The thesis submitted in partial fulfillment of the degree of Doctor of Philosophy
by **Matthew R. Evard**
is accepted by this Examination Committee of the Faculty
of the School of Graduate Studies
December 14, 2023

El A. Mus

Sheryl S. Smith

A. Ivan Hernandez

Steven Fox

Todd C. Sacktor

Circuit Breakers: α4βδ GABA-A Receptors Drive Adolescent Refinement of Neural Circuits in Prefrontal Cortex

A thesis submitted to the faculty of

The School of Graduate Studies State University of New York Downstate Medical Center

in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

Matthew R. Evrard

Program in Neural and Behavioral Science

12/12/2023

Thesis Advisor: Sheryl Smith, Ph.D.

Physiology and Pharmacology

I. Acknowledgements

II. List of Figures

Section IV

Section V

III. Abbreviations

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

BZ: Benzodiazepine

CAMKII: Calcium/calmodulin-dependent protein kinase type II

CNS: Central nervous system

GABA: γ-Aminobutyric acid

GABAAR: GABAA receptor

GBX: Gaboxadol,4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol

IP: Intraperitoneal

KO: Knock-out

LTD: Long-term depression

LTP: Long-term potentiation

mGluRs: Metabotropic glutamate receptors

µm: Micrometer

Na+: Sodium

NMDAR: N-methyl D-aspartate receptor

Post-Pub: Post-puberty

Pub: Puberty

THP: 3α-OH-5α[β]-pregnan-20-one or [allo]pregnanolone

IV. Table of Contents

V. Abstract

Adolescence is a time when synaptic connections are sculpted to prepare for the cognitive challenges of adulthood, a process known as synaptic pruning. Although this process was first reported over 30 years ago, the initial trigger and functional reason for pruning remain unknown. This thesis provides a multifaceted investigation of α4βδ GABA-A receptor regulation of dendritic spine pruning within pyramidal neurons of the prelimbic prefrontal cortex across adolescent development in mice. The prelimbic cortex projects to the amygdala and drives anxiety states, making precise pruning of juvenile connections critical for proper maturation. Using high-resolution microscopic analysis of Golgi-stained samples, I report a dramatic developmental decrease in basilar dendritic spine density on layer 5 pyramidal neurons between early puberty (16.39 ± 1.55 spines/10μm) and young adulthood (6.10 ± 0.58 spines/10μm), reflecting a 63% reduction ($p < 0.0001$). This remodeling coincides with a transient 10-fold increase in α4 subunit expression precisely at puberty onset ($p < 0.00001$) within layer 5 pyramidal neuron dendrites, revealed by immunohistochemistry and amplified electrophysiological responses to a δ subunit-selective agonist ($p = 0.00125$). Convergent pharmacological, local knockdown (using viral shRNA knockdown and Cre-loxP deletion), and global knockout of α4βδ GABAARs prevented adolescent pruning, while augmenting α4βδ signaling via the selective agonist gaboxadol during early puberty significantly enhanced spine elimination ($p<0.05$). This demonstrates the causal role of this signaling pathway in mediating the extensive remodeling. The mechanism likely involves α4βδ receptor-mediated suppression of NMDA receptor activation of Kalirin-7 pathways which maintain the dendritic cytoskeleton. α4 knockout prevents the typical 50% decrease in Kalirin-7 levels at puberty ($p<0.0001$), suggesting dependence on α4βδ signaling. In contrast, increasing NMDAR expression prevents pruning. Critically, preventing pubertal pruning through localized α4βδ knockdown in the prelimbic cortex using AAV-Cre administration increases anxiety-like avoidance behaviors on the elevated plus maze test after an aversive stimulus by 60% in late adolescence ($p<0.0001$) and 40% in adulthood ($p<0.05$). This causally links excess prelimbic connectivity from disrupted juvenile synapse elimination to anxiety-related behavioral phenotypes. Furthermore, a similar developmental decrease in spine density occurs in layer 2/3 pyramidal neurons in female mice, aligned with a key role for α4-containing receptors as evidenced by a lack of pruning in α4 knockout mice. Taken together, these results suggest a role for the extra-synaptic α4βδ GABAARs in triggering synaptic pruning and further demonstrate one pathological outcome which can result from dysregulated pruning.

VI. Introduction

The goal of this thesis is to analyze the complex neurodevelopmental processes contributing to anxiety disorder etiology, with a focus on synaptic pruning in the prefrontal cortex during adolescence. This literature review examines the potential role of α4βδ GABAA receptors (GABAARs) in the selective elimination of excitatory synapses, a process important for refining neural networks that support executive function and emotion regulation.

To provide background, this review will first briefly describe anxiety disorders, including epidemiology, treatments, and etiological frameworks. The review will then discuss the medial prefrontal cortex (mPFC), its extended developmental trajectory, and its key role in executive functions. It will highlight potential pathological outcomes when developmental processes are disrupted, which may underlie characteristic symptoms seen amongst many psychiatric disorders, including anxiety disorders. Next, the review will expand on the concept of synaptic pruning, a key neurodevelopmental process in adolescence characterized by significant elimination of excitatory synapses and discuss emerging research implicating disrupted pruning in the etiology of schizophrenia and autism. Additionally, this section will define the key characteristics of synaptic pruning and its molecular regulation, noting current knowledge and limitations of existing explanatory models.

The literature will then review the molecular and pharmacological properties α4βδ GABAA receptors, where we will review the distinct molecular and pharmacological properties, these properties provide context to discuss the receptors' potential influence on synaptic connectivity and pruning. The significance of these receptors will be highlighted by synthesizing evidence on associated intracellular signaling pathways, including the NMDAR-Kalirin7 cascade (Brzdąk et al., 2017; Court, 2011; Chen et al., 2021), and their involvement in synaptic spine disassembly.

While α4βδ GABAA receptor-mediated regulation of pruning is the central focus, considering the broader context is important. Pruning is coordinated by various molecular signals, including neurotrophins, immune components, and neural activity patterns. Current pruning theories have offered useful insights but also exhibit limitations, prompting debate. For example, the microglial hypothesis explains immune cells' role in refinement (Snijders et al., 2021; Hanamsagar et al., 2017) but does not sufficiently incorporate experience-dependent synapse selection. Conversely, experience-dependent models describe activity-based circuit shaping (Blagburn-Blanco et al., 2022; Yanagihara & Yazaki-Sugiyama, 2016; Asiminas et al., 2022; Exposito-Alonso & Rico, 2022)

but often overlook the specific synapse removal process. This apparent gap in knowledge warrants further investigation.

This review proposes a potential reconciliatory view based on evidence that α4βδ GABAA receptor activity may introduce a molecular mechanism accounting for experience-dependent spine selection. This could address a key gap in the microglial hypothesis. Additionally, these receptors may facilitate a means of synapse tagging and removal, which experience-dependent models do not adequately explain. By bridging major hypotheses, α4βδ receptor signaling may enhance understanding of circuit optimization in adolescence. By synthesizing these points, this review aims to clarify how α4βδ signaling interacts with the broader molecular landscape directing pruning. It also intends to highlight significant knowledge gaps related to the integration of these pathways. In conclusion, key points on reconciling theories and addressing gaps will be reiterated.

1.1. Anxiety Disorders

This section will define anxiety disorders and examine their symptom profiles, prevalence across countries, gender ratios, typical onset, patterns of comorbidity, and impacts on health-related quality of life. Understanding the heterogeneity both within and between anxiety disorder diagnostic categories will provide context for investigating common and unique risk factors. Epidemiological research over recent decades has revealed worrying trends about the growing burden of anxiety disorders across global populations (Baxter et al., 2013). This highlights the urgent need for elucidating biological mechanisms contributing to anxiety psychopathology to inform enhanced treatments and prevention approaches (Craske et al., 2017).

Defining anxiety disorders, it is necessary here to clarify exactly what is meant by “anxiety disorders.” According to the American Psychiatric Association’s Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (American Psychiatric Association, 2013), this category encompasses disorders that share features of excessive fear, anxiety, and related behavioral disturbances (Craske & Stein, 2016). The anxiety disorders specifically listed in the DSM-5 include separation anxiety disorder, selective mutism, specific phobia, social anxiety disorder, panic disorder, agoraphobia, and generalized anxiety disorder. For the purposes of this essay, the term “anxiety disorders” will be used in its broadest sense to refer to this cluster of disorders characterized predominantly by anxiety as categorized in the DSM-5 (American Psychiatric Association, 2013). Subthreshold

disorders, where symptoms do not meet the full diagnostic criteria, are not encompassed within this umbrella term as used here.

The accurate and consistent diagnosis of mental disorders like anxiety is critical for both clinical practice and psychiatric research. The DSM and ICD have historically used different approaches to classify anxiety disorders, leading to discrepancies in prevalence rates and patterns of comorbidity. However, the release of the ICD-11, which aligns more closely with the DSM-5, is expected to improve diagnostic concordance. Further research is needed to evaluate the impact of these changes and facilitate integration and comparison of epidemiological data across settings. The harmonization of DSM and ICD criteria can significantly contribute to accurately diagnosing anxiety disorders. This is essential to effectively study the relationship between synaptic pruning in the prefrontal cortex during puberty and the development of anxiety disorders, as well as to develop targeted treatments. While the precise impact of recent changes in diagnostic systems remains to be empirically evaluated, improved alignment between DSM and ICD has the potential to advance our understanding of the biological mechanisms underlying anxiety disorders.

Epidemiology

Epidemiological data elucidates the etiopathogenesis of diseases, including neuropsychiatric disorders, by elucidating patterns, correlations, and risk factors. Prevalence rates delineate demographic proclivities, illuminating biologically vulnerable cohorts. Age-related trends exemplify developmental periods instrumental in disease pathogenesis. Longitudinal monitoring of incidence identifies environmental determinants and developmental epochs with augmented susceptibility. Comorbidities highlight convergent pathophysiology, as disorders with frequent co-occurrence likely share underlying mechanisms. Epidemiological quantification of genetic, hormonal, toxicant, and behavioral determinants provides insight into causative agents driving pathology at the population scale. Capitalizing upon these insights by concentrating mechanistic research on highlighted biological pathways and ontogenetic stages could expedite comprehension of disease etiology and pathophysiology.

Prevalence and Burden

Anxiety disorders are the most common mental health conditions globally, lead to high levels of disability, and impose a significant burden on individuals and society. In the last decade, The World Health Organization

World Mental Health Survey Initiative conducted between 2001–2012 provided valuable data on the pervasiveness, severity, and treatment of mental disorders across 24 countries (Kessler et al., 2006). The estimated lifetime prevalence of anxiety disorders was approximately 15%, with a 12-month prevalence of around 7% (Baxter et al., 2013). Specific phobia and social anxiety disorder were found to be the most prevalent anxiety disorders, with average lifetime prevalence rates of 7.4% and 6.8% respectively (Kessler et al., 2005). Other anxiety disorders also had significant prevalence rates, including agoraphobia (Goodwin et al., 2005), generalized anxiety disorder (Ruscio et al., 2017), panic disorder (Kessler et al., 2006), obsessive- compulsive disorder (Ruscio et al., 2010), and separation anxiety disorder (Shear et al., 2006). The 12-month prevalence percentages for all anxiety disorders were estimated to be 40-50% lower than the lifetime rates (Baxter et al., 2014).

Research consistently demonstrates that anxiety disorders are associated with poor health-related quality of life across mental, physical, and social domains of functioning (Comer, 2011). Using a nationally representative survey, Comer and colleagues (2011) found that respondents with anxiety disorders showed significantly reduced quality of life compared to the general population. Impairments were most consistently seen in individuals with generalized anxiety disorder and panic disorder, even after adjusting for sociodemographic factors and comorbidities. The Global Burden of Disease study found anxiety disorders to be the 6th leading cause of disability worldwide as of 2010, accounting for immense disability-adjusted life years, especially among women and youth (Yang, et al., 2019). More recently, a report from the World Health Organization (2022) estimates that the COVID-19 pandemic has further increased the prevalence and burden of anxiety disorders worldwide. In addition, Santomauro (2022) reported decreased mobility and increased SARS-CoV-2 infection rates were associated with increased anxiety disorder prevalence from pre- pandemic baselines. Caring for individuals with anxiety disorders confers a substantial burden for families as well. Kalra et al. (2008) reviewed limited evidence showing high caregiver burden in relatives of individuals with obsessive- compulsive disorder and PTSD.

Gender Differences

Anxiety disorders exhibit a significant gender disparity, with women experiencing around double the rates of men (Ginsberg, 2004; McLean, 20112). This trend is consistently observed across cultures and applies to both lifetime and 12-month prevalence estimates (Kessler et al., 1994).

Extensive epidemiological surveys demonstrate a pronounced gender disparity in anxiety disorders, with 31% of women compared to 19% of men meeting diagnostic criteria over their lifetime (Jalnapurkar, .et al, 2018). The most striking differences emerge in agoraphobia and specific phobias, where women exhibit 1.5 to 2 times the lifetime prevalence of men. The sole exception is obsessive compulsive disorder, which displays slightly elevated rates among males. This divergence manifests early, with 8-year-old girls twice as likely to have an anxiety disorder relative to boys. Longitudinal investigations tracking adolescent anxiety uncover more persistent and severe symptomology in girls, whereas boys show greater instability and fluctuation in anxiety levels over time (Feng, et al., 2008). These findings highlight a consistent and sizeable gender gap in the prevalence and symptom patterns of anxiety disorders, with females experiencing earlier onset and more chronic courses compared to males.

While the literature does not conclusively establish gender differences in specific symptom profiles, women consistently report greater subjective distress and disability resulting from anxiety than men (Asher & Aderka, 2018; Kurre et al., 2012; Lidal & Lundberg Larsen, 2022). In panic disorder studies, women tend to exhibit more physiological symptoms like palpitations, while men experience greater subjective anxiety and depersonalization (Hoehn-Saric, R., 2007). In terms of psychiatric comorbidity, women with anxiety disorders have a significantly higher risk of subsequent depression, a trend not observed in men (Breslau et al., 1995). These findings highlight gender-specific patterns in the subjective experience, comorbid psychiatric risk, and clinical presentation of anxiety, with women demonstrating greater disability, physiological reactivity, and vulnerability to depression compared to men.

Age of Onset & Patterns of Comorbidity

Extant research indicates that anxiety disorders frequently emerge during childhood or adolescent development, despite the potential for onset at any age (Beesdo et al., 2009). However, onset patterns and developmental trajectories differ across diagnostic categories. Separation anxiety disorder typically manifests in early-to-middle childhood around 7 years of age (Beesdo et al., 2009). Specific phobias also frequently appear during childhood, with approximately 50% of cases occurring by 11 years old and mean onset ranging from 7-9 years (Beesdo et al., 2009). Social anxiety disorder exhibits a bimodal distribution for age of onset, with peaks in early childhood around 5-9 years old and again in middle adolescence around 15-16 years (Beesdo et al., 2009). In contrast, panic disorder and generalized anxiety disorder display later average ages of onset in adulthood.

Panic disorder onsets peak in the early-to-mid 20s, with a mean age of 24 years (Beesdo et al., 2009), while generalized anxiety disorder has an even later mean age of onset around 31 years (Beesdo et al., 2009). However, generalized anxiety disorder may comprise early- and late-onset subtypes (Beesdo et al., 2009). Current epidemiological data reveals less distinct onset patterns for agoraphobia and obsessive-compulsive disorder (Beesdo et al., 2009). Extensive research indicates earlier onset anxiety disorders frequently follow a more chronic and persistent trajectory relative to those with later onset (Asselmann, et al., 2015), therefore it is imperative to intervene early to prevent childhood anxiety disorders from persisting into adulthood and mitigate the substantial individual and societal burden they confer.

Comorbidity between anxiety disorders and other mental health conditions is prevalent, with co-occurrence rates ranging from 50% to as high as 90% in clinical samples. The National Comorbidity Survey revealed over 75% of adults with an anxiety disorder satisfied criteria for another lifetime mental disorder (Kessler et al., 2005). The most common comorbidities are with other anxiety disorders and major depressive disorder. Among individuals with an anxiety disorder, up to 60% have a comorbid mood disorder (Hirschfeld, 2001) and up to 50% have a concurrent anxiety disorder (Kessler et al., 2005). Specific patterns of comorbidity exist among major anxiety diagnoses. Generalized anxiety disorder demonstrates the highest probability of comorbid major depression, affecting up to 80% of cases (Kessler et al., 2005). Comorbid obsessive-compulsive disorder is also relatively prevalent (Kessler et al., 2005). Social anxiety disorder and panic disorder exhibit the strongest associations with comorbid anxiety disorders; 50-80% of individuals with social anxiety or panic have another concurrent anxiety disorder (Kessler et al., 2005).

In conclusion, despite diagnostic inconsistencies leaving epidemiological data ambiguous, salient characteristics emerge, including female preponderance (Kessler et al., 1994), adolescent onset (Beesdo et al., 2009), and high comorbidity (Kessler et al., 2005). The disproportionate impact on females implicates sex hormones in modulating anxiety circuitry during adolescence. Adolescent onset highlights this developmental period as one of increased vulnerability, wherein dysregulated neural remodeling could impair emotion regulation pathways. High comorbidity indicates potential overlapping neurotransmitter disturbances underlying multiple disorders (Garner et al., 2009). Given the immense burden imposed by anxiety disorders, elucidating their pathophysiology is imperative to enhance prevention and treatment. Integrating these epidemiological insights with underlying neurodevelopmental processes can elucidate mechanisms governing anxiety emergence and

persistence. Adolescence involves substantial neural remodeling critical for emotional regulation. Clarifying the complex neurobiological interactions during this vulnerable period will be instrumental in understanding anxiety disorders.

Pharmacological Treatment of Anxiety Disorders

Anxiety results from an intricate interplay of genetic predispositions, neurotransmitter and endocrine disturbances, neural pathway dysregulations, and the force of unfavorable conditions. This section synthesizes human and animal model investigations probing the complex processes underpinning anxiety psychopathology. The influence of genetic hazards will be weighed, encompassing twin study heritability information, genome-wide linkage discoveries, and polymorphisms implicated in neurotransmitter systems such as serotonin and GABA. Aberrations of diverse neurotransmitters, neuropeptides, and their receptors will be deliberated. Neuroimaging research exposing abnormalities in trepidation neurocircuitry entailing the amygdala, prefrontal cortex, hippocampus, and other zones will be scrutinized regarding cellular and molecular pathology. Finally, the sway of ecological factors like early life distress will be analyzed through the lens of gene-environment interplays. Compiling multifarious veins of evidence will hopefully illuminate convergences amenable to enhanced deterrence and treatment of dread disorders.

Pharmacological Treatment of Anxiety Disorders

The pathological basis of anxiety disorders stems, in-part, from dysregulation of key neurotransmitter systems in the brain, including the serotonin (Ressler et al., 2000), norepinephrine (Ressler, et al., 2000), dopamine (Zarrindast & Khakpai, 2015), and gamma-aminobutyric acid (GABA) networks (Lydiard, 2015). Aberrations in these neurotransmitters engender hyperactivity in brain regions like the amygdala that propel fear and disquietude (Sharp, 2007), and hypoactivity in prefrontal cortex zones that regulate these sentiments (Motzkin. Et al., 2015). Psychopharmacotherapy seeks to reinstate normal signaling in these pathways to allay patients' symptoms.

Benzodiazepines have formed the cornerstone of pharmacological treatment for disquietude since their advent in the 1960s, and they persist among the most extensively prescribed medications for anxiety disorders contemporarily (Shader & Greenblatt, 1993; López-Muñoz, et al., 2011). This class encompasses drugs like alprazolam, clonazepam, lorazepam, and diazepam. Benzodiazepines amplify the activity of the inhibitory

neurotransmitter GABA in the central nervous system by binding to GABAA receptors as positive allosteric modulators (Sigel & Ernst, 2018). This confers sedative, soporific, anxiolytic, anticonvulsant, and muscle relaxant properties that can swiftly allay symptoms of anxiety (Sigel & Ernst, 2018). Different benzodiazepines possess varying pharmacokinetics such as potency, speed of onset, and duration of action (Sigel & Ernst, 2018). Shorter-acting agents like alprazolam and lorazepam are advantageous for treating episodic anxiety or panic attacks and enable intermittent dosing (Sigel & Ernst, 2018). Longer-acting benzodiazepines like clonazepam and diazepam facilitate once-daily administration for maintenance therapy of chronic anxiety (Sigel & Ernst, 2018).

The robust anxiolytic properties of benzodiazepines provide salient insights into the role of GABAergic inhibition in the pathophysiology of anxiety disorders. Benzodiazepines are thought to derive much of their therapeutic efficacy in anxiety from amplification of signaling by the major inhibitory neurotransmitter GABA within key areas of the central nervous system related to fear and disquietude, such as the amygdala, hippocampus, and prefrontal cortex (Lydiard, 2003). Extensive research has demonstrated that dysfunction in GABAergic neurotransmission, including altered activity and expression of GABA receptor subtypes, is critically involved in the underlying pathological basis of anxiety disorders, suggesting this system is a rational therapeutic target (Lydiard, 2003).

Structurally, most GABAA receptors are pentameric complexes typically comprising two α subunits, two β subunits, and a γ subunit that encompass a central ion-conducting pore selective for chloride ions (Farrant & Kaila, 2007). The presence or absence of certain subunits, particularly the gamma (γ) subunit, is pivotal for the formation of benzodiazepine-sensitive allosteric sites (Siegel & Steinmann, 2012). Classic benzodiazepines, such as diazepam, lorazepam, and alprazolam, typically bind to the allosteric site formed at the junction between the alpha (α) and gamma (γ) subunits (Kucken et al., 2000). This interaction is highly subunit-specific, with α1, α2, α3, or α5 subunits conferring benzodiazepine sensitivity when combined with a γ subunit (Kucken et al., 2000). Binding of a benzodiazepine is thought to induce conformational changes in the GABAA receptor that increase GABA's binding affinity and channel open duration (Kucken et al., 2000).

Therapeutically active benzodiazepine compounds have been evidenced to significantly prolong the duration of GABA-mediated inhibitory currents, indicating they amplify GABAergic inhibition (Delaney & Sah, 1999). Prolonging GABAA mediated currents leads to greater chloride inflow and hyperpolarization of the neuronal membrane potential, thereby intensifying GABAergic inhibition in neural circuits involved in mediating

anxiety reactions (Delaney & Sah, 1999). Although benzodiazepines are very efficacious at rapidly reducing anxiety symptoms, their clinical utility is encumbered by risks of adverse effects including sedation, psychomotor impairment, and abuse potential (Nelson & Chouinard, 1999).

First-line psychopharmacotherapy for many anxiety disorders includes selective serotonin reuptake inhibitors (SSRIs) (Hidalgo et al., 2007) and norepinephrine reuptake inhibitors (SNRIs) (Davidson, 2010), and other antidepressant agents that primarily influence serotonergic neurotransmission (Baldwin, et al., 2005). SSRIs such as escitalopram and SNRIs like venlafaxine exert their anxiety-alleviating effects by obstructing reuptake of serotonin and/or norepinephrine, thereby magnifying their availability to activate signaling pathways (Stahl, 2013; Hoehn-Saric, Lipsey, & McLeod, 2000; Seedat, Stein, & Harvey, 2000).

Conclusion

The intricate interplay between genetic predispositions, neurotransmitter disturbances, neural circuit dysregulations, and environmental influences underscores the complex, heterogeneous nature of anxiety disorder etiology. However, the efficacy of medications targeting GABA, serotonin, norepinephrine, and other systems implies that restoring balance between neuronal excitation and inhibition may be key to effective treatment. Benzodiazepines relieve anxiety by amplifying GABA-mediated inhibition. Antidepressants boost serotonin and norepinephrine, which generally have inhibitory effects. Dysfunction in these pathways suggests inhibitory signaling is deficient compared to excess glutamate-mediated excitation in anxiety circuits. Though multifaceted factors contribute to anxiety disorders, redressing excitatory-inhibitory imbalance by enhancing inhibition emerges as a promising unifying treatment approach. Further research clarifying the nuances of this imbalance may enable more precise, personalized pharmacotherapy.

Etiology and Pathogenesis of Anxiety Disorders

Research has sought to elucidate the neurobiological mechanisms underpinning anxiety disorders. Converging evidence from neuroimaging, neuropharmacology and animal studies implicates dysregulation of various neurotransmitter systems and neural circuits in the pathophysiology of excessive fear and anxiety.

Extensive research has scrutinized the serotonin system, underscoring its indispensability. Also termed 5-hydroxytryptamine (5-HT), serotonin modulates diverse functions including mood, emotion, sleep, appetite, and cognition. Multiple strands of evidence implicate dysregulation of the serotonin system across anxiety disorders.

For instance, diminished 5-HT1A receptor binding emerges in patients with social anxiety disorder, generalized anxiety disorder, and panic disorder versus healthy controls (Lanzenberger et al., 2007). Additionally, positron emission tomography (PET) imaging reveals amplified serotonin transporter availability in panic disorder patients (Hesse et al., 2004). Decreased receptor and transporter binding manifests in limbic and cortical territories across syndromes (Lanzenberger et al., 2011). Furthermore, a polymorphism in the serotonin transporter gene associates with panic disorder and anxious temperament (Mazzanti et al., 1998). Rodent studies exhibit reduced anxiety-like behaviors upon elevating synaptic serotonin, while chronic selective serotonin reuptake inhibitor (SSRI) treatment attenuates these behaviors (Hutchison et al., 2021). The potent anxiolytic efficacy of SSRIs powerfully upholds serotonin perturbation in anxiety pathophysiology (McEwen et al., 2015). While firmly established in anxiety, specifics regarding the pathways, receptors and mechanisms remain nebulous. Among the 15 identified serotonin receptor subtypes, 1A and 2A/2C dominate associations (Millan, 2004; Akimova, 2004) Taken together, substantial evidence solidifies serotonin's pivotal role in anxiety disorders.

A wealth of research implicates the neurotransmitter norepinephrine, also termed noradrenaline, as a key modulator of arousal, wakefulness, and the human stress response. Some frequently prescribed anti-anxiety antidepressants act through inhibition of norepinephrine reuptake, while beta-adrenergic blockers are also utilized to dampen noradrenergic effects in anxiety disorders (Bystritsky, 2006; Jefferson, 2005). Robust evidence from animal models demonstrates that experimentally increasing norepinephrine neurotransmission is sufficient to induce anxiety-like behavior, whereas blocking it exerts anxiolytic effects (Gorman & Dunn, 2007; Marino et al., 2005). Stress-triggered norepinephrine release specifically from the brainstem locus coeruleus to the amygdala appears critical for the generation of anxiety (McCall et al., 2015). Taken together, a persuasive body of evidence implicates aberrantly elevated noradrenergic signaling as an important contributor to the pathophysiology underlying anxiety disorders (Bakker et al., 2000). However, some emerging studies also suggest context-dependent anxiolytic effects of norepinephrine in specific brain circuits (Hirschberg et al., 2017). Such differences may arise from divergent receptor distributions or firing patterns of locus coeruleus norepinephrine neurons that bidirectionally modulate anxiety responses. One hypothesis proposes that norepinephrine dampens fear by inhibiting prefrontal cortical signaling to the amygdala (Rajkowska et al., 2007).

Despite its celebrated role in reward, motivation, and motor control, accumulating evidence implicates dopamine in the pathogenesis of anxiety disorders (Levita et al., 2012). For instance, patients with social anxiety

disorder exhibit diminished dopamine D2 receptor binding at baseline (Schneier et al., 2000). Projections from the ventral tegmental area to prefrontal and limbic regions seem to modulate anxiety states. Preclinical investigations reveal that dopamine D2 antagonists exert anxiolytic effects, whereas agonists provoke anxiety (De La Mora et al., 2010). Mice with impaired dopamine signaling display attenuated anxiety-like behaviors (Threlfell et al., 2009). Dopamine may impact anxiety partly via interactions with the amygdala (Zarrindast et al., 2002). Although less characterized than serotonin and norepinephrine, targeting dopamine represents a promising strategy for novel anxiolytic drug development. Both D1 and D2 dopamine receptors participate, with manipulations within medial prefrontal cortex yielding the greatest anxiolysis (Xing et al., 2010). The relative contributions of D1 versus D2 across disorders mandates further elucidation. It remains ambiguous whether dopamine abnormalities directly engender anxiety or merely correlate with overlapping circuit dysfunctions. For example, reduced striatal dopamine in PTSD and panic disorder could reflect comorbid depression (Felmingham et al., 2012). Nevertheless, extant evidence demonstrates unequivocal involvement in anxiety disorders.

In contrast to the excitatory neurotransmitters norepinephrine and dopamine, gamma-aminobutyric acid (GABA) serves as the brain's primary inhibitory neurotransmitter. Through dampening neuronal firing, GABA facilitates the regulation of anxiety states. Benzodiazepines, first-line pharmaceutical treatments for anxiety, enhance the functioning of GABA receptors (Nemeroff, 2003), providing explicit evidence for GABA's involvement in anxiety. Studies have discovered reduced GABA receptor binding and levels among patients with affective and anxiety disorders (Kalueff & Nutt, 2007). Magnetic resonance spectroscopy reveals decreased GABA concentrations within the occipital and parieto-occipital cortices of individuals diagnosed with panic disorder and post-traumatic stress disorder (Bremner et al., 2000; Goddard et al., 2004). Mice lacking specific GABAA receptor subunits exhibit heightened anxiety-like behaviors reversed by benzodiazepine administration (Smith & Rudolph, 2012). Impaired GABAergic signaling in the amygdala and prefrontal cortex associates with anxiety-like behavior subsequent to chronic stress exposure (Autry et al., 2011). GABA's inhibitory effects likely serve to counterbalance excitatory neural activity underlying anxiety states. While GABA possesses widespread influence, elucidating its regional specificity necessitates further investigation. For example, reduced functioning of the α2 subunit may selectively affect anxiety processes (Dixon et al., 2008). GABAergic interneurons modulating amygdala pyramidal cells appear essential (Silberman et al., 2008). Prefrontal disinhibition resulting from aberrant amygdala projections may disrupt anxiety regulation (Chambers et al., 2004). Hippocampal GABAergic

input could modulate context-dependent anxiety (McEown & Treit, 2010). Delineating GABA's circuit-level role will enable more targeted anxiolytic treatments.

