression of the $GABA_AR$ α5 in the cortex (Follesa et al., 1998). In addition, several studies reported changes in $GABA_AR$ subunit expression in progesterone withdrawal models, mainly an increase in α4 and δ subunit expression (Smith et al., 1998; Sundstrom-Poromaa et al., 2002). Here we report a decrease in $\delta GABA_AR$ expression in day 18 pregnant mice in several different brain regions, such as the dentate gyrus, CA1, striatum, and thalamus. A recent report demonstrated a different pattern of $\delta GABA_AR$ regulation during late pregnancy in the rat (Sanna et al., 2009), suggesting perhaps that $\delta GABA_AR$ regulation throughout pregnancy and postpartum may be species specific. However, this study used a different antibody, untested for specificity in *Gabrd*$^{-/-}$ animals, to quantify changes in $\delta GABA_AR$ immunoreactivity in the dentate gyrus granule cell layer, which is not the typical localization of these receptors (Peng et al., 2004). Our findings demonstrate alterations in $\delta GABA_AR$ expression in the dentate gyrus molecular layer, which is where this protein is most abundant (Peng et al., 2004).

Our data suggest that alterations in $\delta GABA_ARs$, specifically, are essential for the changes in neuronal excitability during preg-

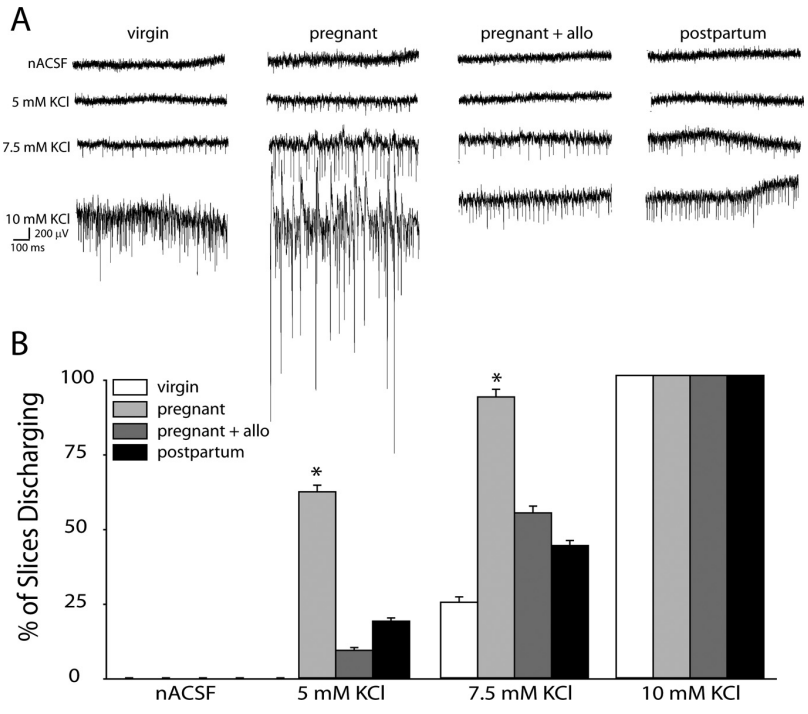

Figure 6. Network hyperexcitability during pregnancy in the absence of allopregnanolone. ***A***, Representative traces in the CA1 region of the hippocampus in nACSF and 5, 7.5, and 10 mM extracellular KCl in virgin, pregnant, and postpartum wild-type mice. ***B***, The average percentage of slices discharging at increasing concentrations of extracellular KCl was increased in pregnant mice compared with virgin and postpartum mice. This increase in excitability was not evident in the presence of physiological concentrations of allopregnanolone (allo) (100 nM). *$p < 0.05$ using a one-way ANOVA at each concentration of KCl compared with virgin wild-type slices in nACSF.

nancy. The evidence that allopregnanolone restores neuronal excitability to virgin levels further suggests a role for δ $GABA_A$Rs in neuronal excitability changes during pregnancy. Even more convincing is the lack of neuronal excitability alterations during pregnancy in *Gabrd*$^{-/-}$ mice. Although not statistically significant, there is a trend toward more hyperexcitability, evident from a slight shift in the I/O curve, during pregnancy in *Gabrd*$^{-/-}$ mice, which could be attributable to decreased expression of the $GABA_A$R γ2 receptor subunit during pregnancy (Maguire and Mody, 2008). The similarity of the synaptic I/O curves in wild-type and *Gabrd*$^{-/-}$ mice is most likely attributable to the lower stimulation intensities used to elicit similar population responses in the two preparations (Table 2), consistent with the loss of δ$GABA_A$R-mediated tonic inhibition in these mice and the significant contribution of tonic inhibition to overall excitability (Farrant and Nusser, 2005). In addition, the partial compensation of the tonic conductance by α5$GABA_A$Rs in dentate gyrus granule cells of *Gabrd*$^{-/-}$ mice may also dampen the differences between the I/O curves recorded in the two genotypes (Glykys et al., 2008).

The hyperexcitability in *Gabrd*$^{-/-}$ mice becomes evident during depolarization in response to elevated extracellular KCl. Virgin *Gabrd*$^{-/-}$ mice are hyperexcitable, and pregnancy does not significantly alter the excitability in these animals. The virgin *Gabrd*$^{-/-}$ mice exhibit epileptiform activity that consists of a large depolarization with epileptiform bursts. In contrast to wild-type mice, in which the epileptiform activity is characterized by synchronous population discharges at regular intervals, the epileptiform activity in *Gabrd*$^{-/-}$ mice consists of a large depolarization with high-frequency discharges reminiscent of epileptiform bursts. The type of epileptiform activity in *Gabrd*$^{-/-}$ mice is unlike that induced in wild-type slices, suggesting that there may be a different mechanism underlying the epileptogenesis. However, this hypothesis requires additional, intensive investigation that is outside the scope of this study and will be investigated in the future.

Our previous study demonstrated that changes in δ$GABA_A$R expression that occur over the ovarian cycle and in response to stress were mediated by steroid hormone-derived neurosteroids (Maguire and Mody, 2007). However, we cannot exclude the contribution of other physiological changes that occur during pregnancy, such as increases in estrogen, oxytocin, corticosterone, and prolactin levels. Estrogens are thought to be proconvulsant, whereas progesterone is thought to act as an anticonvulsant. Estrogen is known to increase hippocampal spine density (Gould et al., 1990; Woolley and McEwen, 1993) and excitatory synapses (Woolley and McEwen, 1992; Woolley et al., 1997) and to alter the sensitivity of NMDA receptors to glutamate (Woolley et al., 1997). In addition, oxytocin and vasopressin can both exert distinct effects on neuronal excitability in the CNS. For instance, in the amygdala, vasopressin is excitatory and inhibited by oxytocin (Huber et al., 2005) (for review, see Raggenbass, 2008), demonstrating that many other physiological changes associated with pregnancy can exert effects on neuronal excitability. Although we cannot entirely rule out the possibility of other physiological factors affecting neuronal excitability during pregnancy, the restoration of neuronal excitability to pre-pregnancy levels by physiological levels of allopregnanolone suggests that neurosteroids and their site of action, the δ$GABA_A$Rs, play a significant role in mediating changes in neuronal excitability associated with pregnancy.

Changes in neuronal excitability have been reported in epileptic women during pregnancy with conflicting results (Schmidt, 1982). Seizure frequency in patients with epilepsy is often difficult to assess attributable to patients noncompliance with drug regimen or changes in the pharmacokinetics of the antiepileptic medications during pregnancy. In addition, experimental studies in animals have been primarily limited because of the use of chemical convulsants, which have inherent problems when working with pregnant animals, including changes in dosing, absorption, and metabolism during pregnancy compared with virgin animals. However, in a kindling model of epileptogenesis, there was no difference in threshold to kindle animals between pregnant mice and controls (Holmes and Weber, 1985). In this study, we systematically assessed changes in network excitability *in vitro* and *in vivo*, and our findings demonstrate that pregnancy does not alter network excitability or seizure frequency in the intact animal.

The factors underlying the changes in neuronal excitability during pregnancy have been proposed to result from stress, changes in sleep patterns, metabolic factors, respiratory changes,

noncompliance with drugs, changes in the pharmacokinetics of drugs, and changes in seizure propensity during pregnancy (for review, see Swartjes and van Geijn, 1998). Although changes in seizure propensity have been proposed to occur during pregnancy, there is little evidence of intrinsic changes that would render the brain more susceptible to seizures. Our study elucidates changes in GABAergic inhibition during pregnancy that may play a role in excitability changes during pregnancy, including the decrease in $\delta GABA_A Rs$ in the dentate gyrus, similar to that observed previously by Western blot analysis (Maguire and Mody, 2008) and a decrease in the striatum and the thalamus. However, we did not find a change in $\delta GABA_A R$ expression in the cortex during pregnancy, suggesting that the changes in receptor expression are brain region specific, and their sensitivity to allopregnanolone may also be under region-specific control by yet unknown mechanisms.

Our study is the first to assess changes in network excitability during pregnancy and demonstrates no inherent propensity for hyperexcitability during this hormonally labile period. However, our data also suggest that hyperexcitability during pregnancy has the opportunity to manifest itself if there are abnormal changes in neurosteroid levels or if the receptors for neurosteroid action, namely the $\delta GABA_A Rs$, are not properly regulated. In the face of high levels of $\delta GABA_A R$-targeting neurosteroids throughout pregnancy, the levels of these receptors are decreased to maintain the pre-pregnancy level of inhibition. If neurosteroid levels decline, inhibition will decrease and likely result in hyperexcitability. Similarly, if the levels of $\delta GABA_A Rs$ decrease too much, inhibition will decrease and the brain will be rendered hyperexcitable.

Our data demonstrate a physiological homeostatic mechanism whereby the brain maintains a steady level of excitability throughout pregnancy. In addition, the importance of the GABAergic system in maintaining a constant level of neuronal I/O function throughout pregnancy suggests that the antiepileptic drugs gabapentin, gabitril, and lamotrigine may be better treatments for women with epilepsy because they target the function of $GABA_A Rs$, which may be directly involved in the pathophysiology. These and other drugs acting by similar mechanisms may be particularly useful in the treatment of women sensitive to the steroid hormone-associated changes in seizure frequency.

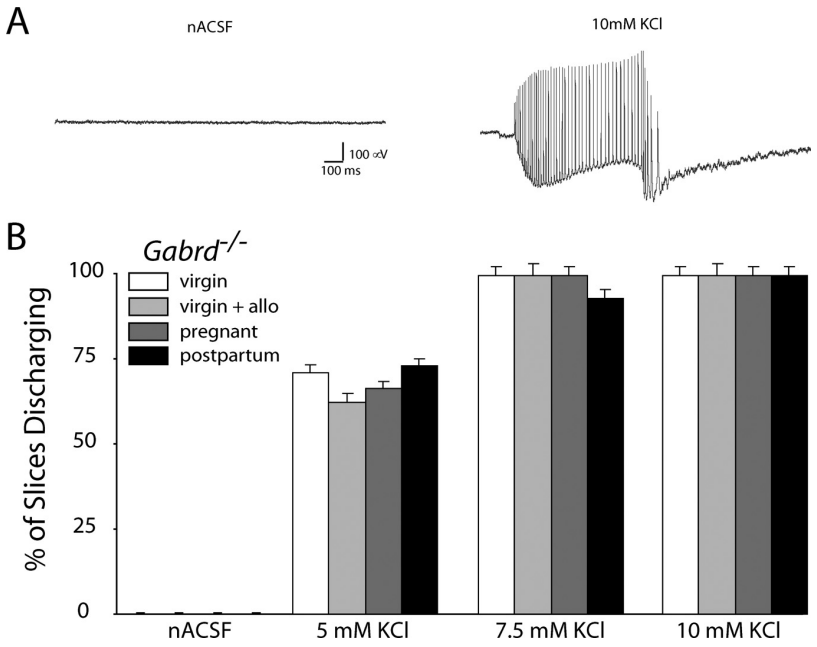

Figure 7. Network hyperexcitability in *Gabrd*$^{-/-}$ mice. ***A***, Representative traces in the CA1 region of the hippocampus in *Gabrd*$^{-/-}$ mice in nACSF and 10 mM extracellular KCl. ***B***, The average percentage of slices discharging at increasing concentrations of extracellular KCl was not significantly different in pregnant mice compared with virgin and postpartum mice. Physiological levels of allopregnanolone (allo) (100 nM) had no effect on excitability in *Gabrd*$^{-/-}$ mice. No significance was detected at any concentration of KCl using a one-way ANOVA.

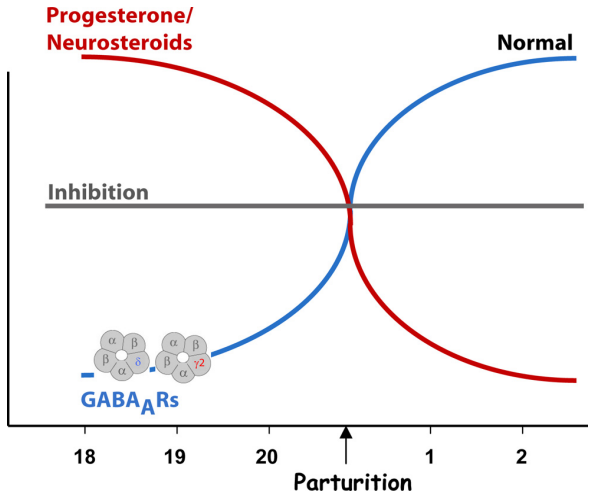

Figure 8. A model of $GABA_A R$ plasticity throughout pregnancy and the postpartum period. In the face of robust levels of neurosteroids throughout pregnancy, $GABA_A Rs$ must become downregulated to maintain an ideal level of inhibition throughout pregnancy and prevent sedation and/or anesthesia in gravid mammals. At parturition, neurosteroid levels abruptly decline, and the expression levels of $GABA_A Rs$ must be recovered to maintain the ideal level of inhibition throughout the postpartum period. $GABA_A R$ regulation represents a homeostatic mechanism to maintain an ideal level of inhibition in the face of changing neurosteroid levels throughout pregnancy and postpartum.

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CHAPTER 5

ALTERED GAMMA OSCILLATIONS DURING PREGNANCY THROUGH LOSS OF δ SUBUNIT-CONTAINING $GABA_A$ RECEPTORS ON PARVALBUMIN INTERNEURONS

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Altered gamma oscillations during pregnancy through loss of δ subunit-containing $GABA_A$ receptors on parvalbumin interneurons

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Gamma (γ) oscillations (30–120 Hz), an emergent property of neuronal networks, correlate with memory, cognition and encoding. In the hippocampal CA3 region, locally generated γ oscillations emerge through feedback between inhibitory parvalbumin-positive basket cells (PV+BCs) and the principal (pyramidal) cells. PV+BCs express δ-subunit-containing $GABA_A$Rs (δ-$GABA_A$Rs) and NMDA receptors (NMDA-Rs) that balance the frequency of γ oscillations. Neuroactive steroids (NS), such as the progesterone-derived (3α,5α)-3-hydroxy-pregnan-20-one (allopregnanolone; ALLO), modulate the expression of δ-$GABA_A$Rs and the tonic conductance they mediate. Pregnancy produces large increases in ALLO and brain-region-specific homeostatic changes in δ-$GABA_A$Rs expression. Here we show that in CA3, where most PV+ interneurons (INs) express δ-$GABA_A$Rs, expression of δ-$GABA_A$Rs on INs diminishes during pregnancy, but reverts to control levels within 48 h postpartum. These anatomical findings were corroborated by a pregnancy-related increase in the frequency of kainate-induced CA3 γ oscillations *in vitro* that could be countered by the NMDA-R antagonists D-AP5 and PPDA. Mimicking the typical hormonal conditions during pregnancy by supplementing 100 nM ALLO lowered the γ frequencies to levels found in virgin or postpartum mice. Our findings show that states of altered NS levels (e.g., pregnancy) may provoke perturbations in γ oscillatory activity through direct effects on the GABAergic system, and underscore the importance of δ-$GABA_A$Rs homeostatic plasticity in maintaining constant network output despite large hormonal changes. Inaccurate coupling of NS levels to δ-$GABA_A$R expression may facilitate abnormal neurological and psychiatric conditions such as epilepsy, post-partum depression, and post-partum psychosis, thus providing insights into potential new treatments.

Keywords: gamma oscillations, pregnancy, neurosteroids, $GABA_A$ receptors, delta subunit, CA3 interneurons, parvalbumin, tonic inhibition

INTRODUCTION

Oscillations in cortical local field potentials in the γ-frequency band (30–120 Hz) reflect coordinated neuronal activity that manifests during different processing tasks such as memory and sensory encoding, and are considered important in adaptive functional organization of neuronal assemblies, spike-time dependent synaptic plasticity, and neurological performance (Singer, 1993; Paulsen and Moser, 1998; Sederberg et al., 2003; Montgomery and Buzsáki, 2007).

Frequency and power of γ oscillations result from a synchronized feedback dialogue between excitatory neurons and perisomatic inhibitory interneurons (INs). In particular, PV+BCs are the major contributors to the generation of γ oscillations, their activity is both necessary and sufficient to drive the rhythm, albeit other IN types may also play a regulatory role (Hájos et al., 2004; Mann et al., 2005; Cardin et al., 2009; Buzsáki and Wang, 2012). We have previously shown how γ oscillation frequency recorded in the CA3 *in vitro* is controlled by a δ-$GABA_A$Rs-mediated tonic conductance of INs, that is dynamically balanced by an NMDA-R-mediated tonic excitation (Mann and Mody, 2010). Unlike its fast, synaptic $GABA_A$Rs-mediated phasic counterpart, tonic inhibition is a slow persistent inhibitory conductance that is activated by ambient GABA, is mediated by extrasynaptic $GABA_A$Rs, and decreases overall neuronal excitability by hyperpolarization or shunting inhibition (Brickley and Mody, 2012).

In the hippocampus, δ-$GABA_A$Rs are predominantly expressed on dentate gyrus granule cells (DGGCs) and INs (Sperk et al., 1997). Regardless of cell specificity, all δ-$GABA_A$Rs are uniquely sensitive to NS, including the progesterone-derived ALLO. NS are potent modulators of tonic inhibition, which they amplify by increasing GABA efficacy on δ-$GABA_A$Rs (Stell et al., 2003; Meera et al., 2009). Moreover, NS can modulate network excitability by modifying δ-$GABA_A$Rs surface expression (Maguire et al., 2005; Maguire and Mody, 2007), albeit candidate molecular mechanisms remain unknown. In mammals, brain ALLO follows oscillations in plasma progesterone, and during the last third of pregnancy, they both reach concentrations two orders of magnitude higher than any other physiological state

(Paul and Purdy, 1992; Concas et al., 1998). These changes are paralleled by a downregulation of δ-GABA$_A$Rs in DGGCs and CA1 pyramidal cells, in a compensatory homeostatic mechanism, which if altered, may lead to great imbalances in network excitability and postpartum behavior (Maguire and Mody, 2008; Maguire et al., 2009).

Thus far, δ-GABA$_A$Rs plasticity has been reported only in neurons expressing α4-GABA$_A$R (Smith et al., 1998; Sundström Poromaa et al., 2002; Maguire et al., 2005, 2009) that is considered natural partner of δ-GABA$_A$R (McKernan and Whiting, 1996; Sur et al., 1999) in the forebrain. It is unclear whether INs, which usually express δ-GABA$_A$Rs assembled with α1-GABA$_A$Rs (Glykys et al., 2007) go through the same NS-related modifications. Moreover, as δ-GABA$_A$Rs-mediated tonic conductance on PV+BCs controls γ oscillation frequency, it remains an open question whether γ oscillations are modulated during pregnancy. Deficits in PV+BCs output and consequent changes in γ oscillations have been reported in schizophrenia and may cause memory perturbations (Haenschel et al., 2009; Minzenberg et al., 2010). At the same time, the vast clinical evidence for pregnancy and postpartum-related psychiatric and neurological disturbances that vary from memory impairments to postpartum psychosis (Poser et al., 1986; Sit et al., 2006; Henry and Rendell, 2007) may also indicate a state of unrest in γ oscillations.

Our objective was to assess pregnancy-related δ-GABA$_A$Rs plasticity in INs and possible changes in γ oscillations. We show that CA3 PV+INs express δ-GABA$_A$Rs that become diminished during pregnancy. These anatomical findings are validated by a functional increase in kainate-induced γ oscillation frequencies driven by an IN-specific NMDA-R-dependent mechanism. Consistent with a very rapid plasticity process, pre-pregnancy δ-GABA$_A$R expression and γ oscillation frequencies are restored already at 48 h postpartum. The homeostatic nature of these alterations is demonstrated by our findings that physiological levels of ALLO found during pregnancy revert γ oscillations frequencies to control values.

MATERIALS AND METHODS

ANIMAL HANDLING

This study used adult (9–15 weeks of age) C57Bl/6 and mice lacking δ-GABA$_A$Rs (*Gabrd*$^{-/-}$ mice, on C57Bl/6 background) were housed with *ad libitum* access to food and water under the care of the UCLA Division of Laboratory Animal Medicine (DLAM). Mice were maintained on a light/dark cycle of 12 h, and all experiments were performed during the light period. Stress was minimized by moving the animals to the experimental area in their home cage at least 2 h prior to use during which time they were never handled. Virgin mice were anovulatory non-cycling females, pregnant mice were day-18 first time pregnant, postpartum mice were first time dams 48 h after parturition, and only if the pups were fed and cared for. Genotyping was performed by Transnetyx.

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Deeply anesthetized mice were transcardially perfused with 50 ml of 4% paraformaldehyde in 0.12 M phosphate buffer, at room temperature pH 7.3. Brains were postfixed overnight at 4°C, cryoprotected in 30% sucrose solution in Millonigs modified PBS, embedded in OCT compound (Andwin Scientific) on dry ice, and sectioned at −16°C with a cryostat (coronal, 35 μm). δ-GABA$_A$Rs stains were processed under non-permeabilizing conditions in order to stain for membrane localized, functionally relevant receptors. Slices from virgin, pregnant and postpartum mice were processed in parallel. For diaminobenzidine (DAB) δ-GABA$_A$Rs stain: endogenous peroxidases were quenched in methanol and 3% H_2O_2, 30 min. Slices were blocked in 10% normal goat serum (NGS), 2 h, incubated first with anti-δ-GABA$_A$R antibody that recognizes the extracellular N-terminus of δ-GABA$_A$R (1:500 in 10% NGS; generous gift from Dr. Werner Sieghart, Medizinische Universität, Wien, Austria) overnight, then with biotinylated goat anti-rabbit antibody (1:200 in 10% NGS; Vector Laboratories), 4 h. Signal was amplified with HRP-conjugated avidin enzyme complex (ABC Elite; Vector Laboratories), 30 min, then developed with DAB (Vector Lab). For DAB PV stain: 1.5%H_2O_2 in TBS, 10% normal horse serum (NHS) and 0.3% Triton X-100 as blocking agent, mouse anti-PV (1:5000 in 1:50 NHS; Swant), biotinylated horse anti-mouse (1:200 in 1:30 NHS; Vector Laboratories). For δ-GABA$_A$Rs-PV double labeling: slices were incubated for 70 min at 90°C in a citrate buffer solution (0.05 M sodium citrate in 1% NaCl, pH 8.6) for antigen retrieval. Blocking was done in 10% NGS and 0.3% Triton X-100, 2 h. Slices were incubated in anti-δ-GABA$_A$R and anti-PV (1:100; 1:5000 respectively, 0.1% NaN3 in TBS) for 4 days at room temperature, then in Cy3 and Cy5-conjugated goat anti-rabbit and anti-mouse antibodies, 2 h (1:750; Millipore). Slices were mounted on Superfrost Plus slides (Fisher Scientific), dehydrated (DAB stains only) and coverslipped with DPX Mountant for Histology (DAB stains, Sigma-Aldrich) or Fluoromount G (fluorescence stains, Southern Biotechnology).