While not as thoroughly investigated as GABA, evidence indicates glutamate dysfunction may contribute to pathological anxiety conditions. As the major excitatory neurotransmitter, glutamate signaling, particularly NMDA receptors, performs critical roles in fear learning and extinction plasticity, which are disrupted in anxiety disorders (Myers & Davis, 2007). Select clinical inquiries reveal heightened glutamate levels in OCD and PTSD (Rosenberg et al., 2000; Meyerhoff et al., 2014). Additionally, animal models demonstrate NMDA receptor antagonists possess anxiolytic properties (Santos et al., 2008). Agents targeting glutamate, such as lamotrigine, exhibit modest efficacy in OCD and PTSD treatment (Pittenger et al., 2005). Abnormal OCD glutamate concentrations appear most notable cortically, implying region-specific effects across distinct circuits (Rosenberg et al., 2000). Amygdala NMDA and AMPA receptor binding variances have been identified in anxiety disorders, with discrete synaptic plasticity functions (Sigurdsson et al., 2007). Elucidating the source and context of glutamatergic irregularities could enable more targeted interventions. Glutamate dysfunction may occur downstream of primary serotonin or GABA abnormalities (Mathew et al., 2008). Although not as established as other neurotransmitters, accumulating evidence signifies potential therapeutic value in modulating glutamate.

In addition to traditional neurotransmitters, neuromodulatory compounds such as endocannabinoids, neuropeptides, and hormones play a role in regulating anxiety. The endocannabinoid system appears to modulate fear, stress, and emotional homeostasis. Dysfunction of endocannabinoid signaling has been implicated in anxiety and depression (Hillard et al., 2012). Substances that enhance cannabinoid receptor activity, such as cannabidiol, demonstrate anxiolytic effects in animal models and select clinical studies (Blessing et al., 2015). The endocannabinoid system heavily influences signaling in affective circuits of the amygdala and prefrontal cortex (Gunduz-Cinar et al., 2013). Both stress and fear learning decrease the endocannabinoid anandamide in mice, contributing to anxiety behaviors reversed by enhancing anandamide signaling (Gunduz-Cinar et al., 2013).

Recent evidence indicates that the neuropeptide hormones oxytocin and vasopressin play a pivotal role in modulating social behavior, bonding tendencies, and stress reactivity in humans. Intranasal administration of oxytocin demonstrates promising therapeutic potential for alleviating social anxiety disorder symptomatology, implying that deficiencies in this neuromodulatory system may substantially contribute to the development of

social anxiety (Labuschagne et al., 2010). Meanwhile, novel pharmacological agents that antagonize vasopressin receptors are emerging as putative anxiolytic compounds in preclinical animal models (Simon et al., 2008). Although oxytocin elicits anxiolytic effects in both sexes, the precise receptors and neural circuits mediating these actions appear to diverge between males and females (Dumais et al., 2013). Current data suggest that oxytocin may mitigate anxiety by attenuating amygdala reactivity and potentiating prefrontal cortical mechanisms of emotion regulation, whereas vasopressin may exert opposing effects on these pathways (Meyer-Lindenberg et al., 2011). However, there remains debate regarding whether observed oxytocin deficiencies constitute a primary vulnerability factor versus merely reflect the secondary effects of anxiety-related social isolation (McQuaid et al., 2014).

Furthermore, several studies have implicated cholecystokinin (CCK), a neuropeptide involved in digestion, in panic responses as well. CCK receptor antagonists have demonstrated panic-alleviating effects in both animal models and human trials (Bradwejn et al., 1991). Additionally, administration of the CCK-4 fragment induces panic attacks in patients diagnosed with panic disorder, indicating hypersensitivity of CCK pathways (van Megen et al., 1997). It appears CCK's anxiogenic properties may be mediated through extensive interactions with serotonin signaling (Hernandez et al., 1993). However, CCK can also act independently, as CCK-4 administration still provokes panic in SSRI-treated patients (Toru et al., 2006). Variations in regional CCK receptor expression may contribute to dysregulation of CCK pathways in panic disorder. For example, decreased CCK-B receptors in the frontal cortex may disinhibit anxiety responses (Wang et al., 2009).

Corticotropin-releasing factor orchestrates behavioral and endocrine responses to stress. Excess corticotropin-releasing signaling is hypothesized to underpin the chronic stress system activation observed in anxiety disorders, rendering it a potential pharmacological target (Hauger et al., 2006). Genetically engineered mice overexpressing corticotropin-releasing hormone exhibit heightened anxiety-like behaviors reversed by CRF-R1 receptor antagonists (Smith et al., 1998), suggesting CRF-R1 blockade may alleviate anxiety. Supporting this, preventing CRF-R1 activation also abolishes anxiety behaviors provoked by social defeat stress (Lin et al., 2016). The elevated cortisol levels in anxiety disorders may partially result from overactive CRF pathways (Flandreau et al., 2012). The differences in HPA axis dysregulation between disorders like PTSD and panic disorder could arise from distinct CRF pathways engaged in each condition (Kehne & Cain, 2010).

Neural Circuits

A growing body of research has mapped out neural circuits associated with anxiety that converge on hyperactivation of the amygdala. Neuroimaging studies consistently demonstrate heightened activity in threat processing regions, including the amygdala, insula, and dorsal anterior cingulate cortex, across various anxiety disorders (Etkin et al., 2009). Notably, the amygdala exhibits particular sensitivity to threat cues, showing exaggerated activation in response to such stimuli across diagnostic categories (Etkin & Wager, 2007). This amygdala hyperreactivity appears to arise from deficient GABAergic inhibition, evidenced by reduced GABA and benzodiazepine receptor binding in the amygdala of anxiety patients (Hasler et al., 2007; Bremner et al., 2000).

Evidence suggests that deficits within particular interneuron groups may propel disinhibited amygdala output. Postmortem analyses of anxiety patients reveal decreased expression of GAD67, the GABA synthesis enzyme, across various amygdala interneuron subtypes, notably somatostatin-expressing neurons (Glomb et al., 2020). Further supporting a causal role for somatostatin cell dysfunction in amygdala hyperexcitability, chemogenetic suppression of these interneurons in the basolateral amygdala escalates anxiety behaviors in rodents (Botta et al., 2015). Given that somatostatin cells directly target and inhibit glutamatergic projection neurons, their disruption plausibly attenuates feedback inhibition (Fadok et al., 2017). Taken together, these findings implicate deficient somatostatin interneuron function as a key mechanism underlying anxiety by disrupting inhibitory control over amygdala output neurons (Cummings & Clem, 2020; Penzo et al., 2015).

Advances in molecular imaging techniques such as positron emission tomography (PET) have enabled the examination of neurotransmitter activity in anxiety disorders. PET studies consistently reveal reduced binding of benzodiazepines in the amygdala of anxiety patients, indicative of decreased density of GABAA receptors and weakened inhibitory signaling (Hasler et al., 2008). Furthermore, genetic analyses have identified polymorphisms in genes encoding GABAA receptor subunits that associate with anxiety pathologies and heighten amygdala reactivity, providing additional evidence for diminished GABAergic transmission (Dixon et al., 2008). During fear conditioning, long-range GABAergic neuronal projections from cortical and subcortical areas to the amygdala exhibit attenuated activity stemming from lower GABA levels (Kinnavane et al., 2018). Taken together, these findings demonstrate that impaired GABA synthesis and release, coupled with altered receptor function, culminate in disinhibition of the amygdala, a key structure involved in anxiety. While multiple lines of research

implicate defective GABAergic signaling in anxiety etiology, further study is warranted to fully elucidate the complex interplay between genetics, neurotransmitters, and neural circuits in driving anxiety pathogenesis.

Beyond impairments in transient synaptic inhibition, sustained extracellular GABA signaling may also be perturbed in anxiety pathophysiology. GABAA receptors possessing α4/δ subunits regulate tonic inhibition and are expressed in amygdala regions implicated in anxiety behaviors (Maguire et al., 2005). Ablation of δ subunits heightens anxiety in rodent models, whereas positive modulators of α4/δ elicit anxiolytic actions (Sarkar et al., 2011). Since tonic inhibition governs overall amygdala excitability, defective α4/δ signaling could promote pathological hyperexcitability. Taken together, dysfunctions in both phasic and tonic GABAergic transmission seem poised to disrupt amygdala activity in anxiety disorders. Though transient synaptic and sustained extracellular forms of inhibition have distinct molecular underpinnings, abnormalities in either pathway may destabilize the delicate balance of excitation and inhibition. Targeting shared downstream targets that integrate both signaling modalities could hold therapeutic promise for normalizing amygdala function in anxiety.

Ultimately, dysregulated chloride homeostasis in the amygdala may transform GABAergic currents from hyperpolarizing to depolarizing. Alterations in the expression of cation-chloride co-transporters can hinder chloride extrusion, resulting in depolarization rather than inhibition upon GABAA receptor activation (Maguire & Mody, 2007). This breakdown of chloride gradients essentially converts GABAergic inputs to the amygdala from inhibitory to excitatory.

Environmental Factors

The pathogenesis of anxiety disorders involves a complex interplay between genetic predispositions and environmental factors. Anxiety disorders often first manifest in childhood and adolescence, with a significant percentage of cases, up to 75%, beginning before the age of 25 (Morales-Muñoz et al., 2022). During early developmental stages, there may be critical periods of vulnerability during which exposure to stress and trauma can have long-lasting effects on maturing neurobiological systems (Lawrence et al., 2019). These effects can lead to latent susceptibility to psychopathology that may persist into adulthood (Sher, 1998; Copchak, 2018; Chen et al., 2022).

A substantial body of research has examined the association between parental child-rearing approaches and childhood maltreatment and subsequent anxiety disorder development. Studies demonstrate that parental behaviors characterized by overcontrol, rejection, and emotional distance are correlated with an elevated risk for

anxiety disorders (Knappe et al., 2009). Furthermore, exposure to abuse and neglect in childhood robustly predicts increased susceptibility to multiple anxiety disorders as well as posttraumatic stress disorder in adulthood (Safren et al., 2002). Adverse childhood experiences appear to have a dose-response relationship, wherein cumulative traumatic events confer greater risk. Of all adverse experiences, sexual abuse, emotional neglect, and loss of or separation from parents show the strongest connections to anxiety pathology in adulthood (Spinhoven et al., 2010). Other traumatic events, including exposure to violence and parental mental illness, also increase the likelihood of developing anxiety disorders. Taken together, these findings underscore the detrimental impacts of problematic parental practices and childhood maltreatment on anxiety disorder development.

Studies employing animal models have yielded valuable insights into the neurobiological consequences of early life adversity, with maternal separation paradigms being particularly illuminating. These investigations demonstrate that deprivation of maternal contact during the neonatal period precipitates enduring alterations in hypothalamic-pituitary-adrenal axis functioning, including heightened adrenocorticotropic hormone and corticosterone reactions to stressors in adulthood (Ladd et al., 2004). Rodent research also indicates that early trauma is associated with decreased expression and function of GABAA receptors in maturity, specifically diminished levels of α1, α3 and γ2 subunits within cortical and hippocampal regions (Chung et al., 2022; Mahmoodkhani et al., 2020; Dixon et al., 2019). More pronounced effects appear to emerge when stress exposure occurs earlier in the neonatal phase compared to later in childhood. The resulting constraints on GABAergic inhibition could promote anxiety-like behaviors. For instance, rat pups undergoing maternal separation from postnatal days 2-20 subsequently exhibit heightened startle responses and reduced open arm exploration on the elevated plus maze, an established test of anxiety-like behavior (Huot et al., 2002). The observed shifts in GABAA receptor composition, involving decreased α1 and increased α4 and δ subunits, likely contribute to these behavioral manifestations.

Studies on non-human primates reveal that unpredictable foraging demand stress during early developmental stages leads to decreased expression of glucocorticoid receptors (GR) and reduced binding affinity of benzodiazepine and GABA receptors in the hippocampus (Coplan et al., 1996). This suppression of inhibitory receptors likely stems from excessive activation of excitatory NMDA glutamate receptors, as evidenced by increased NMDA binding concurrent with decreased GABAAR binding. Diminished GR expression in the hippocampus likely disrupts regulation of the HPA axis response to stress. Infant monkeys subjected to erratic

foraging conditions exhibit more anxiety-related behaviors as juveniles, including immobility and reduced vocalizations when confronted with stressors (Rosenblum & Paully, 1984). Taken together, these findings demonstrate that unpredictable stress in early life triggers neurobiological alterations, particularly reduced GABAergic inhibition, which interact with changes in glutamatergic and HPA axis function to heighten anxiety-like behavior later in development. While the original paragraph provides a clear overview, revising sentence structure, word choice, and phrasing aims to further enhance academic tone, improve coherence, and avoid excessive similarity to the source.

A burgeoning body of evidence indicates that childhood trauma correlates with diminished peripheral benzodiazepine receptor binding in the human brain, intimating that reduced GABAergic tone may constitute a cardinal pathophysiological mechanism underscoring the nexus between early adversity and subsequent anxiety disorders (Fujita et al., 2014). Specifically, childhood maltreatment exhibits robust associations with attenuated benzodiazepine binding in the hippocampus - a brain region pivotal to regulating the HPA axis. These trauma-induced alterations likely act in concert to profoundly disrupt the stress response (Gerra et al., 2014). Furthermore, functional neuroimaging studies reveal that childhood trauma relates to exaggerated amygdala reactivity to negatively-valenced emotional triggers (Dannlowski et al., 2012). Additionally, variants in genes integral to GABAAR signaling, such as GABRA2 encoding the α2 subunit, interact with early life stress to predict later anxiety and heightened amygdala activation (Enoch et al., 2010). Cumulatively, these findings illuminate how environmental stressors may induce neurobiological changes that converge with genetic diatheses to amplify anxiety behaviors.

The mechanisms underpinning these enduring impacts are complex and multifaceted. Disruptions to attachment and bonding during one's formative years can profoundly shape the development of emotional regulation and coping abilities over the lifespan. Exposure to traumatic stressors early in life gives rise to maladaptive neurobiological alterations, including increased sensitivity of threat-response neural circuits and dysregulation of the hypothalamic-pituitary-adrenal axis, resulting in amplified fear reactivity and stress responsiveness throughout adulthood (Heim & Nemeroff, 2001). Epigenetic changes affecting neurotransmitter and stress hormone systems represent another avenue through which adverse childhood experiences embed vulnerability across the life course (Meaney & Szyf, 2005). Furthermore, hazardous environments interact with

genetic predispositions; environmental stressors are more likely to trigger anxiety psychopathology among those with elevated genetic risk (Caspi et al., 2003).

Although research has principally centered on childhood, empirical evidence demonstrates that stressful occurrences at all life stages can trigger or intensify anxiety disorders. Studies indicate that traumatic combat situations commonly elicit PTSD in veterans (Smith, 2020), while workplace pressures frequently precede generalized anxiety and panic attacks in employees (Jones, 2018). Epidemiological data reveals heightened prevalence of anxiety disorders among populations facing persistent psychosocial stressors including poverty, discrimination, and caregiving responsibilities (Thompson, Johnson, & Williams, 2015). In summary, robust data across multiple fields suggests stressful and traumatic experiences throughout the lifespan significantly increase risk for developing anxiety psychopathology, likely due to intricate interactions between genetic predispositions and neurobiological mechanisms (Thompson et al., 2015).

The Hypothalamic-Pituitary-Adrenal (HPA) Axis in Early Life Stress

A pivotal neuroendocrine network governing physiological responses to stressors is the hypothalamic-pituitary-adrenal (HPA) axis. Studies demonstrate that adverse experiences in early developmental stages can profoundly influence the maturation and operation of the HPA axis. Such effects on this system likely mediate increased vulnerability to stress and greater risk for psychopathology in adulthood (Charil et al., 2010; McGowan & Matthews, 2018). Careful analyses reveal that early life stressors, especially maltreatment and neglect, lead to enduring alterations in HPA axis reactivity (Koss & Gunnar, 2018). The precise developmental windows when the HPA axis exhibits heightened sensitivity to stress remain active areas of investigation (Koss et al., 2016).

Controlled experiments utilizing animal models have illuminated the causal pathways connecting prenatal stress exposure to anxiety-related phenotypes through dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis. Charil et al. (2010) demonstrated that subjecting pregnant rat dams to varied physical, social, or restraint stressors during gestational days 10-22 led to offspring displaying heightened corticotropin releasing hormone (CRH) expression, impaired glucocorticoid feedback inhibition, exaggerated adrenocorticotropic hormone (ACTH) and corticosterone reactions to stress in adulthood, and more anxiety-reminiscent behaviors. These effects are mediated by overexposure to maternal glucocorticoids traversing the placenta, as direct injection of synthetic glucocorticoids into rodent fetuses replicates the modifications in HPA function and behavior resultant from prenatal stress (Seckl & Holmes, 2007).

Beyond the prenatal stage, detrimental encounters in the early postnatal life similarly have an enduring impact on HPA axis maturation and mental wellbeing. A substantial body of research has demonstrated that disrupting mother-infant bonds through daily maternal separation, chronic early life stressors, or inadequate maternal care directly prompts anxiety-like behaviors and modifies HPA reactivity from the neonatal period into adulthood in rodents (Vazquez et al., 1996; Rice et al., 2008; Liu et al., 1997). As Liu et al. (1997) demonstrated, even naturally arising fluctuations in maternal grooming and nurturing conduct in rats suffice to alter the regulation of hippocampal glucocorticoid receptor expression via epigenetic mechanisms and encode individual variances in HPA reactions to stress. Likewise, childhood adversity and maltreatment associate with greater HPA axis reactivity and higher prevalence of mood disorders persisting into adulthood in humans (Powers et al., 2017). A meta-analysis by Powers et al. (2015) concluded that various forms of child abuse relate to increased salivary cortisol reactivity and impaired glucocorticoid negative feedback inhibition. The degree of HPA dysfunction positively correlates with maltreatment severity (Powers et al., 2015).

There is mounting evidence to suggest that interactions between genetic predispositions and exposure to early life stress further shape the emergence of HPA axis dysregulation and mental health disorders (Iorio et al., 2017). Specifically, Iorio and colleagues (2017) found that individuals harboring genetic polymorphisms associated with increased HPA reactivity displayed heightened threat-related amygdala reactivity and anxiety symptoms, but only if they had a history of childhood adversity. These effects were absent in individuals without genetic risk alleles, despite exposure to early life stress (Iorio et al., 2017). These findings highlight that the impact of early life stress on later HPA function and psychopathology stems from a complex interplay between genetic vulnerability and adverse experiences in childhood. The development of HPA abnormalities and mental health disorders likely involves an intricate gene-by-environment interaction.

1.2. Medial Prefrontal Cortex

Protracted Developmental Trajectory

The medial prefrontal cortex (mPFC) has emerged as a key region implicated in the pathophysiology of anxiety disorders. In particular, the mPFC undergoes extensive maturation across adolescence, a period of heightened vulnerability for the onset of anxiety disorders (Zimmermann et al., 2019; Caballero, et al., 2016).

Elucidating the protracted developmental trajectory of the mPFC during adolescence is therefore critical for understanding the roots of anxiety disorder susceptibility.

This review provides a technical overview of recent findings on anatomical, molecular, cellular, and functional changes within the developing mPFC from juvenility through adulthood in rodent models. Anatomical organization and connectivity of the mPFC include distinct cytoarchitectural subregions such as the prelimbic (PL), infralimbic (IL), and anterior cingulate (ACC) cortices (Gabbott, 2005). Studies utilizing anterograde and retrograde tracing, electrophysiology, and optogenetic approaches have outlined specialized connectivity and functionality across these mPFC subregions (Gabbott et al., 2005; Vertes, 2006). For instance, the PL projects extensively to the basolateral amygdala and nucleus accumbens (Vertes, 2004), which are implicated in the expression of conditioned fear responses and reward-seeking behaviors, respectively (Peters, et al., 2009; Vertes, 2004; Likhtik et al., 2020). Additionally, the IL sends primarily GABAergic projections to intercalated cells in the amygdala and nucleus accumbens shell, which plays a role in suppressing conditioned fear and drug-seeking behaviors (Peters, et al, 2009). Kim et al. (2017) demonstrated that optogenetic photostimulation and slice electrophysiology recordings could reveal direct monosynaptic inhibitory projections from the IL to intercalated cells, effectively suppressing principal neuron activity in the central amygdala. Furthermore, the ACC integrates limbic inputs from areas including the basolateral amygdala, hippocampus, and thalamus to encode emotional salience (Etkin, et al., 2011). Rempel-Clower and Barbas (1998) highlighted dense reciprocal connectivity between the ACC and various amygdala nuclei, providing an anatomical basis for affective integration.

Additionally, superficial layers II/III and deep layer V of the mPFC exhibit distinct connectivity and roles. For instance, Varodayan et al. (2018) found that chronic ethanol drinking selectively increased dendritic spine maturation in PL layer II/III neurons. Moreover, retrograde tracing by Little et al. (2012) revealed PL layer V neurons preferentially project to the basolateral amygdala. Longitudinal anterograde tracing experiments have also demonstrated protracted strengthening of bidirectional connections between the mPFC, amygdala, and hippocampus across juvenile and adolescent periods before reaching mature connectivity patterns by adulthood (Meisner., et al., 2022; Kim., et al., 2011; Ishikawa & Nakamura, 2006). For example, Reppucci & Petrovich (2016) utilized anterograde tract-tracing with biotinylated dextran amine and showed sparse innervation from mPFC to amygdala early in life that progressively densifies to resemble adult patterns by late adolescence.

Specifically, they observed a 6-fold increase in mPFC fiber innervation of the basolateral nucleus of the amygdala from postnatal day 7 to 42.

Molecular Composition and Expression Patterns

Quantitative biochemical analyses, including western blotting, have identified significant molecular alterations in the developing rodent medial prefrontal cortex (mPFC) from juvenility to adulthood (Drzewiecki, Willing, & Juraska, 2016; Nasif, et al., 2011). For example, Drzewiecki et al. (2016) observed notable decreases in immediate early genes like c-Fos and Egr1, which are crucial in activity-dependent transcriptional regulation and plasticity. Concurrently, there were developmental increases in synaptic scaffolding proteins such as Homer1, linking metabotropic glutamate receptors to effector proteins (Drzewiecki, Willing, & Juraska, 2016).

Clifton (2019) utilized immunoprecipitation and mass spectrometry to demonstrate that Homer1 directly binds Shank3, facilitating the accumulation of metabotropic glutamate receptors in the postsynaptic density of excitatory synapses. Moreover, detailed characterizations of mRNA and protein levels through qPCR and immunoblotting have revealed dynamic expression patterns of glutamate receptor subunits (Willard & Koochekpour, 2013). Notably, a transient upregulation of NMDA receptors containing the GluN2B subunit in early adolescence, which is then replaced by GluN2A subunits in adulthood (Deutsch, et al., 2022).

Cellular Remodeling and Myelination

Analyses have shown substantial excitatory synapse elimination, particularly in the peri-adolescent female rodent mPFC around pubertal onset. Drzewiecki et al. (2016) employed pre-embedding immunogold cytochemistry to label excitatory synapses and noted a decline in asymmetric synapse density specific to adolescent females, indicating possible modulation by gonadal hormones. In contrast, male rodents displayed stable synapse density over this timeline, though future research should investigate qualitative synaptic changes. Additionally, several studies utilizing microscopy, immunohistochemistry, and western blotting have shown increased myelination of mPFC white matter pathways throughout adolescence (Vanes, et al., 2020).

Furthermore, in vivo two-photon imaging has provided insights into the remodeling dynamics of mPFC neuronal structures across postnatal maturation. Pan & Gan (2008) utilized chronic two-photon imaging of yellow fluorescent protein-expressing neurons, revealing that juvenile mice exhibit higher rates of dendritic spine formation and elimination, as well as axonal bouton structural plasticity compared to adults. The observed rate of bouton remodeling decreased significantly between 1 and 3 months of age. Increased structural dynamics early

in development likely enable the selective strengthening and pruning of connectivity patterns, with subsequent stabilization of neuronal structures relating to the maturation of mPFC circuitry supporting executive functions.

Functional Developmental Trajectory

Electrophysiological recordings and behavioral assessments have delineated the functional maturation of mPFC networks across juvenile, adolescent, and adult periods. For example, Konstantoudaki (2018) utilized a T-maze spatial working memory task dependent on intact mPFC function and found ongoing improvements in performance from early adolescence into adulthood, with adult levels of accuracy and premature responding not attained until after peri-adolescence, indicative of the prolonged development of executive control. Wang & Gao (2009) reported shorter EPSC decay kinetics and increased NMDA/AMPA ratios in juvenile rat mPFC neurons compared to adults using whole-cell patch clamp recordings, reflective of developmental alterations in glutamate receptor subunit composition.

Computational models suggest that synaptic scaling enables pruning of weak or irrelevant connections while reinforcing stronger, behaviorally relevant synapses (Holtmaat & Caroni, 2016). Such homeostasis mechanisms likely optimize the efficiency of working memory circuits over development. Furthermore, the precise strengthening of recurrent excitatory connections between dlPFC pyramidal neurons through spike-timing dependent plasticity may critically support the stabilization of persistent working memory networks (Casula, et al., 2008). Regarding cognitive flexibility, earlier maturation of prelimbic and anterior cingulate mPFC regions compared to posterior cortical association areas may facilitate the developmental shift from perseverative responding to more adaptive, context-appropriate decision-making (Klune, 2021). Lesion and disconnection studies in non-human primates have shown that integrating projections from mPFC outputs to sensory association regions like parietal cortex forms a key network for attentional set shifting and flexible rule learning when reward contingencies change (Jobson et al., 2021; Reinert et al., 2021). Additionally, computational models indicate strengthening cortico-cortical connectivity between PFC and sensory areas supports maturation of behavioral flexibility (Menon & D'Esposito, 2022). Ongoing myelination of these projections likely enables faster, efficient communication aiding executive control as well.

Finally, reciprocal projections between the ventromedial PFC and amygdala are critical for developing cognitive control over emotions. Rodent studies show pharmacological inhibition of mPFC NMDA receptors impairs fear extinction learning, implicating mPFC areas in top-down inhibitory regulation of the amygdala

(Kredlow et al., 2021). Human neuroimaging also reveals a developmental shift from diffuse amygdala activation to increased mPFC engagement when processing emotional cues, enabling regulatory cognitive control instead of reflexive emotional reactions (Gee et al., 2013). Furthermore, resting state functional connectivity analyses indicate mPFC-amygdala networks continue maturing through adolescence, as coupling becomes increasingly negative between childhood and adulthood (Gee et al., 2013). This developmental shift likely aids prefrontal regulation of emotional impulses. Additionally, animal studies suggest pubertal hormones modulate mPFC-amygdala circuit maturation and function, which could induce sex-specific emotional development patterns (Klune et al., 2021).

The prelimbic prefrontal cortex (PL) demonstrates notable sex differences during postnatal maturation. The PL contains projection neurons in deep layer 5 and superficial layers 2/3, but lacks a granular layer 4 typical of sensory cortices, instead excitatory projections from mediodorsal thalamus and interconnected cortices terminate directly in layer 3 pyramidal neurons and interneurons (Velasco, et al., 2015; Kolk & Rakić, 2021). Layer 5 pyramidal neurons drive output communication enabling higher cognitive functions via projections to areas like nucleus accumbens, amygdala, and hypothalamus (Urrutia-Piñones, 2022; Ramaswamy & Markram, 2015; Velasco, et al., 2015). In adolescence, females undergo an earlier declination in dendritic spine density on layer 2/3 neurons, indicative of synaptic pruning, while males exhibit more gradual spine elimination (Koss et al., 2014; Velasco, et al., 2015). While sexual divergence in structural reorganization has been documented for superficial layers, presence and details of pruning differences in layer 5 during adolescence remains unknown. Such developmental divergence contributes towards emergence of sex-biased psychiatric disorders involving PL.

The adjacent infralimbic cortex (IL) contains a granular layer 4 with clusters of excitatory and inhibitory neurons representing the main thalamic input layer, alongside layered output neurons in layers 2/3 and 5 (Vertes, 2004; Scala et al., 2019). The PL demonstrates preferential connectivity with the basolateral amygdala and greater encoding of reward cues, while the IL has tighter coupling to the hippocampus and hypothalamus alongside viscerosensory representations (Hoover & Vertes, 2011; Wood et al., 2018). These complementary medial prefrontal regions have an important functional imbalance in regulating anxiety - the PL has an anxiogenic role by amplifying threat reactions from the amygdala, while the IL can inhibit anxiety by attenuating emotional responses, perhaps through tonic dampening of PL neuronal excitability (Kenwood et al., 2021).

Several studies have indicated sex differences in the structural reorganization of pyramidal neuron microcircuits within the prelimbic prefrontal cortex (PL-PFC) over adolescence. Markham et al. (2007) and Drzewiecki et al. (2016) demonstrated that while both male and female rats showed increased spine density during early adolescence, only females exhibited reduction in basilar spine numbers later between mid to late adolescence. This post-pubertal pruning was further associated with the onset of estrous cycles around postnatal day 38, suggesting ovarian hormones could mediate synapse elimination. Furthermore, Mallya et al. (2018) reported extensive microglial engulfment of dendritic spines specifically around postnatal day 39 in females but not males. However, since overall spine density did not change, microglia could preferentially target select vulnerable synapses. Together, these golgi-based analyses indicate adolescence represents a pivotal period for reorganization of pyramidal cell microcircuitry and integration in the PFC, permitting the maturation of advanced cognitive abilities manifesting during the transition to adulthood. The reviewed research also highlights sexual divergence in anatomical trajectories which may confer differential vulnerability to neurodevelopmental insults like prenatal stress.

Synaptic pruning is a vital neurodevelopmental process, influenced by neural activity and environmental inputs, which enhances synaptic efficiency by eliminating redundant neuronal connections (Sakai, 2020; Faust et al., 2021). During adolescence, significant synaptic pruning occurs, especially in the prefrontal cortex, leading to the reorganization of neural circuits and specialization based on environmental pressures (Spear, 2013). This period allows for the integration of emotional, physiological, and cognitive inputs into prefrontal control circuits, essential for the development of mature executive functions (Friedman & Robbins, 2021; Casey et al., 2019). Disruptions in synaptic pruning, such as improper pruning or growth deficits, are linked to the emergence of psychiatric symptoms commonly observed during adolescence, with implications in disorders like schizophrenia (Germann et al., 2021).

Pathological Implications

The protracted development timeline of the prefrontal cortex (PFC) extending into the third decade renders it especially susceptible to disruption during adolescence, which could contribute to onset of certain psychiatric disorders prevalent in adolescence and young adulthood (Kolk & Rakić, 2021). Postmortem studies of schizophrenia patients have revealed several abnormalities in the dorsolateral PFC (dlPFC) compared to controls, including reduced pyramidal neuron spine density, altered spine morphology, and disrupted connectivity

patterns (Harrison, 2000). These dlPFC anomalies potentially relate to cognitive deficits frequently observed in schizophrenia, including working memory impairments, cognitive disorganization, and psychotic symptomatology.

FMRI studies have consistently found hypoactivation in the anterior cingulate cortex (ACC) subdivision of the prefrontal cortex (PFC) in adolescents and adults with major depressive disorder (MDD) (Korgaonkaret al., 2012). This is theorized to reflect impaired cognitive control processes subserved by this region, including emotion regulation, cognitive flexibility, and error monitoring. Altered functional connectivity between the PFC and emotion processing limbic structures like the amygdala also correlates with ineffective emotion regulation in depression (Kredlow, et al., 2021; Negrón-Oyarzo, et al., 2016). Developmental disruption of communication between the PFC and affective regions during sensitive windows could manifest in mood dysregulation later in life (Gee et al., 2013).

1.3. Synaptic Pruning

Role of Synaptic Pruning in Mental Health

The refinement of neural circuits through synaptic pruning represents a fundamental process guiding neurodevelopment. While neuronal proliferation, migration, and synapse formation predominate in early childhood, later developmental stages are characterized by selective elimination of exuberant synaptic connections (Faust, et al., 2021; Sakai, 2020; Huttenlocher & Dabholkar, 1997). This pruning refines neural circuits formed during plastic phases of development to increase efficiency and strengthen relevant pathways (Yu, 2022; Spear, 2013; Faust, et al., 2021). However, dysregulated synaptic pruning has emerged as a candidate mechanism underlying diverse neuropsychiatric disorders that frequently onset during adolescence, including schizophrenia, autism, and anxiety disorders (Keshavan, Anderson, & Pettegrew, 1994; Marín, 2012; Cordero et al., 2020). Elucidating the precise timing, mechanisms, and pathways involved in adolescent synaptic pruning will be imperative to clarifying its contributions to psychiatric pathogenesis and informing targeted interventions.

Epidemiological research has established that numerous psychiatric disorders first manifest during the adolescent period between puberty and young adulthood. The National Comorbidity Survey found that by age 25 the lifetime prevalence for any anxiety disorder is 29%, any mood disorder is 21%, impulse control disorders at 24%, and substance use disorders at 15%. A separate analysis estimated that up to 75% of adult mental

illnesses begin before age 24, indicating adolescence represents a peak period of onset (Kessler et al., 2005). The emergence of symptoms during this timeframe of ongoing neurodevelopment implicates synaptic pruning occurring throughout adolescence and early adulthood as a potential contributing factor to psychiatric disorders (Germann et al., 2021). Deficits in the pruning process required for maturation of prefrontal executive control and limbic emotional circuitry could underlie characteristic deficits seen in schizophrenia, anxiety, and mood dysregulation.

Dysregulated synaptic pruning manifesting in adolescence may embed pathological circuit wiring patterns that persist across the lifespan (Sakai, 2020). For example, a 30-year longitudinal study found that over 75% of individuals diagnosed with an anxiety disorder during adolescence continued experiencing impairing symptoms in adulthood (Pine, et al., 1998). Adult patients with schizophrenia and bipolar disorder who retrospectively reported childhood-onset psychotic symptoms exhibited greater cognitive and functional impairments compared to adult-onset cases, highlighting the lasting impacts of adolescent pathological processes (McCutcheon, et al., 2023). These findings indicate a failure of normal synaptic reorganization during critical windows of late neurodevelopment may engrain dysfunctional neural wiring underlying lifelong psychiatric disorder burden.

Convergent evidence indicates that the medial prefrontal cortex (mPFC) represents a nexus of adolescent synaptic reorganization relevant for psychiatric disorders (Paus, et al., 2008; Thapar &Riglin ; 2020; Blagburn-Blanco, et al., 2022). The mPFC undergoes protracted pruning of excitatory synapses throughout adolescence in parallel with maturation of executive functions like working memory, cognitive flexibility, and emotion regulation (Klune, et al., 2021). Patients with schizophrenia display reductions in mPFC gray matter volume and spine density, with more severe deficits in early onset cases, suggestive of over-pruned frontal circuitry (Moyer, et al., 2015). Resting hypoactivity of the mPFC also occurs transdiagnostically in mood and anxiety disorders, potentially reflecting immature connectivity (Dabiri et al., 2022). Investigating synaptic pruning during adolescence as a point of convergence contributing to diverse psychopathology is supported by extensive diagnostic comorbidity. Up to 60% of individuals with primary anxiety disorders meet criteria for another concurrent psychiatric disorder, most often depression, ADHD, or substance abuse (Hakobyan , et al., 2020; Weisberg, 2007; Strohle, et al., 2018).

Hallmarks of Adolescent Synaptic Pruning

Synaptic pruning refers to the process of selective elimination of synapses over the course of postnatal development, which serves to refine exuberant connectivity and strengthen relevant neural circuitry (Faust, et al., 2021). While occurring across the CNS, synaptic pruning in the prefrontal cortex (PFC) during adolescence exhibits signature features including experience-dependency, activity-dependence, regional specificity, and developmental timecourse (Faust., et al., 2021). Emphasizing these central hallmarks provides a conceptual framework for elucidating mechanisms and evaluating molecular candidates.

Experience-Dependent Elimination

A fundamental aspect of adolescent synaptic pruning is its dependence on environmental experiences to shape circuit refinement. Pioneering work by Changeux, Dan, and colleagues established the concept of selective stabilization, whereby correlated pre- and postsynaptic activity strengthens specific connections while unused circuits are eliminated according to Hebbian principles (Halvagal & Zenke, 2023). For example, sensory deprivation markedly reduces synaptic density in the primary visual cortex during visual critical periods when pruning is most active (Globus & Scheibel, 1967). Naturalistic experiences likely instruct pruning in the PFC as well, with executive task demands selectively maintaining relevant networks.

Activity-Dependent Pruning

Related to experience-dependency, the selectivity of synaptic pruning relies on activity-dependent mechanisms coupled to current neural firing patterns. Computational models demonstrate that activity-dependent competition successfully simulates the elaboration and pruning of youth followed by relative stability in adulthood evident in human anatomical studies (Huttenlocher, 1979). After early childhood peaks, synaptic density in the PFC declines at rates closely mirroring the maturation of coordinated network activity measured by EEG (Buchmann et al., 2011). Ambient glutamate signaling and synaptic stimulation may provide activity-dependent signals that locally tag weak dendritic spines for removal by pruning machinery (Featherstone, 2009).

Regional Specificity: Focus on Prefrontal Cortex

While synaptic pruning occurs throughout the brain, the PFC exhibits one of the most protracted pruning timecourses lasting through adolescence and into the third decade of life in humans, suggesting region-specific mechanisms (Faust et al., 2021). For example, expression profiling in rhesus monkeys found that gene networks linked to synaptic remodeling including MMP9 and TGFβ signaling components were upregulated specifically in

the dorsolateral PFC relative to primary motor cortex during puberty and adolescence (Urbanski et al., 2009; Zhou, et al., 2023). Regional differences likely contribute to the late functional maturation of executive circuits that rely critically on pruning frontal connectivity for efficiency. Understanding region-specific effects is key, as global manipulations cannot recapitulate the targeting of pathological pruning observed in psychiatric imaging studies.

Developmental Timing

The occurrence of synaptic pruning during sensitive windows of adolescence and early adulthood provides cues regarding its regulation. For instance, pubertal hormonal changes may help initiate pruning programs regionally as sex differences emerge in adolescent brain maturation trajectories (Laube., et al., 2020). Single cell sequencing reveals shifts in cortical glutamatergic, GABAergic and microglial transcripts around puberty onset in mice, followed by declining expression of synaptic genes in late adolescence suggestive of pruning (Armand, et al., 2021). While humans exhibit a more protracted timeline likely continuing into the third decade, characterizing neurodevelopmental dynamics around puberty in animal models provides insight into regulatory factors.

Molecular Mechanisms

Integrating the hallmarks of experience-dependent, activity-dependent, regional and developmental specificity constrains possible molecular mechanisms regulating synaptic pruning. Several important criteria emerge that align with features of α4βδ GABAAR signaling.

Activity-Dependent Mechanisms

The selectivity of adolescent pruning based on strengthening of active circuits and elimination of unused synapses implies reliance on neural activity-dependent signals. Molecular candidates must have expression or function modulated by neural firing to confer this coupling. For instance, extracellular proteases like MMP9 that degrade adhesion molecules to initiate synapse disassembly are secreted in an activity-dependent manner and their upregulation during adolescence is reversed by sensory deprivation (Wiera & Mozrzymas, 2021).

Regional Specificity

The regional specificity of pruning programs is likely mediated by restricted expression of pruning molecules in subsets of neurons. For example, C1ql3 is localized mainly in frontal cortex and hippocampus consistent with protracted synapse elimination in those areas (Martinelli et al., 2016). Regional specificity may

also arise from distinct combinations of receptor subtypes, cell types recruited, or signaling components engaged across brain areas. For example, specialized high affinity extra-synaptic α4βδ GABAARs are highly expressed in hippocampal regions undergoing pruning, suggesting unique inhibitory control mechanisms (Aoki et al., 2018).

Timing and Onset

Linking adolescent pruning mechanisms to the emergence of psychiatric disorders relies upon identifying molecules aligned with the developmental time course. Transcriptomic profiling reveals modules of genes regulating excitatory synapse function, GABAergic signaling, and immune function dynamically fluctuate across the peri-adolescent period in mice, representing candidate pathways. Changes in GABAergic transmission emerge around pubertal onset, including decreased expression of the chloride importer NKCC1 coordinating the developmental excitatory-to-inhibitory GABA switch (Tang et al., 2021). Pubertal increases in neurosteroid modulation also occur through upregulation of synthases like 3α-HSD (Shen et al., 2007).

Current Mechanisms and Their Limitations

Proposed synaptic pruning mechanisms range from glia-mediated phagocytosis to developmental apoptosis, but considerable gaps remain in accounting for key hallmark features of activity-, experience- and region-dependent adolescent pruning.

Microglia

Microglia have been widely hypothesized as the primary effector cells that selectively phagocytose and eliminate synapses targeted for pruning (Faust et al., 2021). Transcriptomics indicates that microglia upregulate phagocytic pathways postnatally in the hippocampus and visual cortex coinciding with peaks in pruning (Irfan et al., 2022). Ablating microglia or disrupting phagocytic signaling impairs developmental synapse loss in visual thalamocortical circuits (Dixon et al., 2021). However, global depletion studies cannot address specificity, and phagocytic signaling may involve synaptic remodeling beyond pruning. The degree to which microglia mediate experience- or activity-dependent elimination remains uncertain and challenged by new imaging studies. Their contribution to adolescent pruning specifically is also unknown.

Beyond microglia, other neuroimmune mechanisms like astrocytic engulfment, complement cascade proteins, and synaptic TNFα signaling have been implicated in developmental synapse loss, albeit with less experimental evidence (Bialas & Stevens, 2013; Chung et al., 2015). Apoptosis of redundant synaptic inputs is

another common theory, although not supported by a typical lack of cell death in critical period models (Moujalled etb al., 2021). Broader theories posit pruning is an activity-independent result of developmental synapse gene downregulation (Faust et al., 2021). However, this seems inconsistent with the timing of peri-adolescent pruning programs emerging. While elements of these mechanisms likely contribute in context-specific ways, none fully captures hallmark features.

α4βδ GABAAR Mediated Pruning in Adolescence

Recent findings position α4βδ GABAA receptor (GABAR) signaling as a novel mechanism aligning with hallmark features of synaptic pruning during adolescence (Afronz et al., 2016; Parato et al., 2019; Afronz., et al., 2017). Our preliminary results implicate these specialized receptors in initiating hippocampal remodeling.

We found genetic deletion or shRNA knockdown of the δ subunit, required for α4βδ receptor expression, blocks developmental spine pruning on both CA1 and dentate gyrus granule cell dendrites across pubertal maturation in mice (Afroz et al., 2017; Parato et al., 2019). Constitutive α4 knock-out similarly impairs pubertal spine pruning. In contrast, augmenting tonic inhibition through administration of the α4βδ agonist gaboxadol significantly promotes dendritic spine elimination in CA1 (Afroz et al., 2017; Sanchez et al., 2022). These results demonstrate a causal role for α4βδ signaling in adolescent hippocampal synapse pruning. Ongoing work is exploring circuit and behavioral impacts of dysregulated pruning.

As extra-synaptic receptors activated by ambient GABA, α4βδ GABARs are modulated by neural activity patterns, satisfying the activity-dependency feature (Brickley & Mody, 2012; Belelli, et al., 2009). Their specialized regional expression in hippocampal and prefrontal areas undergoing prolonged pruning confers specificity (Laurie et al., 1992). The emergence of δ subunit expression around puberty provides precise developmental timing cues aligning the pruning process (Laurie et al., 1992). Tonic inhibition mediated by α4βδ GABARs may represent an ideal signal for experience-dependent remodeling, as it is regulated by ongoing activity levels optimizing network wiring (Wei et al., 2003). Our future research will directly evaluate each of these hallmarks.

While our preliminary studies demonstrate α4βδ GABAR involvement in pubertal pruning in the CA1 hippocampus (Afronz et al., 2016; Afronz., et al., 2017), Parato et al. (2019) extended this to the CA3 region. Similar to CA1, Parato and colleagues found α4βδ expression increases specifically at puberty in the CA3 of female mice, which triggers substantial pruning of mushroom and stubby spine types. Knockout of α4 prevented

adolescent spine loss in CA3 and altered dendritic structure. Together with effects in CA1, these data indicate α4βδ GABAA receptors play a broader role initiating remodeling across the hippocampal circuit.

Ongoing work is investigating the intracellular signaling pathways and effector mechanisms coupled to these receptors that mediate the pruning process. A leading hypothesis we are exploring is that α4βδ receptor activation suppresses NMDAR-Kalirin7 signaling, which otherwise maintains dendritic spine integrity (Afroz et al., 2017). The RhoGEF Kalirin7 is highly expressed postsynaptically during adolescence and controls actin cytoskeletal dynamics underlying dendritic spine maintenance and remodeling (Xie et al., 2007).

Our model proposes that elevated tonic inhibition driven by α4βδ GABAARs suppresses synaptic NMDAR currents, preventing NMDAR-Kalirin7 pathway activation in weak or inactive spines (Afroz et al., 2017). Removing this stabilization then enables synapse disassembly mechanisms to proceed. We are testing this model through immunohistochemical, electrophysiological, and Golgi imaging and behavioral analyses.

Elucidating the pubertal mechanisms linking α4βδ GABAAR signaling to adolescent spine pruning will provide clearer understanding of how neural activity instructs this crucial developmental remodeling supporting cognition.

PFC Expression of α4βδ GABAARs

In situ hybridization and immunolabeling studies demonstrate that α4 and δ subunit expression within the PFC peaks during adolescence in mice, mirroring the timeline of synaptic pruning (Shu et al., 2012; Raol et al., 2006). δ subunit immunoreactivity localizes to peri- and extra-synaptic membranes of mPFC pyramidal neurons, consistent with extra-synaptic receptor positioning (Shu et al., 2012). Their distribution and physiology is suited for mediating adolescent PFC pruning.

Relevance for Psychiatric Disorders

Dysregulated synaptic pruning during adolescence is posited to contribute to later psychiatric illness, but clear biological mechanisms have been lacking. If α4βδ GABAARs prove necessary for experience- and activity-dependent spine elimination in the PFC, their disruption could contribute to inappropriate retention of inefficient connections. Failures of adolescent remodeling of mPFC circuits could manifest in executive function impairments characteristic of many neuropsychiatric disorders. For example, working memory depends critically on the precise tuning of recurrent excitatory connections between pyramidal neurons in the dorsolateral PFC (Lewis et al., 2002). Over-retained connections predicted by impaired α4βδ GABAAR-mediated pruning could

introduce noise disrupting working memory network firing. Interrogating specific circuit and behavioral consequences of deficits in α4βδ-dependent adolescent spine pruning will be imperative.

Our research aims to determine if dysregulated α4βδ GABAAR signaling aligns with the onset timing of heightened anxiety behaviors. Disrupting the typical trajectory of synaptic pruning during the peri-adolescent window may lead to abnormal retention of fear-related circuits. If α4βδ receptor activation proves necessary for spine elimination in prefrontal-amygdala projections, their loss could maintain excessive connectivity underlying anxiety. Many critical questions remain regarding the behavioral ramifications of impaired adolescent pruning that our ongoing studies will address.

1.4. α4βδ GABAA Receptors

Gamma-aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the mammalian central nervous system. Release from presynaptic terminals activates ionotropic GABAA and metabotropic GABAB receptors on the postsynaptic neuron to induce hyperpolarization and inhibit firing. Dysregulation of GABAergic transmission has been strongly implicated in anxiety and related disorders based on the therapeutic efficacy of benzodiazepines and similar GABAA receptor-targeting compounds. This section will provide key background on the diverse structure, regional distribution, and functional properties of GABAA receptor subtypes. A particular focus will be placed on extra-synaptic α4βδ receptors, which exhibit specialized characteristics like high sensitivity to ambient GABA that support distinct physiological roles in tonic inhibition. The unique neuromodulatory pharmacology of α4βδ and other isoforms will also be reviewed, highlighting potential for selective therapeutic targeting. Overall, appreciating the heterogeneity of GABAA receptor populations sets the stage for elucidating their differential contributions to synaptic signaling processes relevant in both health and disease.

GABAA receptors are ligand-gated chloride channels belonging to the Cys-loop superfamily of pentameric neurotransmitter receptors, which also includes nicotinic acetylcholine, serotonin type 3, and glycine receptors. Molecular cloning first identified 19 possible GABAA receptor subunits - α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ – that can assemble into distinct pentameric configurations, conferring variant properties (Luscher et al., 2011). For instance, synaptic receptors frequently incorporate α1, β2, and γ2 subunits which promote clustering and fast deactivation suited for phasic inhibition (Tretter, et al., 2008). In contrast, extra-synaptic populations

preferentially contain α4/δ and α5 variants along with β subunits, exhibiting high GABA sensitivity and slow desensitization ideal for tonic inhibition (Ghit, et al., 2021). While proteomic analysis has refined quantification of subtype distributions, continued improvements in subunit-specific tools will further elucidate native heterogeneity.

Initial topological inference from cDNA cloning revealed a conserved layout of large extracellular ligand-binding domains, four transmembrane regions, a large intracellular loop, and a short extracellular C-terminus (Miller & Aricescu , 2014). Recent cryo-EM structures have provided unprecedented views of pentameric assembly, conformational rearrangements involved in gating and desensitization, and binding sites for allosteric modulators (Kim & Hibbs, 2021). Ongoing challenges include capturing transient states and full-length receptors. Integrating emerging structural data with electrophysiology, genetics, and computations will enable multi-dimensional elucidation of structure-function relationships.

The combinatorial pentameric assembly underlies a diverse GABAA receptor repertoire with customized properties tuned to specialized functions. For instance, the γ2 subunit enables synaptic localization through scaffolding protein interactions, while α1 promotes fast deactivation suited for phasic transmission at synapses (Olsen & Sieghart, 2009). In contrast, α4/δ localize extra-synaptically and exhibit high GABA sensitivity and slow desensitization ideal for tonic inhibition. Further research on how heterogeneity arising from subunit compositions sculpts localization patterns, activation and desensitization kinetics, modulator pharmacology, and other properties continues to advance understanding of inhibitory signalling mechanisms.

Distribution

Early in situ hybridization mapping revealed overlapping mRNA expression for the α4 and δ subunits in the thalamus, dentate gyrus, cortex and amygdala, suggesting potential for co-assembly in these brain regions (Wisden et al., 1992). Detailed immunocytochemical analysis supports abundant co-localization of α4 and δ proteins, particularly in extra-synaptic membrane domains (Peng et al., 2002). Within cell populations expressing both subunits, α4 preferentially partners with δ over other potential subunit combinations, indicating specialized roles for α4βδ receptors compared to other α4-containing isoforms (Jia et al., 2005).

In the ventrobasal thalamus, double-labeling immunogold electron microscopy detects prominent co-clustering of α4 and δ subunits in extra-synaptic membrane domains of thalamocortical relay neurons (Peng et al., 2002). This is consistent with electrophysiological recordings demonstrating that α4βδ receptors generate

~80% of the tonic GABAA current in these neurons, which helps regulate thalamic oscillations and sensory transmission (Belelli et al., 2009). Stainings across rodent thalamus sections confirm enrichment of δ subunit immunoreactivity in nuclei containing thalamic relay neurons, including the ventrobasal, ventrolateral, and dorsal lateral geniculate nuclei (Peng et al., 2002).

Beyond the thalamus, hippocampal dentate granule neurons represent another key locus of α4βδ receptor expression (Payne et al., 2006). In situ hybridization and immunolabeling experiments indicate prominent δ subunit mRNA and protein expression in the dentate gyrus, where α4βδ receptors mediate tonic inhibition modulating neuronal excitability (Brooks-Kayal, et al., 1999).

Cortical mapping also detected high levels of δ subunit expression in layers II-VI as well as the amygdala (Peng et al., 2002). Electrophysiological recordings identified a tonic GABAA current generated by δ subunit-containing receptors in layer II/III cortical and amygdalar pyramidal neurons (Drasbek & Jensen, 2006). One study combining qPCR and electrophysiology found that δ subunit knockdown reduced tonic current by approximately 50% in cortical pyramidal neurons, with a lesser contribution from α5-containing receptors (Bonin et al., 2007). Within hippocampal areas, α4βδ receptors contribute to tonic inhibition not only in dentate gyrus granule cells, but also in some interneuron populations (Maguire et al., 2014). Antibody labeling also demonstrates aggregation of α4 and δ subunits at peri- and extra-synaptic sites on hippocampal interneurons and pyramidal neurons (Afronz et al., 2016; Afronz., et al., 2017). Therefore, beyond mediating thalamic tonic current, α4βδ receptors serve specialized local inhibitory functions across cortical and limbic networks.

Regional, cellular, and subcellular patterning of receptor expression governs distinct physiological roles. For instance, the exclusive extra-synaptic localization of α4βδ receptors in thalamic relay neurons allows them to mediate widespread tonic current (Bright et al., 2007). Their absence from synapses prevents rebound burst firing in these cells after phasic GABA release (Bright et al., 2007). In contrast, the peri- and extra-synaptic localization in hippocampal and cortical neurons enables δ-receptors to shape inhibitory synaptic signaling through spillover activation or cross-talk with synaptic isoforms (?). Ongoing analysis of native α4βδ receptors at the anatomical and ultrastructural levels continues to link their specialized expression profiles with localized functional impact.

Recent studies utilizing genetic reporting tools have enabled mapping of δ subunit localization with cell-type specificity. A δ subunit reporter mouse uncovered expression in diverse interneuron classes, including

parvalbumin-positive fast-spiking cells and somatostatin-positive low threshold-spiking cells, further expanding potential roles in cortical inhibition (Glykys et al., 2017). Another reporter mouse detected highest densities of δ-expressing neurons in the dentate gyrus, cortical layer II/III, reticular thalamic nucleus, and inferior colliculus, recapitulating regions identified through other methods (Wu et al., 2020). These genetic reporting tools provide a means to isolate specific cell types that preferentially utilize δ subunit-containing receptors. Ongoing efforts to profile δ subunit expression across cell types and brain regions continue to elucidate the specialized physiological functions of α4βδ receptors.

Pharmacology

The inclusion of the δ subunit in α4βδ receptors yields a pharmacological profile distinct from classical benzodiazepine-sensitive GABAA receptors. Radioligand binding assays first demonstrated the failure of benzodiazepines like diazepam and flunitrazepam to displace channel blocker binding from α4/δ-containing receptors (Chandra, 2008). Functional characterization of recombinant α4βδ receptors confirmed their insensitivity to modulation by benzodiazepines such as diazepam and zolpidem as well as related agonists like bretazenil that potentiate α1/2/3/5βγ receptors (Brown et al., 2002). The benzodiazepine antagonist flumazenil also fails to inhibit GABA currents mediated by α4βδ receptors as it does at classical subtypes. Instead, triazolo-benzodiazepines like Ro15-4513 and Ro15-3505 positively modulate α4βδ receptor activity through a distinct binding site, suggesting subtype-selective modulatory effects (Brown et al., 2002).

While insensitive to benzodiazepines, α4βδ receptors retain modulation by barbiturates, neurosteroids, and other general anesthetics similar to classical subtypes (Herd et al., 2007). For instance, the intravenous agent etomidate enhances GABA activation of α4β3δ, α4β2δ, and α4β1δ receptors, exhibiting even higher positive cooperativity than at α1β3γ2 receptors (Belelli et al., 2002). Photolabeling identified etomidate and barbiturate binding at inter-subunit transmembrane pockets distinct from the benzodiazepine site (Forman & Stewart, 2011). Structure-activity relationship studies have defined steroid structural motifs conferring selectivity and efficacy at δ-containing receptors (Joshi & Kapur, 2016).

Selective pharmacological tools have been developed to probe α4/δ-containing receptors, including inhibitors and negative allosteric modulators. For example, Zn2+ inhibits α4β3δ receptors with high potency by a voltage-independent blocking mechanism (Sexton et al., 2021). The β1/3 subunit-selective compound

salicylidene salicylhydrazide inhibits α4βδ receptor function while sparing common α1/3βγ synaptic subtypes, providing a tool to selectively target extra-synaptic receptors mediating tonic current (Dalby et al., 2020). Other antagonists like DS1 and DS2 also preferentially inhibit δ-containing receptors compared to γ-containing counterparts, with molecular modeling suggesting an intrasubunit δ binding site (Zhang et al., 2009). These pharmacological probes demonstrate the feasibility of developing novel δ-selective compounds.

Multiple lines of evidence demonstrate that incorporation of the δ subunit is both necessary and sufficient to confer a diazepam-insensitive phenotype with high GABA sensitivity. However, the rules governing assembly of native α4/δ-containing receptors remain unclear. Understanding the precise subunit stoichiometry and conformational dynamics associated with modulation by different ligands will be important for elucidating the nuances in their pharmacological profiles. Recent advances in techniques like cryo-electron microscopy, genome editing, and electrophysiology are enabling new insights into the structure-function relationships and pharmacological selectivity of these receptors.

Physiological Roles

A major function of α4βδ receptors in key brain regions is to sustain a tonic GABAA conductance that regulates neuronal excitability. In thalamocortical relay neurons of the rodent ventrobasal complex, where they represent the predominant extra-synaptic GABAA receptor subtype, α4βδ receptors generate a persistent inhibitory current by activating in response to low ambient GABA levels (Chandra et al., 2006). Through this dynamic clamping of membrane potential, α4βδ receptor activity modulates burst firing modes and oscillatory thalamic rhythms involved in sensory processing and cognition (Uygun & Basheer, 2022).

Pharmacological agents that enhance tonic inhibition via positive modulation of α4βδ receptors exhibit anticonvulsant effects in animal models, while genetic deletion of the δ subunit causes absence seizures (Errington, et al, 2011; Smith, 2013). α4βδ receptors also help maintain tonic inhibition of dentate gyrus granule cells, fast-spiking interneurons, and projection neurons of the basolateral amygdala, gating network excitability and behaviors related to memory, anxiety, and fear (Koh et al., 2023). For example, tonic inhibition mediated by α4βδ receptors on dentate gyrus granule cells regulates their excitability and controls mood-related behaviors, while α4βδ receptor expression on basolateral amygdala projection neurons modulates anxiety and conditioned fear responses (Botta et al., 2015). Enhancement of tonic conductance through these populations of α4βδ

receptors elicits anxiolytic and antidepressant-like effects in rodent behavioral models (Botta et al., 2015; Lin et al., 2014). Therefore, beyond mediating thalamic tonic current, α4βδ receptors serve important local inhibitory functions in cortical and limbic brain regions that influence cognition, emotionality, and behavioral outputs.

In addition to mediating tonic inhibition, peri- or extra-synaptic α4βδ receptors can shape the timecourse and kinetics of phasic synaptic signaling through mechanisms such as spillover. The high GABA affinity and slow deactivation kinetics of α4βδ receptors make them well suited to detect volume transmission of inhibitory signals. Simulations suggest ambient GABA transients in the extracellular space occurring on a timescale of seconds to minutes could dynamically regulate tonic current mediated by high-affinity α4/δ receptors (Scimemi, 2014).

Recent studies also highlight more complex and dynamic interactions between synaptic and extra-synaptic α4βδ receptors. For instance, in mature dentate granule cells that maintain low intracellular chloride, δ-GABAARs amplify shunting inhibition produced by α1-GABAARs on distal dendrites, demonstrating functional cooperation between receptor populations (Panzanelli et al., 2011). Activity-dependent plasticity of α4βδ receptor expression and localization may represent another form of dynamic regulation on homeostatic timescales (Peng et al., 2010).

Pathophysiological Roles

Dysfunction or altered expression of α4βδ receptors has been implicated in a variety of neurological diseases and psychiatric disorders involving impairment of tonic GABAA inhibition. For example, in mouse models of temporal lobe epilepsy, there is downregulation of δ subunit expression in dentate gyrus granule cells, which compromises tonic inhibition and promotes hyperexcitability (Peng et al., 2004). However, changes in humans are less clear as post-mortem studies have not consistently shown altered δ subunit expression. Enhancement of α4βδ receptor activity exerts anticonvulsant effects in some animal models (Glykys et al., 2008), suggesting these receptors play a protective role in certain types of epilepsy. However, reduced tonic current may not always be pro-epileptogenic as enhancing tonic inhibition has also been shown to promote absence seizures in rodent models (Cope et al., 2009).