MICROSCOPY AND DENSITOMETRIC ANALYSIS

For bright field microscopy, digital images were taken with an Axioskop 2 Microscope and an AxioCam digital camera system and AxioVision 4.8 software (Zeiss). For the same magnification and the same staining images were taken under identical conditions of brightness and exposure time. The region of interest in CA3 was the whole CA3 stratum pyramidale (SP). The intensity of labeling was measured as optical density of the region of interest using NIH ImageJ software (**Figure 4**). As we did not find significant differences in background staining (which was minimal) between experimental groups, background subtraction was deemed unnecessary. For fluorescent microscopy, images were collected using a confocal microscope (Leica TCS-SP, Mannheim, Germany) equipped with Plan Fluor objectives connected to a camera (DP70, Olympus), and Leica confocal and DP70 camera software. Digital projection images of 35 μm *z*-stacks were assembled and analyzed using NIH ImageJ software. All images were captured under the same light intensity and exposure limits.

SLICE PREPARATION AND ELECTROPHYSIOLOGY

Mice were anesthetized with halothane and decapitated following UCLA Chancellor's Animal Research Committee protocol. Horizontal 350 μm hippocampal slices were cut on a

Leica VT1000S Vibratome in ice-cold N-Methyl-D-Glucamine (NMDG)-based HEPES-buffered cutting solution, containing in mM: NMDG 135, D-glucose 10, $MgCl_2$ 4, $CaCl_2$ 0.5, KCl 1, KH_2PO_4 1.2, HEPES 26, Given the highly lipophilic nature 7.3–7.4, 295–297 mOsm/L, bubbled with 100% O_2. Slices were incubated for 30 min at 32°C in an interface chamber in modified sucrose aCSF, containing in mM: NaCl 85, D-glucose 25, sucrose 55, KCl 2.5, NaH_2PO_4 1.25, $CaCl_2$ 0.5, $MgCl_2$ 4, $NaHCO_3$ 26, pH 7.3–7.4 when bubbled with 95% O_2, 5% CO_2. Modified aCSF was slowly substituted for normal aCSF at 32°C, containing in mM: NaCl 126, D-glucose 10, $MgCl_2$ 2, $CaCl_2$ 2, KCl 2.5, NaH_2PO_4 1.25, Na Pyruvate 1.5, L-Glutamine 1, $NaHCO_3$ 26, pH 7.3–7.4 when bubbled with 95% O_2, 5% CO_2. Recordings were done in an interface chamber at 35°C perfused with normal aCSF and 50 nM kainic acid (Tocris). Oscillatory network activity was recorded in CA3 SP with the use of a patch pipette filled with nACSF connected to a patch clamp amplifier (A-M Systems Inc., model 3000) sampled at 4096 Hz, band-pass filtered between 0.1 and 3,000 Hz, and an instrumentation amplifier (Brownlee BP Precision, model 210A). Field potentials were recorded using EVAN (custom-designed LabView-based software from Thotec) and analyzed with a custom written procedure (Wavemetrics, IGOR Pro 6.22A). Peak frequencies, power at peak frequency and total power were derived from the corresponding power spectral densities, calculated from 180 s period averages. Before and after drug treatment values are derived from the average power spectral density (psd) of 180 s periods before drug perfusion and 10 min after. D-AP5, PPDA, ALLO and finasteride were purchased from Tocris. ALLO and finasteride were dissolved in DMSO (final vehicle concentration 0.01%). Morlet wavelet transform was used to illustrate the power of γ-frequency band in time as previously described (Mann and Mody, 2010).

CORTICOSTERONE MEASUREMENTS

Whole blood was collected at decapitation and plasma isolated by high-speed centrifugation. Plasma corticosterone levels were measured by enzyme-linked immunosorbent assay (Enzo Life Sciences). Absorbance was measured at 405 nm, and sample values derived from fitted standard binding curve. All samples and standards were run in parallel in the same plate.

DATA ANALYSIS

All data is shown as mean ± SEM. Statistical significance was determined at the 95% confidence interval with the use of statistical tests specified in each section.

RESULTS

PV IMMUNOREACTIVITY REMAINS UNCHANGED IN THE CA3 AT DIFFERENT GESTATIONAL STATES

We first tested for gestational state-related anatomical alterations in PV+BCs. These INs display characteristic anatomical and firing properties and typically express the calcium-binding protein parvalbumin (PV), which is used as their anatomical hallmark (Klausberger et al., 2005, 2003; Freund and Katona, 2007). Although they are not the only hippocampal INs to express PV, they comprise the majority of PV+ INs (Baude et al., 2006), and together with cholecystokinin-expressing BCs (CCK+BCs) they are the only PV+INs to innervate the perisomatic region of principal cells (Freund and Katona, 2007; Klausberger and Somogyi, 2008). Until more cell-type specific protein markers are described, hippocampal SP PV immunolabeling is a good approximation to investigate PV+BCs anatomical distribution.

Immunohistochemistry of immunoperoxidase staining for PV shows numerous PV immunopositive cell bodies and dense terminals surrounding the principal cells in hippocampal areas CA1, DG and CA3, consistent with previously reported distribution of PV staining in these structures (**Figure 1A**) (Gao and Fritschy, 1994; Freund and Buzsaki, 1996). CA3 PV immunoreactivity is preserved across the three experimental gestational groups. In order to assess potential changes specifically in PV+BCs innervation we carried out a densitometric analysis of PV staining in the SP, an area where most PV+ boutons belong to PV+BCs (Klausberger and Somogyi, 2008). No modification in CA3 PV plexus was detected through optical density (OD) measurements of CA3 SP (**Figures 1B**, **5**).

The CA3 is an ideal brain region for the investigation of modulations in γ oscillations dependent on δ-GABA$_A$Rs expression in INs. Here not only γ oscillations are locally generated, but also δ-GABA$_A$Rs are exclusively expressed on INs as CA3 pyramidal cells tonic conductance is sustained solely by α5-GABA$_A$Rs (Sperk et al., 1997; Glykys and Mody, 2006; Mann and Mody, 2010). In contrast, CA1 pyramidal cells and DGGCs use a combination of

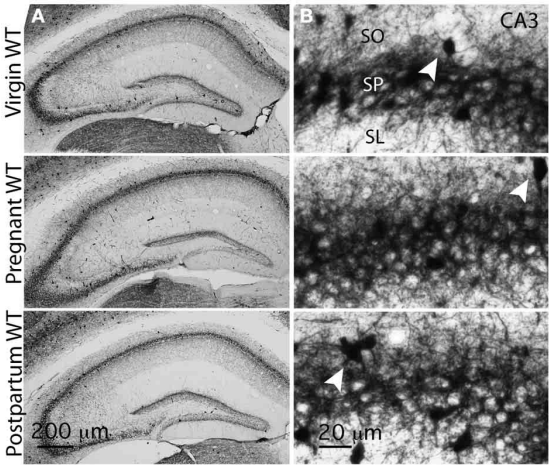

FIGURE 1 | PV distribution remains unchanged throughout the different gestational states. (A) Representative bright-field images of whole hippocampal DAB staining for PV in virgin, pregnant and postpartum WT mice. PV+ terminals innervate CA1 and CA3 pyramidal cells and dentate gyrus granule cells, and form a plexus that wraps around their somata and proximal dendrites. **(B)** Representative high-magnification images of CA3 PV plexus in virgin, pregnant and postpartum WT mice. PV+IN somata are clearly visible within the stratum pyramidale (SP) and in its immediate vicinity (initial portion of stratum oriens SO and stratum lucidum SL), arrowheads. Optical density measurements in CA3 SP show no difference across gestational groups (in arbitrary units AU, mean ± SEM: virgin = 193.6 ± 0.9; pregnant = 192.9 ± 1.9; postpartum = 196.1 ± 1.7; n = 12, 10, 8 slices and n = 3 mice for each group.). One-Way ANOVA; $p = 0.32$, $F_{(2, 27)} = 1.175$.

δ-GABA$_A$Rs and α-GABA$_A$Rs for their tonic conductance (Glykys et al., 2008), therefore making it hard to differentiate network effects of δ-GABA$_A$Rs modulation on principal cells from that of INs.

CA3 PV+ INs EXPRESS δ-GABA$_A$Rs

CA3 γ oscillation frequency is controlled by a δ-GABA$_A$Rs mediated tonic conductance of INs (Mann and Mody, 2010). PV+INs in the dentate gyrus have been shown to abundantly express δ-GABA$_A$Rs (Yu et al., 2013). Alterations in these receptors during pregnancy could result in changes in γ frequency oscillations. Therefore, we sought to examine the presence of δ-GABA$_A$Rs on PV+INs of the CA3 region, where kainate-dependent γ oscillations can be readily induced *in vitro*.

Functional evidence of δ-GABA$_A$Rs expression on most CA3 INs has been described (Mann and Mody, 2010), but a detailed anatomical study of δ-GABA$_A$Rs expression in specific types of IN of the hippocampus has yet to be done. Here we demonstrate a large overlap of the two immunoreactivities in the somata of INs in area CA3 of the hippocampus (**Figures 2A,B**). Of 93 INs identified in 6 sections (35 μm thick) 3 sections apart in an area spanning across CA3-SP and 30 μm around it toward stratum oriens (SO) or stratum lucidum (SL), where most PV+BC somata are located, 85.2 ± 3.3% expressed both PV and δ-GABA$_A$Rs, 11.7 ± 2.4% only PV and 3 ± 1.4% only δ-GABA$_A$Rs. In the same slices, of the 31 labeled INs in the distal SO and stratum radiatum (SR), 38.9 ± 9.3% expressed both PV and δ-GABA$_A$Rs, 31 ± 7.2% only PV and 30.1 ± 7.7% only δ-GABA$_A$Rs. A χ^2 analysis shows a highly significant difference between the two distributions. $\chi^2 = 80.5$, $p < 0.0001$, 2df. (**Figure 2**). Our peri-SP distribution is consistent with the findings of a recent work that showed almost complete overlap of PV and δ-GABA$_A$Rs immunolabeling in DG INs proximal to the GC layer (Yu et al., 2013). Specificity of δ-GABA$_A$Rs antisera was demonstrated with the use of a *Gabrd*$^{-/-}$ male mouse as a negative control (**Figure 2C**). The importance of this control is particularly relevant for δ-GABA$_A$Rs immunolabeling, as a commercially available δ-GABA$_A$Rs specific antibody (Santa Cruz Biotechnology, SC-31438) showed unspecific binding in brain slices from *Gabrd*$^{-/-}$ mice (Ferando et al., 2009).

Our results clearly show evidence for δ-GABA$_A$Rs expression by CA3 PV+ INs. Additionally, as shown in **Figure 2A**, colabeling is also evident in hippocampal area CA1 INs and neuropil and the DG (data not shown).

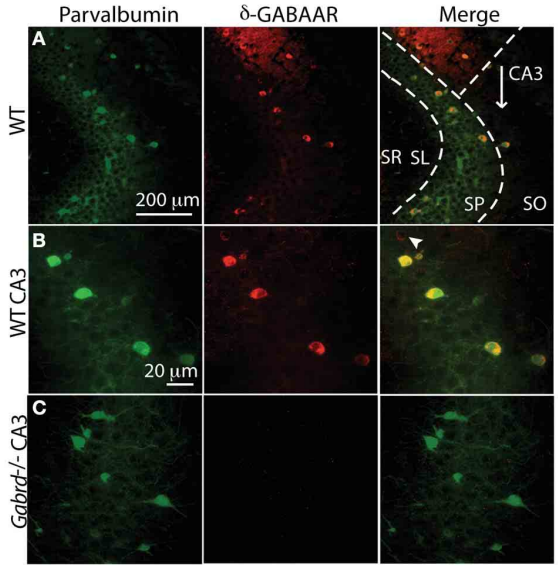

FIGURE 2 | The majority of CA3 PV+ interneurons also express δ-GABA$_A$Rs. (A,B) Immunohistochemical evidence of δ-GABA$_A$Rs expression on CA3 PV interneurons in a WT mouse, by confocal microscopy. Green: PV, red: δ-GABA$_A$Rs, yellow: colocalization. **(A,B)** PV and δ-GABA$_A$Rs immunolabeling is strongest at the somata and it is clearly present and more faint in the processes around CA3 pyramidal cells. Most PV+ INs in the stratum pyramidale (SP) also co-localize δ-GABA$_A$Rs; very rare interneurons are δ-GABA$_A$Rs+ and PV+ (**B**, white arrowhead). Most CA3 PV+ and/or δ-GABA$_A$Rs+ INs are concentrated around the SP, whereas they are sparser in stratum oriens (SO) stratum lucidum (SL) and stratum radiatum (SR). **(C)** Specificity of δ-GABA$_A$Rs immunolabeling is confirmed by the lack of δ-GABA$_A$R staining in a *Gabrd*$^{-/-}$ mouse. No significant change in PV labeling is found in *Gabrd*$^{-/-}$ mice.

SURFACE δ-GABA$_A$Rs EXPRESSION DECREASES DURING PREGNANCY IN INs OF THE PYRAMIDAL CELL LAYER

Pregnancy-related δ-GABA$_A$Rs plasticity has been previously described in hippocampal principal neurons (Maguire et al., 2009). To assess whether modulation of δ-GABA$_A$R expression in INs also plays a role in physiological and pathophysiological alterations during pregnancy and the postpartum, we stained slices of pregnant mice in parallel with slices of virgin and postpartum mice with δ-GABA$_A$R-specific antisera (**Figure 3**). Pregnant mice were used for experiments at day-18 of pregnancy, in order to study long-term brain exposure to high levels of NS. Virgin mice were anovulatory, in order to avoid estrus cycle-linked modifications in δ-GABA$_A$Rs previously described in the hippocampus (Maguire et al., 2005). Postpartum mice were used 48 h after parturition, when blood progesterone and ALLO levels become normalized to pre-pregnancy values (Concas et al., 1998).

Neuroactive steroid levels fluctuate with plasma progesterone and corticosterone levels. δ-GABA$_A$R expression is modified at different time points in the ovarian cycle, and is influenced by short periods of acute stress (Maguire and Mody, 2007). In order to control for potential stress-related variations across animal groups, corticosterone plasma levels were measured. We found levels and variability similar to those previously reported for C57Bl/6 WT mice and *Gabrd*$^{-/-}$ mice (Sarkar et al., 2011). No differences were found across groups (in ng/ml: virgin = 34.6 ± 16.3, pregnant = 41.6 ± 10.4, postpartum = 29.8 ± 16.5, *Gabrd*$^{-/-}$ = 13.6 ± 3.2). One-Way ANOVA; $p = 0.6159$, $F_{(3,\ 27)} = 0.6075$.

In terms of δ-GABA$_A$Rs anatomical distribution, while there is functional evidence for the existence of axonal δ-GABA$_A$Rs on CA3 mossy fiber boutons (Trigo et al., 2008; Ruiz et al., 2010), we found δ-GABA$_A$Rs immunoreactivity in hippocampal CA3 area to be restricted to the somata of INs and their terminal fields,

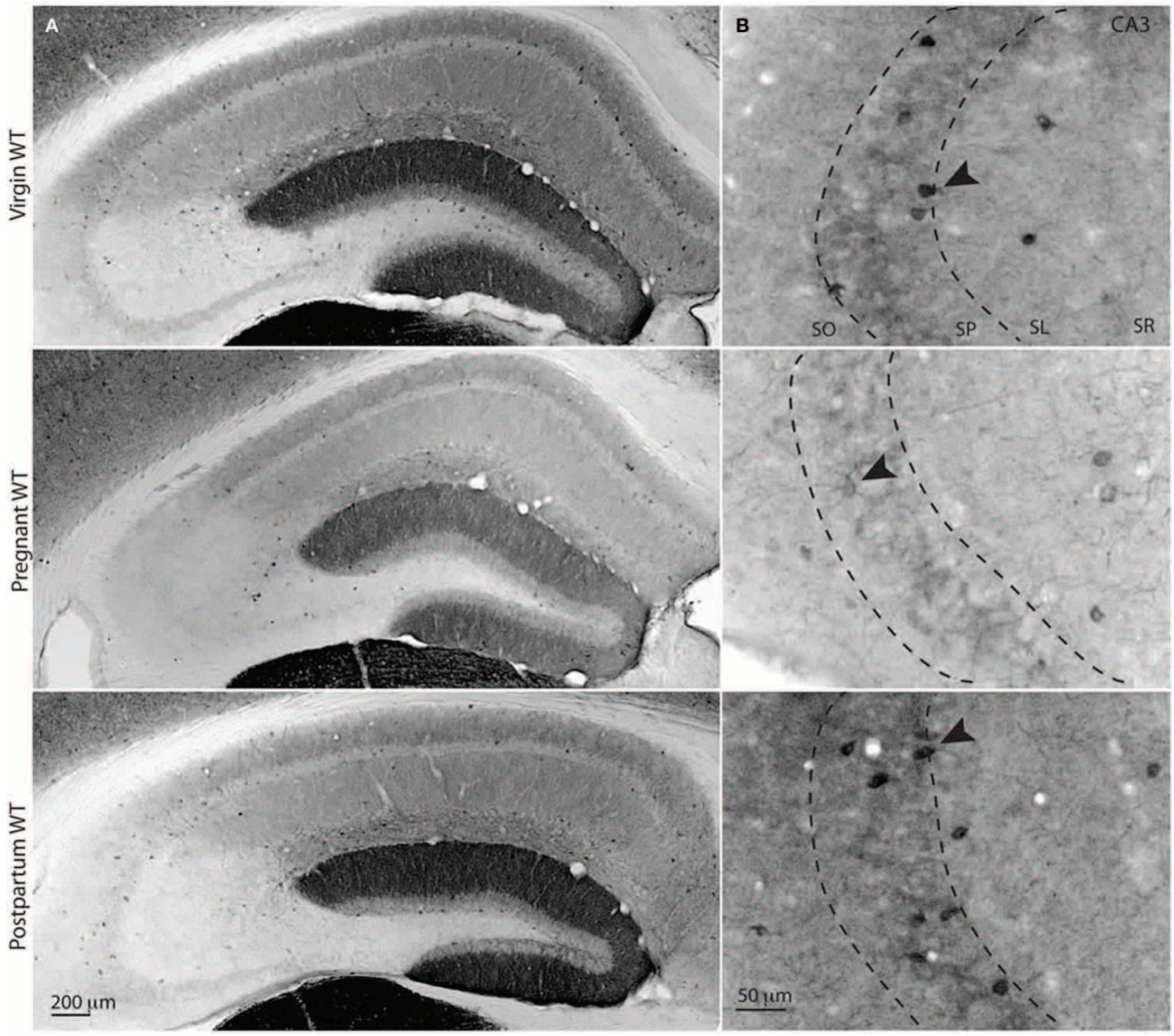

FIGURE 3 | Surface δ-GABA$_A$Rs expression is reduced during pregnancy in CA3 stratum pyramidale interneurons. (A,B) Representative bright-field images of whole hippocampal DAB staining for δ-GABA$_A$Rs in virgin, pregnant and postpartum (48 h) WT mice. Surface staining of functionally relevant receptors was obtained by processing tissues under non-permeabilizing conditions. In the hippocampus δ-GABA$_A$Rs are mostly expressed on dentate gyrus granule cell dendrites (forming dentate molecular layer), many hippocampal INs, and CA1 principal cells basal and apical dendrites. **(A)** Surface δ-GABA$_A$Rs expression is reduced in hippocampal CA1 pyramidal cells and dentate gyrus granule cells, as previously reported, and this is reflected in a lighter staining of the neuropil in CA1 and DG. **(B)** δ-GABA$_A$Rs expression in CA3 is exclusively found in interneuron somata, dendrites (black arrowheads) and in the interneuronal processes surrounding CA3 pyramidal cells (dotted lines show the boundaries of CA3 stratum pyramidale). In pregnant animals, CA3 δ-GABA$_A$Rs surface expression is reduced, as suggested by lighter staining. Optical density measurements in CA3 SP are in AU mean ± SEM: virgin = 45.2 ± 2.4; pregnant = 15.7 ± 2.3; postpartum, 48.5 ± 1.7 CA3-SP total OD of bright-field images; $n = 17$, 10, 16 slices and $n = 3$ mice for each group. One-Way ANOVA followed by Tukey's multiple comparisons test; $p < 0.0001$ for virgin and pregnant and for pregnant and postpartum, and $p > 0.05$ for virgin and postpartum, $F_{(2,\ 40)} = 58.95$.

which finely extend along the pyramidal cell layer as previously reported (Sperk et al., 1997; Peng et al., 2004).

Our findings show that most δ-GABA$_A$Rs labeled interneuronal somata were localized in close proximity (within 30 μm) or within the CA3-SP. Interestingly, under non-permeabilizing conditions we find a significant decrease in the δ-GABA$_A$R staining of hippocampal CA3 INs of pregnant mice, suggestive of a downregulation of functionally relevant δ-GABA$_A$Rs on the surface of INs. Surface expression measured as optical density reverted to pre-pregnancy levels already 48 h postpartum (**Figures 3**, **5**).

We have previously described a functionally relevant decrease in δ-GABA$_A$R immunostaining in hippocampal principal cells (DG molecular layer and CA1 SO and SR) during pregnancy

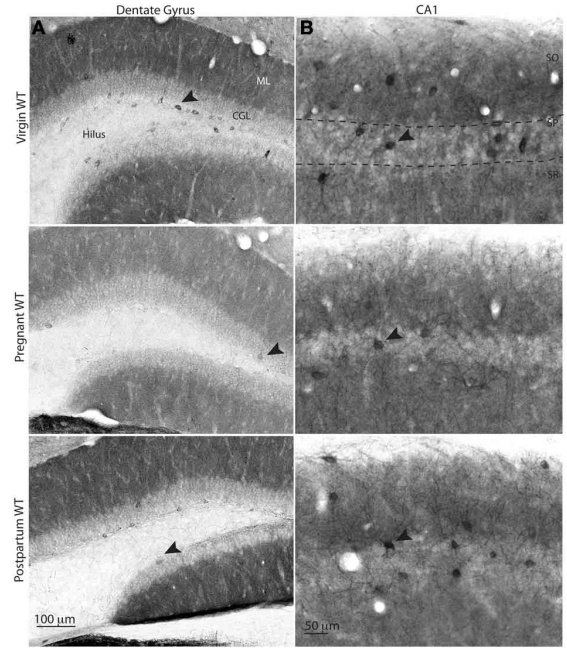

FIGURE 4 | δ-GABA$_A$R immunolabeling of interneurons in the dentate gyrus and area CA1 of the hippocampus at different gestational states. Representative bright-field images of δ-GABA$_A$R staining in the dentate gyurs **(A)** and CA1 **(B)**. δ-GABA$_A$R specific immunolabeling shows decreased staining of the INs that localize in the inner part of the granule cell layer and within CA1 stratum pyramidale (arrowheads). Some δ-GABA$_A$R labeled INs are visible in the molecular layer of the dentate gyrus in virgin and postpartum animals, whereas no labeled INs are visible in slices from pregnant animals in this area.

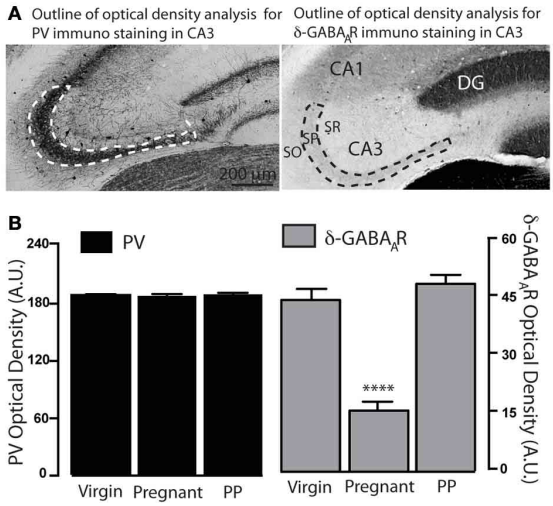

FIGURE 5 | Semi quantitative optical density analysis of PV and δ-GABA$_A$R immunolabeling across different gestational states. (A) Representative bright-field images of CA3 PV and δ-GABA$_A$R-specific DAB staining. Dotted lines delimit the boundaries of the area analyzed (CA3 stratum pyramidale) for optical density measurements. **(B)** Optical density measurements (arbitrary units, A.U.) show a significant reduction in δ-GABA$_A$R expression in pregnant animals. No difference is found in PV expression across gestational states. Asterisks denote significance, $p < 0.0001$.