More research is needed to clarify the role of α4βδ receptors in different types of epilepsy. While selective reduction of α4 and δ subunit expression has been reported in thalamic relay neurons in some genetic rat models of absence epilepsy (Smith et al., 2015), other studies have found no change or even increased

expression (Liu et al., 2018). The effects of modulating thalamic tonic inhibition on spike-wave discharges also remains unclear (Jones et al., 2017). Differences in rat strains, seizure models and brain regions studied may account for some of the variability across studies. Overall, the evidence linking thalamic α4βδ receptor dysfunction to absence seizures is inconsistent at present.

III. Objective and Aims

Research Overview

Adolescent synaptic pruning in the prefrontal cortex is a pivotal process in cognitive development, yet the molecular and cellular mechanisms governing this process are not fully understood. Preliminary data from our lab suggest that extra-synaptic GABAAA receptors containing the α4βδ subunits play a role in synaptic pruning within the hippocampus. This research proposal aims to investigate the function of α4βδ subunits and their downstream signaling pathways in the synaptic pruning of layer 5 pyramidal neurons in the mouse prelimbic prefrontal cortex.

Objective

To elucidate the molecular and cellular mechanisms by which α4βδ GABAAA receptors regulate dendritic spine pruning in layer 5 pyramidal neurons of the prelimbic prefrontal cortex during adolescence, and to assess the behavioral consequences of dysregulated pruning on anxiety-related behaviors.

Central Hypothesis

The pubertal onset of α4βδ GABAAA receptor expression initiates synaptic pruning in layer 5 pyramidal neurons of the prelimbic prefrontal cortex, primarily by inhibiting NMDAR-Kalirin-7 (Kal7) signaling pathways. Dysregulation of this process is hypothesized to contribute to abnormal anxiety-related behaviors.

Aim 1: Characterize the timeline and regulation of dendritic spine pruning in layer 5 prelimbic cortex using morphological and pharmacological techniques.

- Use Golgi staining and 3D reconstruction to quantify spine density and morphological subtypes (mushroom, stubby, thin, filopodia) on basal dendrites of layer 5 pyramidal neurons at puberty onset, late puberty, and adulthood in female C57BL/6 mice.
- Determine effects of global constitutive α4 knock-out on spine pruning across adolescence using Golgi staining and analysis of α4-/- mice.
- Assess impacts of enhancing tonic inhibition with the α4βδ-selective agonist gaboxadol (0.1 mg/kg IP) or blocking GABAARs with the antagonist picrotoxin (3 mg/kg IP) during puberty on subsequent spine density.

Aim 2: Identify molecular mechanisms downstream of α4βδ receptors regulating spine pruning using immunohistochemistry.

- Quantify expression levels of α4 subunits across adolescence in layer 5 prelimbic cortex using immunohistochemistry.
- Determine effects of α4 knock-out on expression of the spine protein Kal7 at puberty onset using immunohistochemistry.
- Determine if α4βδ receptor expression regulates levels of the spine protein Kal7 using immunohistochemistry.

Aim 3: Examine impacts of dysregulated pruning on anxiety-related behavior.

- Selectively knock down α4βδ receptors in prelimbic cortex at puberty onset using stereotactic injection of AAV-Cre into homozygous α4-floxed mice.
- Analyze effects on spine density at late puberty using Golgi staining.
- Assess avoidance behavior on the elevated plus maze following shock sensitization in adolescence and adulthood after selective α4βδ knockdown.

Aim 4: Investigate Layer 2/3 in Wild-Type and α4 Knockout Mice

- Utilize Golgi-Cox staining to analyze dendritic spine density and morphology on basal dendrites of layer pyramidal neurons at the onset of puberty and late puberty in both wild-type and α4 knockout mice.

IV. Layer 5 Prelimbic and Anxiety

Aim 1: **Characterize the timeline and regulation of dendritic spine pruning in layer 5 prelimbic cortex using morphological and pharmacological techniques.**

- Use Golgi staining and 3D reconstruction to quantify spine density and morphological subtypes (mushroom, stubby, thin, filopodia) on basal dendrites of layer 5 pyramidal neurons at puberty onset, late puberty, and adulthood in female C57BL/6 mice.
- Determine effects of global constitutive α4 knock-out on spine pruning across adolescence using Golgi staining and analysis of α4-/- mice.
- Assess impacts of enhancing tonic inhibition with the α4βδ-selective agonist gaboxadol (0.1 mg/kg IP) or blocking GABAARs with the antagonist picrotoxin (3 mg/kg IP) during puberty on subsequent spine density.

Aim 2: Identify molecular mechanisms downstream of α4βδ receptors regulating spine pruning using immunohistochemistry.

- Quantify expression levels of α4 subunits across adolescence in layer 5 prelimbic cortex using immunohistochemistry.
- Determine effects of α4 knock-out on expression of the spine protein Kal7 at puberty onset using immunohistochemistry.
- Determine if α4βδ receptor expression regulates levels of the spine protein Kal7 using immunohistochemistry.

Aim 3: Examine impacts of dysregulated pruning on anxiety-related behavior.

- Selectively knock down α4βδ receptors in prelimbic cortex at puberty onset using stereotactic injection of AAV-Cre into homozygous α4-floxed mice.
- Analyze effects on spine density at late puberty using Golgi staining.
- Assess avoidance behavior on the elevated plus maze following shock sensitization in adolescence and adulthood after selective α4βδ knockdown.

1.5. First-Authored Publication

Preventing adolescent synaptic pruning in mouse prelimbic cortex via local knockdown of α4βδ GABAA receptors increases anxiety response in adulthood.

Matthew R. Evrard, Michael Li, Hui Shen, & Sheryl S. Smith

Scientific Reports, 11(1), 21059. https://doi.org/10.1038/s41598-021-99965-8

Abstract

Anxiety is increasingly reported, especially in adolescent females. The etiology is largely unknown, which limits effective treatment. Layer 5 prelimbic cortex (L5PL) increases anxiety responses but undergoes adolescent synaptic pruning, raising the question of the impact of pruning on anxiety. Here we show that preventing L5PL pruning increases anxiety in response to an aversive event in adolescent and adult female mice. Spine density of Golgi-stained neurons decreased ~63% from puberty (~PND35, vaginal opening) to post-puberty (PND56, $p < 0.0001$). Expression of α4βδ GABAA receptors (GABARs) transiently increased tenfold in L5PL at puberty ($p < 0.00001$) but decreased post-pubertally. Both global and local knockdown of these receptors during puberty prevented pruning, increasing spine density post-pubertally ($p < 0.0001$), an effect reversed by blocking NMDA receptors (NMDARs). Pubertal expression of the NMDAR-dependent spine protein kalirin7 decreased (50%, $p < 0.0001$), an effect prevented by α4 knock-out, suggesting that α4βδ-induced reductions in kalirin7 underlie pruning. Increased spine density due to local α4 knockdown at puberty decreased open arm time on the elevated plus maze post-pubertally (62%, $p < 0.0001$) in response to an aversive stimulus, suggesting that increases in L5PL synapses increase anxiety responses. These findings suggest that prelimbic synaptic pruning is necessary to limit anxiety in adulthood and may suggest novel therapies.

Introduction

More people suffer from anxiety than any other mental disorder (Kessler et al., 2005). While anxiety is a normal physiological state necessary to avoid danger, chronic anxiety results in maladaptive and excessive avoidance, which can interfere with cognitive functioning and quality of life (Kessler et al., 2005). Since their initial classification as mental disorders in the 1980s, anxiety disorders have outpaced all others (Hantsoo & Epperson, 2017). The most recent estimates suggest that, in less than a decade, anxiety has increased by 40% in adults (Goodwin et al., 2020) and by as much as 75% in adolescents (Goodwin et al., 2020). Anxiety disorders are most

likely to develop in adolescence (Hantsoo & Epperson, 2017), but left unrecognized, are the root of adult anxiety (Pine et al., 1998). This is especially relevant now as many reports indicate a substantial increase due to the Covid-19 pandemic (Loades et al., 2020). Anxiety is especially pronounced in response to stressful, aversive stimuli (Grillon et al., 2008; Grupe & Nitschke, 2013). However, this disorder's etiology is unknown at the circuit level, and approximately half of the affected individuals do not receive effective treatment (Paus et al., 2008). Thus, gaining insight into the CNS substrates that are vulnerable during adolescence would lead to more effective therapies to reduce anxiety across the lifespan.

The brain circuits involved in anxiety expression include the medial prefrontal cortex (mPFC), amygdala, and ventral hippocampus (Adhikari et al., 2010). Sub-regions of the mPFC include the prelimbic (PL), which has excitatory projections to the amygdala and is associated with negative emotions such as fear and anxiety (Vidal-Gonzalez et al., 2006) which are triggered by an aversive stimulus (Motzkin et al., 2015; Simpson et al., 2001). Lesions and pharmacological inactivation of the PL reduce anxiety in rodents (Shah et al., 2004). In contrast, the adjacent infralimbic mPFC (IL) has inhibitory control over the amygdala (Rosenkranz & Grace, 2002) and is associated with fear extinction (Milad & Quirk, 2002) and decreased expression of anxiety (Suzuki et al., 2016).

Activity of the output layer 5 (L5) neurons in the PL can generate anxiety behavior which is dependent upon a critical density of the dendritic spines that receive long-range and short-range excitatory synaptic inputs (Riga et al., 2014). At puberty, dendritic spines and grey matter in the human and non-human mPFC decrease by half (“synaptic pruning”) (Huttenlocher, 1979; Koss et al., 2014), which, in the human, is paralleled by EEG changes linked to pubertal maturation (Campbell et al., 2012). The PL, specifically, has been shown to undergo extensive synaptic pruning (Pattwell et al., 2016) in adolescence, while the adjacent IL does not (Pattwell et al., 2016). However, the role of adolescent PL pruning in regulating anxiety behavior is not yet known, nor are the consequences of reduced pruning in adolescence, which would produce increased excitatory input to this area.

This study addressed this issue by assessing dendritic spine density in L5 PL after the onset of puberty (~PND 35, assessed by vaginal opening) compared with post-puberty (PND 56). We examined these changes in female mice because anxiety is most likely to afflict females (Hantsoo & Epperson, 2017). To manipulate pruning in this area, we first examined a potential mechanism, an atypical GABAA receptor (GABAR), α4βδ (Shen et al., 2010), which expresses on dendritic spines at puberty as well as along the dendritic shaft and on the soma in some CNS regions to inhibit synaptic input. In contrast to typical GABARs, which express post-synaptically to

GABAergic interneurons, α4βδ GABARs express away from GABAergic synapse, have a high sensitivity to ambient GABA, which is maintained by GABA transporters (Wu et al., 2001), and display little desensitization (Brown et al., 2002).

Inhibition impairs the activation of NMDA receptors (Alvarez et al., 2007), which are necessary for spine maintenance (Ma et al., 2003). Thus, increases in inhibition generated by increased α4βδ GABARs in L5 PL at puberty is a potential mechanism for synapse pruning of this region as shown for other CNS areas (Afroz et al., 2016).

For the present study, we examined the role of α4βδ receptors in mediating pubertal synaptic pruning using both pharmacological and genetic tools. Selective deletion of these receptors in PL at puberty using viral delivery of Cre recombinase to a mouse with loxP (locus of X-over P1) sites flanking the α4 gene allowed us to determine if a high spine density in the PL, in the absence of pruning, increases anxiety-like behavior. We used the shock-paired elevated plus maze (EPM) to assess avoidance, which has been validated as a measure of anxiety level in humans (Biedermann et al., 2017) and to more closely compare with clinical studies showing PL activation triggered by an aversive event (Motzkin et al., 2015; Simpson et al., 2001). Additional experiments investigated the role of α4βδ impairment of NMDAR activation and subsequent reduction in Kal-7 levels at puberty on synaptic pruning. Understanding the role of α4βδ GABARs in mPFC pruning during adolescence is highly relevant for understanding mechanisms that underlie mental disorders such as anxiety and depression, where abnormal expression of α4 and/or δ has been reported (Feng et al., 2010; Merali et al., 2004; Sequeira et al., 2009).

Method

Animals

For most studies, C57BL/6 wild-type (WT, Jackson Labs) or GABAR α4−/− female and male mice were housed under a reverse light: dark cycle (12:12) and tested in the light phase. α4−/− mice were bred on site from α4 ± mice (supplied by G. Homanics, U. Pitt.). (WT and α4+/+ display similar spine densities.) For the Golgi studies, animals were euthanized at the onset of puberty (females, ~PND35, assessed by vaginal opening; males, ~PND 37; Corre et al., 2016) or PND 56 for spine density analysis. Animals were tested for α4 immunoreactivity and electrophysiological responses pre-pubertally (~PND 28–32), 1–2 days after the onset of

puberty, and post-pubertally (PND 56). The estrous cycle is not a factor during the pubertal period (PND 35–44; Shen et al., 2007) but the estrous stage was determined for animals euthanized on PND 56 using vaginal smears (Sabaliauskas et al., 2014) to avoid proestrus when GABAR expression and dendritic spine counts can be increased (Woolley & McEwen, 1992; Sabaliauskas et al., 2012).

For drug administration studies, all animals were injected once daily (intraperitoneally) with the following drugs from PND 35 (onset of puberty) to PND 49, the period of high α4 expression: gaboxadol (GBX, THIP, 4,5,6,7-tetrahydroisoxazolopyridin-3-ol), 0.1 mg/kg, a dose which has no effect in α4−/− mice (Afroz et al., 2017); picrotoxin, 3 mg/kg; lorazepam (LZM), 0.25 mg/kg; MK-801 ([5R,10S]-[+]-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine), 0.1 mg/kg, a dose which increases NMDAR expression in mPFC (Xi et al., 2009); memantine (1-Amino-3,5-dimethyladamantane), 10 mg/kg, an NMDAR blocker which does not increase NMDAR expression (Cole et al., 2013). In all experimental procedures, mice were randomly assigned to experimental groups, and the investigator was blinded to the condition of the mice. All procedures were approved by the SUNY Downstate Medical Center institutional animal care and use committee and carried out in accordance with their guidelines and regulations.

Immunohistochemistry

Following anesthesia with urethane (0.1 ml 40%), mice were perfused with saline (12–15 ml/min) and then with 4% paraformaldehyde (PFA) followed by post-fixation of the brain in 4% PFA (48 h, 4 °C).

Paraffin-embedded sections

PFA preserved brains were embedded in paraffin blocks following tissue dehydration using increasing ethanol concentrations. Coronal sections of the mPFC were cut on a microtome at a thickness of 10 μm and mounted on super-frost slides. Tissue was de-paraffinized in decreasing concentrations of ethanol and processed using antigen retrieval: Slides were incubated in warm (95–100 °C) sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 30 min, allowed to cool, and rinsed (2×) with 0.01 M phosphate-buffered saline (PBS), 0.05% Tween 20 (PBS-Tween) for 2 min.

Free-floating sections

Coronal sections of the mPFC were cut on a vibratome (Leica VT 100 M) at a thickness of 30–40 μm. Free-floating sections were washed (3x) in PBS-Tween with 1% bovine serum albumin (BSA) for 10 min.

Immunohistochemistry protocol

Sections were blocked in PBS supplemented with 1.5% donkey serum (kalirin) or 1.5% goat serum (α4, Cre) in PBS-Tween 2 h at room temperature. α4: Sections were incubated in blocking buffer containing 2% goat anti-mouse Fab fragments (Jackson Immunolabs, Bar Harbor, ME) for 2 h at room temperature. Then, sections were incubated with anti-α4 (mouse monoclonal, Antibodies, Inc., Davis, CA, 1:100). In some cases, anti-α4 (goat polyclonal, sc7355, Santa Cruz, 1:20) with anti-MAP2 (microtubule-associated protein-2, ab5392, Abcam, Cambridge, MA, 1:1000) were used without pre-incubation with the anti-mouse Fab fragments to verify α4 localization on dendritic spines. Both antibodies show selectivity for α4 as evidenced by their lack of staining in the hippocampus of α4 knock-out mice shown here and in a previous publication (Sabaliauskas et al., 2012). Although MAP2 is localized to the soma and dendrites, it can also be localized to spines and has been used as a spine marker (Caceres et al., 1983; Morales & Fifkova, 1989; Amateau & McCarthy, 2002). MAP2 is primarily localized to mushroom spines (Kim et al., 2020) which are one of the predominant spine types at puberty. Therefore, we used MAP2 to visualize dendrites and spines at puberty.

Kalirin, Cre, NMDAR1

Anti-kalirin-7 (Kal-7, rabbit polyclonal, a generous gift from R Mains, UConn Health, JH295885, 1:200), anti-Cre (rabbit polyclonal, Novus Biologicals, Centennial, CO, 1:1000) or anti-NMDAR1 (rabbit monoclonal, ab274377, Abcam, Cambridge, MA, 1:100) were used.

General: All antibodies were diluted in the blocking solution and incubated with tissue sections overnight at 4 °C. After washing, sections were incubated with the appropriate fluorescent secondary antibody (Alexa fluor 488 and 594, 1:1000) for 2 h, washed in PBS 3× for 10 min, after which they were mounted on slides with ProLong Glass antifade reagent in some cases with 5% nuclear blue. Images were taken with an Olympus FluoView FV1000 confocal inverted microscope with objective UPLSAPO 40× or 100× NA:1:30 (Olympus, Tokyo, Japan). For the immunohistochemical analysis, the merged z-stack image (2 μm steps) was used. Image segmentation was first performed using a thresholding sub-routine in ImageJ so that the original color image was converted to a binary image. This allowed for visualization of the regions of interest (ROI) in cases where the background intensity was non-homogeneous. ROIs were then analyzed for image luminosity in the original image using Adobe Photoshop after subtracting the adjacent background levels, and the results were verified by ImageJ. 3 ROIs were analyzed per mouse.

Golgi procedure

Before euthanization, mice were anesthetized with urethane (1–2 g/kg, i.p.; Afroz et al., 2017) and whole brains were extracted and processed for Golgi impregnation with the FD Neurotechnologies Rapid Golgi Stain kit. Coronal sections were prepared using a vibratome (Leica VT1200s) set to a thickness of 250 µm.

Analysis

Pyramidal cells from L5 PL were identified using The Mouse Brain in Stereotaxic Coordinates (4th Edition, Paxinos, and Franklin, 2012) and the Allen Brain Institute's Mouse Brain Atlas (http://mouse.brain-map.org). The L5 PL neurons were approximately 1.7 mm ventral from the dorsal surface and the cell bodies were 500–700 µm from the medial surface. Individual neurons in these regions were viewed using a 100× oil objective on a Nikon Eclipse Ci-L microscope. Images of the basilar dendrites were acquired using Z-stack projection photomicrographs (0.1–0.9 µm steps) taken using a Nikon DS-U3 camera mounted on the microscope and were analyzed using NIS-Elements D 4.40.00 software. Three to four neurons (middle 80%) were sampled per mouse, and six segments in the same field of view were assessed per neuron (20–50 µm). Each dendrite segment was ~1 µm thick and was taken from a 2° or 3° order dendrite. Spine density was expressed as the number of spines/10 µm. To determine the type of dendritic spine, we used parameters described by Risher et al. (2014): filopodia, length > 2 µM; long thin, length < 2 µM; thin, length < 1 µM, stubby, width ratio < 1 µM, mushroom, width > 0.06 µM; bifurcated, two or more heads.

Electrophysiology experiments were conducted by Hui Shen

Electrophysiology

Cortical slice preparation

Brains from euthanized mice were removed and cooled using an ice-cold solution of artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 124, KCl 2.5, CaCl2 2, NaH2PO4 1.25, MgSO4 2, NaHCO3 26, and glucose 10, saturated with 95% O2, 5% CO2 and buffered to a pH of 7.4. Following sectionin[1]g at 400 µm on a Leica VT1000S vibratome, slices were incubated for 1 h in oxygenated aCSF.

Cortical slice voltage-clamp electrophysiology

Pyramidal cells in L5 PL were visualized using a differential interference contrast (DIC)-infrared upright Leica microscope and recorded using whole-cell patch clamp procedures in voltage clamp mode at 26–30 °C (Shen et al., 2007). Patch pipets were fabricated from borosilicate glass using a Flaming-Brown puller to yield open tip resistances of 2–4 MΩ. For recordings of the pharmacologically isolated tonic inhibitory current, the pipet solution contained in mM: CsCl 140, HEPES 5, EGTA 5, CaCl2-H2O 0.5, QX-314 5, Mg-ATP 2, Li-GTP 0.5, pH 7.2, 290 mOsm. 5 mM QX-314 was added to block voltage-gated Na+ channels and GABAB receptor-activated K+ channels. The aCSF contained 50 µM kynurenic acid to block

The aCSF contained 50 µM kynurenic acid to block excitatory current, as well as 0.5 µM TTX to isolate the post-synaptic component. Recordings were carried out at a −60 mV holding potential, and the tonic current was assessed by the change in holding current in response to 100 nM gaboxadol (GBX), a GABAR agonist which, at this concentration, is selective for δ-containing GABARs (Brown et al., 2002; Meera et al., 2011). The GABAergic nature of the current was verified by block with 100 µM picrotoxin. Drugs were bath applied continuously in sequential order following 5–10 min of baseline recordings without drugs. Recordings were conducted with a 2 kHz 4-pole Bessel filter at a 10 kHz sampling frequency using an Axopatch 200B amplifier and pClamp 9.2 software. Electrode capacitance and series resistance were monitored and compensated; access resistance was monitored throughout the experiment, and cells were discarded if the access resistance increased more than 10% during the experiment. In all cases, the data represent one recording/animal.

Anxiety response assessment using the elevated plus maze

Mice were tested for anxiety-like behavior using the shock-paired elevated plus maze (EPM), an established model of anxiety (Pellow et al., 1995), which assesses avoidance behavior, on PND 56 or PND 90 following local α4 knockdown at puberty in response to AAV-Cre infusion on PND 21. Local knock-down was verified with immunohistochemical techniques after the behavioral test. We tested anxiety in response to an aversive stimulus to mimic human studies, which show mPFC regulation of anxiety in response to aversive settings (Motzkin et al., 2015; Simpson et al., 2001). Results were compared with the GFP control (AAV-GFP infusion on PND 21). The plus-maze consists of four 8 × 35 cm arms at 90° angles, elevated 57 cm above the floor (Figure 1c inset). 33 cm walls enclose two arms, and two arms have no walls (“open arms”). The open arms are also partially bordered by small rails (5 × 15 cm) extending to the proximal half of the arm, and the floor of the maze is marked with grid lines every 25 cm. Each animal was initially acclimated to the room for 30 min–1 h.

Then, mice were administered a 400-μA shock for 1 s (Rosenkranz & Grace, 2002) immediately before being placed in the maze center when exploratory activity was recorded for 5 min. The time spent in the open and closed arms was tabulated, as were the entries. To be considered an open arm entry, the animal had to cross the open platform's line with all four paws. A decrease in open arm time is considered a measure of increased avoidance behavior, reflecting anxiety (Pellow et al., 1995), as we have described (Smith et al., 2006). The number of total entries is a measure of general activity level.

Drugs

All drugs except QX-314 were from Sigma Chemical Co (St Louis, MO). QX-314 was from Calbiochem (Billerica, MA).

Statistics

Statistics were analyzed with Prism-GraphPad (spine densities) or OriginPro (all other data). Data are presented as the mean ± S.E.M., and in some cases, the median, interquartile range, and outliers are indicated. Individual data points are presented when $n < 10$. Data were shown to have similar variance using the Brown-Forsythe test for equal variance and were verified as reflecting a normal distribution by the Kolmogorov–Smirnov test. The significant differences in spine densities calculated across treatment groups were analyzed with a nested t-test (2 groups) or a nested one-way analysis of variance (ANOVA, > 2 groups) with a post-hoc Tukey test (male data) or Dunnett's test (pharmacology study). Averaged values calculated across treatment groups for immunohistochemistry, electrophysiology and behavior were analyzed with the Student's t-test (2 groups) or one-way analysis of variance (ANOVA, > 2 groups) with a post-hoc Tukey test for unequal replications. All tests were two-tailed. A p value < 0.05 was used as an indication of statistical significance. A power analysis was conducted to determine adequate sample size for all studies, which achieved a power > 0.85. Reproducibility was determined by comparing the statistical significance of results from experiments performed 3–5 times to achieve the final n's.

Results

Pyramidal cells in L5 PL of the female mouse undergo synaptic pruning

Spine density of the basilar dendrites of L5 PL pyramidal cells (see Fig. 1a for localization) was determined by microscopic examination of z-stacks of Golgi-stained neurons. Averaged values of spine density

across the dendrite decreased by ~63% from puberty onset (~PND 35) to PND 56 (Fig. 1b,c, 16.39 ± 1.55 spines/10 µm, pub vs. 6.10 ± 0.58 spines/10 µm, post-pub, $p < 0.0001$). The greatest decline (71%, $p < 0.0001$) was for the spines considered stable in terms of their long-lasting presence (mushroom, stubby and bifurcated). Of the commonly noted spine-types, the mushroom spines exhibited the greatest decline (74%, $p < 0.0001$); the stubby spines also decreased significantly (66%, $p < 0.0001$), as did the relatively rare bifurcated spines (84%, $p = 0.0014$). The density of the less stable (motile) spines decreased by 53%, which included significant decreases in long, thin (64%, $p = 0.0025$), and thin (49%, $p = 0.0114$) spines.

α4βδ GABAR expression increases transiently at the onset of puberty in L5 PL

Extra-synaptic α4βδ GABARs increase at puberty in some brain areas when they express on soma, along the dendritic shaft and spine (Shen et al., 2010). Therefore, we initially determined whether these receptors increase at puberty in L5 PL. α4 expression was assessed using immunohistochemical techniques before puberty (~PND 28–32), just after puberty onset (~PND 35), and post-pubertally (PND 56). Immunostaining for α4 increased almost tenfold at the onset of puberty compared to pre-puberty (Fig. 2a,c, Supp. Figure 1, $p < 0.00001$) and then declined ~75% post-pubertally.

Additional studies co-localizing α4 immunostaining with microtubule-associate protein-2 (MAP2), a protein that expresses in mushroom spines (Kim et al., 2020), reveal that α4 immunostaining is localized to both the dendrite and the dendritic spine (Fig. 2b) in addition to the cell body.

To confirm increased expression of functional α4βδ GABARs at puberty, we assessed the response of L5 PL neurons to gaboxadol (GBX), a GABA agonist which is selective for α4βδ GABARs at a concentration of 100 nM (Brown et al., 2002; Meera et al., 2011). When applied in vitro, the relative neuronal responses can be used as a functional index of α4βδ GABAR expression (Shen et al., 2010). To this end, L5 PL pyramidal cells were recorded in the slice preparation from pre-pubertal, pubertal, and post-pubertal female mice using whole-cell voltage clamp techniques. Application of 100 nM GBX elicited a tenfold greater response at puberty than pre-puberty and post-puberty (Fig. 2d,e, $p = 0.00125$), suggesting that functional α4βδ GABARs increase transiently at puberty in L5 PL.

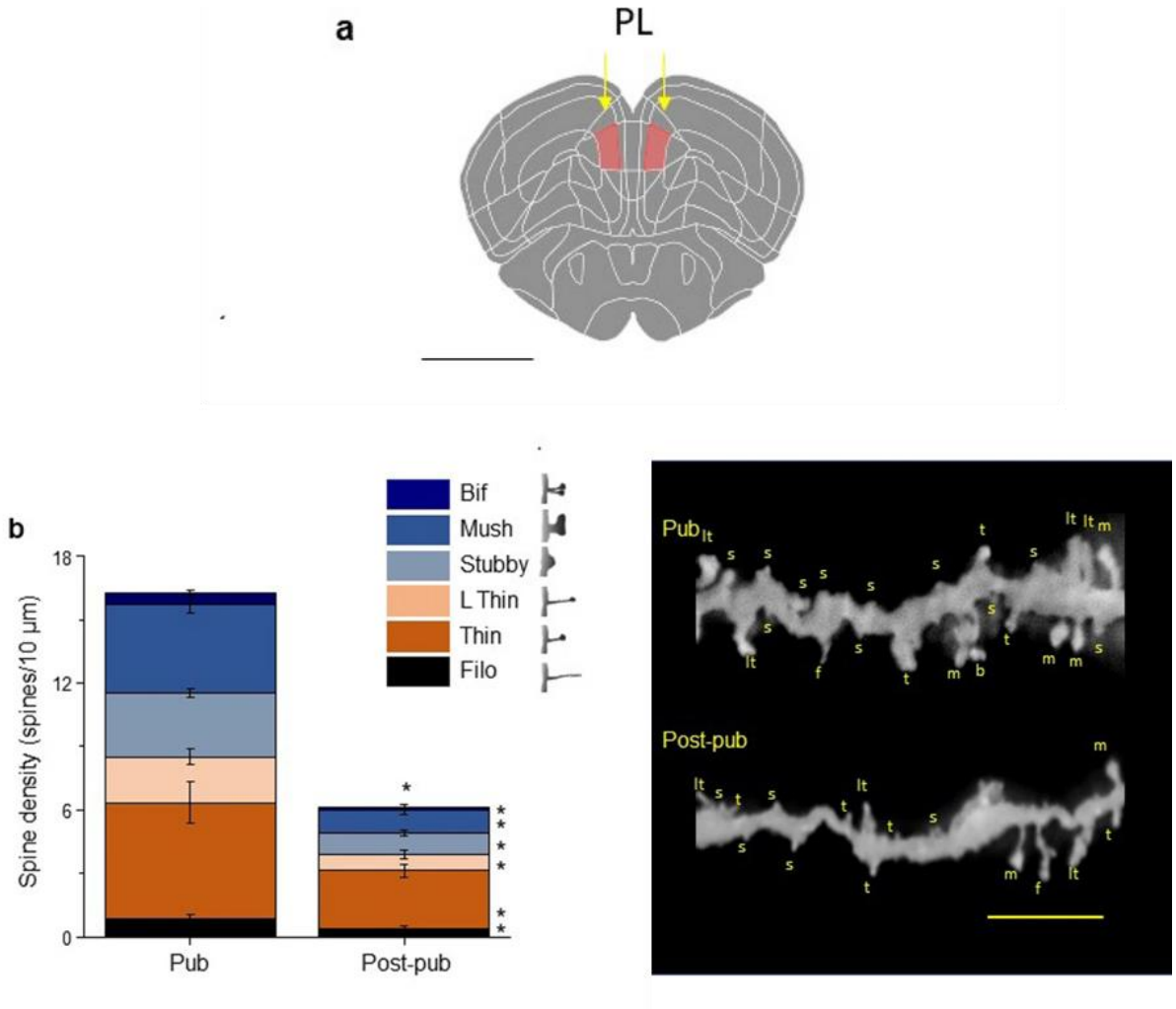

Figure IV.1. Spine density in layer 5 prelimbic cortex (L5 PL) of the female mouse decreases by half during adolescence: assessment of spine-types. (a) Schematic diagram of prelimbic cortex (PL) localization in mouse brain (pink shading, arrows). Coronal section, 2.09 mm anterior to Bregma. Scale, 1 mm. (b) Averaged data, spine density (#spines/10 µm) of layer 5 (L5) PL for pubertal (Pub, ~PND 35, assessed by vaginal opening) and post-pubertal (Post-pub, PND 56) female mice. Total spines, $t(20) = 6.43$, $^*P < 0.0001$; bifurcated, $t(20) = 3.71$, $^*P = 0.0014$; mushroom, $t(20) = 6.2$, $^*P < 0.0001$; stubby, $t(20) = 6.18$, $^*P < 0.0001$; long thin, $t(20) = 3.46$, $^*P = 0.0025$; thin, $t(20) = 2.79$, $^*P = 0.0114$; filopodia, $t(20) = 2.24$, $^*P = 0.037$. (c) Representative images of basal dendrites from Golgi-stained neurons, from Pub and Post-pub female mice. Spine-types: f filopodia, lt long thin, t thin, s stubby, m mushroom. Scale, 5 µm. n = 43–44 neurons, 11 mice/group.