(Maguire et al., 2009). Here we confirmed these modifications (**Figure 4**). Additionally, we demonstrate a brain-region specific upregulation to pre-pregnancy levels in the same areas during postpartum, consistent with previously described postpartum normalization of δ-GABA$_A$Rs by use of whole hippocampal Western blot analysis (Maguire and Mody, 2008). Interestingly, similar to our findings in the CA3 region, the staining of INs in the CA1 and DG regions is also suggestive of surface δ-GABA$_A$Rs downregulation during pregnancy, as δ-GABA$_A$R-labeled INs consistently appear less numerous and less strongly immunoreactive (**Figure 4**). Moreover, just like in the CA3 region, downregulation of δ-GABA$_A$Rs on INs reverted to pre-pregnancy levels in the immediate postpartum. In the DG, labeled INs were mostly localized around the inner granule cell layer, and some sparse INs could be found in the molecular layer as previously described (Peng et al., 2004; Glykys et al., 2007) (**Figure 4**). Similar findings are also evident in hippocampal area CA1, where most affected INs appear those in the immediate proximity of the pyramidal cell layer.

In CA3, CA1 and DG anatomical localization of those INs in which δ-GABA$_A$R expression is mostly affected during pregnancy is suggestive of BCs, as cell bodies of this class of INs are normally found in close proximity of the principal cell layer, in an ideal position to make preferential contacts with their perisomatic region (Freund and Buzsaki, 1996; Klausberger and Somogyi, 2008).

In vitro CA3 γ OSCILLATION FREQUENCY IS INCREASED DURING PREGNANCY

In the light of our anatomical findings we decided to examine a PV+BCs dependent network behavior, γ oscillations, as their frequency is controlled by δ-GABA$_A$R-mediated tonic inhibition of INs. As a result, *in vitro* experiments on *Gabrd*$^{-/-}$ mice show a constitutively higher frequency both in cholinergically-induced and kainate-induced γ oscillations in the CA3, compared to WT mice (Mann and Mody, 2010). Given these previous findings, we addressed the question whether δ-GABA$_A$R plasticity on CA3 INs of pregnant mice has functional consequences on CA3 γ oscillations. Oscillations are defined by their frequency and power and result from the periodically timed feedback interaction between INs and principal neurons (Mann et al., 2005).

We found a statistically significant increase in the peak frequency of kainate-induced γ oscillations in slices obtained from pregnant mice (**Figures 6A–C**). This resembled the increased frequency found in *Gabrd*$^{-/-}$ virgin mice. γ oscillations in slices from virgin and postpartum WT mice have similar, lower frequencies (**Figures 6A–C**). We found no differences across groups in power at peak frequency or in total power (between 30 and 120 Hz) (**Table 1**). These findings are consistent with the previously reported similar power of γ oscillations of WT and *Gabrd*$^{-/-}$ males (Mann and Mody, 2010). Statistical significance

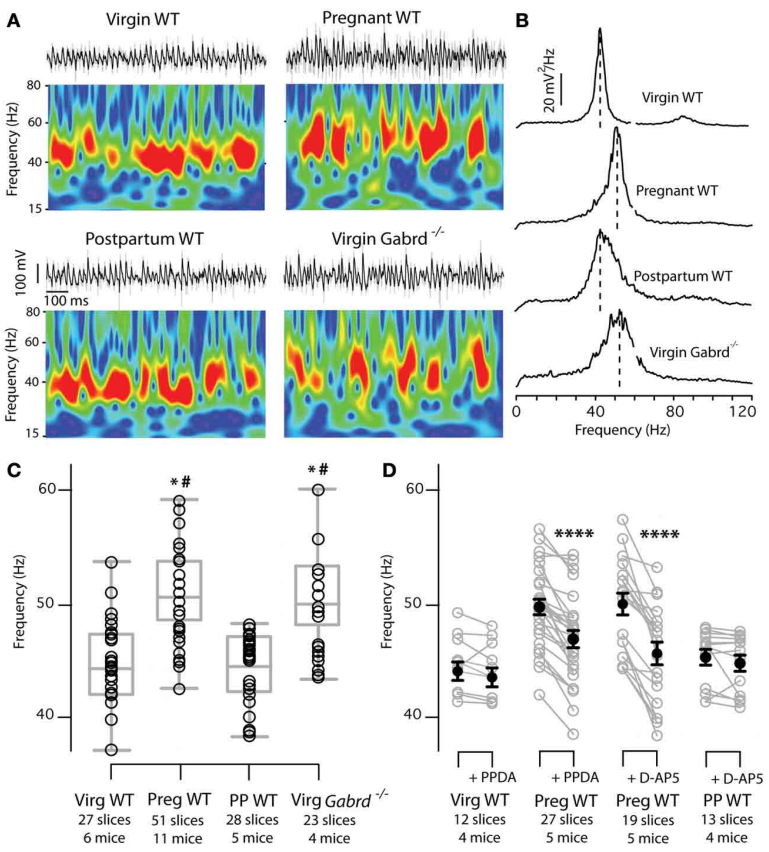

FIGURE 6 | γ Oscillations frequency is increased in the CA3 of pregnant animals in the absence of physiological ALLO levels. Increased CA3 γ oscillations frequency in slices of WT pregnant mice is sustained by NMDA-Rs activation on interneurons. **(A)** Kainate induced γ oscillations (50 nM) recorded extracellularly in CA3 stratum pyramidale at different gestational states show higher frequency in slices of WT pregnant and *Gabrd*$^{-/-}$ virgin mice compared to slices from WT virgin and postpartum mice. Upper traces: representative 1 s epochs of LFPs band-pass filtered between 15 and 120 Hz (black) and raw traces (gray). Morlet wavelet transforms of the corresponding traces show γ oscillatory behavior. Warmer colors represent higher power, and the same scale has been used for all four wavelets. **(B)** Plots of power spectral density calculated over 180 s periods of the same recordings as in **(A)**. Note increased γ frequency in slices from pregnant and *Gabrd*$^{-/-}$ mice. No differences are found in power at peak frequency or total power (30–120 Hz) **(C)**, Box plots showing peak frequencies for the four experimental groups. Each dot symbolizes the peak frequency of one slice calculated as the frequency with the highest power in a 180 s period power spectral density as in **(B)**. Box plots represent mean, 25th and 75th percentile, and largest and smallest values. Significance established by One-Way ANOVA followed by Turkey's multiple comparisons test. $^{*}p < 0.0001$ between WT pregnant and WT virgin or WT postpartum respectively. $^{\#}p < 0.0001$ between *Gabrd*$^{-/-}$ virgin and WT virgin or WT postpartum respectively. **(D)** In slices of WT pregnant mice bath application of the NMDA-R subunit-unspecific antagonists D-AP5 (25 μM) or GluN2D-containing NMDA-Rs specific antagonist PPDA (1μM) decreases γ oscillations frequency to WT virgin and WT postpartum (PP) values. The same drugs have no effect on γ frequencies of slices of WT virgin or WT postpartum mice. Mean frequency ± SEM in Hz, significance established by two-tailed paired *t*-test: pregnant PPDA = 49.7 ± 0.7 to 46.9 ± 0.7, $p < 0.0001$, $n = 27$ slices, 5 mice; virgin PPDA = 44.0 ± 0.8 to 43.5 ± 0.9 $p = 0.1$, $n = 12$ slices, 4 mice; pregnant D-AP5 = 49.8 ± 0.96 to 45.6 ± 1.0 $p < 0.0001$, $n = 19$ slices, 5 mice; postpartum D-AP5 = 45. 5 ± 0.68 to 45.1 ± 0.68 $p = 0.2$, $n = 13$ slices, 4 mice. Asterisks denote significance. *n*'s for each group are reported in the figure.

was determined by One-Way ANOVA followed by Tukey's multiple comparisons test for peak frequency: $p < 0.0001$, $F_{(3,\ 125)} = 28.12$; for power at peak frequency: $p = 0.32$, $F_{(3,\ 125)} = 1.168$; for total power (30–120 Hz): $p = 0.27$, $F_{(3,\ 125)} = 1.338$.

For each gestational state we tested for individual variability or slice location differences (septal through temporal) in peak frequency, power at peak frequency and total power by One-Way ANOVA, and found no significant differences. All *p*-values > 0.05, F(DFn, DFd) for peak frequency, power at peak frequency and total power (30–120 Hz) as follows: virgin WT, individual variability $F_{(5,\ 21)} = 1.267$, 1.136, 1.966; slice location $F_{(5,\ 21)} = 0.418$, 0.69, 0.9. Pregnant WT, individual variability $F_{(10,\ 40)} = 1.86$, 0.66, 1.27; slice location $F_{(6,\ 44)} = 1.13$, 1.27, 1.73. Postpartum WT, individual variability $F_{(4,\ 23)} = 1.5$, 0.66, 0.65; slice location $F_{(6,\ 21)} = 1.56$, 0.72, 0.66. Virgin *Gabrd*$^{-/-}$, individual variability $F_{(3,\ 19)} = 0.28$, 1.38, 1.68; slice location, $F_{(5,\ 17)} = 0.18$, 1.16, 0.98. These findings are consistent with the idea that kainate-induced γ

Table 1 | Details of γ oscillations characteristics by gestational state and genotype.

	Virgin WT		Virgin *Gabrd*$^{-/-}$	Pregnant WT					Postpartum WT	
	Control	+PPDA (1 μM)	Control	Control	+D-AP5 (25 μM)	+PPDA (1 μM)	+DMSO	+ALLO (100 nM)	Control	+D-AP5 (25 μM)
Frequency (Hz)	44.9 ± 0.7	43.5 ± 0.8	50.5 ± 0.8*	50.8 ± 0.5*	45.6 ± 1*	46.9 ± 0.7*	50 ± 1*	45.2 ± 1*	44.6 ± 0.6	45.1 ± 0.7
Power at peak frequency (E-11 V^2s^{-1})	4.87 ± 1.05	3.96 ± 1.24	4.6 ± 0.93	7.22 ± 1.3	7.53 ± 1.38	5.19 ± 1.49	6.34 ± 1.33	10.2 ± 5.94	5.05 ± 0.99	4.52 ± 1.31
Total power 30–120 s^{-1} (E-10 V^2)	8.54 ± 1.32	8.23 ± 1.94	10.7 ± 1.76	14.1 ± 2.25	14.3 ± 2.35	11.5 ± 2.79	13.7 ± 3.02	12.1*E* ± 2.97	11.4 ± 1.9	10.8 ± 3
n slices (mice)	27 (6)	12 (4)	23 (4)	51 (11)	19 (5)	27 (5)	21 (5)	14 (5)	28 (5)	13 (3)

Summary of mean values and SEM for each of the gestational states and genotypes derived from the corresponding 180 s period averages power spectral densities. γ oscillations were characterized by their peak frequency, power at peak frequency and total power (30–120 Hz). Asterisks denote significance calculated by One-Way ANOVA followed by Turkey's multiple comparisons test (for controls), or two-tailed paired t-test (for DAP-5 and PPDA treatment), or two-tailed unpaired t-test (for DMSO and ALLO incubation).

oscillations *in vitro* are homogeneous throughout the septo-temporal axis of the hippocampus and vary little across different animals.

Our electrophysiological results corroborate the anatomical finding of decreased CA3 interneuronal δ-GABA$_A$R expression in pregnant mice with an increase in the frequency of kainate-induced γ oscillations during pregnancy. Interestingly, γ power remained unchanged across the experimental groups, suggesting that only γ oscillations frequency is under the control of interneuronal δ-GABA$_A$Rs. Additionally, we showed that γ band oscillations are increased in frequency in *Gabrd*$^{-/-}$ females as it was previously demonstrated in *Gabrd*$^{-/-}$ males (Mann and Mody, 2010).

INCREASED γ OSCILLATION FREQUENCY RESULTS FROM IMBALANCE BETWEEN ACTIVATION OF NMDA-Rs AND δ-GABA$_A$Rs ON INs

The switch of γ oscillations to higher frequencies in the face of reduced δ-GABA$_A$Rs on CA3 INs most likely results from a reduced tonic GABA conductance in these cells, which translates into an enhanced NMDA-R-mediated tonic excitation (Mann and Mody, 2010). This is thought to be a control mechanism that allows for ample modulatory ability and a large dynamic range of the γ oscillations *in vivo*. Although at near physiological temperature (35°C) *in vitro* CA3 γ oscillations are quite stereotyped, there is large variability of γ frequencies *in vivo* within the same animal (Colgin et al., 2009). An equilibrium between tonic inhibition and excitation on the neurons that generate and maintain the oscillations may be a mechanism by which γ frequencies are modulated (Mann and Mody, 2010).

In order to determine if a similar balancing mechanism is also responsible for the increased γ frequency found in slices from pregnant mice, we tested the effect on γ frequencies of two NMDA-R antagonists: the wide spectrum antagonist 2-amino-5-phosphonopentanoic acid (D-AP5), and a drug (2S*,3R*)-1-(phenanthren-2-carbonyl)piperazine-2,3-dicarboxylic acid (PPDA) that at low concentrations preferentially antagonizes GluN2D-containing NMDA-Rs (Feng et al., 2004; Morley et al., 2005; Lozovaya et al., 2004). As interneuronal NMDA-Rs are particularly enriched in GluN2D subunits (Monyer et al., 1994; Standaert et al., 1996), the use of the latter compound addresses whether the increased γ oscillation frequency results from the activation of NMDA-Rs on INs. D-AP5 (25 μM) significantly reduced γ frequency in slices of pregnant mice to levels comparable to pre-pregnancy values (**Figure 6D**; **Table 1**). At the same time, D-AP5 was ineffective in modifying γ frequency in slices of postpartum mice.

Consistent with the D-AP5 findings, PPDA (1 μM) decreased γ oscillation frequency in slices from pregnant mice (**Figure 6D**; **Table 1**) and was ineffective in modifying γ oscillation frequency in slices from virgin mice. D-AP5 and PPDA had no effects on power at peak frequency and total power (between 30 and 120 Hz), $p > 0.05$ by two-tailed paired *t*-test (**Table 1**). One-Way ANOVA comparison of the 4 frequency groups after NMDA-R block (pregnant after D-AP5, pregnant after PPDA, virgin after PPDA, postpartum after DAP-5) shows no difference across groups, $p = 0.16$; $F_{(3,\ 99)} = 1.752$.

These data demonstrate that the increased γ oscillation frequency observed in slices of pregnant mice is sustained by a PV+INs specific mechanism involving enhanced NMDA-R-mediated tonic excitation following the reduction of the δ-GABA$_A$Rs-mediated tonic conductance. This appears to be the result of the pregnancy-related downregulation of δ-GABA$_A$Rs in INs. The increase in γ frequency is thus mediated by a mechanism similar to that described for *Gabrd*$^{-/-}$ mice (Mann and Mody, 2010). The virgin and postpartum experimental groups were not sensitive to NMDA-R blockade, consistent with the idea that the activation of δ-GABA$_A$Rs on INs is sufficient to keep the tonic excitation in check.

EXPOSURE TO ALLO LEVELS FOUND IN PREGNANCY (100 nM) LOWERS THE FREQUENCY OF γ OSCILLATIONS TO PRE-PREGNANCY VALUES

Given the highly lipophilic nature of NS, it was suggested that they may access their binding sites on GABA$_A$Rs after accumulation and lateral diffusion in the plasma membrane (Chisari et al., 2010). Whether during *in vitro* preparations of brain slices NS dissolved in plasma membranes are completely washed off remains to be fully established although some evidence would suggest at least partial depletion. Several *in vitro* experiments using finasteride, an inhibitor of 5α-reductase (a key enzyme in the local NS synthesis pathway), have unveiled the existence of NS synthesis in slices (Belelli and Lambert, 2005). This suggests that NS synthetized prior to the enzymatic block are either degraded or washed off during *in vitro* incubation. To confirm this depletion in our slices, in a separate set of experiments in slices from WT males we noticed a significant increase in γ oscillation frequency after 30 min of incubation in 1 μM finasteride compared to slices incubated in vehicle alone (**Figure 7**). As δ-GABA$_A$Rs respond poorly to GABA in the absence of NS, this finding is consistent with the wash-out of NS from slices, following pharmacological blockade of local NS synthesis. NS presence in slices likely result from continuous enzymatic conversion of local precursors, namely steroids synthetized *de novo* from cholesterol (Rupprecht et al., 2010), rather than the *in vivo* NS still bound to the plasma membrane after slice preparation. Since during pregnancy most of brain ALLO is derived from plasma progesterone (Paul and Purdy, 1992; Concas et al., 1998), it is reasonable to assume that slices incubated in a progesterone- and ALLO-free nACSF will be devoid of the NS levels found in the brains of pregnant mice. Consequently, *in vitro* brain preparations of pregnant mice will suffer an acute withdrawal of NS from plasma precursors, making synthesis from local precursors the only enzymatic pathway for maintaining NS levels. In support of this idea, we previously published evidence of altered slice excitability in slices from pregnant mice in the absence of physiological pregnancy levels of ALLO (Maguire et al., 2009).

Therefore, in order to determine whether interneuronal δ-GABA$_A$Rs downregulation and the correlated increase in γ frequency during pregnancy could be ascribed to a homeostatic mechanism which counterbalanced increased NS levels with δ-GABA$_A$Rs downregulation, we tested the effects of physiological pregnancy levels of ALLO (100 nM) (Paul and Purdy, 1992; Concas et al., 1998) on γ oscillations frequency. Slices of pregnant mice were incubated from the time of cutting to the time of recording in either vehicle (0.01% DMSO) or 100 nM ALLO. Slices from the same animal were randomly assigned to either one experimental group and oscillations were recorded. We found that slices incubated in DMSO had γ oscillation peak frequencies similar to those recorded in slices of pregnant mice not exposed to the vehicle (two-tailed unpaired *t*-test, $p = 0.2$). Moreover, 100 nM ALLO was capable of significantly lowering peak frequency to values comparable to virgin and postpartum slices, $p < 0.0001$ by two-tailed unpaired *t*-test (**Table 1**; **Figure 8**). Exposure to ALLO did not affect power at peak frequency or total power (between 30 and 120 Hz). These findings demonstrate that under experimental conditions similar to physiological states during pregnancy, network output remains constant. In particular, γ oscillation frequencies are regulated by the levels of brain NS and the amount of δ-GABA$_A$Rs expressed on INs. The inability to appropriately and timely regulate δ-GABA$_A$Rs expression on INs or NS synthesis may predispose to or facilitate states of altered network oscillatory activity.

DISCUSSION

This study demonstrates a homeostatic δ-GABA$_A$R plasticity in mouse hippocampal INs during pregnancy and postpartum. Immunohistochemical findings showed a transient δ-GABA$_A$Rs downregulation in INs during the last third of pregnancy, which was fully reversible in the early postpartum. This led to altered network dynamics after the acute *in vitro* withdrawal of the high levels of ALLO found in pregnancy manifested in increased γ oscillation frequency in the hippocampal CA3 region of pregnant mice. This increase was fully reversible either by blocking interneuron-specific NMDA-Rs, or by restoring ALLO levels in the slices. Our findings are consistent with the idea that a δ-GABA$_A$R-mediated tonic conductance of CA3 INs controls γ oscillation frequency by modulation of NMDA-R-mediated tonic excitation (Mann and Mody, 2010).

The observation that gamma oscillation frequency in slices from mice with partially downregulated δ-GABA$_A$R expression closely resembles gamma oscillation frequencies found in slices obtained from *Gabrd*$^{-/-}$ mice may not be unexpected (see similar dentate excitability between *Gabrd*$^{-/-}$ and pregnant WT in the absence of ALLO, in Maguire et al., 2009). It is possible that in the total absence of δ-GABA$_A$Rs, other tonically active GABA$_A$Rs may be upregulated, but this hypothesis will require further investigation. Our findings in the present paper about the effects of partial δ-GABA$_A$Rs reduction in PV+ INs during pregnancy were validated in mice heterozygous for δ-GABA$_A$R expression only in PV+ cells (data not shown). In these mice, as in pregnant mice, *in vitro* γ oscillations are significantly faster compared to WT mice. Thus, it is possible that a partial reduction δ-GABA$_A$Rs in PV+INs results in a full activation of NMDA-Rs in the same cells that can be no longer enhanced by further deletion of δ-GABA(A)Rs.

In the CNS δ-GABA$_A$Rs are found on both principal cells and INs. In neocortical INs, δ-GABA$_A$Rs are thought to be mostly expressed in neurogliaform cells (Oláh et al., 2009). Nevertheless, the modulation of γ oscillation frequency by a THIP-sensitive (synthetic δ-GABA$_A$R-specific agonist) tonic current of CA3 INs (Mann and Mody, 2010), suggests that in the hippocampus δ-GABA$_A$Rs expression is also present in other types of IN, at least in PV+BCs, the IN type mainly responsible for generation of γ oscillations (Buzsáki and Wang, 2012). The anatomical confinement of δ-GABA$_A$Rs to PV+INs, evidenced by the very low ratio (3%) of PV+negative δ-GABA$_A$Rs expressing INs around the SP, also suggests that CCK+BCs do not express δ-GABA$_A$Rs. If confirmed by more detailed future studies, this finding could open interesting functional implications particularly since hippocampal CCK+BCs do not seem to express α1-GABA$_A$Rs (Gao and Fritschy, 1994), a natural partner of δ-GABA$_A$Rs in INs (Glykys et al., 2007). Endogenous and exogenous modulators of δ-GABA$_A$R-mediated tonic conductance, such as NS and EtOH, by influencing only PV+BCs through δ-GABA$_A$Rs, will dynamically

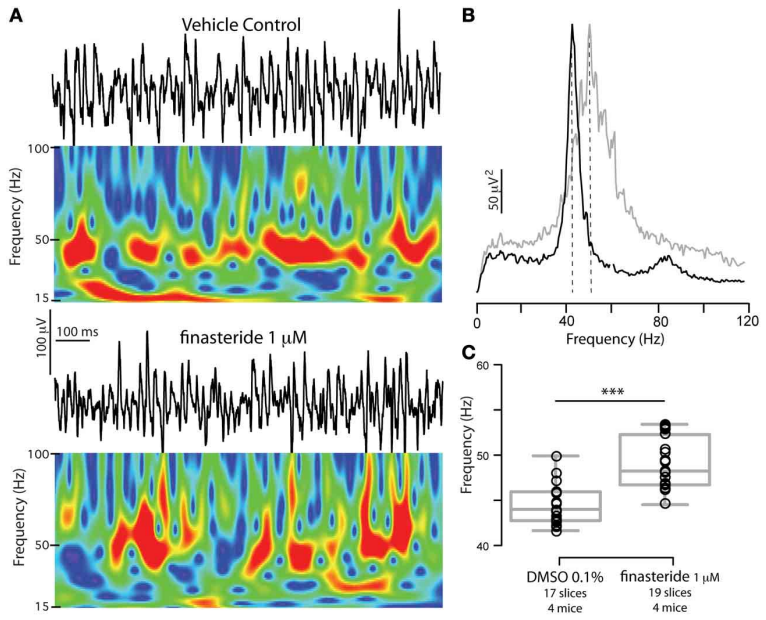

FIGURE 7 | Blocking neurosteroidogenesis increases γ oscillations frequency in slices from WT mice. (A) Representative 1 s epoch of local field potential oscillations and corresponding Morlet Wavelets in the CA3 region of hippocampal slices obtained from a WT adult male mouse in vehicle (DMSO 0.01%) or in 1 μM finasteride (after 30 min incubation). **(B)** Power spectral densities of the same recordings (average of 180 s) for DMSO 0.01% (black) and finasteride (gray). **(C)** Peak frequencies of γ oscillations recorded in either vehicle or 1 μM finasteride. The latter have significantly higher peak frequencies. Box plots represent mean, 25th and 75th percentile, and largest and smallest values. Mean peak frequency ± SEM in Hz: DMSO 0.01% = 44.5 ± 0.5, finasteride = 49.1 ± 0.6 $p < 0.0001$, two-tailed unpaired *t*-test. Asterisks denote significance ($p < 0.05$). No differences were found in power at peak frequency, $p = 0.5$, and total power (30–120 Hz), $p = 1$. *n*'s for each group are reported in the figure.

shift the weight between the two types of perisomatic inhibition (Freund and Katona, 2007). It is remarkable that PV+BCs, defined as the "orderly clockwork" of the hippocampus compared to the "variable fine-tuning" role of CCK+BCs (Freund and Katona, 2007), would preferentially express δ-GABA$_A$Rs sensitizing them to constantly changing molecules (e.g., NS). If PV+BCs use NS for instantaneous modifications of network dynamics in response to behavioral needs, the compensatory downregulation in δ-GABA$_A$Rs expression during pregnancy suggest that network oscillatory activity is indeed a functional neuronal output that is kept under strict control in terms of consistency, reliability, and "order." PV+BCs are not the only PV+expressing INs in CA3, although (at least in CA1) they are the majority (Baude et al., 2006). Axoaxonic cells and bistratified cells are other PV+INs, but these INs don't participate much in γ oscillations (Gulyás et al., 2010; Dugladze et al., 2012). Therefore, they are unlikely to be involved in changes in γ activity following δ-GABA$_A$Rs modulation. Moreover, these latter PV+INs have been shown to express substantially less extrasynaptic α1-GABA$_A$Rs and consequently have lower levels of tonic inhibition (Baude et al., 2006; Gao and Fritschy, 1994).