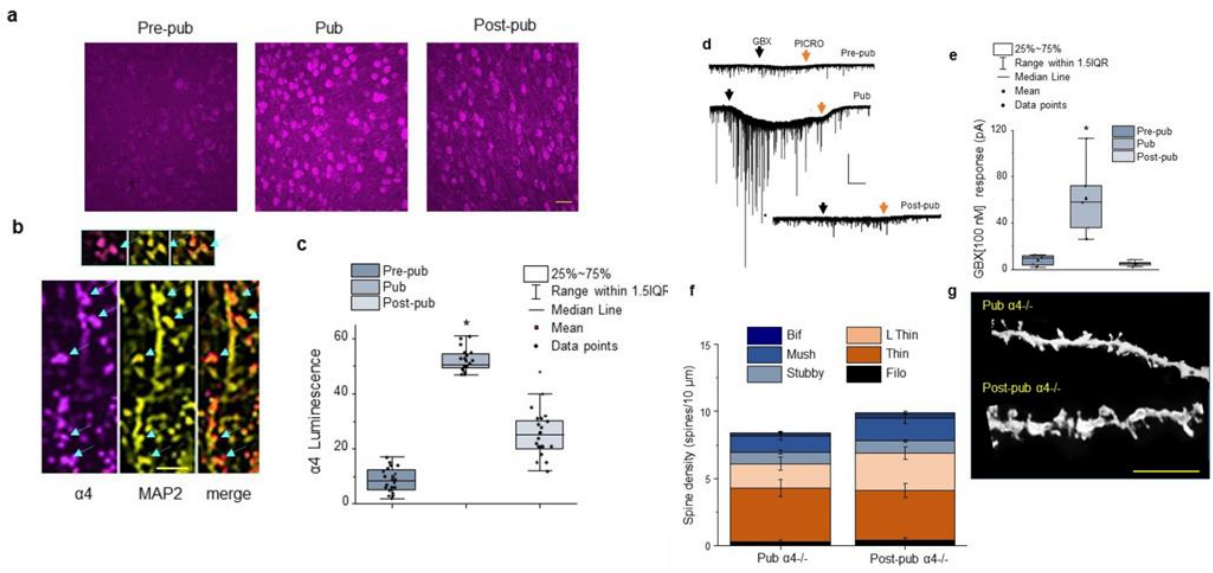

Figure IV.2 Increases in α4βδ GABAR expression in L5 PL at puberty are necessary for synaptic pruning in female mice. (a) Representative images, α4 immunostaining (magenta), L5 PL pyramidal cells from pre-pubertal (pre-pub, left), pubertal (pub, center), and post-pubertal (post-pub, right) female mice (α4, magenta). Scale, 100 μm. Z-stack sequences used for merged images are presented in Supp. Figure 1. (b) Representative images, α4 (magenta, left), MAP2 (yellow, middle), and merged (orange, right) on the dendrites and spines (arrows) of pyramidal cell in L5 PL. from a pubertal mouse. Inset, Co-localization of α4 and MAP2 on a spine (arrow). Scale, 2 μm. (c) Averaged data, mean, median, and interquartile range (IQR). $F(2,57) = 248.9$, $P < 0.00001$, $^*P < 0.05$ vs. other groups. n = 15 neurons, 10 mice/group. (d) Representative whole cell voltage-clamp recordings of L5 PL pyramidal cell response to 100 nM gaboxadol (GBX, black arrow) from Pre-pub, Pub, and Post-pub female mice. PICRO (picrotoxin, 100 μM, red arrow). Scale, 50 s, 200 pA. (e) Averaged data, mean, median, interquartile range, and individual data points for GBX responses. $F(2,12) = 12.28$, $^*P = 0.00125$. $^*P < 0.05$ vs. other groups. n = 5 mice/group. (f) Averaged data, spine density (#spines/10 μm) of L5 PL from a Pub and Post-pub female α4-/- mouse. (g) Representative images, Golgi-stained dendrites from pub and post-pub female α4-/- mice. Scale, 5 μm. n = 13–31 neurons, 8 mice/group.

Synaptic pruning of L5 PL pyramidal cells is prevented by knock-out of the GABAR α4 subunit

The increase in α4βδ GABARs on L5 PL pyramidal cells at puberty raised the possibility that these receptors may play a role in L5 PL synaptic pruning. Thus, we assessed spine density in pubertal and post-pubertal α4−/− female mice. Unlike the wild-type mouse, total spine density did not significantly change from puberty to post-puberty in L5 PL pyramidal cells from the global α4 knock-out mouse (Fig. 2f,g), nor did spine density for any of the different spine types assessed.

Adolescent synaptic pruning of male L5 PL is a result of increased α4βδ GABAR expression

We also investigated synaptic pruning in the male L5 PL. Spine density in the male L5 PL decreased by ~53% in adolescence ($p < 0.0001$) with significant decreases in stubby, thin and long, thin spines ($p < 0.03$;

Supp. Figure 2), while expression of the GABAR α4 subunit in L5 PL pyramidal cells increased sixfold ($p < 0.00001$) at puberty (Supp. Figure 2) compared to pre-puberty, before declining 75% by PND 56 ($p < 0.00001$. Total spine density did not change significantly during adolescence in the male α4−/− mouse (Supp. Figure 2), although there was a significant ($p = 0.0076$) 59% decrease in the long, thin spines.

Electrophysiology experiments were conducted by Hui Shen

Effects of pharmacological manipulation of GABARs on synaptic pruning

We tested whether systemic pharmacological manipulation of GABARs at the circuit level during the pubertal period (PND 35-49) alters PL spine density at PND 56. Systemic administration of the non-selective GABAR antagonist picrotoxin, at a dose sub-threshold for seizure induction, for 2 weeks beginning at the onset of puberty (~PND 35, 3 mg/kg, i.p., daily, Fig. 3a inset) increased spine density by ~3.5-fold compared to vehicle ($p < 0.0001$, Fig. 3a,b) on PND 56, with greatest increases (almost threefold, $p < 0.0001$) in the mushroom spines. Significant increases in the filopodia ($p = 0.011$), thin spines ($p = 0.0019$), and stubby spines ($p < 0.0001$) were also observed, suggesting a role of GABARs in PL pruning.

In order to potentiate current gated by α4βδ GABARs during puberty, GBX was injected across the same pubertal period at a dose (0.1 mg/kg, i.p.) selective for α4βδ GABARs (Afroz et al., 2017). Augmenting α4βδ inhibition in this way produced a 47% decrease in density of the thin spines on PND 56 ($p = 0.045$, Fig. 3a,b), confirming that α4βδ GABARs, at the network level, play a role in adolescent pruning of L5 PL.

In the cortex, GABAergic interneurons have synaptic contacts on dendritic spines (Kawaguchi & Kubota, 1997), which activate GABARs containing γ2 rather than δ, raising the possibility that pruning is altered by these receptors. Thus, we injected lorazepam (LZM, 0.25 mg/kg, i.p.), a benzodiazepine positive allosteric modulator of the GABAR subtypes found at synaptic sites containing α(1-3,5)βxγ2 (Sigel & Luscher, 2011). LZM administration across the pubertal period did not alter spine density (Fig. 3a,b). Taken together, these data suggest that α4βδ GABARs trigger synaptic pruning in L5 PL, and that synaptic α1βγ2 GABARs do not contribute to this process.

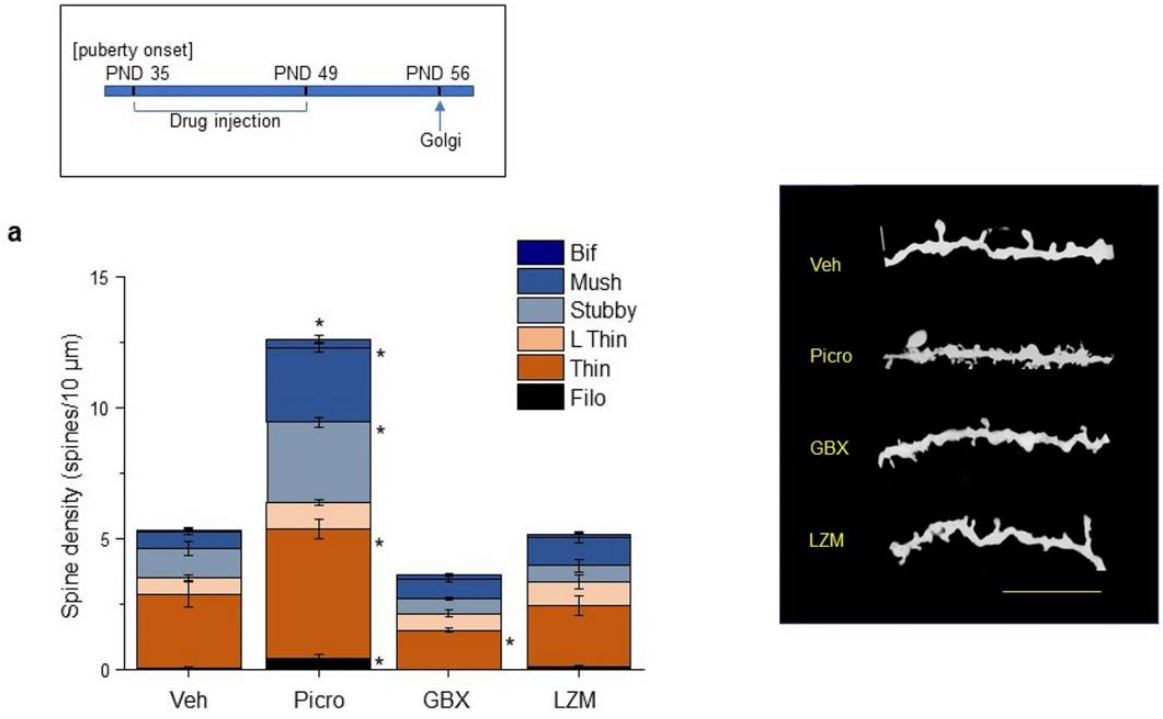

Figure IV.3. ***Pharmacological manipulation of GABAR-gated current during puberty alters spine density post-pubertally.*** Inset, timeline of drug administration during the pubertal period of high α4 expression (PND 35–49) using picrotoxin (PICRO, 3 mg/kg, i.p.), gaboxadol (GBX, 0.1 mg/kg,i.p.), lorazepam (LZM, 0.25 mg/kg, i.p.) or vehicle (VEH). Mice were euthanized for Golgi procedures on PND 56. (a) Averaged data, Total spines, $F(3,22) = 119.5$, $P < 0.0001$ [Veh vs. Picro, $^*P < 0.0001$]; mushroom, $F(3,22) = 25.53$, $P < 0.0001$ [Veh vs. Picro, $^*P < 0.0001$]; stubby, $F(3,22) = 43.76$, $P < 0.0001$ [Veh vs. Picro, $^*P < 0.0001$]; thin, $F(3,22) = 15.45$, $P < 0.0001$ [Veh vs. Picro, $^*P = 0.0019$; Veh vs. GBX, $^*P = 0.0445$]; filopodia, $F(3,22) = 4.71$, $P = 0.011$ [Veh vs. Picro, $^*P < 0.02$]. (b) Representative images of basal dendrites from Golgi-stained neurons from Post-pub female WT mice treated with the indicated drugs during puberty. Scale, 10 µm. n = 14–23 neurons, 7 mice/group.

Role of NMDARs in synaptic pruning of L5 PL

α4βδ GABARs impair the activation of NMDARs (Alvarez et al., 2007), which are necessary for spine maintenance (Ma et al., 2003). Thus, we tested whether over-expressing NMDARs during the pubertal increase in α4βδ expression (~PND 35–49) would increase spine density on PND 56. Administration of a low dose of MK-801 (0.1 mg/kg, i.p.), paradoxically increases NMDAR expression by more than twofold in L5PL after 5 days ($p < 0.0001$, Supp. Figure 3) as a compensatory response (Xi et al., 2009). MK-801 treatment during the pubertal period prevented adolescent pruning in wild-type mice (Fig. 4a,b), similar to α4 knock-out, resulting in spine densities which were almost threefold greater than the PND 56 vehicle control ($p = 0.0002$). The greatest increase in commonly observed spine types was for the mushroom spines, which increased sevenfold above

control levels ($p = 0.0007$), while the thin spines increased by twofold ($p = 0.0027$). The bifurcated spines were also significantly increased ($p = 0.0002$).

We then used the NMDAR blocker memantine, which does not increase NMDAR expression (Cole et al., 2013) due to its high binding affinity (Bresink et al., 1995), to block NMDARs during puberty in α4−/− mice which previously exhibited impaired pruning (Fig. 4c,d). This treatment resulted in spine densities of approximately one-third those of the α4−/− control ($p = 0.0003$) at PND 56. All spine-type densities were significantly lower after memantine treatment except for the stubby spines, with the greatest effects on the mushroom spines ($p = 0.001$), long thin spines ($p = 0.0003$), and filopodia ($p < 0.0001$). These data suggest that NMDAR activity plays a role in synaptic pruning of L5 PL.

Expression of the spine protein Kal-7 decreases at puberty in wild-type but not α4−/− L5 PL

The spine protein Kal-7 is necessary for spine maintenance (Ma et al., 2003). Therefore, we assessed expression levels of this protein in L5 PL of wild-type and α4−/− mice before puberty, during puberty, and post-pubertally with immunohistochemical techniques.

Kal-7 expression in L5 PL was unchanged at the onset of puberty (vaginal opening, PND 35) but decreased 75% by PND 40 ($p < 0.0001$) compared to pre-pubertal values (Fig. 4e,f, Supp. Figure 4), assessed in wild-type mice. However, Kal-7 expression levels partially recovered on PND 56, increasing by almost twofold ($p < 0.0001$), suggesting an inverse correlation with α4 expression.

Kal-7 expression in pubertal (PND 36–40) α4−/− mice was fivefold greater than comparable expression in pubertal wild-type mice of the same age ($p < 0.0001$), implicating α4βδ receptors as the cause for the decline in Kal-7 expression at puberty.

Local pubertal α4 knockdown increases PL spine density at PND 56

Because the α4 global knock-out is not selective for the PL, stereotaxic virus injections were used to selectively knockdown α4 in the PL at puberty to confirm its role in pruning: AAV-Cre or AAV-GFP (control) was injected into the PL of PND 21 transgenic mice with loxP sites flanking the α4 gene. Immunohistochemical

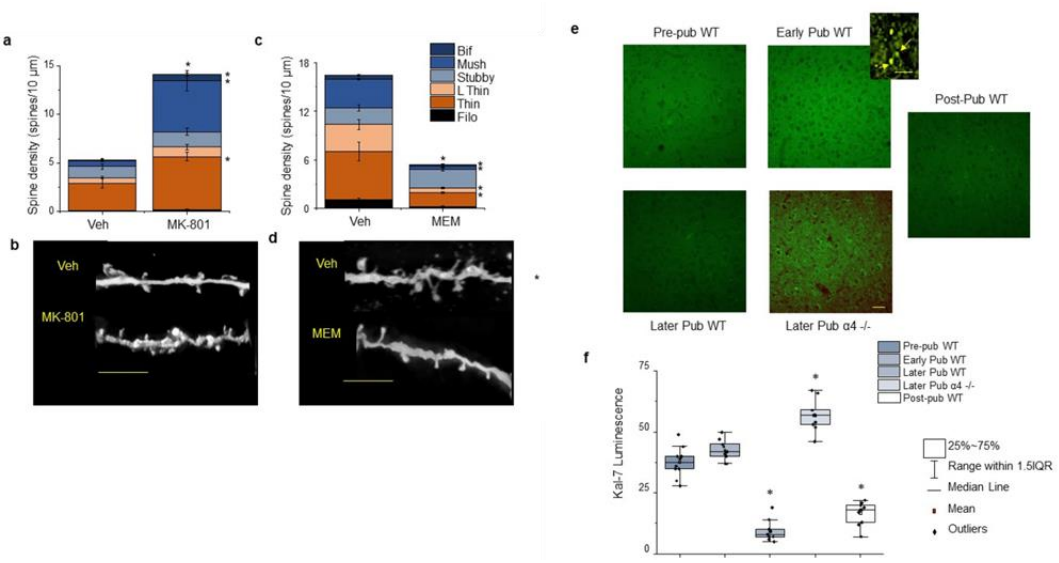

Figure IV.4 ***Pubertal state and α4 knock-out: Effects on NMDA receptor-regulated pruning and expression of the spine protein kalirin-7.*** (a) Averaged data, spine density (#spines/10 µm) of post-pubertal (PND 56) female WT mice treated during the pubertal period (PND 35–49, inset) with MK-801 (0.1 mg/kg, i.p.) which paradoxically increases NMDA receptor expression in mPFC[34] (Supp. Figure 3). Total spines, $t(12) = 5.32$, $^{*}P = 0.0002$; bifurcated, $t(12) = 4.16$, $^{*}P = 0.0002$; mushroom, $t(12) = 4.53$, $^{*}P = 0.0007$; stubby, $t(12) = 1.1$, $P = 0.206$; thin, $t(12) = 3.76$, $^{*}P = 0.0027$; long thin, $t(12) = 1.25$, $^{*}P = 0.0027$. (b) Representative images of basal dendrites from Golgi-stained neurons, from the indicated groups. Scale, 5 µm. n = 18–21 neurons, 7 mice/group. (c) Averaged data, spine density of post-pubertal female $\alpha4^{-/-}$ mice treated during the pubertal period (PND 35–49) with memantine (0.1 mg/kg, i.p.) an NMDA receptor antagonist. Total spines, $t(18) = 4.54$, $^{*}P = 0.0003$; bifurcated, $t(18) = 2.6$, $^{*}P = 0.018$; mushroom, $t(18) = 3.87$, $^{*}P = 0.0011$; long thin, $t(18) = 4.53$, $^{*}P = 0.0003$; thin, $t(18) = 3.69$, $^{*}P = 0.0017$; filopodia, $t(18) = 5.46$, $^{*}P < 0.0001$. (d) Representative images of basal dendrites from Golgi-stained neurons, from the indicated groups. Scale, 5 µm. n = 29–41 neurons, 10 mice/group. (e) Representative images, kalirin-7 (Kal-7, green) immunostaining of L5 PL from pre-pubertal (pre-pub), early pubertal (early pub, day of vaginal opening, later pubertal (later pub) and post-pubertal (post-pub) WT female mice as well as from a pubertal $\alpha4^{-/-}$ mouse (Later Pub $\alpha4^{-/-}$). Scale, 100 µm. Inset, spine localization of Kal-7. Scale, 4 µm. (Z-stack sequences used for merged images are presented in Supp. Figure 4). (f) Averaged data, mean, median and interquartile range (IQR) from the indicated groups. $F(4,45) = 139$, $P < 0.0001$; $^{*}P < 0.05$ vs. other unstarred groups. n = 20 neurons, 9 mice/group.

analysis verified that the infusions targeted the PL (Fig. 5a) and induced Cre recombinase (Fig. 5b) by PND 35. α4 staining in L5 PL was almost undetectable in the AAV-Cre group on PND 35 compared to the robust staining of the AAV-GFP group, which was 26-fold greater than for the AAV-Cre group ($p < 0.00001$, Fig. 5c,d, Supp. Figure 5a), assessed after puberty onset (PND 35–38) demonstrating successful α4 knockdown. In contrast, α4 staining was not altered in the IL (Supp. Figure 5b). Local knock-down of α4 using local AAV-Cre infusion was also associated with a ~two-fold increase in expression of Kal-7 (Fig. 5e,f, Supp. Figure 6, $p < 0.00001$) compared to the GFP control, suggesting that α4βδ GABARs regulate Kal-7 expression.

Local knockdown of α4βδ GABARs in PL during puberty increases anxiety responses to an aversive stimulus post-pubertally

In order to determine the behavioral consequence of increased spine density in L5 PL as a result of reduced pubertal pruning in the absence of α4 expression, we assessed avoidance behavior post-pubertally at PND 56 as well as in adulthood (PND 90) following local α4 knockdown in the PL during puberty (Fig. 5a inset). The PL has been shown to increase anxiety responses (Vidal-Gonzalez et al., 2006), which can be assessed by measuring avoidance behavior on the elevated plus-maze (EPM, Fig. 6c inset). Decreased time spent on the open arm reflects an increase in anxiety-like behavior. Shock-pairing was used to increase the paradigm's aversive quality, as we have demonstrated (Smith et al., 2006) to parallel clinical studies showing mPFC regulation of anxiety responses to an aversive stimulus (Motzkin et al., 2015; Simpson et al., 2001). Increases in PL spine density produced by local α4 knockdown (AAV-Cre) were associated with a 62% decrease in open arm time on the EPM when tested on PND 56 ($p = 0.0031$, Fig. 6c), and a ~40% decrease in open arm time when tested at PND 90 ($p = 0.0366$, Fig. 6c) in a separate group of animals, compared to AAV-GFP injected control mice. These results suggest that increased spine density in L5 of the PL cortex contributes to increased avoidance behavior, a measure of increased anxiety. However, the number of total entries, a measure of locomotor activity, was not altered by AAV- Cre infusion at either testing age (Fig. 6d).

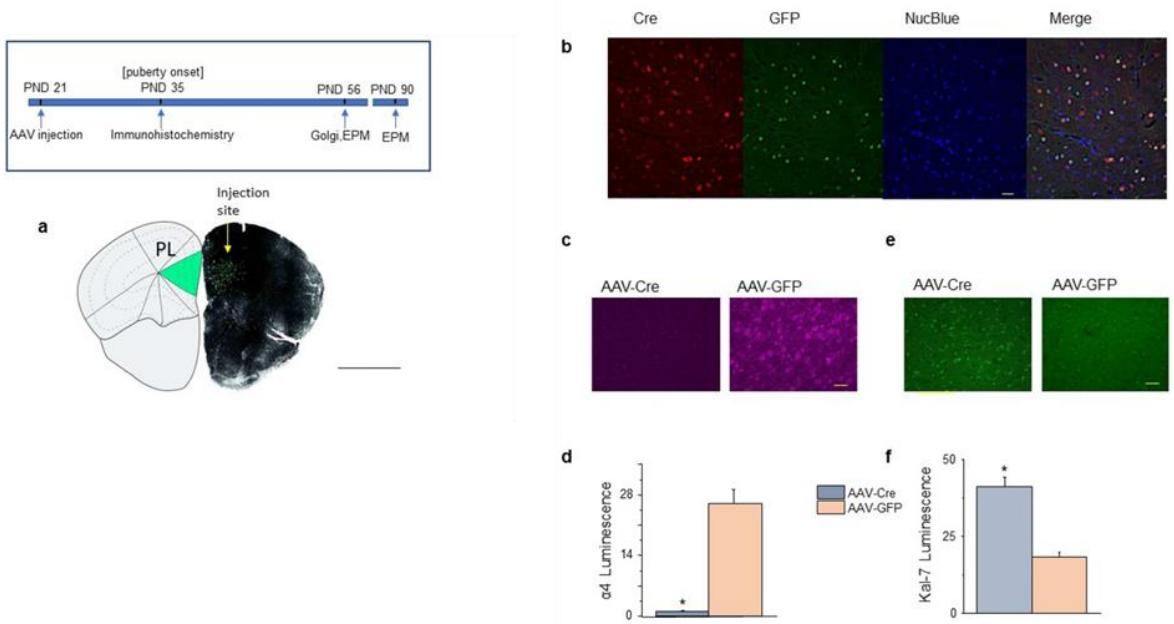

Figure IV.5 ***Local infusion of AAV-Cre into PL on PND 21 results in Cre expression and α4 knockdown in PND 35 female mice.*** Inset, Timeline showing the day of AAV injection (PND 21) when transgenic female mice expressing LoxP sites flanking the GABRA4 gene were injected bilaterally into the PL with adeno-associated virus (AAV)-Cre recombinase or AAV-green fluorescent protein (GFP) and testing (PND 35, α4 immunohistochemistry; PND 56, Golgi spine protocol; PND 56 and 90, anxiety tested using the EPM). (a) Left, schematic showing PL (green). Right, Representative image at PND 35 of GFP staining restricted to the PL (green) boundaries following injection of AAV-Cre/GFP at PND 21. Scale, 2067 μm. This coronal slice was taken 1.767 μm anterior to Bregma. (b) Representative images (×40) at PND 35 following injection of AAV-Cre/GFP at PND 21 of Cre recombinase (Cre) immunostaining (left to right, Cre, GFP, nuclear blue (Nuc blue), merged); Scale, 50 μm. (c) Representative images of α4 immunostaining at PND 35 after infusion of AAV-Cre or AAV-GFP on PND 21 . Scale, 100 μm. (Z-stack sequence, Supp. Figure 5). (d) Averaged data, $t(18) = 7.27$, $^*P < 0.00001$. $n = 10$ neurons, 5 mice/group. (e) Representative images of Kal-7 immunostaining after infusion of AAV-Cre or AAV-GFP on PND 21. Scale, 100 μm. (Z-stack sequence, Supp. Figure 6). (f) Averaged data, $t(18) = 6.36$, $^*P < 0.00001$. $n = 10$ neurons, 4 mice/group.

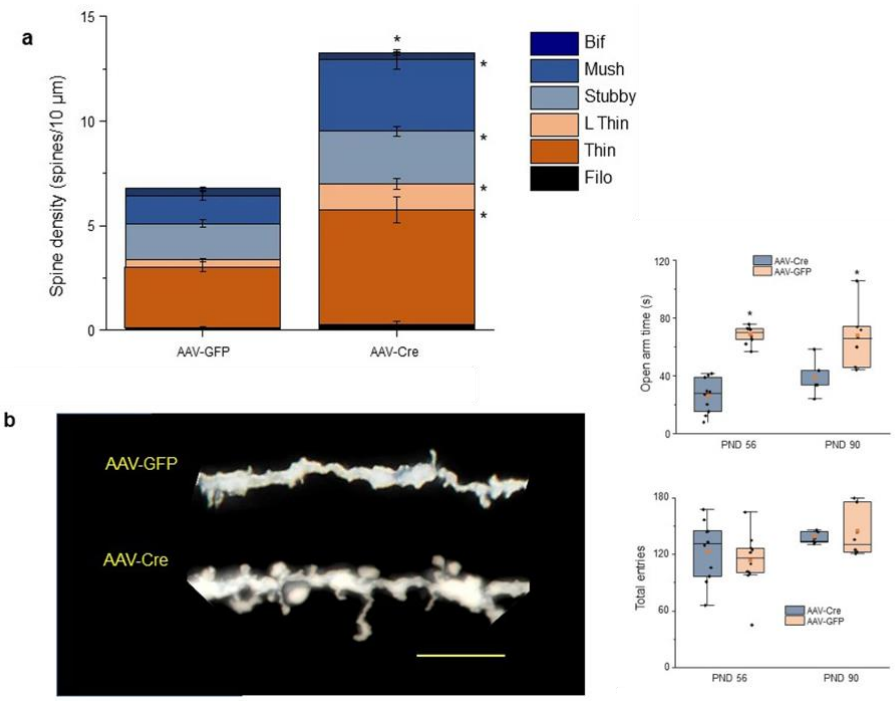

Figure IV.6 Local α4 knockdown at puberty increases spine density of L5 PL pyramidal cells at PND 56 and increases the anxiety response to an aversive stimulus. (a) Averaged data, spine density (#spines/10 µm) in post-pubertal (PND 56) female L5 PL following local α4 knockdown with AAV-Cre (right) compared to the AAV-GFP control (left). Total spines, $t(16) = 4.94$, $^*P = 0.0001$; mushroom, $t(16) = 3.89$, $^*P = 0.0013$; stubby, $t(16) = 2.38$, $^*P = 0.03$; thin, $t(16) = 3.63$, $^*P = 0.0023$; long thin, $t(16) = 3.13$, $^*P = 0.0065$. (b) Representative images of basal dendrites from Golgi-stained neurons from the indicated groups. Scale, 5 µm. n = 36 neurons, 9 mice/group. (c) Inset, schematic drawing of elevated plus maze (EPM; arms, 30 cm long, 5 cm wide, elevated 40 cm): white, open arms, half enclosed by walls 7 cm high, extending 15 cm from the center; black, closed arms enclosed by walls 15.25 cm high for the entire length of the arm. (c) Mean (red square), median line, 25–75% interquartile range (IQR), SEM, and individual data points for open arm time in the EPM for PND 56 or PND 90 female mice injected with AAV-GFP or AAV-Cre in the PL on PND 21. PND 56, $t(18) = 9.97$, $^*P < 0.00001$; PND 90, $t(9) = 2.45$, $^*P = 0.0366$. A decrease in open arm time is an increase in avoidance behavior which reflects anxiety. (d) Mean (red square), median line, 25–75% interquartile range (IQR), SEM, and individual data points for total crossings in the EPM, measuring locomotor activity. n = 10 mice/group (PND 56); n = 5–6 mice/group (PND 90).