The relationship between δ-GABA$_A$R plasticity and its partnership with various α subunits is an important issue. We have previously shown how swings in brain NS content will affect network output not only by increasing neuronal tonic conductance, but also by regulating surface δ-GABA$_A$Rs expression (Maguire et al., 2005; Maguire and Mody, 2007). Similar modulations in α4-GABA$_A$Rs, the specific partner of δ-GABA$_A$Rs in principal neurons, have also been observed (Smith et al., 1998; Follesa et al., 2001). In addition, studies in *Gabra4*$^{-/-}$ mice showed how surface δ-GABA$_A$Rs expression in excitatory neurons depends on the presence of α4-GABA$_A$Rs (Glykys et al., 2007; Chandra et al., 2006). These findings together with the established plastic nature of α4-GABA$_A$Rs (Roberts et al., 2005, 2006) led to the notion that perhaps the δ-GABA$_A$R plasticity observed during altered NS levels depends on α4-GABA$_A$Rs (Shen et al., 2007, 2010; Kuver et al., 2012). However, in INs δ-GABA$_A$Rs naturally pair-up with α1-GABA$_A$Rs (Glykys et al., 2007) and although δ/α1-GABA$_A$Rs are as sensitive to NS as δ/α4-GABA$_A$Rs (Bianchi and Macdonald, 2003; Meera et al., 2009) the question whether δ/α1-GABA$_A$R expression is also regulated following NS oscillations remained open. Ours is the first report of δ-GABA$_A$R plasticity in neurons with no α4-GABA$_A$Rs. Although δ-GABA$_A$R upregulation in molecular layer INs of the DG has been proposed in a mouse model of epilepsy, a concurrent reduction in DG neuropil labeling made quantification somewhat difficult (Peng et al., 2004).

Acute exposure to levels of NS found in pregnancy leads to sedation and anesthesia (Child et al., 1971; Carl et al., 1990; Rupprecht, 2003), hence during pregnancy the mammalian brain faces the challenge to maintain an overall functional network, despite large hormonal changes. Indeed many women experience mild to severe disturbances in their neurological performance,

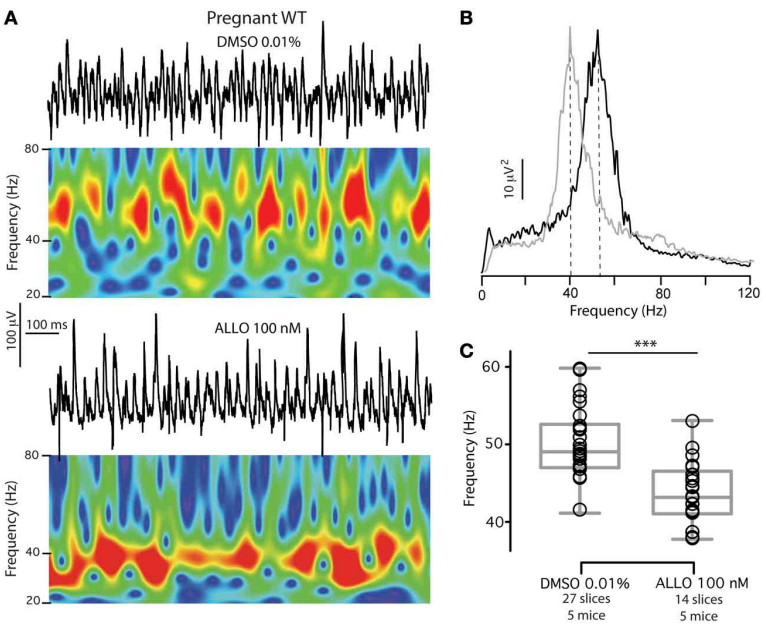

FIGURE 8 | Exposure to ALLO levels found in pregnancy (100 nM) reverts CA3 γ oscillations frequency in pregnant mice to control values. **(A)** Representative 1 s epoch of local field potential oscillations and corresponding Morlet wavelet transforms of 1 s epochs of local field potentials recorded in the presence of 50 nM kainate depict γ oscillatory behavior over time. Slices from the same pregnant WT animal were incubated and recorded in either vehicle (DMSO 0.01%) or ALLO 100 nM. Slices incubated in vehicle show significantly higher γ oscillations frequency compared to slices incubated in ALLO. **(B)** Power spectral densities of the same recordings (average of 180 s) for DMSO 0.01% (black) and ALLO (gray). **(C)** Box plots summarizing peak frequencies of the two experimental groups and representing mean, 25th and 75th percentile, and largest and smallest values. *n*'s for each group are reported in the figure. Significance established by two-tailed unpaired *t*-test, $p < 0.0001$.

mostly during times of fast rise or drop in progesterone and its neuroactive metabolites (Poser et al., 1986). We have previously proposed a model for δ-GABA$_A$Rs downregulation in excitatory neurons as a homeostatic mechanism of adaptation that allows these cells to maintain a constant level of excitability throughout pregnancy (Maguire et al., 2009).

δ-GABA$_A$Rs plasticity on DGGC or DG INs can be ruled out as possible player in the observed increased frequency of CA3 γ oscillations. In fact, Although DG can sustain gamma oscillatory activity which can couple with that of CA3 (Akam et al., 2012), *in vivo* and *in vitro* studies have shown how the DG doesn't host an endogenous oscillator, on the contrary it needs intact afferent entorhinal connections in order to oscillate at γ frequency (Bragin et al., 1995; Csicsvari et al., 2003). In addition, in (Mann and Mody, 2010), some experiments were done after isolating CA3 from the DG without any apparent effects on the characteristics of γ oscillations in CA3. Lastly, in the presence of 50 nM kainate the LFP activity in the DG is unaffected. For these reasons we propose the observed shift in frequency depends solely on δ-GABA$_A$Rs modulation on CA3 interneurons.

Here we describe a pregnancy-dependent loss of δ-GABA$_A$Rs in CA3-SP INs, which was reversible within 48 h postpartum, and seems to be homeostatic in nature. In addition to the δ-GABA$_A$R downregulation in CA3-SP INs, we show similar changes in CA1-SP and DG-INs. Numerous cortical INs also express δ-GABA$_A$Rs, and it is likely that δ-GABA$_A$Rs plasticity during pregnancy occurs in some or all of these cells. Identification of the specific types of INs modifying δ-GABA$_A$Rs expression during pregnancy or other periods of steroid hormone changes (ovarian cycle or stress), and the functional consequences of this plasticity will require further studies. Although the IN-δ-GABA$_A$R plasticity during pregnancy and the postpartum period likely affects different IN types, the increase in γ oscillations frequency is the functional consequence of pregnancy-related δ-GABA$_A$Rs loss specific to PV+BCs. The expression of PV in BCs decreases in patients with schizophrenia (Lewis et al., 2005, 2012), and this is correlated with modifications in γ oscillation activity, although the exact underlying mechanism remains to be established (Uhlhaas and Singer, 2010, 2012). The changes in γ oscillatory activity during pregnancy in our study did not result from changes in PV immunoreactivity across different gestational states, but from the plasticity of a specific GABA$_A$R subunit on these INs. Other, still to be uncovered, molecular and cellular modifications in PV+INs may also contribute to altered γ oscillatory activity and may lead to convergent psychiatric syndromes.

Slices prepared from pregnant mice are subject to an artificial acute NS withdrawal as plasma-derived precursors and NS are washed out during nASCF perfusion. Addition of 100 nM ALLO to nACSF completely restored γ oscillation frequencies to virgin and postpartum values consistent with the homeostatic nature

of IN-δ-GABA$_A$Rs downregulation during pregnancy and with the dependency of γ oscillations frequency on a NS-regulated δ-GABA$_A$Rs system. Modifications in network activity are only revealed after abrupt *in vitro* ALLO withdrawal indicating the natural propensity of the network to adapt to large hormonal swings. However, this inherent plasticity may expose the brain to ineffective network oscillatory dynamics in the case of exaggerated or untimely NS modifications, or to inadequate adjustment of δ-GABA$_A$Rs expression. Moreover, the physiological control of network dynamics exerted by NS fluctuations could be potentially less adaptable during pregnancy. A recent report showed concentration-dependent dual effects of GABA on the inhibitory or excitatory nature of IN tonic conductance (Song et al., 2011). In our study we did not perform direct tonic GABA conductance recordings from PV+BCs, but the complete reversibility of γ oscillation frequency to lower values after NMDA-R blockade in INs when IN δ-GABA$_A$Rs are diminished is consistent with an inhibitory role of the tonic GABA conductance in PV+BCs. During pregnancy, CA3 PV+BCs must have a decreased inhibitory (hyperpolarizing or shunting) tonic GABA conductance, which is normalized by 100 nM ALLO, and is capable to antagonize the tonic NMDA-R-mediated excitation of these cells.

The molecular pathways involved in the dynamic plasticity of δ-GABA$_A$Rs remain unknown and will constitute the subject of future investigations. Fast modifications in the endocytosis machinery may play a role in the initial δ-GABA$_A$Rs downregulation (Gonzalez et al., 2012). Interestingly it seems that long-term exposure to NMDA induces δ-GABA$_A$R mRNA expression in cultured neurons (Gault and Siegel, 1997). A similar mechanism may play a role in postpartum upregulation of δ-GABA$_A$Rs to virgin levels in INs.

Changes in γ oscillations have been reported in various neurological and psychiatric disorders (Uhlhaas and Singer, 2010, 2012), and range from poor mnemonic performance to psychosis and schizophrenia. Here we show how diminished δ-GABA$_A$Rs and increased NS levels are balanced during pregnancy and postpartum so that the tonic inhibition of PV+BCs and ultimately γ oscillation frequency are kept constant. Several symptoms typical of pregnancy and postpartum pathology may be ascribed to altered γ oscillations. If postpartum depression is a condition resulting from a mismatch between rapidly plummeting NS levels and the need to restore the number of δ-GABA$_A$Rs to pre-pregnancy levels (Maguire and Mody, 2008), then the plasticity of IN δ-GABA$_A$Rs may also follow a similar course in such pathological conditions. Accordingly, in schizophrenic patients the abnormal γ activity and the high occurrence of depressive behaviors may be a sign of comorbidity between these two conditions (Buckley et al., 2009). As cortical γ activity can be easily recorded with scalp EEG, changes in these oscillations in subjects predisposed to postpartum depression and epilepsy may help identify patients at risk, and could serve to devise δ-GABA$_A$R-specific pharmacological strategies for treating some of the major symptoms of the disease.

CONCLUDING REMARKS

We have demonstrated a homeostatic down-regulation of δ-GABA$_A$Rs in PV+ INs in late pregnancy. We thus established that these cells control the surface expression of δ-GABA$_A$Rs without expressing the highly plastic α4 subunit partner of δ-GABA$_A$Rs. We provide evidence that γ oscillation frequency recorded *in vitro* is artificially increased in slices of pregnant animals because of the acute withdrawal from plasma precursors. Adding back the levels of NS found in pregnancy normalizes γ frequency, showing the finely balanced homeostatic reduction in δ-GABA$_A$Rs expression. Giving the large amount of evidence linking altered γ oscillations with dysfunctional network processing, our findings have the potential to define neurological performance and precipitate preexisting neurological and psychiatric conditions during and after pregnancy. Milder and shorter NS fluctuations such as those typical of the ovarian cycle and stress could also modify δ-GABA$_A$Rs expression on PV+ INs and consequently influence network oscillatory behavior, depending on the accuracy of time coupling of NS to δ-GABA$_A$Rs expression. The δ-GABA$_A$Rs plasticity on PV+ INs may not be restricted to hippocampal area CA3, making it highly probable that similar modulations of γ oscillations take place on a wider scale. Recent discovery of differential pharmacology between α4/δ-GABA$_A$Rs and α1/δ-GABA$_A$Rs (Jensen et al., 2013) may help elucidate of their role in the control of emergent properties of neuronal networks in brain areas where both receptor combinations are present.

AUTHOR CONTRIBUTIONS

Isabella Ferando and Istvan Mody designed research, Isabella Ferando performed experiments, Isabella Ferando and Istvan Mody analyzed data, Isabella Ferando and Istvan Mody wrote the paper.

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CHAPTER 6

OVARIAN CYCLE-LINKED PLASTICITY OF δ-GABA$_A$ RECEPTOR SUBUNITS IN HIPPOCAMPAL INTERNEURONS AFFECTS GAMMA OSCILLATIONS *IN VIVO*

6.1 Summary

GABA$_A$receptors containing δ subunits (δ-GABA$_A$Rs) are GABA-gated ion channels with extra- and perisynaptic localization, strong sensitivity to neurosteroids (NS), and a high degree of plasticity. In selective brain regions they are expressed on specific principal cells and interneurons (INs), and generate a tonic conductance that controls neuronal excitability and oscillations. Plasticity of δ-GABA$_A$Rs in principal cells has been described during

states of altered NS synthesis including acute stress, puberty, ovarian cycle, pregnancy and the postpartum period, with direct consequences on neuronal excitability and network dynamics. The defining network events implicated in cognitive function, memory formation and encoding are γ oscillations (30-120 Hz), a well-timed loop of excitation and inhibition between principal cells and PV-expressing INs (PV+INs). The δ-$GABA_A$Rs of INs can modify γ oscillations, and a lower expression of δ-$GABA_A$Rs on INs during pregnancy alters γ frequency recorded *in vitro*. The ovarian cycle is another physiological event with large fluctuations in NS levels and δ-$GABA_A$Rs. Stages of the cycle are paralleled by swings in memory performance, cognitive function, and mood in both humans and rodents. Here we show δ-$GABA_A$Rs changes during the mouse ovarian cycle in hippocampal cell types, with enhanced expression during diestrus in principal cells and specific INs. The plasticity of δ-$GABA_A$Rs on PV+INs decreases the magnitude of γ oscillations continuously recorded in area CA1 throughout several days *in vivo* during diestrus and increases it during estrus. Such recurring changes in γ magnitude were not observed in non-cycling wild-type (WT) females, cycling females lacking δ-$GABA_A$Rs only on PV+INs (PV-$Gabrd^{-/-}$), and in male mice during a time course equivalent to the ovarian cycle. Our findings may explain the impaired memory and cognitive performance experienced by women with premenstrual syndrome (PMS) or premenstrual dysphoric disorder (PMDD).

6.2 Introduction

There are numerous reports about women experiencing fluctuating cognitive and neuropsychological functions during specific stages of the menstrual cycle. During the luteal phase, when progesterone levels are high, some women may experience different levels of dysthymia, irritability, anxiety, impaired working and emotional memory. All of these symptoms are inevitably aggravated in patients with PMDD (Man et al., 1999; Sveindóttir and Bäckstrøm, 2000; Bäckström et al., 2003; Reed et al., 2008; Ertman et al., 2011; Rapkin and Akopians,

2012; Yen et al., 2012; Bayer et al., 2014). Although all of these conditions can be hardly ascribed to a single mechanism, ovarian cycle-linked plasticity of δ-$GABA_A$Rs and resulting effects on tonic inhibition have been implicated in modifications in anxiety and memory performance in rodents (Maguire et al., 2005; Cushman et al., 2014). Moreover, patients with PMDD seem to be less sensitive to GABAergic modulation during their luteal phase, which led to hypothesize a luteal deficit of $GABA_A$Rs plasticity (Bäckström et al., 2003; Maguire et al., 2005).

The δ-$GABA_A$Rs are high affinity, low efficacy non-synaptic $GABA_A$ receptors with a high sensitivity to NS (Brickley and Mody, 2012). During times of altered NS levels, δ-$GABA_A$Rs expression changes in a direction that seems to depend on the timing of NS fluctuations. For instance, δ-$GABA_A$Rs plasticity has been observed during the ovarian cycle, pregnancy, the postpartum, puberty and acute stress, with direct effects on neuronal excitability and network activity. In particular, δ-$GABA_A$Rs plasticity has been reported in both principal cells and INs in different rodent brain areas including the hippocampus, different nuclei of the thalamus and the periaqueductal grey (Lovick et al., 2005; Brack and Lovick, 2007; Maguire and Mody, 2007, 2008; Maguire et al., 2009; Ferando and Mody, 2013a; Smith, 2013; MacKenzie and Maguire, 2014).

The tonic conductance mediated by δ-$GABA_A$Rs constitutes a powerful constrain over gain of neuronal signal transmission both in principal cells and in INs (Mody and Pearce, 2004; Semyanov et al., 2004; Farrant and Nusser, 2005; Walker and Semyanov, 2008; Song et al., 2011). The δ-$GABA_A$Rs of hippocampal INs modulate γ oscillations frequency *in vitro* (Mann and Mody, 2010; Ferando and Mody, 2013a). The γ oscillations are a periodic network activity (30-120 Hz) that can be recorded in different brain areas during certain wakefulness states and REM sleep, and arise from a synchronized excitation and inhibition loop between principal cells and PV+INs, which have a critical role in initiating and maintaining local oscillations (Sohal et al., 2009; Wulff et al., 2009; Korotkova et al., 2010; Carlén et al., 2011; Zheng et al., 2011; Lasztoczi and Klausberger, 2014). These oscillations are thought

to enable encoding and memory formation in discrete neuronal network, to facilitate spike-time dependent plasticity, and are considered to play an important role in the physiology of learning and memory (Colgin and Moser, 2010; Uhlhaas et al., 2011; Buzsáki and Wang, 2012; Uhlhaas and Singer, 2012).

In a recent study in mice we showed a homeostatic pregnancy-related down-regulation of δ-GABA$_A$Rs in CA3 stratum pyramidale (SP) INs which led to an increase in the frequency of γ oscillations recorded *in vitro* (Ferando and Mody, 2013a), in a manner similar to what has been observed in *Gabrd*$^{-/-}$ mice (Mann and Mody, 2010). However, the effects of δ-GABA$_A$R plasticity of INs on network activity and dynamics in the intact brain remain to be elucidated. In this study we show ovarian cycle-linked alterations in δ-GABA$_A$R expression in hippocampal CA1 and CA3 SP INs, on dentate gyrus granule cells (DGGCs) and pyramidal cells of the CA1, with increased expression during the high progesterone stage of diestrus, and decreased expression in estrus. These changes correlate with periodic modifications in the magnitude of γ oscillations recorded *in vivo* in CA1 SP of freely moving mice.

Previous studies showed increased anxiety and memory performance in female WT but not in global *Gabrd*$^{-/-}$ mice during estrus (low progesterone phase), while the behavior during diestrus in WT mice closely resembled that of male mice (Maguire et al., 2005; Moore et al., 2010; van Goethem et al., 2012; Cushman et al., 2014). However, δ-GABA$_A$Rs are plastic in both hippocampal principal cells and INs, so that behavioral correlates in global *Gabrd*$^{-/-}$ mice cannot distinguish between receptor changes in specific neuronal subtypes. By using a recently engineered floxed-*Gabrd* mouse (Lee and Maguire, 2013) and the PV-IRES-Cre line (JAX Stock # 008069), we specifically deleted the δ subunits of GABA$_A$Rs from PV+INs (PV-*Gabrd*$^{-/-}$) to examine potential changes in ovarian cycle-linked modifications in γ oscillations magnitude in the absence of δ-GABA$_A$Rs on PV+INs. Our findings identify a possible underlying cause for the different degrees of cognitive impairment experienced by some women at various phases of the ovarian cycle.

6.3 Materials and Methods

6.3.1 Animal Handling

In this study we used adult (15-20 week-old) female and male C57BL/6J mice, WT ($Cre^{-/-}$) or mice lacking δ-$GABA_A$Rs specifically on PV+INs (PV-$Gabrd^{-/-}$), generated by crossing PV-Cre (PV-IRES-Cre line; JAX Stock # 008069) and *Gabrd*-flox mice (generous gift of Dr. Jamie Maguire, Tufts University) (Lee and Maguire, 2013) both back-crossed for > 10 generations on C57BL/6J background. The δ-$GABA_A$Rs ablation from PV+IN was confirmed with immunohistochemical fluorescent double labeling (Ferando and Mody, in preparation, data not shown). Mice were housed with *ad libitum* access to food and water and kept on a 12-hour light/dark cycle, under the care of the UCLA Division of Laboratory Animal Medicine (DLAM). All experiments were performed during the light period and according to a protocol (ARC # 1995-045-53B) approved by the UCLA Chancellor's Animal Research Committee. Genotyping was performed by Transnetyx (Memphis, TN).

6.3.2 Surgery

Surgeries were performed under aseptic conditions on mice weighing 25-30 g. Under isoflurane anesthesia (22.5% in O_2 alone) the animal was mounted into a standard Stoelting instrument stereotaxic frame with blunt ear bars. Body temperature was maintained at 37°C using a rectal probe and a water circulated heating pad. The cranium was exposed through a small midline scalp incision. The bone was dried and three holes were drilled (0.5 mm diameter) in the cranium. With the aid of a micromanipulator, 2 sterilized recording electrodes (PlasticsOne, stainless steel, 125 μm diameter) were lowered into hippocampal CA1 region SP, bilaterally (at stereotaxic coordinates: anteroposterior, AP, 5.5 mm; mediolateral, ML, 1.45 mm; dorsoventral, DV, 1.2 mm from brain surface). The third hole was drilled above the cerebellum to insert the ground/reference electrode. The skull surface was covered by thin layer of cyanoacrylate based glue (Insta-Cure+, Bob Smith Industries) and then dental

acrylate (Ortho-Jet, Lang Dental Manufacturing Co., Inc.) was used to attach the electrode sockets to the skull surface. Immediately after surgery, the mouse was continuously monitored until recovered, as demonstrated by their ability to maintain sternal recumbency and to exhibit purposeful movement. During the recovery period after surgery, warm saline solution (0.01-0.02 ml/g/twice/day) was administered subcutaneously to prevent dehydration. To prevent any infection around the implant we topically administered Neosporin for 7 days. For analgesia, 0.1 mg/kg of buprenorphine was injected subcutaneously prior to surgery. Buprenorphine injections were continued following the surgery every 12 hours for 48 hours.

6.3.3 Ovarian Cycle Induction and Monitoring

Female virgin mice are generally anovulatory or have irregular cycles, unless exposed to male pheromones. In the present study ovarian cycle was induced in previously anovulatory virgin adult mice (15-20 week-old) by a single exposure to male bedding, and monitored by means of vaginal impedance measurements and vaginal smears cytological analysis, as previously described (Ramos et al., 2001; Maguire et al., 2005; Jaramillo et al., 2012; Cushman et al., 2014). Briefly, vaginal impedance was measured daily (Estrus cycle monitor EC40, Fine Science Tools). Daily vaginal smears were fixed in methanol and stained (Giemsa Staining, Fisher Diagnostics). Diestrus and estrus were defined as 3 days prior and 1 day after vaginal impedance peak respectively and by vaginal cytology profile (e.g., Fig.6.5A). Mice were tested in either diestrus (high plasma progesterone) or estrus (low plasma progesterone) phase of their ovarian cycle.