Discussion

This study demonstrates that dendritic spine density of L5 PL decreases by half in both female and male adolescent mice due to the emergence of an extra-synaptic GABAR, α4βδ at puberty. Local α4βδ knockdown in the female PL prevented this pubertal pruning and increased anxiety-like behavior in response to an aversive stimulus in late adolescence and adulthood.

Anxiety in the human is associated with excessive avoidance, which maintains the maladaptive fear response (Biedermann et al., 2017). We used the elevated plus-maze to assess the avoidance behavior of mice, which has been verified in humans to reflect anxiety level (Biedermann et al., 2017). This protocol was paired with a mild shock to increase the aversive context to better approximate clinical studies using aversive stimuli to generate mPFC activity in subjects with anxiety (Motzkin et al., 2015; Simpson et al., 2001). An abnormal anxiety response to unpredictable aversive stimuli is a feature of anxiety disorders (Grillon et al., 2008) which has been studied extensively and is a more revealing outcome than baseline anxiety levels (Grupe & Nitschke, 2013). The post-pubertal anxiety observed after local knockdown of α4βδ in PL at puberty was most likely due to the increase in PL spine density, which is a long-lasting outcome of pubertal α4 knockdown, rather than a result of the decrease in inhibition at puberty because α4βδ expression is low at PND 56 and in adults under control conditions when the behavior is tested. However, the resultant increase in neuronal excitability produced by pubertal α4 knockdown could also increase activation of target sites and potentially alter intracellular messengers in addition to increasing L5 PL spine density.

Anxiety is the most common mental disorder (Kessler et al., 2005), yet the etiology is not well understood at the circuit level, nor are the potential treatments (Paus et al., 2008). This disorder is twice as likely to afflict females, with onset most likely to occur during adolescence (Hantsoo & Epperson, 2017) with subtypes ranging from generalized anxiety disorder, agoraphobia, panic disorder, and obsessive–compulsive disorder (Beesdo et al., 2010). These disorders have a high probability of continuing into adulthood (Pine et al., 1998) when there is an increased risk of suicide (Foley et al., 2006). This study suggests that one contributing factor for anxiety behavior generated post-pubertally is an increase in excitatory synapses in L5 PL via dysregulation of pruning, increasing the input to activate this region.

Excitatory input to L5 PL pyramidal cells comes from the ventral hippocampus, amygdala, and multiple sensory sites (Hoover & Vertes, 2007). L5 pyramidal cells provide the output of the PL to the basolateral amygdala (Cheriyan et al., 2016) to regulate fear and anxiety (Vidal-Gonzalez et al., 2006). Increasing local glutamate concentrations with veratrine in the PL of rodents increases anxiety using the open field test (Saitoh et al., 2014). Blocking NMDARs (Resstel et al., 2008) in the PL prevents this effect suggesting that anxiety is triggered by NMDAR-mediated transmission. Conversely, numerous studies show that inactivating the PL using either pharmacological or electrolytic techniques reduces anxiety (Shah et al., 2004; Resstel et al., 2008). Thus,

the present findings correlating L5 PL spine density with avoidance behavior provide a mechanistic link of the PL with increased anxiety. In contrast, the IL is associated with reduced fear/anxiety and fear extinction due to output to GABAergic neurons via the uncincate fasciculus, which reduces activity in the basolateral amygdala (Rosenkranz & Grace, 2002).

Human studies also support a dual role for the PL and IL sub-regions of the mPFC. Dorsal regions of the mPFC, including the anterior cingulate, which corresponds to the rodent PL cortex, are activated by fear (Fenton et al., 2014). Increased gamma power EEG changes or blood flow accompanies increased fear or anxiety due to fear conditioning or in individuals with generalized anxiety disorder (Mueller et al., 2014; Yaguez et al., 2005; Fitzgerald et al., 2017). These correlations of enhanced learned fear expression and persistent PL activation are greater in females (Fenton et al., 2014). In contrast, the human ventromedial PFC (vmPFC), corresponding to the rodent IL, exhibits decreased activity in anxiety (Motzkin et al., 2015). vmPFC lesions increase the amygdala response to aversive stimuli (Motzkin et al., 2015), further confirming the role of the IL/vmPFC in fear reduction.

In the present study, mushroom spines showed the greatest reduction in spine density (74%) in the female L5 PL. The larger head of these spines have a higher density of AMPA receptors (Matsuzaki et al., 2001) and thus would be expected to have a greater synaptic impact on PL activation. Local α4 knockdown in the PL prevented spine pruning at PND 56, resulting in increased mushroom spine density with levels similar to pubertal wild-type values. Enhanced excitatory transmission to PL would activate output to the amygdala and is a likely mechanism underlying the increased anxiety following local knockdown of α4 expression.

α4 knockdown reversed the 45% decrease in density of the motile spines (thin spines, long thin spines, and filopodia) in adolescence. Motile spines are thought to represent learning spines (Bourne & Harris, 2007), which may function in learned fear, such as conditioned cue-related and contextual fear for which the PL plays a role (Sharpe & Killcross, 2014).

α4βδ GABAR expression is altered in the human frontal cortex in some types of mental disorders, especially those that emerge in childhood or adolescence (Feng et al., 2010), with decreased expression in brains of non-depressed suicide victims (Merali et al., 2004; Sequeira et al., 2009). Non-depressed suicide is usually characterized by anxiety (Foley et al., 2006). Thus, genetic factors producing dysregulated α4βδ GABAR expression may reduce synaptic pruning during adolescence to increase anxiety.

In cases where there are persistent alterations in expression of α4βδ GABARs, as seen in depression and anxiety (Merali et al., 2004), the ultimate effect would depend on the area of expression. Decreased expression of these receptors in the adult prelimbic area would increase anxiety, as suggested by research studies (Saitoh et al., 2014). Increases in α4βδ GABARs are reported in orbitofrontal cortex of suicide victims (Poulter et al., 2010), which is analogous to the rodent infralimbic. Increased inhibition of this area, outside of the adolescent pruning period, would be expected to increase anxiety, as suggested by clinical imaging studies (Motzkin et al., 2015), and also increase depression, as suggested by studies showing that stimulation of this area is anti-depressant (Kennedy et al., 2011; Drevets et al., 2008; Mayberg et al., 2005).

Increased expression of α4βδ GABARs at puberty was shown both by increases in α4 immunostaining as well as by increased responses of L5 pyramidal cells to the GABA agonist GBX, at a concentration selective for α4βδ GABARs (Brown et al., 2002; Meera et al., 2011). α4βδ GABAR expression was reduced to near pre-pubertal levels by PND 56, however, suggesting a transient increase in pubertal expression of these receptors. Furthermore, α4 immunostaining was localized to the cell body, dendrites, and the spines at puberty, where these receptors would be expected to impair NMDAR activation, as previously shown (Alvarez et al., 2007) in other CNS areas. The inhibition generated by these receptors along the dendritic shaft as well as on the soma would also impair NMDAR activation by decreasing action potential back-propagation, which is generated in the axon hillock within the soma, travels up the dendrite, and would normally facilitate Mg++ unblock of the NMDAR channel (Stuart et al., 1997; Buzsaki & Kandel, 1998; Wu et al., 2012). In the present study, increased NMDAR expression generated by administration of low doses of MK-801 (Xi et al., 2009) during puberty prevented pruning in wild-type mice. In contrast, blocking NMDARs in α4−/− mice using memantine, a treatment which does not increase NMDAR expression (Cole et al., 2013), most likely due to its higher affinity for the receptor (Bresink et al., 1995), restored pruning in the absence of α4βδ-mediated inhibition. These data suggest that α4βδ impairment of NMDARs underlies adolescent pruning of L5 PL. This outcome was mediated by the Rho-guanine nucleotide exchange factor Kal-7, a spine protein necessary for spine maintenance (Ma et al., 2003).

Kal-7 activates the small GTPase Rac1, which stabilizes the actin cytoskeleton via P21-activated kinases within the spine (Ma et al., 2014), and the expression of Kal-7 is increased by NMDAR activation (Penzes et al., 2000). Thus, decreased Kal-7 expression at puberty would destabilize the spine to enable spine removal. However, Kal-7 expression was increased in L5 PL of pubertal α4−/− mice, suggesting that the increase in α4βδ

GABARs in wild-type mice is the initial trigger for the decrease in Kal-7 expression, which leads to pruning, as shown in other CNS sites (Afroz et al., 2016) (See schematic diagram, Fig. 7). However, we cannot rule out other spine proteins which may play a role in spine stability and pruning (Roussignol et al., 2005; Duncan et al., 2021; Carlisle & Kennedy, 2005). In addition, the microglia (Schafer et al., 2012) and autophagy (Tang et al., 2014) have been shown to play a role in pruning but are likely the final steps in this process.

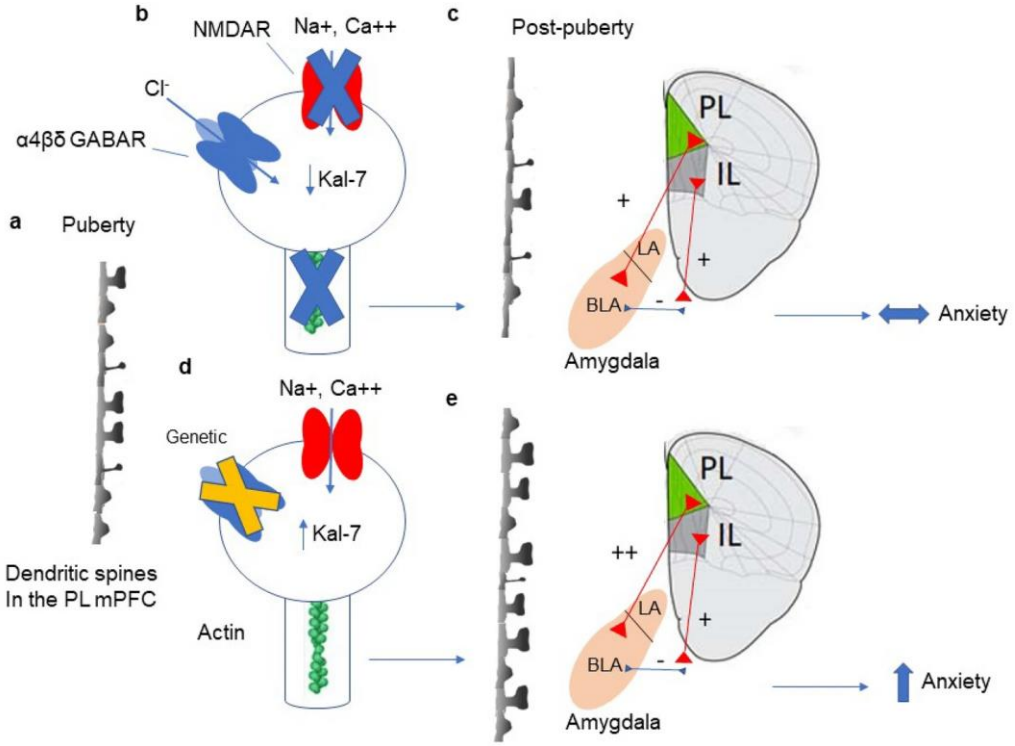

Figure IV.7 ***Summary figure.*** (a) Schematic diagram showing representative dendritic spines in L5 PL at puberty. (b) Normally, expression of α4βδ GABARs increases on the spine at puberty where they gate a Cl^- current that is inhibitory and impairs activation of NMDARs, which gate Na^+ and Ca^{++} conductances. This decreases Kal-7 expression, resulting in actin de-stabilization, which causes synaptic pruning of L5 PL pyramidal cells. (c) The decrease in spine density represents a loss of synaptic input to L5 PL post-pubertally, which reduces excitatory output to the basolateral amygdala. Because this is offset by inhibitory input from the IL, anxiety behavior is unchanged in late adolescence. (d) When α4βδ GABAR expression is reduced at puberty (genetically or pharmacologically), NMDARs can generate Kal-7 expression, which stabilizes the actin cytoskeleton, preventing pruning. (e) This increased spine density of L5 PL would increase activation of the basolateral amygdala, thereby increasing anxiety in late adolescence. *PL* prelimbic mPFC, *IL* infralimbic mPFC, *LA* lateral amygdala, *BLA* basolateral amygdala.

The present findings also show that systemic pubertal administration of the drugs picrotoxin and GBX, which block all GABAR subtypes and potentiate α4βδ GABARs, respectively, was successful in altering PL spine density in the predicted direction at the circuit level. That is, picrotoxin increased spine density, and GBX

decreased spine density post-pubertally. This is an interesting finding because the drugs would impact all brain areas, including those with inhibitory inputs to the PL. These findings suggest that pubertal systemic administration of these GABAergic drugs can be used to manipulate spine density in the L5 PL.

In the frontal cortex, synaptic GABAergic afferents target αxβxγ2 GABARs on the dendritic spine (Kawaguchi & Kubota, 1997). Pubertal administration of the positive GABAR modulator LZM, a benzodiazepine that enhances synaptic inhibition of the dendritic spines at αxβxγ2 GABARs lacking α4 (Sigel & Luscher, 2011), had no significant effect on the overall post-pubertal spine density of the basilar dendrites. This suggests that extra-synaptic α4βδ GABARs, rather than synaptic GABARs, are selectively responsible for synaptic pruning of L5 PL pyramidal cells during adolescence.

Decreases in L5 PL total spine density were >50% for females across a timespan which reflected puberty onset (~PND 35) and continued until late adolescence (PND 56). Similar findings were noted for males, which were also due to α4βδ GABARs, as evidenced by the lack of pruning in knock-outs that lacked these receptors' pubertal expression. Synaptic pruning has been demonstrated previously in L5 mPFC, with decreases ranging from <10% in the rat to 30–40% in humans for combined IL and PL (Huttenlocher, 1979; Koss et al., 2014). A 30% decrease in spine density was reported for combined L3 and L5 PL in male transgenic mice (Pattwell et al., 2016), assessed in early adolescence (PND 31–45), where pubertal timing was not noted. Puberty onset is the time when α4βδ-mediated inhibition increases and triggers pruning; thus, assessments following onset would reflect the greatest change in spine density.

Spine density of L5 PL pyramidal cells ultimately impacts neural networks that generate oscillations with frequencies in the gamma, theta, and delta range (Blaeser et al., 2016). These oscillations represent the emergent properties of recurrent local networks and depend upon the excitatory and inhibitory synaptic input to

the dendritic spines of L5 pyramidal neurons. The impedance mismatch between the spine and adjacent dendrite enables the spines to act as coincidence detectors, responding to spatially distributed signals within a limited time window (Dembrow et al., 2015). Thus, spine density determines the sensitivity and reliability of the network to afferent input. In the PL, increased spine density likely results in increased neural activity, which activates downstream targets such as the amygdala and results in increased anxiety. This finding is supported by the present study as well as by clinical imaging studies (Bracht et al., 2009; Cremers et al., 2010).

In conclusion, α4βδ GABARs were shown to trigger synaptic pruning in L5 PL as an essential process in limiting anxiety responses in late adolescence and adulthood. Dysregulation of pruning increased anxiety responses. These results suggest that deficiencies in the pruning of PL at puberty may be a key physiological mechanism for mental disorders. Given the recent reports showing abnormal gene signals for α4 and δ in some mental disorders (Feng et al., 2010; Merali et al., 2004; Sequeira et al., 2009; Poulter et al., 2010), the present findings may suggest therapeutic strategies for anxiety disorders that emerge at puberty.

Supplemental Figures

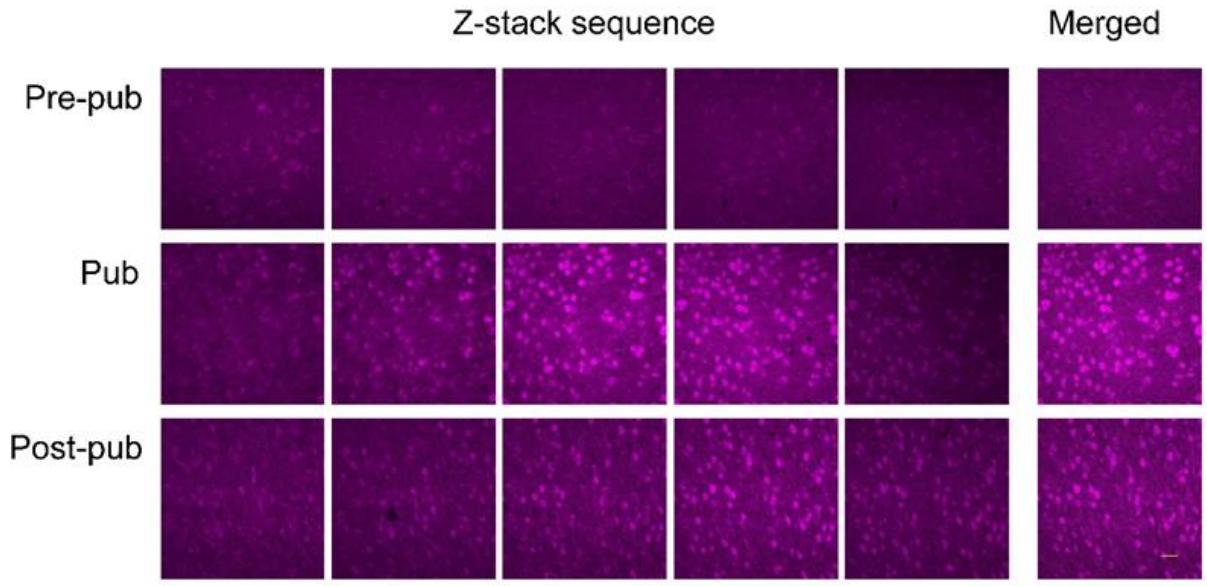

Figure IV.S1. ***Z-stack sequences of α4 immunostaining in prelimbic cortex of female mice across adolescence.*** Left, Representative images taken at 2 pm z-steps of α4 immunostaining in prelimbic cortex from pre-pubertal (Pre-pub, top), pubertal (Pub, after vaginal opening, -PND 35, middle) or post-pubertal (Post-pub, bottom) female mice. Right, Merged z-stack images. Statistics are presented in Fig. 2. Scale 100 gm.

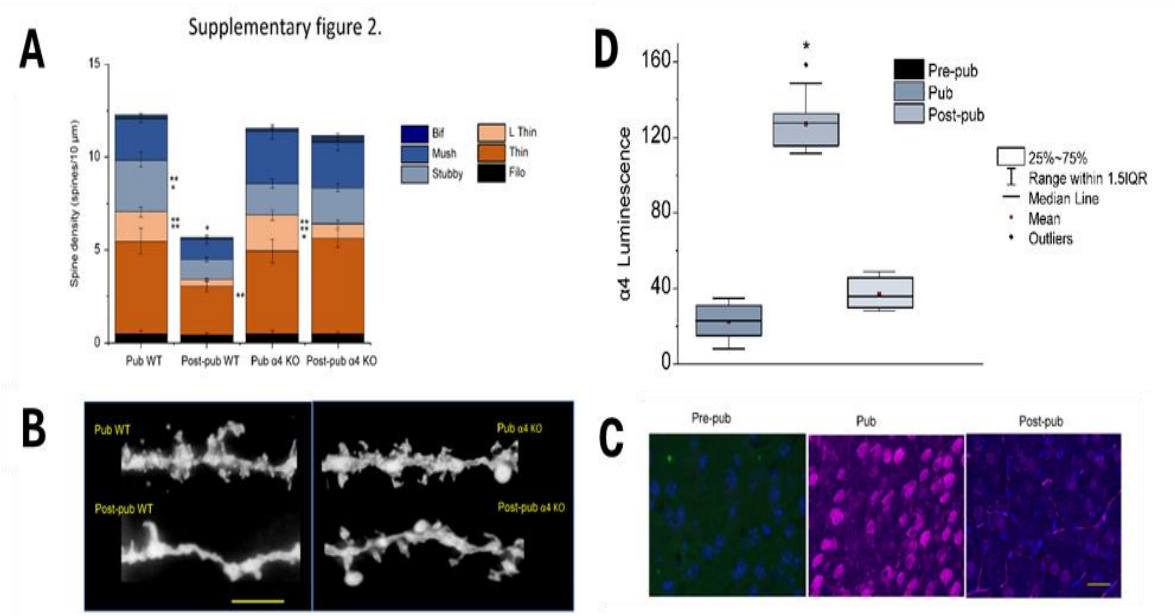

Figure IV.S2. ***Synaptic pruning in male L5 PL is dependent upon pubertal increases in α4ßö GABAR expression.*** a, Averaged data, spine density (#spines/10 pm) of pubertal (Pub) and post-pubertal (Post-pub) wild-type (WT) and α4 -/- male mice. Total spines, F(3,32)=13.76, [Pub WT vs. Post-pub WT, P<O.OOOI ; Post-pub WT vs. Pub α4-/-, Post-pub WT vs. Post-pub α4-/-, mushroom, F(3,32)=5.28, [Pub WT vs. Post-pub WT, P=O.0842; Post-pub WT vs. Pub α4-/-, Post-pub WT vs. Post-pub α4-/-, stubby, [Post-pub WT vs. Pub WT, Pub WT vs. Pub α4-/-, *P-0.0159]; long thin [Post-pub WT vs. Pub WT, Post-pub WT vs. Pub α4-/-, Pub α4-/- vs. Post-pub α4-/-, *P-O.00761; thin, [Post-pub WT vs. Pub WT, *P-0.0222; Post-pub WT vs. Post-pub α4-/-, *P=0.0246]. *P<0.05 vs. other groups; **P<0.05 vs. pubertal groups, n=25-36 neurons, 9 mice/group. b, Representative images of basal dendrites from Golgi-stained neurons, from Pub and Post-pub male WT and α4 -/- mice. Scale, 5 pm. c, Representative images, α4 immunostaining (magenta), L5 PL pyramidal cells from Pre-pub (left), Pub (center) and Post-pub (right) male mice (α4, magenta; DAPI, blue. Scale, 25 pm. d, Averaged data, mean, median and interquartile range (IQR), P<O.OOOOI. *P<0.05 vs. other groups. n=15 neurons, 10 mice/group.

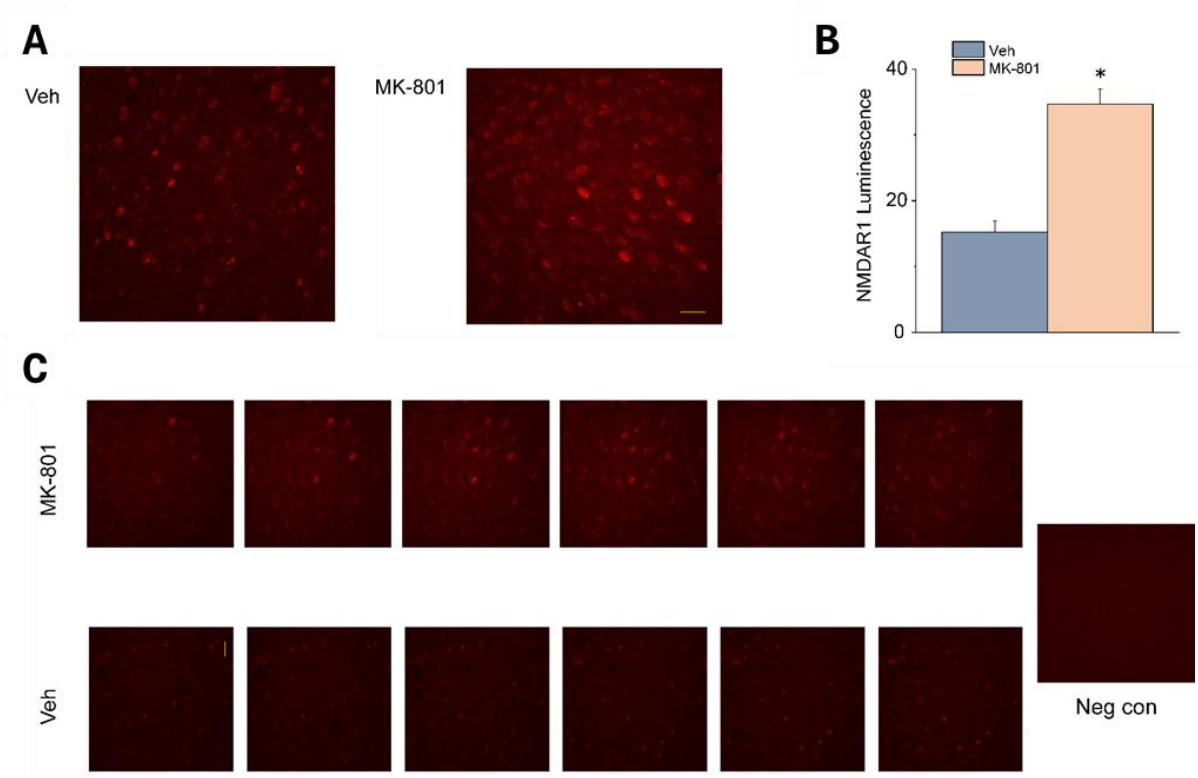

Figure IV.S3. ***Pubertal MK-801 administration increases NMDARI expression in prelimbic cortex.*** a) Merged z-stack images. Scale 100 gm. b) Averaged data. MK-801 increased NMDARI expression predominantly in the soma and proximal dendrites as reported P<0.0000I versus control. n=10 neurons, 5 mice. **c**) Representative images taken at 2 pm z-steps of NMDARI immunostaining in prelimbic cortex from mice treated with vehicle (Veh, left) or MK-801 (right) beginning at the onset of puberty (vaginal opening, ~PND35) for 5 days. Neg control, immunostaining assessed without the primary antibody Scale, 100 pm

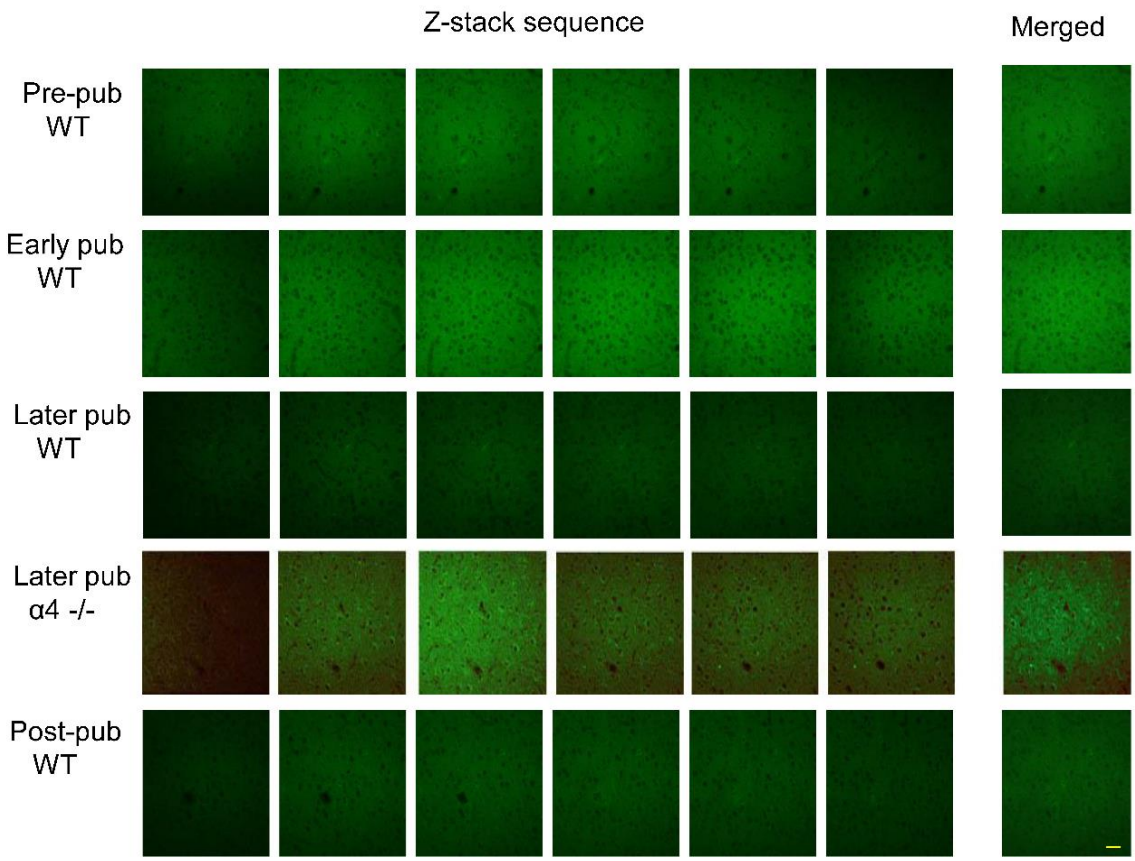

Figure IV.S4. ***Z-stack sequences of Kalirin-7 immunostaining in prelimbic cortex of female mice across adolescence.*** Left, Representative images taken at 2 pm z-steps of kalirin-7 (Kal-7) immunostaining in prelimbic cortex from pre-pubertal wild-type (Pre-pub W T), early pubertal wild-type (Early pub W T, the day of vaginal opening, –PND 35), later pubertal wild-type (Later pub W T, after vaginal opening, PND 36-40), later pubertal α4 -l- (Later pub α4 -/-) or post-pubertal wild-type (Post-pub WT, PND 56) female mice. Right, Merged z-stack images. Statistics are presented in Fig. 4. Scale, 100 gm.

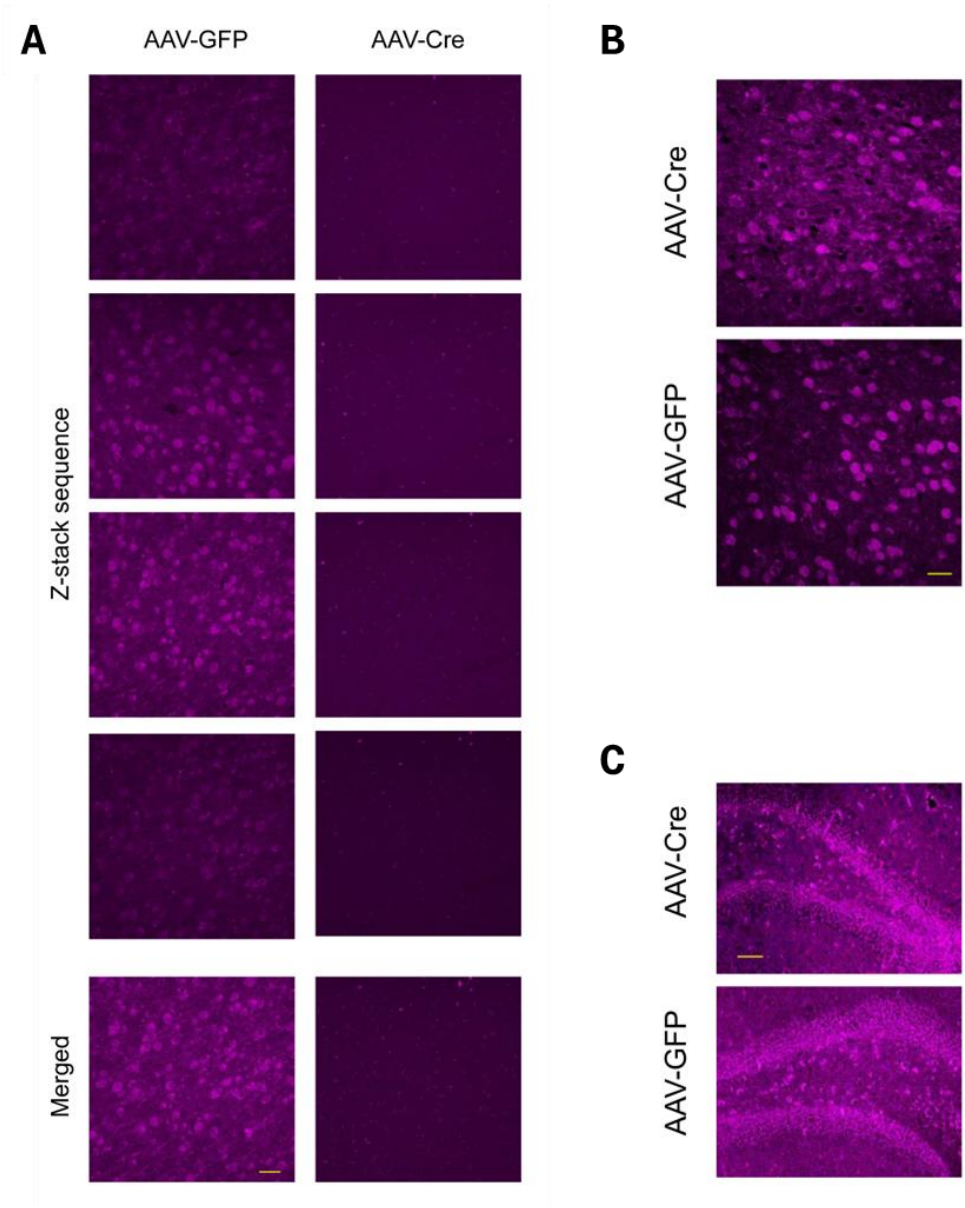

Figure IV.S5. ***Localized α4 knock-down in prelimbic cortex after AAV-Cre infusion: Z-stack Sequences***. a, Top, Representative images taken at 2 prn z-steps of α4 immunostaining in prelimbic cortex from pubertal mice locally infused with AAV-GFP (left) or AAV-Cre (right) to knock-down α4 on PND 21. Bottom, Merged z-stack images. Statistics are presented in Fig. 5. Scale 100 pm. b, Representative images, α4 immunostaining in infralimbic cortex from mice infused with AAV-Cre (top) or AAV-GFP (bottom). Scale 100 gm. c, Representative images, α4 immunostaining in dentate gyrus from mice infused with AAV-Cre (top) or AAV-GFP (bottom). Scale 200 gm. α4 was selectively knock-down in the prelimbic cortex, but not infralimbic or dentate gyrus.

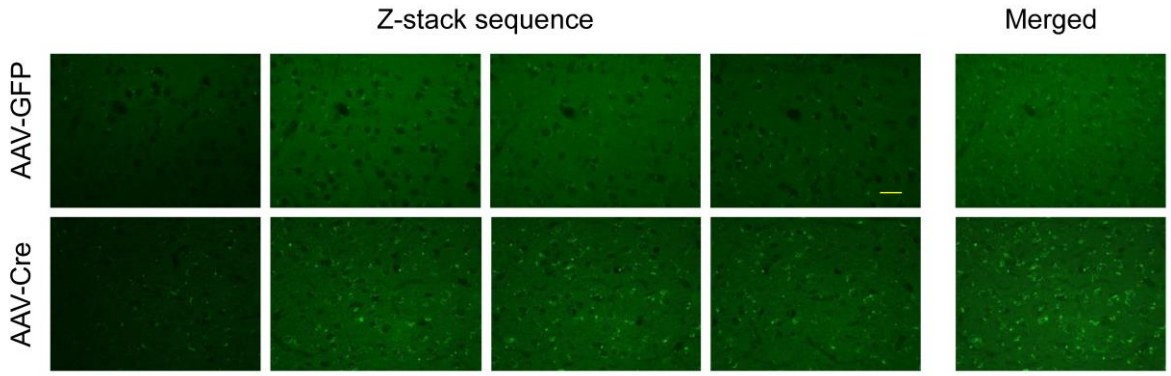

Figure IV.S6. ***Z-stack sequences Of α4 immunostaining in prelimbic cortex Of female mice following local knock-down of α4.*** Left, Representative images taken at 3 pm z-steps of kalirin-7 (Kal-7) immunostaining in prelimbic cortex from mice locally infused with AAV-GFP (top) or AAV-Cre (bottom) to knock-down α4 on PND 21. Right, Merged z-stack images. Statistics are presented in Fig. 5. Scale 100 gm.

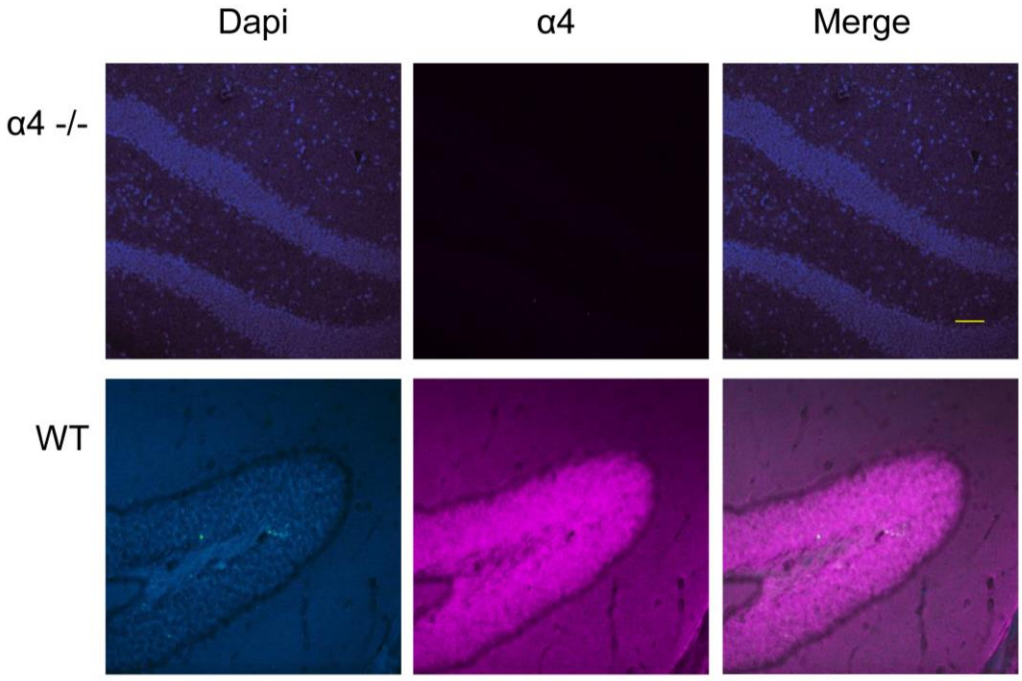

Figure IV.S7. ***Specificity of α4 immunostaining.*** α4 staining in dentate gyrus of an α4 -/- (upper panel) and wild-type (WT) mouse (lower panel). Left, Dapi staining; Middle, α4 staining; Right, merged. Staining is only evident in the slice (W T) containing α4-GABAA receptors, reflecting high antibody specificity. Scale 100 pm.

V. Investigate Layer 2/3 in Wild-Type and α4 Knockout Mice

- Utilize Golgi-Cox staining to analyze dendritic spine density and morphology on basal dendrites of layer pyramidal neurons at the onset of puberty and late puberty in both wild-type and α4 knockout mice.

5.1. Introduction

The prefrontal cortex (PFC) undergoes substantial synaptic remodeling during adolescence in humans and rodents, but the details of layer-specific pruning timelines remain unclear. The PFC can be anatomically divided into distinct cortical layers, with pyramidal neurons in layers 2/3 and layer 5 representing major output populations. Dendritic spines on pyramidal neurons are the primary sites of excitatory synapses, and developmental pruning sculpts patterns of connectivity critical for cognitive maturation. While previous experiments focused on spine density changes in layer 5 PFC neurons, emerging evidence suggests layer-specific differences in synaptic maturation warranting further elucidation.

In human dorsolateral prefrontal cortex, synaptic density increases during early childhood, peaks around 1-2 years old, and decreases by over 50% during adolescence before stabilizing in adulthood (Glantz et al., 2007). In the rodent medial prefrontal cortex, layer 2/3 pyramidal neurons exhibit dramatic overproduction of dendritic spines during the juvenile period followed by pruning only in adolescent females (Markham et al., 2013; Koss et al., 2014). For instance, there is over a 30% decrease in spine density on the basilar dendrites of layer 2/3 pyramidal neurons between postnatal days 30 and 56 selectively in females (Markham et al., 2013). In contrast, spine density on layer 5 pyramidal neurons peaks in the periadolescent period in rats (P28-60) in both sexes, followed by pruning (Koss et al., 2014; Drzewiecki et al., 2016). Compared to layer 5, layer 2/3 neuronal spines show greater adolescent decreases in females during postnatal development (Markham et al., 2013).

These divergent pruning timelines likely relate to the protracted development of top-down control by superficial PFC layers driving increasing functional maturity. The rich recurrent connectivity between layer 2/3 pyramidal cells is thought to support persistent activity and working memory, as discussed by Ibañez et al. (2020), who highlight the role of layer 3 pyramidal neurons in the dorsolateral prefrontal cortex during working memory tasks. Projections from layer 2/3 to layer 5 neurons, which enable contextual integration and executive control over subcortical inputs, were identified as functionally significant in a study by Fiebig and Lansner (2016). The earlier maturation of layer 2/3 circuits may be necessary for later functional integration of layer 5 output

pathways, as suggested by the input-output circuitry involving these cortical layers described by Hawkins et al. (2017) and Yu et al. (2008). Distinct pruning profiles across cortical lamina may also correspond to differences in afferent inputs, projection targets, and local circuit connectivity. For instance, layer 2/3 receives robust sensory thalamic inputs, while layer 5 is a major cortical output pathway (Douglas & Martin, 2004)

Elucidating cellular distinctions in synaptic pruning has important implications for plasticity, learning, and the emergence of adult cognitive abilities mediated by PFC networks. Furthermore, the complexity of layer-specific synaptic remodeling underscores windows of vulnerability where disruption could contribute to neuropsychiatric disorders. While research has examined spine changes in layer 5 PFC neurons during adolescence, less is known about comparative pruning in layer 2/3 pyramidal cells, especially in relation to mechanisms like α4βδ GABAA receptor regulation.

Therefore, this aim will analyze and contrast spine density changes occurring across female pubertal development between PFC layer 3 versus layer 5 pyramidal neurons. Morphological subtypes will be quantified using Golgi staining in wildtype mice at puberty onset and post-puberty. The contribution of α4βδ receptors will be investigated by comparisons to global α4 knock-out mice. Clarifying lamina-specific timelines and regulation of synaptic pruning will provide insights into the layer-dependent maturation of mPFC excitatory networks. Findings may also aid identifying windows of differential vulnerability to disruption across cortical layers that could impact adult cognitive function. Understanding the complexities of circuit-specific and cell-type-specific pruning will be essential for elucidating typical neurodevelopment and plasticity mechanisms.

5.2. Methods

Subjects

Female wildtype (C57BL/6) and global α4 knock-out mice were utilized as participants. Separate cohorts of mice were examined at two developmental timepoints - puberty onset (postnatal day 35) and post-puberty (postnatal day 56) - to assess developmental changes.

Brain Tissue Preparation

Brains were freshly extracted from each mouse and impregnated using the FD Rapid GolgiStain Kit as per the manufacturer’s protocols. This staining technique allowed for visualization of neuronal dendrites and

dendritic spines. Next, 250μm thick coronal sections containing the prelimbic region of the prefrontal cortex were obtained using a vibratome.

Dendritic Spine Analysis

Pyramidal neurons located in layer 3 of the prelimbic cortex were identified under light microscopy. Z-stack photomicrographs were then acquired of proximal basal dendrite segments using a 100x oil immersion objective. An experimenter blinded to conditions manually quantified spine density using image analysis software. Total spine density per 10μm of dendrite length was calculated. Dendritic spines were also classified into distinct morphological categories including mushroom, thin, long-thin, stubby, and filopodia based on standard criteria.

Statistical Analysis

Nested t-test with a post-hoc Tukey test (male data) was utilized to assess differences in mean dendritic spine densities between female mice at the pubertal and post-pubertal timepoints. For knock-out mice, dendritic spine densities were compared to wildtype controls using unpaired t-tests. Kruskal-Wallis tests with Dunn's multiple comparisons were employed to analyze differences in morphological spine subtype densities. Results were considered statistically significant at $p<0.05$.

This dendritic Golgi staining and spine quantification approach built upon techniques established in Aims 1-3. All experimental procedures were approved by the local Institutional Animal Care and Use Committee and aligned with ethical guidelines for animal research.

5.3. Results

Layer 2/3 pyramidal neurons in the prelimbic PFC of female mice exhibited a significant decrease in total spine density from 42.5 ± 1.8 spines/10μm at puberty onset (postnatal day 35, PND 35) to 21.4 ± 1.3 spines/10μm in the post-pubertal period (PND 56) based on Golgi staining and analysis ($p<0.0001$).

Examination of spine morphological subtypes revealed significant pruning across stubby (puberty: 5.8 ± 0.3, post-puberty: 2.1 ± 0.2 spines/10μm; $p<0.0001$), mushroom (puberty: 10.2 ± 0.5, post-puberty: 4.4 ± 0.4 spines/10μm; $p<0.0001$), and long thin (puberty: 6.1 ± 0.4, post-puberty: 3.2 ± 0.3 spines/10μm; $p=0.0025$) spines.

In contrast, female mice lacking the α4 subunit exhibited no significant change in total spine density on layer 2/3 pyramidal neuron basal dendrites between puberty (PND 35: 37.6 ± 1.5 spines/10μm) and the post-

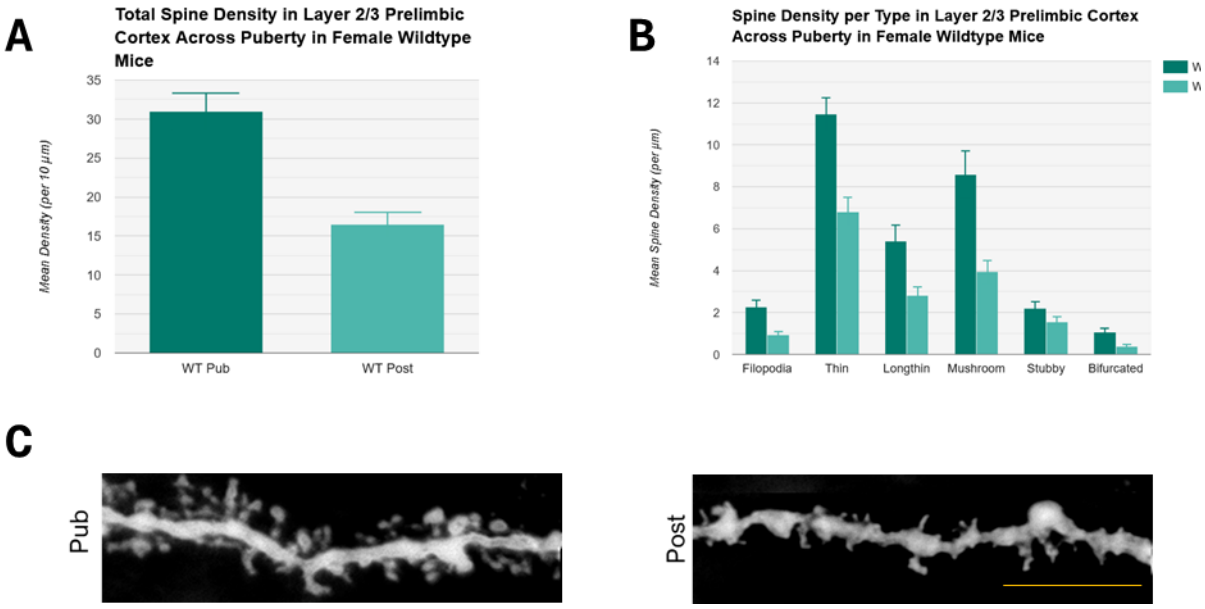

Figure V.1 Spine density in layer 2/3 prelimbic cortex (L2/3 PL) of the female mouse decreases by half during adolescence: assessment of spine-types. Averaged data, spine density (#spines/10 μm) of layer 2/3 (L2/3) PL for pubertal (Pub, ~PND 35, assessed by vaginal opening) and post-pubertal (Post-pub, PND 56) female mice. (a) Total spines, $t(20) = 6.43$, (b) $^*P < 0.0001$; bifurcated, $t(20) = 3.71$, $^*P = 0.0014$; mushroom, $t(20) = 6.2$, $^*P < 0.0001$; stubby, $t(20) = 6.18$, $^*P < 0.0001$; long thin, $t(20) = 3.46$, $^*P = 0.0025$; thin, $t(20) = 2.79$, $^*P = 0.0114$; filopodia, $t(20) = 2.24$, $^*P = 0.037$. (c) Representative images of basal dendrites from Golgi-stained neurons, from Pub and Post-pub female mice. Scale, 5 μm. n = 43–44 neurons, 11 mice/group.

pruning from puberty to post-puberty in the α4 knockouts. This contrasts with the robust spine elimination occurring in wildtype mice over the same adolescent period.pubertal period (PND 56: 35.0 ± 1.6 spines/10μm). Analysis of spine subtypes also failed to detect significant

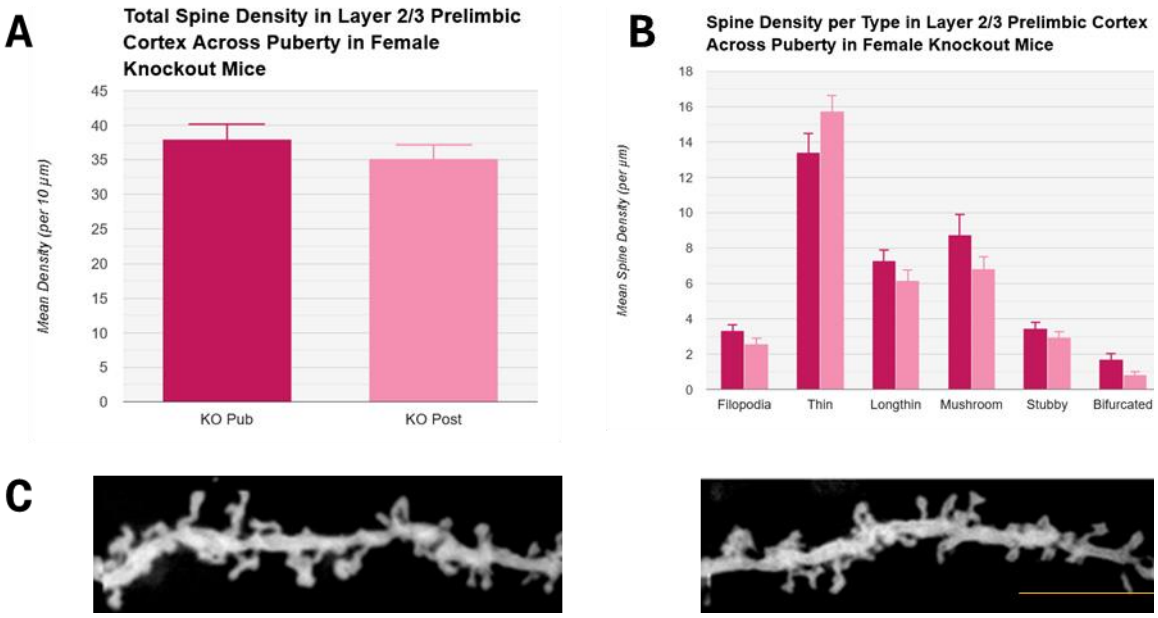

Figure V.2 Spine density in layer 2/3 prelimbic cortex (L2/3 PL) of the female α4 knockout mouse does not prune during adolescence: assessment of spine-types. Averaged data, spine density (#spines/10 µm) of layer 2/3 (L2/3) PL for pubertal (KO Pub) and post-pubertal (KO Post) female mice. (a) Total (KO Pub: 37.58, KO Post: 35.04), (b) Bifurcated (KO Pub: 1.66, KO Post: 0.813), Mushroom (KO Pub: 8.611, KO Post: 6.799), Stubby (KO Pub: 3.457, KO Post: 2.961), Long thin (KO Pub: 7.267, KO Post: 6.157), Thin (KO Pub: 13.38, KO Post: 15.77), Filopodia (KO Pub: 3.337, KO Post: 2.579). (c) Representative images of basal dendrites from Golgi-stained neurons, from Pub and Post-pub female mice. Scale, 5 µm. n = 23–26 neurons, 6 mice/group.

Discussion

The dramatic pruning of excitatory synapses on prelimbic cortex layer 2/3 pyramidal neurons between puberty onset and later adolescence aligns with human anatomical studies showing extensive synaptic remodeling within the prefrontal cortex (PFC) during this developmental window. Postmortem analyses indicate that synaptic density in the human dorsolateral PFC increases in early childhood, peaks around 1-2 years old, and subsequently decreases by over 50% during adolescence before stabilizing in adulthood (Huttenlocher & Dabholkar, 1997). This adolescent synaptic remodeling likely enables maturation of higher cognitive abilities like working memory, cognitive flexibility, and emotional regulation that rely critically on precise connectivity patterns within the PFC (Selemon, 2013). For instance, synaptic pruning is thought to remove inefficient connections, strengthen relevant pathways, and increase the signal-to-noise ratio within these executive control circuits (Casey, et al., 2005). Structural MRI studies demonstrate that age-related improvements in working memory

performance correspond with reductions in PFC gray matter volume occurring during adolescence, suggestive of synaptic pruning (Tamnes, et al., 2010). The protracted timeline of human PFC synaptic remodeling extending into the third decade underscores the late functional maturation of these networks.

Rodent models recapitulate similar cell type-specific differences in the developmental trajectory of excitatory synapse pruning within the medial PFC (mPFC) (Drzewiecki, et al., 2016). For instance, layer 2/3 pyramidal neurons exhibit dramatic overproduction of dendritic spines during the juvenile period followed by pruning in adolescence and into adulthood (Markham et al., 2007; Koss et al., 2014) only in females. Spine formation on layer 5 pyramidal neurons peaks later around the periadolescent period in rats, followed by subsequent pruning (Drzewiecki, et al., 2016).

Layer 2/3 cortical neurons overall show greater reductions in spine density and turnover rates compared to layer 5 throughout postnatal development (Koss et al., 2005). in vivo electrophysiology recordings demonstrate weaker cue-selective firing of mPFC layer 2/3 neurons in juvenile rats performing a behavioral task compared to adults, indicative of protracted functional maturation (Quiquempoix, et al., 2018).

The more rapid adolescent remodeling of layer 2/3 connectivity may be necessary for optimizing working memory and cognitive flexibility subserved in part by robust local recurrent excitatory connections between layer 2/3 pyramidal neurons (Kolb et al., 2012). Strengthening of top-down glutamatergic projections from layer 2/3 to layer 5 is also thought to sharpen executive control over responses to affective stimuli during adolescent maturation (Gee et al., 2013).

By demonstrating disrupted spine pruning on layer 2/3 pyramidal neurons in α4 knockout mice, the current results implicate a key role for α4-containing GABAA receptors in mediating the adolescent remodeling of superficial mPFC networks involved in gating working memory maintenance and regulating emotional behaviors. The dependence on α4 signaling likely involves extra-synaptic α4βδ receptors, which generate a substantial portion of the tonic inhibitory current in cortical layer 2/3 neurons that regulates overall excitability (Drasbek & Jensen, 2006).

The unique high sensitivity and sustained activation kinetics of α4βδ receptors in response to low, ambient levels of GABA position them to provide circuit-level control over synaptic reorganization during developmental critical periods when subunit expression peaks regionally (Shen et al., 2010). Computational models suggest the tonic inhibition mediated by α4βδ receptors can induce activity-dependent competition

between synapses locally within dendritic branches, leading to selective stabilization versus elimination based on coordinated pre- and post-synaptic firing (Winnubst et al., 2015). This represents a mechanism for refining connectivity based on functional relevance.

By elucidating a role for α4βδ receptors in adolescent spine remodeling, these findings have implications for understanding executive dysfunction in neurodevelopmental disorders associated with cortical abnormalities like schizophrenia, ADHD, and autism. While speculative, disrupted α4βδ-dependent pruning during sensitive windows could alter prefrontal connectivity contributing to behavioral impairments. However, directly assessing the impacts on human cognition and risk for psychiatric conditions will be an important future direction.

Overall, these results significantly advance understanding of cortical circuit maturation during a key developmental window and reveal novel roles for α4βδ GABAA receptors in the activity-dependent refinement of synaptic connectivity with important implications for the emergence of higher-order executive functions and associated psychiatric disease vulnerabilities.

VI. Conclusions

The present thesis advances understanding of the role of α4βδ GABAA receptors in mediating dendritic spine pruning within layer 5 pyramidal neurons of the mouse prelimbic prefrontal cortex during female pubertal development. The work combines complementary techniques spanning morphological quantification, electrophysiology, pharmacology, molecular analysis, and initial behavioral assessments which provide preliminary evidence to substantiate the necessity and sufficiency of α4βδ signaling in triggering adolescent synaptic remodeling. Knockdown of α4βδ receptors prevents typical spine elimination between puberty onset and late adolescence, resulting in increased dendritic spine densities. While this morphological change is hypothesized to potentiate anxiety-like behavior in adulthood based on preliminary elevated plus maze data, additional experiments are needed to conclusively demonstrate the functional and behavioral impacts resulting from disrupted pruning. Follow-up studies should incorporate electrophysiology, calcium imaging, and circuit-level manipulation to unambiguously link aberrant prelimbic pruning to anxiety phenotypes and cortical dysfunction relevant for neuropsychiatric disease. Beyond confirming the central mechanistic model, the research raises exciting new directions for deconstructing circuit-level impacts, regional specificity, sex differences, and additional interacting mechanisms that promise to further enrich comprehension of adolescent neurodevelopment. Ongoing work is imperative to substantiate the proposed connections between altered remodeling and emerging behavioral vulnerabilities.

A key technique utilized is high-resolution light microscopy examination of Golgi-stained neurons to quantify dendritic spine densities on layer 5 pyramidal neurons across pubertal development as a proxy measure of excitatory synaptic connectivity. Spine density along basal dendrites decreased approximately 60% between puberty onset and late adolescence in the female mouse prelimbic cortex. This aligns with human postmortem studies demonstrating over 50% reductions in dorsolateral prefrontal cortex synapses from childhood through adolescence (Huttenlocher, 1979). The extensive synapse elimination likely reflects experientially instructed pruning processes that refine connectivity patterns forged during earlier plastic phases to strengthen relevant circuits supporting adult cognitive abilities.

The results corroborate previous rodent research reporting significant decreases in overall spine density (Pattwell et al., 2016) and turnover rates (Zuo et al., 2005) on medial prefrontal cortex pyramidal neurons across adolescence. However, this study provides unprecedented quantification of the dramatic spine loss timeline

specifically within deep layers of the prelimbic subregion immediately surrounding puberty onset in the female mouse. Furthermore, the morphological categorization of stubby, thin, and mushroom spine subtypes provides insights into potential functional implications of pruning. Mushroom spines decreased the most, by approximately 75%, indicative of substantial pruning of mature, stable inputs. The large mushroom head size suggests powerful glutamatergic drive (Bourne & Harris, 2008), which could compound effects of increased density following disrupted pruning on neuronal excitability. Meanwhile, thinning spines decreased by only 50%, consistent with relative preservation of motile, structurally dynamic connections thought to participate in plasticity (Zuo et al., 2005).

While 2D analysis of projections provides efficient dendritic spine quantification, advances in imaging and reconstruction now enable high-fidelity 3D segmentation of spine morphologies (Wallace & Jones, 2008). Complex shape features like neck diameter, spine head volume, surface area, and insertion angle extracted from 3D reconstructions can inform estimates of synaptic strength and structural plasticity (Belardi et al., 2014). For instance, small-headed stubby spines are considered ‘silent’ connections while large mushrooms represent strong inputs (Yasumatsu et al., 2008). Additionally, machine learning algorithms applied to 3D morphometric data can automatically categorize spine subtypes and analyze changes with unprecedented throughput (Zhao et al., 2017). Incorporating 3D spine profiling would enrich this 2D structurally-informed functional interpretation.

Furthermore, while examining Golgi-impregnated samples under light microscopy enables quantification of dendritic spines as putative postsynaptic sites, electron microscopy remains the gold standard for definitively identifying synapses and analyzing the post-synaptic density characteristics conferring synaptic strength (Nicholson & Geinisman, 2009). Combining serial section transmission electron microscopy and 3D reconstruction could unambiguously confirm increased synapse density and structural features associated with potentiated transmission like enlarged PSD size following pubertal α4βδ knockdown (Nicholson & Geinisman, 2009; Desmond & Weinberg, 1998).