6.3.4 *In vivo* chronic simultaneous video and local field potential recordings

Seven to ten days after the animals had fully recovered from the surgery, chronic simultaneous video and local field potential recordings were carried out continuously (24 hrs a day) for 1-3

weeks. Video observation was performed through an infrared USB camera mounted on the top of the recording cage. The video was recorded using the open source iSpy software, which calculates in real time the percentage deviation between consecutive frames and generates a text file (activity data) containing time-stamped information on the percentages of frame-to-frame deviation values.

Local field potentials were recorded with a custom-made miniature dual headstage amplifier connected to the electrode sockets mounted on the animal's head and then wired to an electrical commutator (Dragonfly Inc. or Pinnacle Technology Inc.). The signals from the commutator were fed through a 16-channel extracellular amplifier (A-M Systems model 3500) with a gain of 1,000. Signals were low-pass filtered at 1,000 Hz and sampled at 2,048 s-1 per channel, using a National Instruments A/D board.

Continuous data acquisition was carried out using Igor NIDaq tool (Wavemetrics, Lake Oswego, OR) and data were saved into separate files every week. Activity graphs deriving from the video recordings and corresponding local field potentials were loaded into a custom made software (written in Igor64, Wavemetrics, Lake Oswego, OR) that aligned the two recordings to determine sleep and wake cycles.

6.3.5 Immunohistochemistry and Microscopy

Brains were processed and tissue stained as previously described (Ferando and Mody, 2013a). Briefly, mice were transcardially perfused with 4% paraformaldehyde in 0.12M phosphate buffer, pH 7.3. Fixed brains were sectioned at -16°C with a cryostat (coronal, 35 μm). All sections used for the same analyses (e.g., Fig.6.4C or Fig.6.4D) were processed in parallel.

For diaminobenzidine (DAB) δ-$GABA_A$Rs stain: quenching of endogenous peroxidases was done in H2O2 (3% in methanol, 30 min). Slices were blocked in 10% normal goat serum (NGS), 2h, incubated with anti-δ-$GABA_A$R antibody (1:500; generous gift from Dr. Werner Sieghart, Medizinische Universitt, Wien, Austria) overnight, then with biotinylated goat anti-rabbit antibody (1:200; Vector Laboratories), 4h. Amplification was done with

HRP-conjugated avidin enzyme complex (ABC Elite; Vector Laboratories), 30 min. Signal was developed with DAB (Vector Lab). All steps were done at room temperature.

Bright field microscopy: digital images were collected with an Axioskop 2 Microscope, AxioCam digital camera system and AxioVision 4.8 software (Zeiss). For the same magnification images were taken under identical conditions of brightness and exposure time. Intensity of labeling was measured as optical density (OD) of the region of interest using NIH ImageJ software. For CA1 and CA3 INs the region of interest (ROI) was the soma of all visually identified INs within 30 μm of the pyramidal cell layer, for DGML, CA1 stratum oriens (SO) and radiatum (SR), areas of approximately the same size of identified INs were circled and OD was measured. Reported OD values (represented in arbitrary units, AU) are mean $\pm$ SEM (6.1). Statistical significance was determined with the use of statistical tests specified in each section.

6.3.6 Electrophysiology Data Analysis

Video and local field potential recordings were started after 2-3 days of habituation to the recording home cage. Data were analyzed in 24 hour long epochs. Local field potential recordings were filtered in the δ range (1-4 Hz) and the δ magnitude was calculated using the Hilbert transform. Both activity values deriving from the video data and delta magnitudes were binned at 1 s bin width. The binned activity values were plotted against the binned delta magnitudes for a 24 hour-long session. Based on the point clouds, 3 clusters were separated (low δ + high activity, low δ + low activity, high δ + low activity) and thresholds for δ and activity values between the clusters were determined (Fig. 6.1A).

Using these thresholds a custom made software (written in Igor64, Wavemetrics, Lake Oswego, OR) categorized every second of the long local field potential recording into one of the following 4 groups: movement (activity + low δ), NREM sleep (zero activity + high δ), REM sleep (zero activity + low δ) and a fourth category which consisted of segments that could not be determined (ND) (Fig.6.1B).

The definition of REM sleep was further narrowed by accepting only zero activity + low δ segments longer than 20 s that were adjacent to a segment characterized by high δ + zero activity (putative NREM sleep) phase. The detected REM segments were filtered in the θ (5-12 Hz) and high γ (63-120 Hz) range and θ phases and γ magnitudes were calculated using Hilbert transforms (Fig. 6.2C). θ phase coupled γ amplitudes were determined by calculating the difference between the min and max values of the θ phase triggered average γ magnitude. On Fig. 6.2, Fig. 6.3 and Fig. 6.5 the γ amplitudes were normalized to the mean values across all days and then plotted as a function of days. For male and non-cycling female mice the differences in γ amplitudes were determined at 3 day intervals that approximated the time difference between the estrus and diestrus phases of cycling female mice.

Time-frequency representation of the signal (Fig. 6.2C) was performed using the Morlet wavelet transform. The magnitude of the wavelet transform was plotted as a function of time and frequency, with warmer colors representing increasing magnitude.

6.3.7 Statistics

Data are expressed as mean ± SEM. For group comparisons we used one-way ANOVA with Tukey's post hoc test corrected for multiple comparisons. p values < 0.05 were accepted as significant differences.

6.4 Results

6.4.1 The amplitude of hippocampal γ oscillations recorded *in vivo* fluctuates with phases of the ovarian cycle

In light of current evidence about cognitive performance fluctuations over the ovarian cycle in both women and rodents, it is remarkable that γ oscillations, a network activity that has been implicated in memory and encoding (Singer, 1993; Colgin and Moser, 2010; Uhlhaas

et al., 2011; Buzsáki and Wang, 2012), have not been examined in relationship to the menstrual cycle in women or to the ovarian cycle in rodents. We therefore sought to measure γ oscillations coupled to θ rhythms in cycling female WT mice. We focused on REM θ - γ episodes, because this state is relatively easy to identify, shows consistent θ phase γ amplitude coupling and appears to be linked to emotional information processing and memory consolidation (Montgomery et al., 2008; Walker, 2009; Scheffzük et al., 2011).

To test possible ovarian cycle related changes in γ activity during REM θ-γ episodes, in the first set of experiments continuous video local field potential recordings were performed for 2-3 weeks in cycling female WT mice. REM phases were detected and the average θ phase coupled γ amplitudes were calculated for each consecutive day. Plotting the normalized γ amplitudes revealed a characteristic fluctuation, which correlated with the stage of the ovarian cycle determined by vaginal impedance or cytology (Fig. 6.2A). Comparing the distribution of γ amplitudes for a large number (n $>$ 10/day) of REM segments in estrus and diestrus indicated a shift toward higher γ amplitudes during estrus (averages of normalized γ amplitudes across animals: 1.21 $\pm$ 0.04 for estrus, 0.82 $\pm$ 0.04 for diestrus, n = 2 mice). Comparing the wavelet spectrogram of sample REM segments of a representative estrus and a diestrus day, revealed a more prominent presence of higher γ frequencies during estrus (Fig. 6.2C). The average FFT spectra of all REM phases during an estrus and diestrus day showed a clear shift toward higher γ frequencies (Fig. 6.2D). To investigate this alteration in γ oscillations throughout the study we focused on the θ phase coupled γ activity (γ amplitude) in the frequency range (63 - 120 Hz) where the largest shifts were found in the FFT spectra.

6.4.2 The amplitude of γ oscillations is constant in WT male and non-cycling female mice

To ensure that the dependence of the observed γ rhythm fluctuations on the stages of the ovarian cycle was not a random phenomenon, we also investigated possible alterations in γ

oscillation magnitude over several days in WT males (averages of normalized γ amplitudes across animals: 1.00 ± 0.01, 0.98 ± 0.01 for the first and last days of a shifting 3-day window, n = 2 mice Fig. 3B) and non-cycling females (averages of normalized γ amplitudes across animals: 1.02 ± 0.02, 0.98 ± 0.03 for the first and last days of a shifting 3-day window, n = 2 mice Fig. 3B). During REM, θ coupled γ amplitudes did not reveal fluctuations over similar temporal windows in these 2 groups, demonstrating that in the absence of ovarian cycle-linked hormonal fluctuations there are no periodic changes in γ activity.

6.4.3 The ovarian cycle is associated with δ-GABA$_A$R subunit expression changes in principal cells and interneurons of the hippocampus

We have previously shown how γ oscillations dynamics *in vitro* are controlled by δ-GABA$_A$Rs expressed on PV+INs (Mann and Mody, 2010) and plasticity of these receptors during pregnancy alters network excitability and γ oscillations frequency (Maguire et al., 2009; Ferando and Mody, 2013a). δ-GABA$_A$Rs expression modulation during the ovarian cycle has been described in hippocampal DGGCs with direct consequences on the tonic GABA conductance, anxiety and cognitive performance (Maguire et al., 2005; Maguire and Mody, 2007; Cushman et al., 2014). Specifically, δ-GABA$_A$Rs expression is decreased in the hippocampus of WT mice during estrus, when plasma progesterone levels are low. The tonic GABA conductance recorded in DGGCs is also decreased, and mice exhibit higher degrees of anxiety and trace fear conditioning during this stage of the ovarian cycle, indicating the functional nature of the observed GABA$_A$R plasticity. In these studies, the plasticity of δ-GABA$_A$Rs over the ovarian cycle has been demonstrated in whole hippocampal western blot analyses, and by immunohistochemistry in the periaqueductal grey region (Lovick et al., 2005). In the hippocampus, δ-GABA$_A$Rs are expressed by most principal cells and some INs, and in both cell types they show high levels of plasticity during states of altered NS production (Maguire et al., 2009; Shen et al., 2010; Ferando and Mody, 2013a).

Here, in a broad manner, we addressed the hippocampal neuronal cell-type specificity of ovarian cycle-linked fluctuating expression of δ-GABA$_A$Rs. With the use of δ-GABA$_A$Rs specific antisera, we stained brains of WT female mice perfused at different stages of their ovarian cycle. The cycles were induced and determined as previously described (Maguire et al., 2005). In a separate staining we also compared δ-GABA$_A$Rs expression in CA1 and CA3 SP-INs in cycling WT females to males and non-cycling WT females. All sections were processed in parallel to allow for accurate staining intensity comparisons.

In the hippocampus δ-GABA$_A$Rs are found in the dendritic compartments of DGGCs and to a lesser extent in CA1 PCs, but not CA3 PCs. Moreover, δ-GABA$_A$Rs are expressed by different types of IN, including neurogliaform cells of the DG and lacunosum moleculare and CA3, CA1 and DG PV+INs (Ferando and Mody, 2013b; Yu et al., 2013; Ferando and Mody, 2013a). INs expressing δ-GABA$_A$Rs with their somata located in the SP or within 30 μm around the SP have been shown to have over a 95% chance of being PV+ (Yu et al., 2013; Ferando and Mody, 2013a)

We found that δ-GABA$_A$Rs expression fluctuates over the ovarian cycle in DGGCs, CA1 PCs, and CA1 and CA3 SP INs (Fig. 6.4A, B, C, 6.1). In particular, during times of low plasma progesterone (estrus), staining for δ-GABA$_A$Rs is significantly lower compared to times of high plasma progesterone (diestrus), which is suggestive of a downregulation of δ-GABA$_A$Rs expression during estrus. We found that δ-GABA$_A$Rs expression on CA1 and CA3 SP-INs is similar between diestrus female and male mice, while non-cycling females have a slightly increased expression selectively in CA1 SP-INs (Fig. 6.4D and 6.1).

6.4.4 Ovarian cycle-linked fluctuations in γ oscillations amplitudes depend on the presence of δ-GABA$_A$Rs on PV+INs

Since more than 95% of SP INs that express δ-GABA$_A$Rs also express PV (Ferando and Mody, 2013a), the observed plasticity in δ-GABA$_A$Rs through the ovarian cycle is likely to influence the functioning of PV+INs. Oscillations induced in brain slices in the γ frequency

have been shown to be controlled by δ-GABA$_A$Rs of INs (Mann and Mody, 2010).

In order to address possible functional correlates to the observed δ-GABA$_A$Rs plasticity on CA1 and CA3 SP INs, we generated mice that lack the δ subunit of the GABA$_A$R selectively in PV+INs. These mice lose the great majority of δ-GABA$_A$Rs staining in CA1 and CA3 SP and its close proximity (within 30 μm), which confirms the previously described preferential distribution of δ-GABA$_A$Rs on PV+INs in these areas (Ferando and Mody, in preparation, data not shown). However, the mice have normal ovarian cycles as indicated by the vaginal smears of WT and PV*Gabrd*$^{-/-}$ mice in diestrus and estrus. When induced with litter carrying the smell of male urine, PV*Gabrd*$^{-/-}$ females exhibit regular ovarian cycling that can be ascertained with the use of both vaginal impedance measurements and cytological analysis of vaginal smears Maguire et al. (2005). Their smears are indistinguishable from those of WT mice; i.e., the diestrus phase is characterized by small parabasal cells, large intermediate cells and abundant mucus, while estrus is characterized by large cornified anucleated superficial cells (Fig. 6.5A). Once we established that PV*Gabrd*$^{-/-}$ females have regular ovarian cycles, we went on to measure γ oscillations coupled to θ rhythms during REM sleep periods during the estrus and diestrus stages of the ovarian cycle, as we have done for WT females. Interestingly, we could not observe any fluctuations in γ oscillation magnitude in cycling PV*Gabrd*$^{-/-}$ female mice (averages of normalized γ amplitudes across animals: 1.02 ± 0.04 for estrus, 0.97 ± 0.03 for diestrus, $n = 2$ mice) indicating the requirement of intact δ-GABA$_A$Rs on PV+INs for the observed fluctuations in γ amplitudes. Comparing the difference in γ amplitudes calculated between diestrus and estrus or between days 3 days apart (for explanation see Materials and Methods) in the 4 groups revealed significant fluctuation in the γ amplitudes in cycling WT mice (based on 6 estrus - diestrus days for cycling WT and 4 estrus - diestrus days for cycling PV*Gabrd*$^{-/-}$ mice, 2 animals in each group $F(3,21) = 3.072$, $p < 0.0001$; c.f. Fig. 6.3.).

6.5 Discussion

In this study we describe fluctuations of γ oscillation amplitudes recorded during REM sleep *in vivo* that are coupled to distinct phases of the ovarian cycle. Such periodic fluctuations in γ oscillation amplitudes were not present in male or non-cycling female mice. The γ amplitude fluctuations are inversely related to the level of expression of δ-$GABA_ARs$ hippocampal INs located in the SP, and critically depend on the presence of δ-$GABA_ARs$ on PV+INs. Although the broad shape of oscillation frequency spectra recorded *in vivo* makes it difficult to detect a precise shift in a single coherent frequency peak, we nevertheless noted a shift to the right of the recorded spectra during estrus in WT females, so that higher frequencies became more powerful. This finding is consistent with previous *in vitro* studies describing higher γ oscillations frequency during periods of low δ-$GABA_ARs$ expression on PV+INs (Mann and Mody, 2010; Ferando and Mody, 2013a). Our findings are also consistent with our previous *in vivo* results showing increased γ oscillatory power in the olfactory bulb, after selective ablation of $GABA_ARs$ on INs (Nusser et al., 2001).

As NS fluctuate over the cycle, so does the expression of the highly NS sensitive δ-$GABA_ARs$ in different neurons of the hippocampus. Interestingly, levels of δ-$GABA_ARs$ in hippocampal SP-INs at diestrus are comparable to those found in male mice, whereas δ-$GABA_ARs$ expression decreases during estrus. Non-cycling females appear to have slightly higher δ-$GABA_ARs$ levels than males, selectively on CA1 SP INs. Specific genetic and optogenetic manipulations of PV+INs have cemented their role in the local generation of γ oscillations (Sohal et al., 2009; Wulff et al., 2009; Korotkova et al., 2010; Carlén et al., 2011; Zheng et al., 2011; Lasztoczi and Klausberger, 2014). In line with these findings, our observations merely point out that hippocampal γ oscillation magnitude also depends on the expression of δ-$GABA_ARs$ on these neurons. These receptors are extremely plastic, dramatically changing their expression levels within a few days during hormonal alterations of the ovarian cycle. The precise consequences of fluctuating γ oscillations on memory and cognitive performances may not always be easily predictable, although reports suggest

enhanced memory performance to be correlated with higher γ frequency band magnitude (Lu et al., 2011).

A prediction based on our studies would be that cognitive processes in females would be enhanced during the low progesterone phase of the ovarian cycle (estrus) when γ oscillations are increased. Indeed, several studies reported higher hippocampus-mediated learning and memory performance and increased anxiety levels in female rodents during the estrus phase of the ovarian cycle, whereas animals in diestrus performed similar to males (Maguire et al., 2005; Walf et al., 2006; Moore et al., 2010; van Goethem et al., 2012; Cushman et al., 2014). Although at present it is unknown whether similar alterations in PV+IN δ-GABA$_A$Rs also take place in humans, menstrual cycle-dependent variations in memory performance are not uncommon (Bayer et al., 2014). It also remains to be determined whether a higher cognitive capacity during the preovulatory phase may provide any evolutionary advantage.

In addition to INs, δ-GABA$_A$Rs are also expressed on most principal cells of the hippocampus (Sperk et al., 1997; Glykys et al., 2007; Milenkovic et al., 2013; Ferando and Mody, 2013a). Although δ-GABA$_A$Rs expression on CA1 PCs is modulated across the ovarian cycle (Fig. 6.4C), this does not seem to result in appreciable changes in network level activity, as also supported by previous studies reporting comparable CA1 PC tonic conductances in diestrus and estrus (Maguire et al., 2005). This is not surprising as in these cells 70% of the total tonic inhibition is mediated by α5-GABA$_A$Rs, which have been shown to easily compensate for δ-GABA$_A$Rs loss (Glykys et al., 2008). In contrast, the tonic GABA conductance of hippocampal INs seems to be solely mediated by δ-GABA$_A$Rs (Glykys et al., 2008). It is interesting to note the narrow variance of γ oscillation amplitudes in PV-*Gabrd*$^{-/-}$ mice. This phenomenon will need further investigation, as it is possible that complete lack or insufficient levels of δ-GABA$_A$Rs on PV+INs may allow for restricted degrees of modulation of the γ oscillatory amplitudes, resulting in potentially disruptive effects on cognitive function. Unfortunately, our study using simple single site recordings does not permit accurate comparisons of the γ oscillation amplitudes across animals. Future multi-site recordings and

current source density analyses will be required to ascertain any potential regional differences in the magnitude of γ oscillations between WT and PV-*Gabrd*$^{-/-}$ animals.

The molecular mechanisms responsible for δ-GABA$_A$Rs plasticity during the ovarian cycle, or following steroid fluctuations in general, are unknown but may involve protein phosphorylation, and transcriptional modifications (Choi et al., 2008; Jacob et al., 2008; Abramian et al., 2010; Saliba et al., 2012). Recently intracellular Cl^- itself has been proposed to function as a messenger for plasticity of different GABA$_A$Rs subunits (Succol et al., 2012). Nonetheless, NS synthesis is a necessary event for δ-GABA$_A$Rs modulation over the ovarian cycle (Maguire and Mody, 2007).

The lack of γ oscillation modulation in PV-*Gabrd*$^{-/-}$ *in vivo* suggests that pathological alteration in the normal phsyiology of IN-δ-GABA$_A$Rs through the ovarian cycle may have important consequences on how information is processed at the network level, and may predispose to pathological conditions if combined with aggravating events that lead to altered NS production or inadequate IN δ-GABA$_A$Rs plasticity. Therefore, the development of δ-GABA$_A$Rs specific drugs to selectively control IN function may be a novel future approach to the treatment of these symptoms in women with PMS and PMDD.

6.6 Acknowledgments

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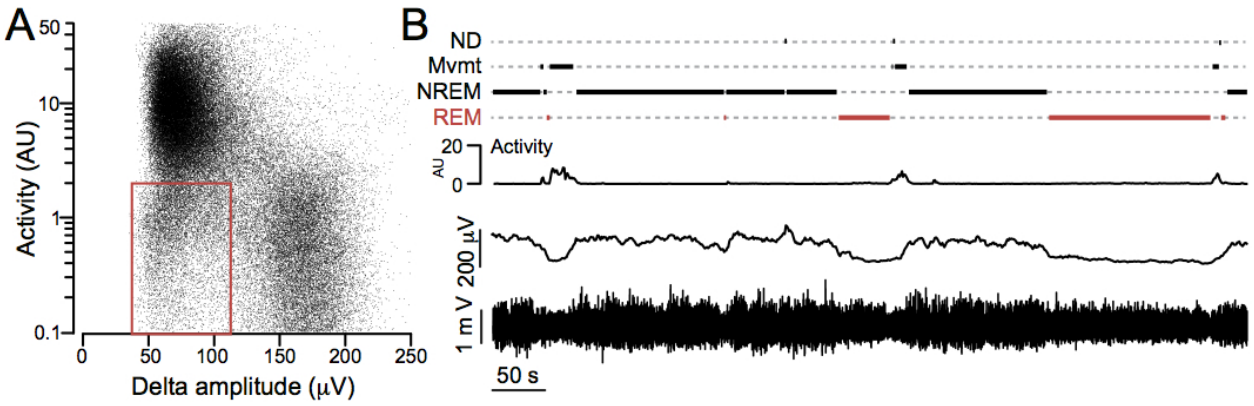
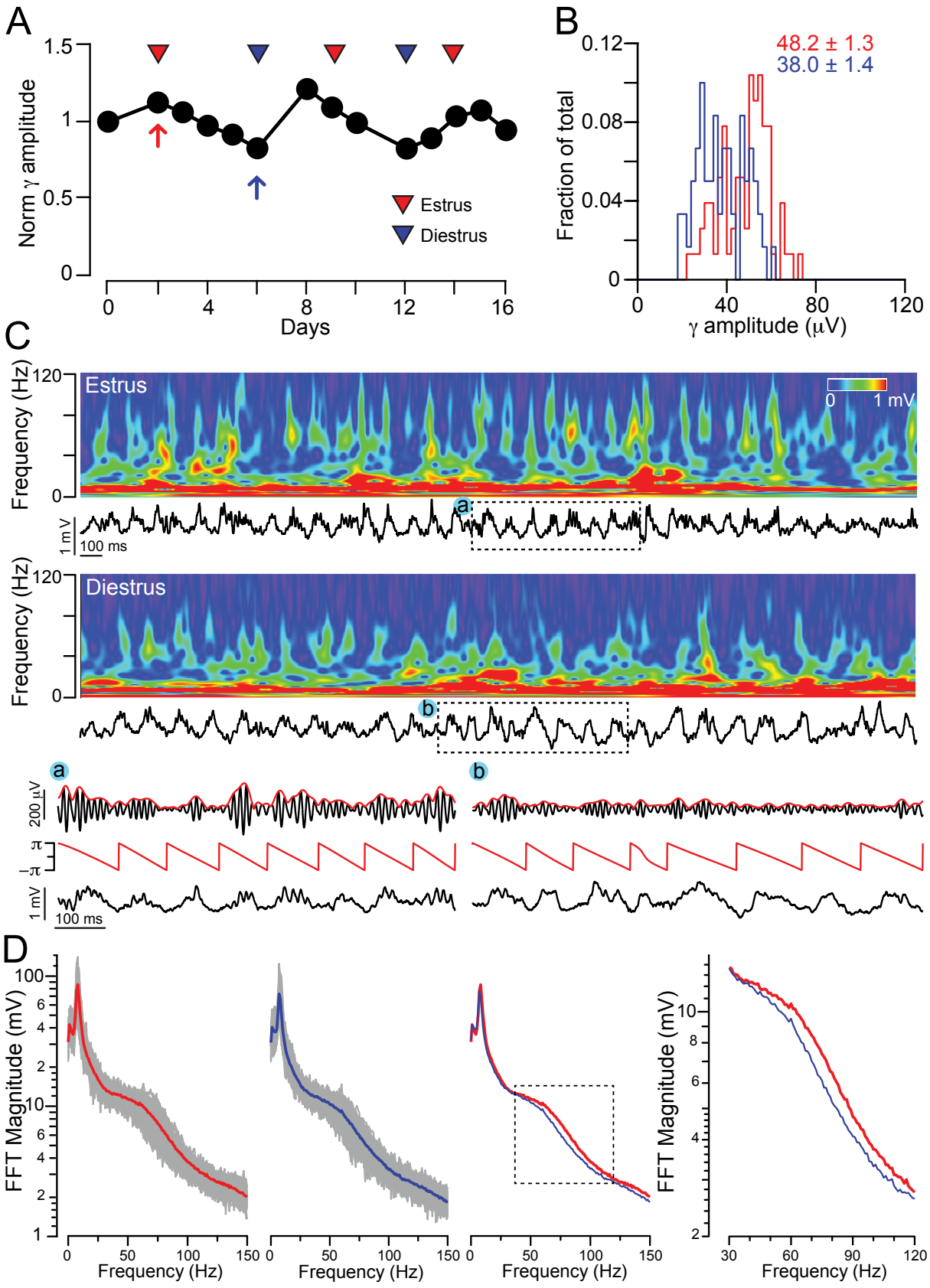


Figure 6.1: Separation of behavioral states based on synchronous video and local field potential recordings. **A**, Activity values plotted against the Hilbert magnitudes in the δ frequency range (1-4 Hz) for each 1 s long epoch (total: 86,400 epochs) during a full day of synchronous video-local field potential recording. Note the appearance of 3 clusters in the point cloud. The red rectangle delineates the point cluster corresponding to the putative REM sleep. **B**, Separation of behavioral states based on combined activity and electrographic thresholds over an 12 min period. Bottom: local field potential recording, above: 1 s binned Hilbert magnitude of the δ-frequency range, above: activity graph, top: step function showing behavioral states based on activity and δ magnitude values (see text for details; REM: REM-sleep, NREM: NREM-sleep, Mvmt: movement, ND: Not determined).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38.0 ± 1.4
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40
80
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Frequency (Hz)
30
60
90
120

Figure 6.2: Fluctuations in REM sleep γ oscillation magnitudes correlate with stages of the ovarian cycle in WT mice. **A**, Diagram showing average normalized θ phase coupled γ amplitudes during REM sleep on consecutive days in a WT cycling female mouse. Red and blue triangles indicate estrus and diestrus days, respectively. **B**, Distribution of γ amplitudes (binned at 2 μV) recorded in all REM phases of a single day of estrus (red) and one of diestrus (blue). The same days are indicated on **A** with red (estrus) and blue (diestrus) arrows. Note the shift of the distribution toward larger values during estrus. Colored numbers indicate mean $\pm$ s.e.m. of the corresponding histograms. **C**, Top rows: 4 s long local field potential recordings with the corresponding wavelet spectra during REM sleep of estrus (top) and diestrus (bottom). (a) and (b): θ phase and γ magnitude components of local field potential segments indicated by dashed rectangles. Below: Hilbert phases of the θ (5-12 Hz) frequency range, filtered trace in the γ frequency range (63 - 120 Hz, black) with the corresponding Hilbert magnitudes (red). **D**, FFT spectra of local field potential recordings of all REM phases during an estrus (leftmost diagram) and a diestrus day (second from left). Grey traces indicate the FFT spectra of individual REM phases, thick lines are the average FFT spectra on an estrus (red) or diestrus (blue) day. Third diagram from left shows the superimposed two average FFT spectra (estrus: red, diestrus: blue) for comparison. The area marked by dotted lines is enlarged on the diagram on the right to show the segments of the average FFT spectra where the largest deviation appears in γ activity.

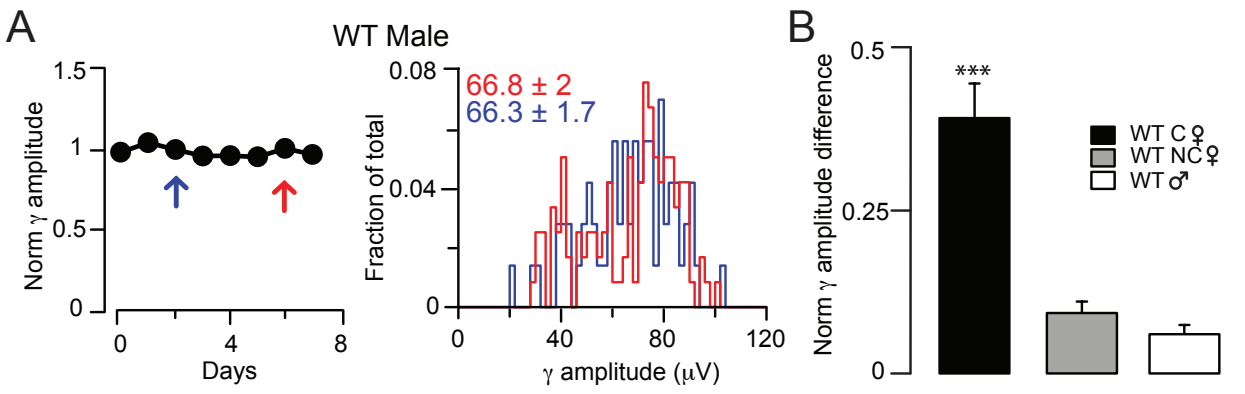

Figure 6.3: Fluctuations in γ oscillation amplitudes recorded during REM sleep are absent in males and non-cycling WT females. **A**, Left: Diagram showing normalized γ amplitudes during REM sleep on consecutive days in a wild type male mouse. Right: Distribution of γ amplitudes (binned at 2 μV) in all REM phases recorded on two separate days 3 days apart. The two days are indicated on the left with red and blue arrows. Colored numbers indicate mean $\pm$ SEM of the corresponding γ amplitude histograms. **B**, Summary data showing the absolute values of the differences between the normalized γ amplitudes during REM sleep in different groups of mice. In the cycling WT females the difference was calculated between days of diestrus and estrus. In WT males and non-cycling females the differences in γ amplitudes were determined at 3-day intervals that approximated the time difference between the estrus and diestrus of cycling female mice. Asterisks indicate significant difference from all other groups in a Tukey's multiple comparison test following a one-way ANOVA.

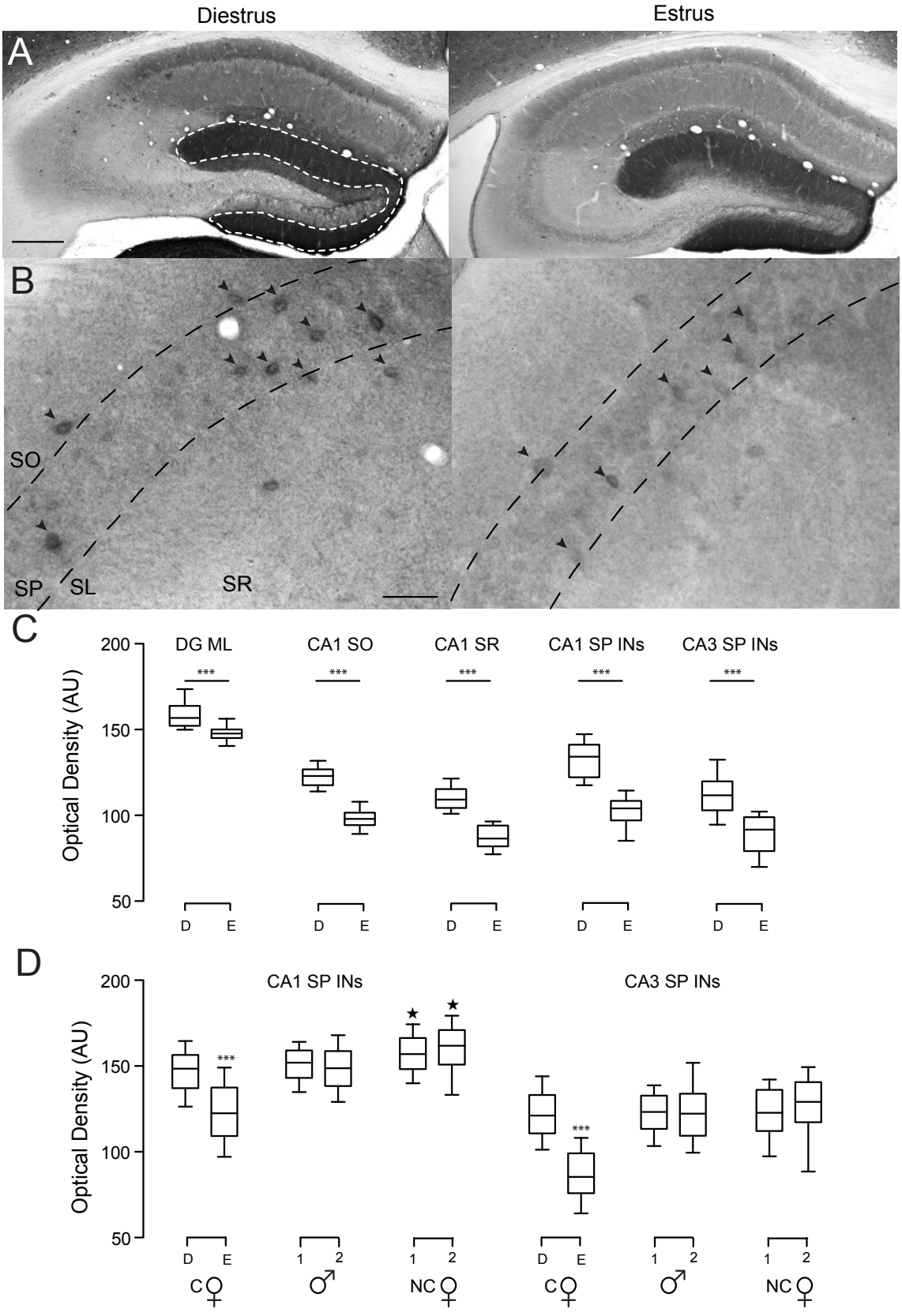
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Figure 6.4: Hippocampal δ-GABA$_A$R plasticity at different stages of the ovarian cycle. Representative bright field images of hippocampal DAB staining showing δ-GABA$_A$R expression patterns in WT cycling females at different stages of the ovarian cycle. In the hippocampus, δ-GABA$_A$Rs are heavily expressed in the molecular layer of the dentate gyrus (DGML) and on numerous INs including neurogliaform cells and PV+INs CA1 strata oriens and radiatum (CA1 SO and SR). Expression in CA1 PC dendrites in the SR is less prominent, but noticeable. **A**, δ-GABA$_A$R expression in DGGCs and CA1 pyramidal cells is lower in estrus compared to diestrus, and this is reflected in the staining intensity in the DGML and CA1 SO and SR. Scale bar 200 μm. **B**, In CA1 and CA3 stratum pyramidale (SP) INs are strongly labeled with δ-GABA$_A$R. Optical densities of δ-GABA$_A$R expression were measured only in these INs (black arrowheads). Labeling of INs is weaker during estrus, which suggests lower δ-GABA$_A$Rs expression on SP-INs (95% of which are PV+INs) during this stage of the ovarian cycle. Scale bar 50 μm. **C**, Optical density measurements are in AU. Box plots represent the 25th and 75th percentile, the line in the middle is the median, and the 10th and 90th percentile are indicated by the error bars. *** denotes $p < 0.0001$ difference from all other groups . **D**, Optical density measurements of CA1 and CA3 SP INs in slices of cycling WT female mice (C ♀) in diestrus (D) or estrus (E), male mice (♂) and non-cycling WT female mice (NC ♀). δ-GABA$_A$Rs expression on SP-INs during estrus is lower than any other group in both CA1 and CA3 (***, $p < 0.0001$); in the CA1 the two NC ♀groups are both higher than any other group in CA1 ($\star$, $p < 0.0001$). Significance levels were established by one-way ANOVA followed by Tukey's multiple comparisons test. All sections for the separate measurements in **C** and **D** were processed together to allow for densitometric comparisons.

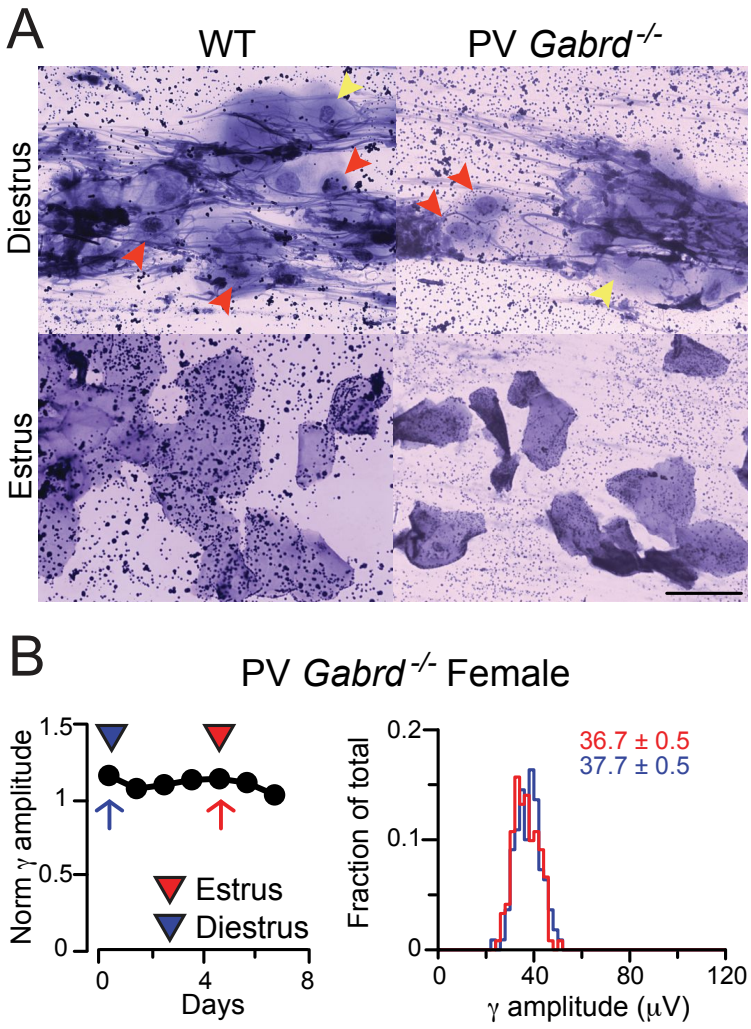

Figure 6.5: Female PV-$Gabrd^{-/-}$ mice cycle regularly but lack ovarian cycle-dependent modulation of REM phase γ oscillation amplitudes. **A**, Ovarian cycle stage was determined by vaginal impedance measurements and cytological analysis of vaginal smears. PV-$Gabrd^{-/-}$ mice had a similar cytological panel to WT mice at different stages. Diestrus was determined by the presence in the smear of abundant mucus, small parabasal cells (red arrowheads) and large intermediate cells (yellow arrowheads). Both cell types are absent in estrus, when smears are typically characterized by large polygonal superficial cells, mostly fully cornified. Scale bar 5 μm. **B**, Left: Diagram showing normalized γ amplitudes during REM sleep on consecutive days in a regularly cycling PV-$Gabrd^{-/-}$ female. Red and blue triangles indicate estrus and diestrus, respectively. Right: Distribution of γ amplitudes (binned at 2 μV) recorded during all REM sleep episodes for an entire day of estrus (red) and one of diestrus (blue). The two days are marked on the left with red (estrus) and blue (diestrus) arrows. Colored numbers indicate mean $\pm$ SEM of the corresponding histograms. Note the narrow variance of the γ amplitude distributions during both phases of the ovarian cycle.

ROI or INs	Status	OD (Mean ± SEM; AU)	ROI or INs (n)	Sections (n)	Mice (n)
DGML	Diestrus	157 ± 2	20	6	2
	Estrus	147.7 ± 1.1*	20	6	2
CA1 SO	Diestrus	122.4 ± 1.4	20	6	2
	Estrus	97.8 ± 1.3*	20	6	2
CA1 SR	Diestrus	109 ± 1.6	20	6	2
	Estrus	86.9 ± 1.5*	20	6	2
CA1 SP INs	Diestrus	132.6 ± 1.4	67	6	2
	Estrus	102 ± 1.4*	64	6	2
CA3 SP INs	Diestrus	112.7 ± 2.1	44	6	2
	Estrus	88 ± 1.7*	55	6	2
CA1 SP INs	Diestrus	145.5 ± 1.4	100	8	2
	Estrus	121.5 ± 1.8*	104	8	2
	Males1	149.5 ± 1.2	107	8	2
	Males2	147.6 ± 1.4	109	8	2
	NC Females1	155.8 ± 1.2*	114	8	2
	NC Females2	177.7 ± 1.6*	113	8	2
CA3 SP INs	Diestrus	119.6 ± 2	63	8	2
	Estrus	84.4 ± 2*	64	8	2
	Males1	120 ± 1.9	63	8	2
	Males2	120.2 ± 2.1	74	8	2
	NC Females1	119.7 ± 2.1	59	8	2
	NC Females2	124.1 ± 2.5	66	8	2

Table 6.1: Details of δ-GABA$_A$Rs expression levels in different cell types of the hippocampus over the ovarian cycle, detected by immunohistochemistry. Summary by hippocampal region of interest (ROI) or INs of optical density mean values in arbitrary units (AU) ± SEM, and n's for diestrus and estrus in WT cycling females, WT non-cycling females and males. Asterisks denote significance (see Fig. 6.4 for statistical tests).

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CHAPTER 7

IN VITRO GAMMA OSCILLATIONS FOLLOWING PARTIAL AND COMPLETE ABLATION OF δ SUBUNIT-CONTAINING $GABA_A$ RECEPTORS FROM PARVALBUMIN INTERNEURONS

7.1 Summary

Perisynaptic and extrasynaptic δ subunit-containing GABAA receptors (δ-$GABA_A$Rs) mediate tonic conductances in many principal cells and interneurons (INs) of the central nervous system. δ-$GABA_A$Rs comprise $\alpha 4$ subunits on principal cells of the neocortex and hippocampus, whereas they usually contain $\alpha 1$ on various INs. δ-$GABA_A$Rs are highly plastic and exhibit a unique pharmacology, being insensitive to benzodiazepines, but sensitive to low

concentrations of neurosteroids (NS). The neuroactive forms of several steroid hormones, NS act on δ-GABA$_A$Rs as potent positive allosteric modulators leading to changes in neuronal excitability and network synchrony. In fact, δ-GABA$_A$Rs on INs have been shown to control the dynamics of γ oscillations. During times of altered NS production, including stress, puberty, ovarian cycle and pregnancy, δ-GABA$_A$R expression varies in different neurons regardless of the α subunits they contain. This plasticity has direct consequences for network functioning. In particular, plasticity of these receptors in pregnancy and the postpartum period affects CA3 γ oscillation frequencies (recorded *in vitro*), and during the ovarian cycle they underlie fluctuations in the amplitude of CA1 γ oscillations (recorded *in vivo*). Most δ-GABA$_A$R-expressing INs in CA3 stratum pyramidale (SP) are parvalbumin (PV)+INs, whose fundamental role in γ oscillation generation and control has been extensively investigated. In this study we reduced or deleted δ-subunits in PV+INs, with the use of a PV/*Cre*-*Gabrd*/floxed genetic system. By means of immunohistochemistry we show that PV expression in PV-*Gabrd*$^{-/-}$ and PV-*Gabrd*$^{+/-}$ mice remains unaltered. Additionally, we confirm the absence of δ-GABA$_A$Rs specifically from PV+INs. We find that in both PV-*Gabrd*$^{-/-}$ and PV-*Gabrd*$^{+/-}$ mice, CA3 γ oscillation frequencies measured *in vitro* were increased compared to littermate controls. The increased frequencies could be lowered to control levels in PV-*Gabrd*$^{+/-}$ by the NS allopregnanolone (3α,5α-tetrahydroprogesterone, ALLO, 100 nM) but not by the synthetic δ-GABA$_A$R positive allosteric modulator 4-Chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl] benzamide (DS-2, 10 μM). This is consistent with the idea that DS-2, in contrast to ALLO, selectively targets $\alpha4/\delta$-GABA$_A$Rs but not the $\alpha1/\delta$-GABA$_A$Rs found on INs. Therefore, development of drugs selective for IN-specific $\alpha1/\delta$-GABA$_A$Rs may be useful in neurological and psychiatric conditions correlated with altered PV+IN function and aberrant γ oscillations.

7.2 Introduction

Tonic inihibition in the cortex is predominantly mediated by extrasynaptic or perisynaptic $GABA_A$ receptors containing either δ or $\alpha 5$ subunits depending on the cell type and the brain region (Brickley and Mody, 2012; Sperk et al., 1997). The physiology and pharmacology of the tonic GABA conductances are of particular interest as these actions of GABA exercise powerful constrains on neuronal excitability and gain (Mody and Pearce, 2004; Farrant and Nusser, 2005). δ-$GABA_A$Rs are highly plastic receptors with a unique pharmacology. They are insensitive to benzodiazepines and sensitive to low concentrations of NS, which act as potent allosteric modulators (Belelli and Lambert, 2005). δ-$GABA_A$Rs are expressed on cortical principal cells, where they selectively contain $\alpha 4$ subunits (Chandra et al., 2006), and on several INs, where the $\alpha 4$ subunits are replaced by $\alpha 1$ subunits (Glykys et al., 2007). An increasing evidence points at δ-$GABA_A$Rs on different neuron types as an important factor in neurological and psychiatric disorders. For example, δ-$GABA_A$Rs plasticity on dentate gyrus granule cells and INs has been described in models of temporal lobe epilepsy (Ferando and Mody, 2012). Polimorphisms in the *GABRD* gene have been found to correlate with major depression and certain epilepsies (Dibbens et al., 2004; Feng et al., 2006, 2010). Homeostatic δ-$GABA_A$Rs plasticity on hippocampal principal cells during pregnancy and the ovarian cycle can modify network excitability and disruptions of this homeostasis has been proposed as a cofactor in PMS, PMDD and postpartum depression (Maguire et al., 2009, 2005; Maguire and Mody, 2008; Lovick et al., 2005; Lovick, 2008; Brack and Lovick, 2007; Lovick, 2006; Smith, 2013; Shen et al., 2010; Griffiths and Lovick, 2005). Moreover, we have previously reported δ-$GABA_A$Rs plasticity on hippocampal cornu ammonis (CA) INs during pregnancy and over the ovarian cycle, with direct consequences on frequency or amplitude of γ oscillations (Ferando and Mody, 2013); (Barth, Ferando, and Mody, unpublished observations). Gamma oscillations (30-120 Hz), a synchronized network activity thought to be involved in learning and memory (Buzsáki and Wang, 2012), are controlled by δ-$GABA_A$Rs expressed by unidentified INs of CA3 SP (Mann and Mody, 2010). Of note, γ oscillations are heavily

dysregulated in schizophrenia, a finding attributed to pathological modifications in PV+INs (Lewis et al., 2005; Uhlhaas and Singer, 2012). Lastly, pregnancy, the postpartum, and the ovarian cycle may be sensitive periods during which pathological alterations in γ oscillations are facilitated. Ideally, therapeutical strategies would target δ-$GABA_A$Rs on principal cells or INs, so that symptoms produced by alterations of δ-$GABA_A$R-mediated tonic inhibition on one or the other cell type may be addressed separately. To this regard, the differential inclusion of $\alpha 4$ or $\alpha 1$ subunits in δ-$GABA_A$Rs in excitatory vs. inhibitory neurons is a promising tool to pharmacologically distinguish between the two cell types. In this paper we address this question by examining the effects of allosteric modulators of δ-$GABA_A$Rs on kainic acid (KA)-induced *in vitro* γ oscillations in CA3 SP of WT, heterozygous (PV-*Gabrd*$^{+/-}$) or knock out (PV-*Gabrd*$^{-/-}$) mice for the δ subunit on PV+INs.

7.3 Materials and Methods

7.3.1 Animal Handling

In this study we used adult (9-15 weeks of age) male mice, either WT littermate controls (referred to as WT) or lacking one or both copies of *Gabrd* gene specifically on PV-INs (PV-*Gabrd*$^{+/-}$ or PV-*Gabrd*$^{-/-}$). These mice were generated by crossing PV-*Cre* (PV-IRES-*Cre* line; JAX Stock # 008069) and *Gabrd*-flox mice (generous gift of Dr. Jamie Maguire, Tufts University) (Lee and Maguire, 2013). All animal experiments were carried out in accordance with the National Institute of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Mice were housed under the care of the division of Laboratory Animal Medicine of the University of California, Los Angeles. Mice were maintained on a light/dark cycle of 12h with ad libitum access to food and water, and all experiments were done during the light cycle. Particular care was taken to minimize stress in the test subjects. In particular, mice were moved to the experimental area in their home cage at least 2 h prior to the experiment. All efforts were made to minimize animal suffering,

to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available. Genotyping was performed by Transnetyx.

7.3.2 Immunohistochemistry and Microscopy

Brains were perfused and tissues processed as previously described (Ferando and Mody, 2013; Maguire et al., 2009). Briefly, deeply anesthetized mice (inhalational isoflurane and sodium pentobarbital, i.p. 100 mg/kg) were transcardially perfused with 50 ml of 4% paraformaldehyde in 0.12 phosphate buffer, pH 7.3. Brains were cryoprotected in 30% sucrose solution in Millonigs modified PBS, frozen and later sectioned (coronal, 35 μm) at -16°C with use of a cryostat. For the same staining all slices were stained in parallel. For diaminobenzidine (DAB) δ-$GABA_A$Rs stain: quenching of endogenous peroxidases, 3% H2O2 in methanol (30 min); blocking, 10% normal goat serum (2 h); primary antibody, anti-δ-$GABA_A$R antibody (1:500 in 10% NGS, overnight; generous gift from Dr. Werner Sieghart, Medizinische Universitt, Wien, Austria); secondary antibody, biotinylated goat anti-rabbit antibody (1:200 in 10% NGS, 4 h; Vector Laboratories). Signal amplification: HRP-conjugated avidin enzyme complex (30 min, ABC Elite; Vector Laboratories). Signal development: DAB (Vector Lab). For δ-$GABA_A$Rs-PV double labeling: antigen retrieval, 70 min at 90°C in a citrate buffer solution (0.05 M sodium citrate in 1% NaCl, pH 8.6); blocking, 10% NGS and 0.3% Triton X-100 (2 h); primary antibodies, anti-δ-$GABA_A$R and anti-PV (1:100; 1:5000 respectively, 0.1% NaN3 in TBS, 4 days); secondary antibodies, Alexa Fluor-647 and Alexa Fluor-488 goat anti-rabbit and anti-mouse antibodies (1:750; Millipore, 1 h). Slices were mounted on Superfrost Plus slides (Fisher Scientific), and coverslipped with DPX Mountant for Histology (DAB stains, Sigma-Aldrich) or Fluoromount G (fluorescence stains, Southern Biotechnology). For bright field microscopy, digital images were taken with an Axioskop 2 Microscope and an AxioCam digital camera system and AxioVision 4.8 software (Zeiss). For the same magnification and the same staining images were taken under identical conditions of brightness and exposure time. For fluorescent microscopy, images were collected using a confocal

microscope (Leica TCS SP2) equipped with Hex PL APO 100x/1.40-0.7 OIL CS objective using LAS AF software (Leica Microsystems). Digital projection images of 35 μm z-stacks were assembled and using NIH ImageJ software. All images were captured under the same light intensity and exposure limits. For optical density measurements, random regions of interest (ROIs) were selected in each slice and analyzed in ImageJ.

7.3.3 Electrophysiology

Hippocampal slices were cut as previously reported (Ferando and Mody, 2013). Briefly mice were anesthetized with isoflurane and decapitated following UCLA Chancellors Animal Research Committee protocol. Horizontal 350 μm slices were cut on a Leica VT1000S Vibratome in ice-cold N-Methyl-D-Glucamine (NMDG)-based HEPES-buffered solution, containing in mM: 135 NMDG, 10 D-glucose, 4 $MgCl_2$, 0.5 $CaCl_2$, 1 KCl, 1.2 KH_2PO_4, 26 HEPES, (bubbled with 100% O_2, pH 7.4, 290 300 mOsm/L). Slices were incubated at 32°C in an interface chamber in a reduced sodium artificial CSF (aCSF), containing in mM: NaCl 85, D-glucose 25, sucrose 55, KCl 2.5, NaH_2PO_4 1.25, $CaCl_2$ 0.5, $MgCl_2$ 4, $NaHCO_3$ 26, pH 7.3-7.4 when bubbled with 95% O_2, 5% CO_2. After 20 min low sodium aCSF was substituted for normal aCSF at 32°C, containing in mM: NaCl 126, D-glucose 10, $MgCl_2$ 2, $CaCl_2$ 2, KCl 2.5, NaH_2PO_4 1.25, Na Pyruvate 1.5, L-Glutamine 1, $NaHCO_3$ 26, pH 7.3-7.4 when bubbled with 95% O_2, 5% CO_2. Recordings were done in an interface chamber at 35°C perfused with normal aCSF and 50 nM kainic acid - KA (Tocris). Oscillatory network activity was recorded in CA3 SP with the use of a patch pipette (3-5 MΩ resistance) filled with nACSF connected to the headstage of an amplifier (A-M Systems Inc., model 3000) where it was band-pass filtered between 0.1 and 1000 Hz. The signal was fed through an instrumentation amplifier (Brownlee BP Precision, model 210A) and sampled at 4096 s^{-1} with a National Instruments A/D board. Field potentials were recorded using EVAN (custom-designed LabView-based software from Thotec) and analyzed with a custom written procedure (Wavemetrics, IGOR Pro 6.22A). Peak frequencies, power at peak frequency and

total power were obtained from the corresponding power spectral densities (psd), calculated from 180 s period averages. ALLO and DS-2 were purchased from Tocris, and were dissolved in DMSO (final vehicle concentration 0.01%). Morlet wavelet transform was used to illustrate the power of γ-frequency band in time as previously described (Mann and Mody, 2010). Evoked field potentials were recorded in the dentate gyrus molecular layer. The perforant path was stimulated every 30 s with a paired pulse 50 ms apart. Average stimulus intensity was 60 μA, 50 μs. Slope was calculated by fitting a straight line to the steepest part of the fEPSP (EVAN). Evoked fEPSP slopes used for determining statistical significance were calculated as mean slope over 5 minutes immediately preceding and during 10-15 minutes after drug application.

7.3.4 Data Analysis

All data shown are expressed as mean ± SEM. The statistical tests used to determine statistical significance are indicated in each section. Statistical significance was defined as $p < 0.05$.

7.4 Results

7.4.1 Expression of δ-GABA$_A$Rs in PV-*Gabrd*$^{+/-}$ and PV-*Gabrd*$^{-/-}$ mice in PV+INs and PV-INs.

Our previous findings show how δ-GABA$_A$Rs downregulation on CA3 INs, such as found during pregnancy, is sufficient to alter KA-induced CA3 γ oscillations (Ferando and Mody, 2013). Interestingly, concentrations of ALLO relevant to pregnancy could normalize γ oscillations in slices of pregnant mice (Ferando and Mody, 2013), raising the question whether a moderate alteration in IN-δ-GABA$_A$Rs expression, in the absence of the large NS alterations typical of pregnancy may alter γ oscillations dynamics. In order to address this question,

we generated a mouse where the δ subunit is knocked down or knocked out specifically from PV+INs, by crossing PV-*Cre* and *Gabrd*-flox mice. We chose this cell type as i) PV+INs (and in particular PV+ basket cells) are responsible for the generation and maintenance of γ oscillations (Carlén et al., 2011; Wulff et al., 2009; Korotkova et al., 2010; Sohal et al., 2009; Zheng et al., 2011; Lasztoczi and Klausberger, 2014), ii) the large majority of PV+INs located in the principal cell layer of the various hippocampal areas also express δ-GABA$_A$Rs, and δ-GABA$_A$Rs expressed in CA3 SP are almost exclusively confined to PV+INs (Yu et al., 2013; Ferando and Mody, 2013; Milenkovic et al., 2013), iii) δ-GABA$_A$Rs on CA3 INs control the frequency of γ oscillations (Mann and Mody, 2010).

Although the *Cre*-lox system is widely used and well established in mouse genetic manipulations (Sauer, 1998), it is important to show the specificity of the ablation of a target gene, as different floxed genes may react incongruously with the same *Cre*-expressing system. With the use of δ-GABA$_A$R-spefic antisera in brain sections of PV-*Gabrd*$^{+/-}$ and PV-*Gabrd*$^{-/-}$ mice, we show a diminished or fully ablated δ-GABA$_A$Rs expression in INs located within 30 μm of CA1 and CA3 SP (Fig. 7.1). This is shown more clearly in the CA3 SP (Fig. 7.1B) where the lack of δ-GABA$_A$Rs expression in CA3 pyramidal cells allows for a clearer visualization of interneuronal staining.

As expected from the specificity of the PV-*Cre* system, in PV-*Gabrd*$^{+/-}$ and PV-*Gabrd*$^{-/-}$ mice δ-GABA$_A$Rs expression remains at WT levels in cells that do not normally express PV. For example, δ-GABA$_A$Rs expression is unaltered in CA1 pyramidal cells and dentate gyrus granule cells, as reflected by the lack of variation in the δ-GABA$_A$R specific staining of CA1 SO and SR, and the molecular layer of the dentate gyrus (DGML), where the dendritic domain of these cells lays and where δ-GABA$_A$Rs are expressed. Optical densities (in arbitrary units) for WT, PV-*Gabrd*$^{+/-}$, and PV-*Gabrd*$^{-/-}$ were, respectively: 132.06 $\pm$ 2.16, 132.5 $\pm$ 1.88, 130.29 $\pm$ 1.33 for the DGML; 75.31 $\pm$ 1.11, 74.49 $\pm$ 2.06, 74.34 $\pm$ 1.17 for the CA1 SP. ($p > 0.05$, one-way ANOVA, n = 20 ROIs in 2 sections, see Materials and Methods).

Since PV+INs are not the only IN types expressing δ-GABA$_A$R, but other INs such as

neurogliaform cells (NGCs) of the neocortex and the hippocampus also heavily express δ-GABA$_A$Rs (Oláh et al., 2009; Faragó et al., 2013), we exmined δ-GABA$_A$Rs distribution in areas where few PV+INs are present. Fig. 7.1C shows INs in CA1 lacunosum moleculare, an area where NGCs are abundant, displaying comparable δ-GABA$_A$R staining in all three experimental groups. The expression of PV in PV+INs is unaffected following pregnancy-induced δ-GABAAR plasticity in these cells (Ferando and Mody, 2013).

Here we also show that the pattern of PV expression and PV+INs localization in CA3 SP remains unaltered following δ-GABA$_A$R ablation from these cells (Fig. 7.2A-B). Moreover, a double-staining experiment with antisera specific for PV and δ-GABA$_A$R confirmed that in PV-*Gabrd*$^{-/-}$ mice PV+INs in CA3 SP do not express δ-GABA$_A$R, whereas in WT mice the overlap of the two antigens is nearly complete (Fig. 7.2B), as previously described (Ferando and Mody, 2013). In PV-*Gabrd*$^{-/-}$ mice INs that expressed δ-GABA$_A$R but not PV could be found in other hippocampal areas, such as the CA3 and CA1 lacunosum moleculare (Fig. 7.2C). These anatomical data demonstrate the specificity of the δ-GABA$_A$R ablation from PV+INs in our mouse model.

7.4.2 Frequency of *in vitro* CA3 γ oscillations following reduction or ablation of δ-GABA$_A$Rs from PV-INs.

We next recorded KA-induced γ oscillations in CA3 SP in all three experimental groups. We have previously shown how the frequency of carbachol-induced and KA-induced CA3 γ oscillations is increased in *Gabrd*$^{-/-}$ mice as a consequence of disinhibition of CA3 SP INs (Mann and Mody, 2010). In the proposed model δ-GABA$_A$Rs on INs control γ oscillations dynamics through modulation of IN excitability. Here we show how in both PV-*Gabrd*$^{+/-}$ and PV-*Gabrd*$^{-/-}$ mice CA3 γ oscillations frequency is increased compared to WT littermate controls, with no effect of oscillations power. Peak frequency (Hz): WT 46 ± 0.8; PV-*Gabrd*$^{+/-}$ 52.2 ± 0.8; PV-*Gabrd*$^{-/-}$ 52 ± 0.7. $p < 0.0001$. $F_{(2,77)} = 15.74$. There were no differences in power at peak frequency or total power (measured between 30-120 Hz).

Powers at peak frequency (xE-11 V^2s^{-1}) were: WT 1.82 ± 0.31; PV-*Gabrd*$^{+/-}$ 5.28 ± 1.59; PV-*Gabrd*$^{-/-}$ 3.08 ± 0.59. p = 0.1. $F_{(2,77)} = 2.37$. Total powers (xE-10 V^2) were: WT 4.93 ± 0.65; PV-*Gabrd*$^{+/-}$ 11.13 ± 3.26; PV-*Gabrd*$^{-/-}$ 7.04 ± 0.95. p = 0.2. $F_{(2,77)} = 1.87$. n (mice, slices) = WT 5, 25; PV-*Gabrd*$^{+/-}$ 7, 33; PV-*Gabrd*$^{-/-}$ 4, 22. Statistical significance determined by One-Way ANOVA followed by Tukeys multiple comparisons test (Fig. 7.3). These findings are consistent with the idea that the observed changes in network dynamics in both *Gabrd*$^{-/-}$ and pregnant WT mice are solely due to changes in δ-GABA$_A$Rs expressed by PV+INs, and that a down-regulation of δ-GABA$_A$Rs in these cells, as it happens during pregnancy in WT mice (Ferando and Mody, 2013), is sufficient to modify network activity.

7.4.3 Differential modulation of γ oscillation frequency by ALLO and DS-2 in slices of PV-*Gabrd*$^{+/-}$ mice.

The increased frequency in γ oscillations found in *Gabrd*$^{-/-}$ mice with global ablation of δ-GABA$_A$Rs is normalized to control levels by selectively decreasing NMDAR-mediated excitation in PV+INs with the NMDA-R antagonists D-AP5 (25 μM) or PPDA (1 μM) (Mann and Mody, 2010). In pregnant WT mice, with a diminished expression of δ-GABA$_A$Rs on CA3 INs, increasing the activity of the remaining δ-GABA$_A$Rs with ALLO (100 nM) also reduces the frequencies to control values (Ferando and Mody, 2013). However, NS potentiate δ-GABA$_A$R-mediated conductances in both INs and principal cells, in the same concentrations range (10-100 nM) (Mann and Mody, 2010; Glykys et al., 2007; Stell et al., 2003), indicating that NS do not efficiently differentiate between α1 and α4/δ-GABA$_A$R. Recently a synthetic δ-GABA$_A$R positive allosteric modulator was developed and a thorough investigation was carried out for its selectivity for different GABA$_A$R subunits (Jensen et al., 2013; Wafford et al., 2009). The magnitude of the DS-2-induced potentiation of GABA responses was dependent on the presence of δ subunits and on the nature of the α but not the β subunits present in δ-GABA$_A$Rs. The compound was most selective for δ-GABA$_A$Rs containing α4 or α6 subunits (Jensen et al., 2013). Moreover, in a recent study done in mice, DS-2 was much

less effective in potentiating tonic conductances in all dentate gyrus INs (including PV+INs) compared to dentate gyrus granule cells (DGGCs) (Wei et al., 2013). These findings have interesting pharmacological implications given the anatomical distribution of δ-GABA$_A$Rs containing α1 or α4 subunits. We tested whether ALLO (100 nM) and DS-2 (10 μM, above the EC50 concentration for α4-α6/δ-GABA$_A$Rs) can differentially modify neuronal network activities modulated by δ-GABA$_A$Rs containing α1 or α4 subunits. We first recorded evoked field EPSPs in the molecular layer of the DG, where network excitability is under strong regulatory control of α4/δ-GABA$_A$Rs expressed by DGGCs. Both drugs decreased the slope of evoked fEPSPs by the same amount, suggesting that at these concentrations they potentiate α4/δ-GABA$_A$Rs in a similar way (% slope decrease were for DS-2: 27.03 $\pm$ 5.9, p ¡ 0.05, n = 5 slices; for ALLO: 29 $\pm$ 5, p ¡ 0.01, n = 5 slices. Significance determined by two-tailed unpaired t-test done on the raw slopes, see Materials and Methods). Lastly we recorded γ oscillations in slices of WT, PV-*Gabrd*$^{+/-}$ and PV-*Gabrd*$^{-/-}$ mice before and after application of ALLO (100 nM) or DS-2 DS-2 (10 μM). Interestingly ALLO but not DS-2 decreased the frequency of γ oscillations to control values in slices of PV-*Gabrd*$^{+/-}$ mice, with no effect on the frequency of γ oscillations in slices WT or PV-*Gabrd*$^{-/-}$ mice (Table 7.1). Neither drug had any effects on power at peak frequency and total power (between 30-120 Hz). These results confirm that CA3 SP γ oscillation frequencies are controlled by δ-GABA$_A$Rs expressed on PV+INs alone, as selectively potentiating α4/δ-GABA$_A$Rs with the use of DS-2 does not result in appreciable changes in γ oscillation frequency in PV-*Gabrd*$^{+/-}$. Taken all together these results indicate that specific pharmacological modulation of δ-GABA$_A$Rs containing either α4 or α1 subunits may provide control over different network behaviors.

7.5 Discussion

In this study we show that δ-GABA$_A$Rs expressed on PV+INs modulate *in vitro* γ oscillations dynamics. By crossing the recently generated *Gabrd*-flox mice (Lee and Maguire,

2013)with PV-*Cre* reporter mice we generated PV-*Gabrd*$^{+/-}$ and PV-*Gabrd*$^{-/-}$ mice, making it possible to investigate the impact of partial and total loss of δ-GABA$_A$Rs expressed on PV+INs on γ oscillations. We targeted PV+INs as they have a central role in the local generation of γ oscillations (Sohal et al., 2009; Carlén et al., 2011; Wulff et al., 2009; Korotkova et al., 2010; Zheng et al., 2011; Lasztoczi and Klausberger, 2014; Mann et al., 2005).

The NMDAR-mediated tonic excitation of hippocampal INs becomes facilitated in *Gabrd*$^{-/-}$ mice (Mann and Mody, 2010) as well as in slices of WT pregnant mice, where δ-GABA$_A$Rs become down-regulated on CA3 SP INs (Ferando and Mody, 2013). In our previous studies on the modulation of γ oscillation frequency we could not unequivocally rule out potential contributions of δ-GABA$_A$Rs on other INs and of presynaptic δ-GABA$_A$R expressed on mossy fiber boutons (Trigo et al., 2008; Ruiz et al., 2010). Although the dentate gyrus is unlikely to participate in CA3 γ oscillations recorded *in vitro*, as it needs intact enthorhinal inputs to generate oscillations in the γ frequency (Bragin et al., 1995; Csicsvari et al., 2003), release from mossy fiber boutons may synchronize local networks. We have now addressed this specificity by demonstrating a clear increase in CA3 γ oscillation frequency both in PV-*Gabrd*$^{+/-}$ and PV-*Gabrd*$^{-/-}$ mice compared to WT littermate controls. Moreover we show how ALLO (100 nM) but not the synthetic positive allosteric modulator DS-2 (10μM) can lower the frequency of γ oscillations in slices of PV-*Gabrd*$^{+/-}$ mice. This is consistent with previous reports of DS-2 being selective for $\alpha4/\delta$-GABA$_A$Rs (Jensen et al., 2013; Wei et al., 2013).

As shown following partial reduction of δ-GABA$_A$Rs expressed by PV+INs during pregnancy (Ferando and Mody, 2013), γ oscillation frequency was increased in slices of PV-*Gabrd*$^{+/-}$ mice. However, no further increase in frequency was noted in PV-*Gabrd*$^{-/-}$ slices where δ-GABA$_A$Rs are altogether missing from PV+INs. A possible explanation for this finding may have to do with the necessity of limiting the frequencies of locally generated γ oscillations. In contrast to γ oscillations recorded *in vitro* which do not usually surpass frequencies beyond 60 Hz, hippocampal γ oscillations recorded *in vivo* have a broader spectrum

and have thus been categorized as slow and fast γ depending on their frequency (usually 60-100 Hz for fast γ, although consensus lacks on the cutoff frequency) (Belluscio et al., 2012). The γ oscillations generated locally have lower frequencies and are tightly controlled not to interfere with long distance inputs (Lasztoczi and Klausberger, 2014). The fast γ oscillations in the hippocampus are thought to be generated by entorhinal inputs (Brun et al., 2002; Colgin et al., 2009). The two types of γ oscillations are involved with different aspects of memory formation and retrieval (Bieri et al., 2014; Yamamoto et al., 2014). Allowing an overlap between locally generated slow and distal input fast γ frequencies may disrupt these processes. Therefore, it is plausible that the highest frequency of the slow γ oscillations locally generated by PV+INs is limited by their tonic excitation. Accordingly, small decreases in δ-$GABA_A$Rs-mediated tonic inhibition would fully release the NMDAR-mediated tonic excitation of PV+INs, such that greater decreases in tonic inhibition will not allow a further increase in γ frequency. Conversely, potentiating the δ-$GABA_A$Rs-mediated tonic inhibition of PV+INs has little effect on reducing γ frequencies, as in WT slices NMDA antagonists have no effect on γ oscillations recorded *in vitro* (Mann and Mody, 2010; Ferando and Mody, 2013). Therefore, the tonic inhibition of PV+INs under control conditions must be sufficient to prevent all NMDAR activation such that a further increase in tonic inhibition is without effect. This hypothesis is fully supported by our previous findings showing that the enhancement of δ-$GABA_A$Rs-mediated tonic inhibition in WT slices could decrease γ frequency only in a low Mg^{2+} modified aCSF when NMDA receptor activity was increased (Mann and Mody, 2010).

We have previously shown how CA3 INs in global *Gabrd*$^{-/-}$ mice lose their tonic conductances with no apparent upregulation of other $GABA_A$Rs subunits that mediate tonic inhibition (Mann and Mody, 2010). Although the recording of tonic GABA conductances in CA3 PV+INs was beyond the scope of this study, based on our previous evidence it is reasonable assume that PV+INs of PV-*Gabrd*$^{-/-}$ mice had no such conductance. Even if PV+INs in these mice compensated for the loss of δ-$GABA_A$Rs by expressing other tonically

active $GABA_A$Rs, a lower GABA sensitivity of the newly expressed receptors may explain their inability to prevent an increase in γ oscillation frequency.

Future studies will be needed to determine the effects of reduced or missing δ-$GABA_A$Rs in PV+INs on *in vivo* network dynamics. Based on our present findings θ oscillations should not be affected, as deleting δ-$GABA_A$Rs in PV+INs does not affect these receptors in other INs (NGFCs and Ivy Cells) that are involved in these lower frequency oscillations (Capogna and Pearce, 2011; Fuentealba et al., 2008). However, as these other INs also express δ-$GABA_A$Rs most likely with α1 subunits, it is important to keep in mind that compounds designed to modify γ oscillations by acting on α1/δ-$GABA_A$Rs receptors of PV+INs will also alter network activities controlled by the other INs. Alterations in θ-γ coupling may be expected in PV-*Gabrd*$^{-/-}$ mice, based on previous work in mice with the $GABA_A$R γ2 subunit selectively deleted from PV+INs. In these mice phasic inhibition of PV+INs was necessary for θ-γ coupling *in vivo*, but the properties of γ oscillations remained unaltered (Wulff et al., 2009).

In this study we specifically focused on γ oscillations recorded in the CA3 region, where δ-$GABA_A$Rs are exclusively found on INs. In other brain areas where both α4-δ and α1-δ/$GABA_A$Rs-expressing neurons are present, non-selective drugs like ALLO would potentiate tonic inhibition on both cell types. Potentiating tonic inhibition on principal cells may decrease the power of γ oscillations, based on the higher γ power found in *Gabra5*$^{-/-}$ mice where the α5-$GABA_A$Rs specific to principal cells are missing (Glykys et al., 2008). Here we found no differences across genotypes in the power of γ oscillations. The relationship between γ oscillations power and frequency is not easily predicted (Jia and Kohn, 2011), as they can be influenced by independent variables such as network volume and single cell properties (Cardin et al., 2009; Pavlov et al., 2014; Mann and Mody, 2010; Buzsáki and Wang, 2012). Our *in vitro* model shows the ability of the CA3 network to modulate γ frequency through NS potentiation of δ-$GABA_A$Rs on PV+INs. This may be an important tool to allow fast responses to instantanous behavioral needs, and may be a mechanism

which facilitates optimization of information processing (Bieri et al., 2014; Lu et al., 2011; Uhlhaas et al., 2011). Moreover, mounting evidence showing a pivotal role of δ-GABA$_A$Rs and their plasticity in many neurological and psychiatric disorders such as epilepsy, anxiety, and postpartum depression may involve the mechanism of γ frequency modulation described in this study. For example, in models of temporal lobe epilepsy, δ-GABA$_A$Rs plasticity has been reported in both principal cells (downregulation) and INs (upregulation) (Peng et al., 2004; Yu et al., 2013; Ferando and Mody, 2012). Moreover, δ-GABA$_A$Rs expression is homeostatically modulated during times of altered NS production in hippocampal principal cells and INs (Maguire et al., 2009; Ferando and Mody, 2013; Maguire et al., 2005; Maguire and Mody, 2007; Lovick et al., 2005; Lovick, 2006), with consequences on network excitability and dynamics. Dysregulations in the control of δ-GABA$_A$Rs plasticity on principal cells or INs have been proposed as contributing factors in some symptoms of postpartum depression, PMS and PMDD and catamenial epilepsy (Maguire and Mody, 2008; Maguire et al., 2005, 2009; Ferando and Mody, 2013; Lovick, 2006), (Barth, Ferando, and Mody, unpublished observations). Moreover disruption of PV+IN function and resulting network dysfunction are well documented in schizophrenia (Uhlhaas and Singer, 2012; Lewis, 2014; Curley and Lewis, 2012; Brickley and Mody, 2012; Lewis et al., 2012).

The selective cell-type dependent localization of δ-GABA$_A$Rs containing α1 or α4 subunits has intriguing implications as it allows for differential pharmacological targeting of various neurons. We have previoulsy shown that α1/δ-GABA$_A$Rs on dentate ML INs were slightly more sensitive to ethanol than the α4/δ-GABA$_A$Rs of DGGCs (Glykys et al., 2007). As α1 and α4/δ-GABA$_A$Rs appear to be equally sensitive to NS (Bianchi and Macdonald, 2003; Meera et al., 2009), these chemicals would be poor candidates for differential therapy. Although to date there are no compounds selective for α1/δ-GABA$_A$Rs, future development of such drugs may have critical clinical applications for the many neurological and psychiatric disorders that involve IN dysfunction and altered γ oscillations.

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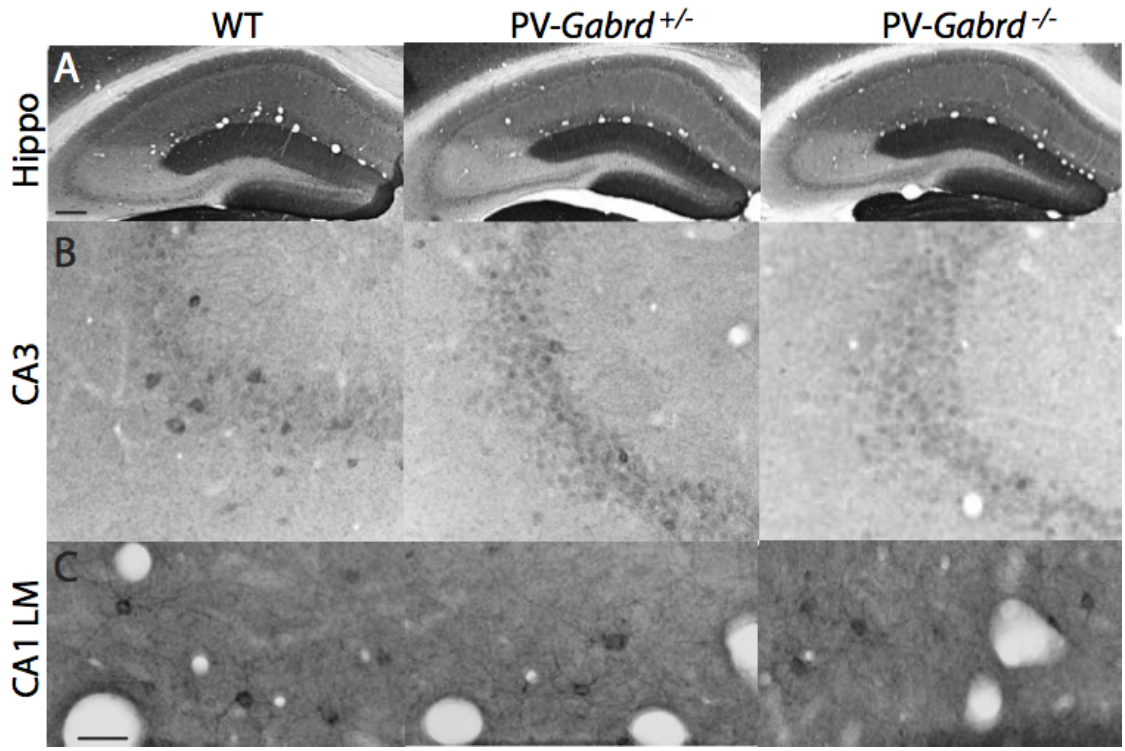

Figure 7.1: δ-GABA$_A$Rs expression in the hippocampal formation of WT, PV-*Gabrd*$^{+/-}$ and PV-*Gabrd*$^{-/-}$ mice. Representative bright field images δ-GABA$_A$Rs staining. (A). Whole hippocampal images show δ-GABA$_A$Rs expression is similar between the three experimental groups in CA1 SO and SR and DGML. (B). High magnification images show hippocampal area CA3. δ-GABA$_A$Rs is exclusively expressed in INs. In PV-*Gabrd*$^{+/-}$ mice theres a partial loss in the δ-GABA$_A$Rs staining of CA3 SP INs, while in PV-*Gabrd*$^{-/-}$ mice very few δ-GABA$_A$Rs+ INs can be found. (C). δ-GABA$_A$Rs staining of interneurons that do not normally express PV remains unchanged in all three experimental groups. Representative image of CA1 LM INs that stain for δ-GABA$_A$Rs in WT, PV-*Gabrd*$^{+/-}$ and PV-*Gabrd*$^{-/-}$ mice. These INs are putative neurogliaform cells, with a radial dentritic tree around a large circular soma. Scale bars = 200 μm, 50 μm.

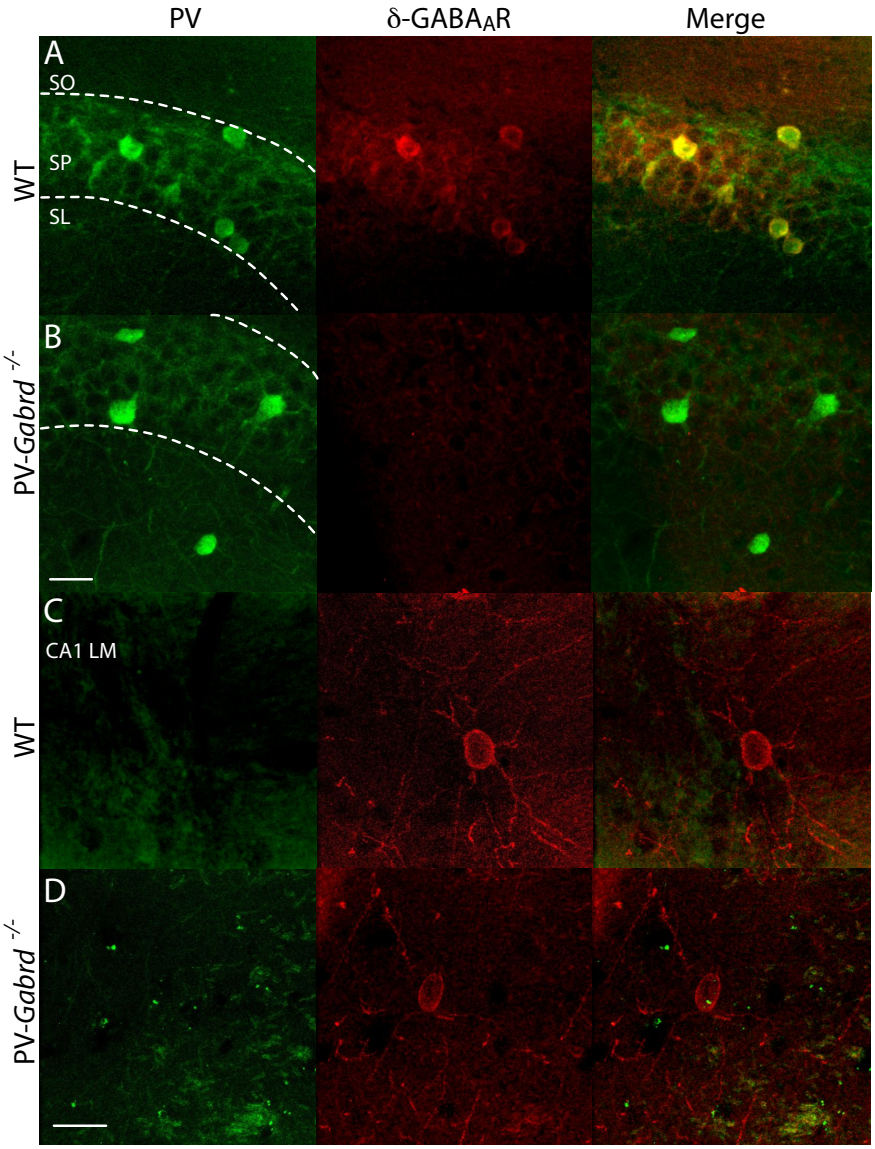

Figure 7.2: δ-GABA$_A$Rs and PV espression in WT and PV-$Gabrd^{-/-}$ mice in two different types of IN. δ-GABA$_A$Rs and PV do not colocalize in PV-$Gabrd^{-/-}$ mice. Green: PV, red: δ-GABA$_A$Rs, yellow: colocalization. (A, B). Representative confocal z-stack projections of hippocampal area CA3 in WT and PV-$Gabrd^{-/-}$ mice. In WT mice PV/δ-GABA$_A$Rs colocalization is almost complete in CA3 stratum pyramidale (thick dotted lines) and peri-stratum pyramidale area (30 μm thin dotted lines), as previously reported (Ferando and Mody, 2013; Yu et al., 2013; Milenkovic et al., 2013) (A). In PV-$Gabrd^{-/-}$ mice no δ-GABA$_A$Rs and PV colocalization could be found (B). (C, D). Representative confocal z-stack projections of of CA1 lacunosum moleculare. Interneurons positive for δ-GABA$_A$Rs do not express PV. Putative neurogliaform cells in WT and PV-$Gabrd^{-/-}$ mice express δ-GABA$_A$Rs but not PV. Scale bars = 20 μm.

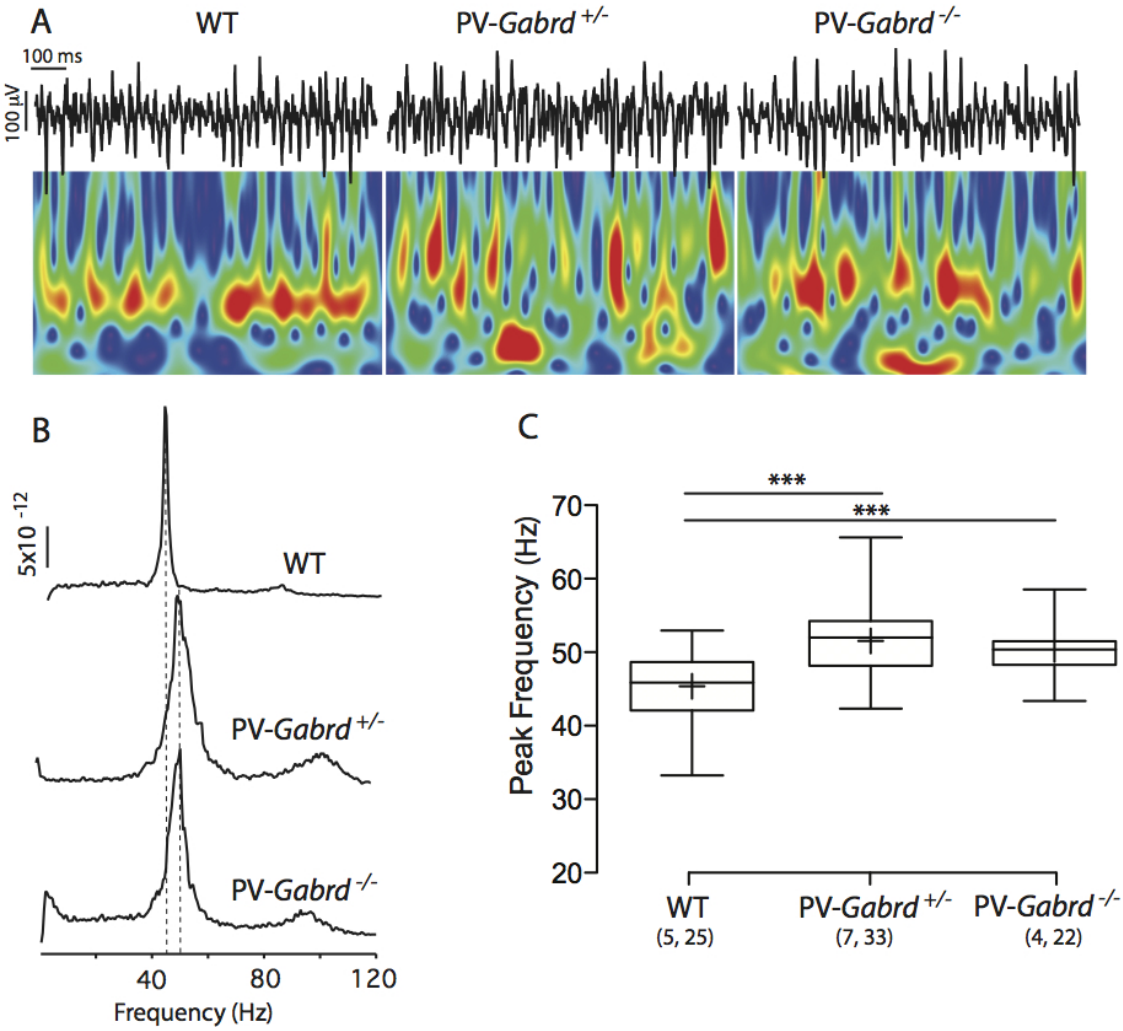

Figure 7.3: Frequency of CA3 SP γ oscillations is increased in slices of PV-$Gabrd^{+/-}$ and PV-$Gabrd^{-/-}$ mice compared to WT controls. (A). KA induced γ oscillations recorded extracellularly in CA3 SP show increased frequency in PV-$Gabrd^{+/-}$ and PV-$Gabrd^{-/-}$ mice. Upper traces, representative 1 s local field potentials band-pass filtered between 15 and 120 Hz. Lower panels, corresponding Morlet Wavelet transforms showing instantaneous LFP oscillatory activity. Warmer colors represent higher power. (B). Power spectral densities calculated over a 180 s period corresponding to the same recordings as in (A). (C). Box plots showing peak frequencies for the three experimental groups. Peak frequency of each slice calculated as the frequency with the highest power in a 180 s period power spectral density as in (B). Median, Mean (+), whiskers represent largest and smallest value, boxes represent 25th and 75th percentile. Significance established by One-Way ANOVA followed by Tukey's multiple comparisons test. ***$p < 0.0001$. n's in mice/slices.

	Peak frequency (Hz)			Power at peak frequency (E-11 V^2s^{-1})			Total power 30-120 Hz (E-10 V^2)		
	WT	PV-*Gabrd*$^{+/-}$	PV-*Gabrd*$^{-/-}$	WT	PV-*Gabrd*$^{+/-}$	PV-*Gabrd*$^{-/-}$	WT	PV-*Gabrd*$^{+/-}$	PV-*Gabrd*$^{-/-}$
No drug	47.8 (0.8) 14; 3	53.4 (1.0) 12; 3	50.8 (0.4) 14; 2	2.4 (0.4) 14; 3	4.2 (1.6) 12; 3	4.1 (0.8) 14; 2	6.6 (0.8) 14; 3	10.7 (4.0) 12; 3	8.5 (1.2) 14; 2
+ ALLO (100 nM)	48.0 (1.2) 7; 2	**47.8*** (1.8) 6; 2	50.9 (0.7) 8; 2	3.6 (0.7) 7; 2	2.5 (1.1) 6; 2	6.1 (1.3) 8; 2	7.37 (1.3) 7; 2	5.2 (1.6) 6; 2	13.9 (3.1) 8; 2
+ DS-2 (10 µM)	47.9 (1.2) 7; 3	54.2 (1.1) 6; 3	51.6 (0.7) 6; 2	2.5 (0.6) 7; 3	7.7 (3.5) 6; 3	2.9 (0.9) 6; 2	6.8 (1.5) 7; 3	16.2 (4.9) 6; 3	7.3 (1.8) 6; 2

Table 7.1: ALLO but not DS-2 reverts the frequency of γ oscillations in slices of PV-*Gabrd*$^{+/-}$ mice to levels found in WT slices. Values represent the mean with SEM in parentheses, and numbers below indicate the number of slices; mice. * indicates significance. Power at peak frequency and total power (calculated at 30-120 Hz) are also indicated. Two-way ANOVA (drug and genotype) showed a significant drug ($p < 0.05$, $F_{(2,71)} = 3.547$), genotype ($p < 0.0001$, $F_{(2,71)} = 12.5$), and interaction ($p < 0.05$, $F_{(4,71)} = 3.247$) effects on peak frequencies. Tukey's post-hoc test showed a significant difference in peak frequencies of PV-*Gabrd*$^{+/-}$ mice before and after ALLO treatment, $p < 0.001$, and between ALLO and DS-2 treatments $p < 0.01$. Two-way ANOVA (drug and genotype) showed no significant effects for power at peak frequency. Drug: $p > 4$ 0.05, $F_{(2,71)} = 0.322$. Genotype: $p > 0.05$, $F_{(2,71)} = 1.67$. Interaction: $p < 0.05$, $F_{(4,71)} = 2.039$. Two-way ANOVA (drug and genotype) showed no significant effects for total power. Drug: $p > 0.05$, $F_{(2,71)} = 0.2784$. Genotype: $p > 0.05$, $F_{(2,71)} = 1.667$. Interaction: $p > 0.05$, $F_{(4,71)} = 2.338$. For the two-way ANOVA tests the no-drug values were grouped from both ALLO and DS-2 experiments. A two-tailed paired *t*-test on values before and after drug treatments showed a significant effect of ALLO $p < 0.05$ but not of DS-2 on reducing γ oscillation frequencies in slices of PV-*Gabrd*$^{+/-}$ mice.

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CHAPTER 8

CONCLUSIONS AND FUTURE DEVELOPMENTS

The present work showed finely regulated δ-$GABA_A$Rs plasticity on both principal cells and interneurons of the hippocampus at times of altered NS production. We showed a δ-$GABA_A$Rs downregulation during the last third of pregnancy, that was quickly reverted to pre-pregnancy levels in the immediate postpartum. The observed plasticity seemed to be homeostatic in nature, as it allowed to maintain levels of excitation and network oscillations comparable to non-pregnant mice. We then showed how during the ovarian cycle, δ-$GABA_A$Rs expression was modulated on hippocampal interneurons, with direct effects on *in vivo* network γ oscillations, a network rhythmic activity thought to underlie many aspects of learning and memory (Buzsáki and Wang, 2012). The last part of this work showed how modulation of δ-$GABA_A$Rs expressed on PV+INs, an interneuron type with a fundamental role in the initiation and maintenance of γ oscillations (Sohal et al., 2009; Carlén et al., 2011; Wulff et al., 2009; Korotkova et al., 2010; Zheng et al., 2011; Lasztoczi and Klausberger, 2014; Mann et al., 2005), is sufficient to control the frequency of such an oscillation.

Moreover network activity sustained by PV+INs and controlled by $\alpha1/\delta$-$GABA_A$Rs could not be modified by a novel synthetic modulator specific for $\alpha4/\delta$-$GABA_A$Rs (Jensen et al., 2013; Wei et al., 2013).

At a time when mounting evidence suggests a central role of tonic inhibition mediated by δ-$GABA_A$Rs in many disorders (Dibbens et al., 2004; Feng et al., 2010; Maguire et al., 2005; Reddy et al., 2001; Maguire and Mody, 2008), this work sheds light on a physiological process, with network consequences, that if altered may predispose or precipitate underlying neurological or psychiatric conditions. For example, alterations in the coupling between δ-$GABA_A$Rs expression levels and NS production may facilitate neuronal network hypo or hyperexcitability. On the other hand, *in vivo* γ oscillations are generally tightly regulated, and such a fine regulation seems to be essential for proper memory formation and retrieval (Lasztoczi and Klausberger, 2014; Bieri et al., 2014). Altering the intrinsic span of inhibition of PV+INs may have large repercussions on memory performance. In addition PV+INs disfunction and consequent network abnormalities have been described in schizophrenia (Lewis et al., 2005; Uhlhaas and Singer, 2010). Modification of the behavior of these interneurons caused by alterations in their δ-$GABA_A$Rs content may play a role in this psychiatric condition. The possibility to selectively alter tonic inhibition on interneurons or principal cells, through the development of drugs that differentially target $\alpha1$ or $\alpha4/\delta$-$GABA_A$Rs, opens intriguing therapeutical applications.

Future work will need to focus on the molecular mechanisms that allow for δ-$GABA_A$Rs plasticity following NS fluctuations. There are many, non necessarily mutually exclusive possibilities. Subunit phosphorylation, transcriptional modifications, NMDA tone and intracellular Cl^- concentration may all play a role in the control of δ-$GABA_A$Rs expression (Gault and Siegel, 1997; Succol et al., 2012; Saliba et al., 2012; Choi et al., 2008).

An interesting, almost unexplored field is that of locally generated NS precursors. Progesterone can be synthesized locally starting from plasma-derived cholesterol in both neurons and glia, through an enzymatic cascade that involves both mitochondria and cytoplasmic

reactions (Belelli and Lambert, 2005; Herd et al., 2007). The rate-limiting factor of this cascade is the Translocator protein - 18kDa (TSPO), that transports cholesterol into the mitochondrial matrix (Rupprecht et al., 2010). TSPO is potentiated by its endogenous agonist DBI (Papadopoulos et al., 1997). Cell-type specific modulations of TSPO expression or activity may greatly influence the amount of tonic inhibition of that particular cell. Interestingly plasma cholesterol levels seem to oscillate over the ovarian cycle (Mumford et al., 2010). These oscillations are likely to influence local NSgenesis, and indirectly δ-$GABA_A$Rs-mediated tonic inhibition and δ-$GABA_A$Rs plasticity.

Lastly, more *in vivo* studies will need to address the many challenging questions that the present work opens, for example regarding the contribution of neuron-type specific tonic inhibition on different behavioral outcomes over the ovarian cycle, pregnancy and stress, and the behavioral consequences of tonic inhibition potentiation or decrease on specific neurons in different brain areas.

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