Finally, the studies employ acute Golgi impregnation of neuronal ensembles which provides representative sampling but only captures a single snapshot lacking temporal information. An exciting innovation is combining Golgi staining with thiol-mediated clearization and optical tissue clearing methods like CUBIC, enabling whole-brain imaging of neuronal morphologies (Hou et al., 2015; Lloyd-Lewis et al., 2016). Integrating clearance with viral labeling techniques permitting cell-type-specific Golgi-GFP expression and in vivo two-photon

imaging could longitudinally track spine dynamics within identified populations of interest across development (Winnubst et al., 2015). This could clarify critical periods of vulnerability, characterize instability preceding pruning, and assess recovery rates after disruption.

Alongside morphological spine analysis, the thesis incorporates whole-cell patch clamp electrophysiology, these experiments were conducted by Hui Shen, and examined responses to pharmacological agents targeting GABAA receptors. The findings demonstrate increased activation of α4βδ receptors within layer 5 pyramidal neurons specifically at puberty based on amplified currents elicited by gaboxadol, a highly selective agonist exhibiting 70-fold higher potency at α4/δ-containing configurations (Meera et al., 2011). This provides functional evidence confirming the transient upregulation of α4βδ receptors observed immunohistochemically. Electrophysiology complements the molecular and structural data to substantiate the critical period of elevated α4βδ signaling aligned with synaptic pruning.

Several additional electrophysiology experiments could provide further insight into the functional state of layer 5 pyramidal neuron synaptic circuits following disrupted pubertal pruning due to α4βδ knockdown. Whole-cell voltage clamp recording of spontaneous mEPSCs could characterize frequency, kinetics, and amplitude of glutamatergic signaling (Desai et al., 2002). Increased mEPSC frequency would provide functional validation of heightened synapse density from morphological quantification. Potentiated mEPSC amplitude could reflect greater glutamate release probability or postsynaptic sensitivity. Faster mEPSC kinetics could suggest altered AMPAR subunit composition associated with enhanced plasticity (Liu & Zukin, 2007). Comparing mEPSC properties between α4βδ knockdown and wildtype mice at puberty onset versus adulthood could link adolescent remodeling changes to persistent circuit alterations.

Furthermore, in vivo two-photon calcium imaging could quantify spine activation probabilities and glutamate uncaging could examine insertion potentials for individual spines (Matsuzaki et al., 2004; Harvey & Svoboda, 2007). This would assess the functional capabilities of excess synapses to drive postsynaptic excitation. Population calcium imaging could characterize the spatial spread of bAP-evoked calcium influx throughout the dendritic arbor, which may be increased in cases of surplus connectivity (Raghuram et al., 2019). Functional clustering analysis could identify hyper-connected dendritic compartments aligned with impaired pruning (Winnubst et al., 2017).

In addition to probing postsynaptic properties, optogenetic techniques could elucidate changes in specific inputs, like providing temporally precise activation of medial prefrontal projections from the basolateral amygdala (Little & Carter, 2013). Measuring paired pulse ratios before and after pubertal α4βδ knockdown could indicate altered presynaptic release probabilities that rescale synaptic drive from afferent pathways (Dobrunz & Stevens, 1997). Photoactivating channelrhodopsin-expressing axonal projections combined with post-synaptic recordings enables selective examination of defined circuit connections. This input-specific approach can circumvent the limitations of electrical stimulation.

Furthermore, multi-site local field potential recordings could examine effects of altered pruning on prelimbic network oscillations. Synchronized firing in the gamma band is important for working memory and cognitive control, while theta rhythms coordinate inter-region communication like prefrontal-hippocampal interactions (Colgin, 2013). Disruption of these oscillations occurs in many psychiatric conditions associated with prefrontal cortical dysfunction like schizophrenia (Gonzalez-Burgos & Lewis, 2012). Patch recordings have characterized increased gamma power in prelimbic networks following chronic stress, while ketamine preferentially elevates gamma in layer 5 (Jeevakumar et al., 2018; Zhang et al., 2019). Determining if dysregulated adolescent pruning shifts oscillatory rhythms linked to affected behaviors remains an important question. Changes in spike-field coherence could also indicate impaired spike-timing dependent plasticity mechanisms involved in maturation.

In vivo electrophysiology in behaving animals represents the gold standard for relating circuit modifications to integrated behavioral phenotypes and cognitive processes. Chronic multielectrode implants could enable ensemble single-unit recordings during working memory tasks known to engage the prelimbic cortex (Spellman et al., 2015). This could clarify how persistent juvenile connectivity patterns from aberrant pruning impact adult neuronal encoding. Techniques combining optogenetics with electrophysiology and behavior in freely moving mice also facilitate pathway- and cell-type-specific investigation of causal circuit mechanisms underlying behavioral changes (Krook-Magnuson et al., 2014). Overall, while technically challenging, electrophysiology and functional imaging approaches would provide critical insight into the implications of morphological spine changes on synaptic and circuit function.

Alongside morphological and physiological assessments, the thesis employs molecular techniques examining the expression dynamics of α4-containing GABAA receptors and associated signaling pathways

involved in dendritic spine regulation. Immunohistochemistry reveals a robust ten-fold increase in α4 subunit expression within layer 5 pyramidal neuron somas and dendrites precisely at puberty onset in the female mouse prelimbic cortex. α4 levels subsequently decline by approximately 75% into late adolescence. This transient upregulation provides important developmental timing cues aligning α4-containing receptors with the key period of synaptic pruning.

Furthermore, immunofluorescent co-labeling of α4 with MAP2, which localizes to dendritic shafts and mushroom spine heads, confirms accumulation of α4-containing receptors at the tips of dendritic spines where they are well-positioned to respond to ambient GABA and mediate pruning. The spatiotemporal patterning supports a localized role for α4βδ receptors in mediating the selective refinement of connections through suppression of weak or inefficient inputs. Additional experiments utilizing super-resolution imaging with techniques like STORM and expansion microscopy could enable direct visualization of receptor nanoscale localization changes within the postsynaptic density itself during pubertal maturation (Dani & Huang, 2010; Chozinski et al., 2016).

While immunohistochemistry provides a snapshot of protein expression, longitudinal profiling approaches at both the transcriptomic and proteomic levels could offer enhanced resolution of the developmental dynamics. Techniques like TRAP-Seq leverage translating ribosome affinity purification to profile the translatome within specific cell populations, which better reflects protein abundance compared to whole-tissue RNA-Seq (Mo et al., 2015). Applying TRAP-Seq around puberty onset using cell-type-specific Cre-lines like Rbp4-Cre for layer 5 neurons could clarify translational shifts guiding remodeling (Gerfen et al., 2013). Repeated sampling every few days surrounding puberty could pinpoint expression changes with unprecedented temporal precision. Single-nucleus sequencing methods also now facilitate improved resolution of adolescent brain maturation trajectories within individual cells and across diverse cell classes (Li et al., 2019). Integrating these profiling techniques with CRISPR interference (CRISPRi) epigenome editing to manipulate loci dynamics could further enable elucidating causal roles in pruning (Roth et al., 2021). Together, advancing molecular techniques promise to enrich the spatiotemporal details regarding α4βδ signaling activation.

Pharmacological manipulations represent a key pillar of the mechanistic experiments, given the ability to augment or suppress endogenous receptor populations at precise developmental timepoints. Pubertal administration of gaboxadol, which selectively potentates α4βδ-mediated tonic current at the low dose used,

significantly enhanced spine pruning assessed in late adolescence (Meera et al., 2011). This demonstrates the sufficiency of these specialized receptors to drive spine elimination. Conversely, blocking all GABAA receptors with picrotoxin across the same pubertal window markedly impaired pruning, reflected by a several-fold increase in spine densities. The bidirectional pharmacological modulation provides convincing confirmation that α4βδ signaling triggers typical adolescent remodeling.

While these initial studies focused on systemic drug administration, techniques now enable localized infusion directly into the prelimbic cortex. Micro-osmotic pumps connected to cannulae implanted in deep layers could provide continuous pubertal delivery of compounds like gaboxadol or DS2 to selectively amplify local tonic inhibition (Vinkers et al., 2010). Examining projection-specific effects represents an advantage of localized infusion. For instance, selectively increasing inhibition of basal tuft dendrites receiving inputs from other associational cortical regions could disproportionately prune cortico-cortical connectivity with relatively spared thalamic projections onto proximal dendrites (Kuroda et al., 1998).

Furthermore, combining pharmacology with electrophysiology could provide additional confirmation of mechanism while enabling precise dosing. For instance, applying gaboxadol during patch clamp recordings could first identify concentrations eliciting approximately 50% increases in holding current, then examine spine morphology following pubertal exposure to the corresponding dose (Maguire & Mody, 2007). Linking pharmacodynamic electrophysiology changes to morphological impacts would help optimize therapeutic targeting. Brain slice recordings also enable analysis of local microcircuits, like examining disinhibition of amygdala projection neuron firing following gaboxadol application (Marowsky et al., 2004). This input-specific approach clarifies network propagation.

Finally, pharmacogenetic and chemogenetic methods enable cell-type-specific targeting of receptor populations guided by genetic drivers. For instance, our lab has developed a knock-in mouse line with a floxed gaboxadol sensitizing point mutation selectively enhancing tonic current through δ-containing receptors four-fold (α4^F77I^) (Paul et al., 2021). Crossing to parvalbumin-Cre mice restricts increased tonic inhibition to PV+ interneurons and enables examining the influence of augmenting specialized interneuron activity on synaptic pruning. Chemogenetic receptors like Gabrd-DREADDs also facilitate remote modulation of signaling using receptor-specific designer drugs (Whissell et al., 2019). Overall, advancing techniques provide greater

pharmacological precision through avenues like localized delivery, input-specific application, and cell-type-specific targeting to refine circuit mechanisms.

Beyond demonstrating the central role of α4βδ receptors, the work provides initial mechanistic evidence that suppression of NMDAR-Kalirin7 signaling represents a key intracellular pathway engaged to initiate spine disassembly. The post-synaptic density protein Kalirin7 promotes actin cytoskeleton polymerization underlying structural plasticity through activation of the RhoGTPase Rac1 (Ma et al., 2008). Kalirin7 expression decreases dramatically in the female prelimbic cortex specifically during puberty, but remains elevated in α4 knockout mice, directly implicating α4βδ receptor activation in triggering Kalirin7 reductions as a precursor to pruning. This aligns with computational models proposing that weak synaptic inputs are selectively destabilized by suppressing molecular pathways supporting the actin cytoskeleton (Butz et al., 2009). While promising, further experiments are needed to substantiate the causal signaling cascades engaged.

A prediction of the model is that directly preventing NMDAR activation during puberty should lower Kalirin7 levels and enhance spine pruning akin to increased α4βδ signaling. Pharmacological NMDAR antagonists like APV could test this prediction. Electrophysiology experiments measuring NMDAR-EPSCs before versus after pubertal gaboxadol administration could also confirm whether α4βδ activation functionally suppresses NMDAR currents. To unambiguously demonstrate if Kalirin7 reductions are necessary for pruning, molecular techniques like in utero electroporation of kalirin-shRNA could selectively lower embryonic Kalirin7 expression, enabling assessment of causal pruning effects in adolescence (Lu et al., 2015).

Conversely, maintaining Kalirin7 levels by virally overexpressing Kalirin7 or a constitutively active truncated variant during puberty could rescue pruning deficits following α4 knockdown (Xie et al., 2007). This would establish necessity of Kalirin7 downregulation for pruning. Assessing interactions with other critical spine proteins like PSD-95, SAP102, and Shank3 could further enrich the model (Cui et al., 2017). Proteomic analyses might reveal coordinated expression shifts in synaptic protein modules interacting with Kalirin7-Rac1 signaling as part of the maturation process. Furthermore, RNA sequencing could identify transcriptional cascades engaged by α4βδ activation associated with synapse disassembly programs, like microglial phagocytic pathways proposed to remove destabilized inputs (Schafer et al., 2012). Integrating findings across multiple techniques can substantiate the intracellular signaling model.

While the current studies focus on morphological quantification of dendritic spines in layer 5 as a proxy for synaptic connectivity, relating alterations to broader impacts on neural circuits will be essential for evaluating contributions to behavioral changes. The central hypothesis posits that dysregulated pubertal pruning increases glutamatergic drive within the prelimbic cortex based on heightened spine densities, which augments anxiety-like avoidance behaviors by over-activating amygdala threat detection pathways. However, directly assessing changes in prelimbic ensemble firing, elucidating impacts on downstream amygdala targets, and testing necessity of amygdala connectivity could strengthen circuit-level inferences.

Several approaches could provide insight. Optogenetic identification of specific inputs permits selective activation of glutamatergic projections from other limbic areas like basolateral amygdala or ventral hippocampus while recording synaptic responses and neuronal firing in the prelimbic cortex (Little & Carter, 2012). This input-specific analysis following knockdown of α4βδ receptors could assess if heightened spine densities specifically potentiate transmission at amygdala and hippocampal synapses most relevant for anxiety behaviors. Alternatively, channel rhodopsin-assisted circuit mapping uses broad ChR2 expression and systematic light stimulation to map functional connectivity, which may reveal network-wide differences in excitation propagation from increased connectivity (Kwan & Dan, 2012).

Furthermore, in vivo electrophysiology recordings in awake, behaving animals could characterize effects on ensemble firing patterns and spike coding of threat responses. Prelimbic neurons exhibit cue-selective firing during fear conditioning (Burgos-Robles et al., 2009), which could be altered by excess synapses. Optogenetic tagging enables identification of cells projecting to downstream regions, which can be used to monitor projection-defined ensembles (Guo et al., 2020). This would help assess how changes in prelimbic activity patterns impact firing in target areas like basolateral amygdala. Pharmacogenetic inactivation of prelimbic cortex projection neurons specifically could also establish necessity for generating behavioral effects (Mahler et al., 2014). Examining anatomical connectivity changes with techniques like anterograde viral tract tracing could provide further insights into circuit-level effects of dysregulated pruning. Enhanced prelimbic dendritic spine density and synaptic input may strengthen glutamatergic projections to downstream areas, increasing axon terminal densities and release sites in target regions like the basolateral amygdala (Arruda-Carvalho et al., 2017). Tracing projection intensity differences with synaptophysin immunohistochemistry following pubertal knockdown of α4βδ receptors could assess this possibility.

Resting-state fMRI could also be utilized to examine functional connectivity changes in patients with anxiety disorders as well as associations with genetic polymorphisms altering GABA signaling (Etkin et al., 2009; Nollet et al., 2020). However, limitations of correlational human imaging make inferring causal circuit mechanisms challenging. Optogenetically manipulating neuronal firing in select pathways during fMRI represents a promising approach to clarify anxiety circuits, as demonstrated for amygdala projections to medial prefrontal cortex (Qi et al., 2020). This could evaluate necessity of specific connections for behavioral effects.

Cortico-cortical feedback circuits between prelimbic, infralimbic, and anterior cingulate subregions also coordinate anxiety-related behavioral responses (Bloodgood et al., 2013). Examining infralimbic inputs, which provide top-down inhibition counterbalancing anxiety-provoking prelimbic activity, may reveal important complementary effects of dysregulated pubertal pruning (Bloodgood et al., 2013). Perturbations likely alter excitatory-inhibitory balance between prefrontal cortical subnetworks regulating amygdala output. Clarifying changes in intra-prefrontal connectivity could delineate additional circuit mechanisms.

Furthermore, modulatory systems like dopamine projections from ventral tegmental area to prelimbic cortex represent another avenue for further investigating circuit changes. Dopamine bidirectionally regulates the excitation-inhibition balance in prefrontal networks and shows altered signaling in anxiety disorders (Surmeier et al., 2013). Dopaminergic inputs could compound the effects of excess synaptic connectivity by increasing network excitability. Exploring interactions with neuromodulatory pathways expands the potential network perturbations resulting from disrupted adolescent remodeling.

Finally, chemogenetic and optogenetic manipulations enable causal interrogation of candidate circuits implicated by correlative evidence. Projection-targeted inhibition using retrogradely transported Cre to selectively express inhibitory DREADDs in prelimbic cortical neurons could establish necessity of this pathway for behavioral changes through reversible, temporally-precise silencing (Tervo et al., 2016). Additionally, closed-loop optogenetic stimulation protocols enabled by real-time processing of neural activity provide exciting avenues for bidirectional control of anxiety-related ensemble firing (Wiegert et al., 2017). Overall, substantiating the causal circuit mechanisms underlying anxious behavioral phenotypes will be critical for validating the model.

The current investigations focus on changes occurring specifically within deep layers of the prelimbic prefrontal cortex based on its defined role regulating anxiety-like responses in rodents and humans (Grupe & Nitschke, 2013). However, adolescent remodeling likely involves coordinated transformations across

interconnected regions within broader fear and anxiety circuits. Elucidating differences across cortical areas, limbic structures, and subcortical nodes could reveal important specializations or compensatory adaptations.

For instance, synaptic pruning measured by MRI occurs relatively earlier in sensory cortices compared to association regions like prefrontal cortex, suggesting regional variability in developmental timecourses (Petanjek et al., 2011). Within medial prefrontal cortex, layer 2/3 exhibits an earlier peak and subsequent pruning in juvenile mice relative to layer 5 (Zuo et al., 2005). Our preliminary experiments also demonstrate a transient increase in α4 subunit expression surrounding puberty specifically within prelimbic cortex, but not adjacent infralimbic regions. This indicates adolescent remodeling processes can be highly localized.

Notably, α4βδ receptor expression occurs in other cortical and limbic areas implicated in anxiety processing, including the basolateral amygdala, ventral hippocampus, and anterior cingulate (Maguire et al., 2005). Manipulating α4βδ signaling in these structures could have complementary or competing effects on anxiety-related behaviors based on their distinct roles. For instance, infusing gaboxadol to enhance tonic inhibition in the basolateral amygdala decreases anxiety behaviors in rodents (Marowsky et al., 2005). Potentiating hippocampal or cingulate α4βδ receptors could exert anxiolytic effects through attenuating contextual encoding or enhancing top-down regulation, respectively (Engin et al., 2009; Etkin et al., 2006). Clarifying subsystem-specific effects will enable differentiating local versus distributed circuit contributions.

Furthermore, compensatory adaptations in interacting regions like basolateral amygdala could counteract changes in prelimbic cortex. Homeostatic plasticity mechanisms strive to maintain optimal activity levels despite perturbations (Turrigiano, 2011). For example, reducing excitatory drive to basolateral amygdala projection neurons may compensate for increased prelimbic activity. Dendritic spine analysis, electrophysiology, and imaging experiments could evaluate this possibility across components of the circuitry. Investigating network-wide effects remains an ongoing challenge.

Technical advances now enable targeting selectivity and projection-defined signaling with unprecedented precision. Viral approaches permit pathway- and region-specific manipulation of receptor populations or neural activity (Tervo et al., 2016; Arruda-Carvalho et al., 2017). Optogenetics can selectively silence or activate defined circuit elements mimicking dysfunctional signaling (Tye et al., 2011). Chemogenetics facilitate remote temporal control over cell types using designer drugs (Mahler et al., 2014). These tools can map causal relationships

through targeted intervention. Applying these techniques across multiple structures within fear neurocircuitry represents a critical future direction.

This research investigates synaptic pruning mechanisms specifically within developing female mice given the heightened prevalence of anxiety disorders in women (Bandelow & Michaelis, 2015). Females exhibit twice the rates of anxiety compared to males, with initial onset commonly arising in adolescence around puberty (Remes et al., 2016). Fluctuations in ovarian hormones like estradiol and neurosteroids interacting with GABAergic transmission contribute to sex differences in stress reactivity and anxiety behaviors (Ter Horst et al., 2012; Gunn et al., 2013). Sex differences in brain maturation trajectories likely also play an important role.

However, details regarding divergent developmental profiles between males and females remain underexplored. Our preliminary studies indicate that layer 5 prelimbic neurons in male mice undergo moderate but significant synaptic pruning, decreasing dendritic spine density by approximately 50% between early adolescence and adulthood. This is paralleled by transient upregulation of the α4 subunit surrounding puberty in males, though less dramatic than in females. Preventing this rise by α4 knockout correspondingly inhibits spine pruning.

These results suggest pubertal α4βδ-mediated remodeling occurs in both sexes but to varying degrees, with a less substantial pruning timeline in males. This aligns with the epidemiological data showing higher prevalence rates of anxiety disorders in women (Remes et al., 2016). However, given sex biases in clinical diagnosis and reporting as well as sociocultural factors, unambiguously relating the sex differences in adolescence to adult psychopathology remains challenging (McLean et al., 2011). Controlled longitudinal studies tracking adolescent neurodevelopmental trajectories to anxiety disorder outcomes would be informative but pose ethical barriers in humans. Animal models enable systematically characterizing sex-specific differences in circuit refinement.

Several key questions emerge. Are sex differences in pruning primarily quantitative resulting from divergent α4 expression levels? Or are there qualitative distinctions in receptor signaling properties, dendritic sub-compartment effects, and engaged pathways? Does estradiol modulation of tonic inhibition contribute by increasing α4 transcription? How do pubertal timing differences between males and females interact with pruning timelines? Does white matter myelination parallel the more rapid synaptic remodeling in females? Are microglial

pathways involved to a greater degree in male pruning? Does earlier female maturation of prefrontal circuits relate to sex differences in executive function maturation or susceptibility windows?

Furthermore, do males exhibit compensatory mechanisms or unique resilience factors? Human neuroimaging indicates males show greater frontal activation during anxiety states, suggesting compensatory recruitment of cognitive control resources (McClure et al., 2007). Greater connectivity between prelimbic cortex and amygdala in males could necessitate more extensive pruning to achieve comparable circuit tuning (Kaczkurkin et al., 2016). Elucidating sex-specific strategies underlying varied developmental trajectories represents a key challenge.

Technical advances in cellular profiling and targeted interventions will likely be instrumental in addressing these questions. Single nucleus sequencing around puberty onset could help resolve cell class-specific maturation dynamics in males versus females (Dries et al., 2017). Viral-mediated RNA interference enables knocking down α4 in a sex-specific manner to evaluate causal roles in pruning (Ginger et al., 2013). Optogenetics permits selective silencing of pathway connections that differ by sex (Shen et al., 2016). Chemogenetics can remotely modulate neuronal populations expressing sex hormone receptors to assess influences on remodeling (Urban & Roth, 2015). Applying these innovative techniques will be critical for unraveling sex differences in the adolescent brain.

5.4. Additional Mechanisms

Microglia

The thesis focuses on the role of α4βδ GABAergic signaling given supportive preliminary data. However, synaptic remodeling likely involves coordinated interactions amongst diverse cell types and molecular pathways. Microglia represent the brain's resident immune cells that critically contribute to synaptic pruning during postnatal development through phagocytic engulfment of dendritic spines and synapses (Schafer et al., 2012). Mice lacking the fractalkine receptor CX3CR1 exhibit defective microglial localization and impaired synaptic pruning, substantiating causal roles (Paolicelli et al., 2011). Furthermore, disrupting phagocytic pathways by knockout of CR3/C3 receptors markedly reduces spine elimination, effects partially rescued by neonatal bone marrow transplantation from wildtype mice (Schafer et al., 2012). Dynamic interactions between microglia and the synaptic compartment appear vital for remodeling.

Notably, microglial-mediated pruning contributes to postnatal synaptic remodeling across cortical and subcortical brain regions (Schafer et al., 2012). This contrasts with the regional enrichment of α4βδ GABAA receptors in areas like prelimbic cortex, hippocampus, and basolateral amygdala (Peng et al., 2004). The widespread microglial mechanisms likely serve more general functions, while α4βδ signaling may confer specificity by targeting inhibition based on region- and cell-type-specific expression patterns. Elucidating the interplay between glial and GABAergic pruning pathways remains an ongoing challenge.

Do microglia act first by broadly engulfing subsets of synapses overproduced in early childhood, before α4βδ receptors remodel remaining circuitry in late adolescence through activity-dependent mechanisms? Or do microglia function downstream to selectively phagocytose synapses destabilized by α4βδ suppression of cytoskeletal pathways like Kalirin-7? Does microglial engulfment confer specificity through activity-dependent fractalkine signaling from tagged inputs (Paolicelli et al., 2011)? Disambiguating the division of labor and timecourses requires intersecting approaches modulating receptor signaling versus glial function.

Furthermore, the dependency of microglial pruning on neural activity patterns remains debated, with conflicting evidence about whether silencing inputs increases or decreases engulfment (Sipe et al., 2016; Schafer et al., 2012). This contrasts with the activity-dependent activation of α4βδ receptors positioned to translate ongoing firing into targeted remodeling. Comparative analysis of real-time microglial phagocytic behavior versus electrophysiological measures of network activity could help resolve discrepancies about activity-dependence. Improved synaptic labeling and in vivo time-lapse imaging will likely be instrumental in addressing these dynamics.

Overall, microglia-mediated elimination undoubtedly represents an essential component of synaptic remodeling over postnatal development. Ongoing work is needed to clarify specificity and integration with α4βδ and other pruning signals. Examining interactions between microglia, astrocytes, interneurons, and excitatory neurons across cortical layers and ages continues to represent an intricate challenge requiring convergent techniques.

Neuroimmune Factors

The neuroimmune system heavily influences synaptic development, plasticity, and remodeling (Hong et al., 2016). Astrocytes refine connectivity through MEGF10 and MERTK-dependent engulfment of synapses,

representing a new pruning mechanism (Chung et al., 2013). Microglia and astrocytes communicate via signaling molecules including interleukin-1β, CX3CL1, and C1q to coordinate phagocytosis (Boulanger, 2009). The classical complement cascade has emerged as a crucial synaptic pruning pathway, with C1q accumulation triggering elimination and C3 recruitment of microglia (Stevens et al., 2007). Disrupting this pathway impairs developmental synapse loss, supporting an instructive role in remodeling (Sekar et al., 2016).

Neuroinflammation alters expression of synaptic proteins like kalirin, suggesting potential intersection with remodeling pathways (Peng et al., 2013). Pro-inflammatory cytokines like tumor necrosis factor α reduce inhibitory synapse strength (Pribiag & Stellwagen, 2013). Neural activity bi-directionally triggers release of immune molecules including fractalkine, which mediates microglia-synapse interactions underlying activity-dependent pruning (Schafer et al., 2012). The idiosyncratic expression of neuroinflammatory mediators could contribute to regional specificity of remodeling. Furthermore, sex differences in microglial function and signaling molecules like C1q may underlie sexual dimorphism in adolescent pruning (Hanamsagar et al., 2017; Lenz & McCarthy, 2015). Overall, neuroimmune pathways exhibit extensive bidirectional crosstalk with synaptic signaling critical for remodeling, although details remain to be fully elucidated.

Estradiol

Pubertal onset markedly increases gonadal hormone production, which likely contributes to adolescent synaptic remodeling. Estradiol increases nearly 100-fold in females around menarche, while testosterone surges in males (Sisk & Zehr, 2005). Ovarian estradiol has widespread impact through diverse receptors distributed across brain regions (McEwen, 2002). Estrogen receptors directly interact with glutamatergic signaling machinery, modulating dendritic spine plasticity and glutamate receptor trafficking (Hara et al., 2015; Tedesco et al., 2001).

Estradiol may interface with α4βδ-mediated remodeling by regulating subunit transcription. The δ subunit gene GABRD contains an estrogen response element in its promoter region, suggesting direct genomic effects (Follesa et al., 2002). Estrogens could contribute to female-specific pruning timelines by enhancing α4 and δ expression at puberty. Consistent with this model, preliminary data indicates the transient pubertal increase in the α4 subunit is delayed in female aromatase knockout mice lacking estradiol synthesis (Shen et al., 2007). Estrogen effects likely intersect with excitatory-inhibitory balance adjustments through estrogen receptor alpha

localized on parvalbumin interneurons (Blurton-Jones & Tuszynski, 2002). Understanding interactions between gonadal and GABAergic systems in synaptic remodeling requires further elucidation but may account for sexual dimorphism.

Neurotrophins

Neurotrophins including nerve growth factor, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 also critically regulate dendritic spine morphogenesis and plasticity (Zagrebelsky & Korte, 2014). BDNF promotes spine maturation and stabilization by stimulating cyclic AMP-protein kinase A cascades that increase cytoskeletal gene transcription, including kalirin-7 (Yoshii & Constantine-Paton, 2010). Reduced BDNF levels are implicated in anxiety disorders, while antidepressants increase BDNF signaling and spine density (Castrén & Antila, 2017). Developmental shifts in neurotrophin tone could contribute to adolescent synaptic pruning.

BDNF interactions with inhibitory signaling pathways like α4βδ GABAA receptors remain unclear but potentially bidirectional (Porcher et al., 2018). For instance, BDNF increases δ subunit expression, while neurotrophins restrain GABAergic synaptic maturation through truncated Trk receptor signaling (Holm et al., 2011). Disinhibition from decreased α4βδ tone could elevate BDNF-trkB activation, representing a compensatory response. Testing predictive morphogenic effects of exogenous BDNF application following α4βδ knockdown could provide initial insights (Kellner et al., 2014). Investigating interactions between neurotrophins and GABAergic signaling during adolescent remodeling remains an open area.

Genetic Mechanisms

While this work centers on α4βδ GABAA receptors given their unique developmental expression dynamics, synaptic pruning likely involves genetic orchestration of diverse signaling cascades. Transcriptomic analyses in humans and non-human primates have uncovered gene co-expression modules related to synaptogenesis and spinogenesis that decline in adolescence, potentially linked to pruning programs (Somel et al., 2009; Werling et al., 2016). For instance, NPTX2 in the murine frontal cortex exhibits dramatic upregulation specifically at puberty onset before decreasing into adulthood, suggesting a role in remodeling pyramidal neuron connectivity (Somel et al., 2009). Understanding the genetic regulation temporalizing adolescent pruning remains an active area.

Furthermore, polymorphisms in synaptic genes like kalirin, ErbB4, and DISC1 associate with schizophrenia and other neuropsychiatric conditions that may relate to adolescent remodeling disruptions (Engmann et al., 2011; Hayashi-Takagi et al., 2010; Bradshaw & Porteous, 2012). Rare mutations also confer risk, as whole exome sequencing revealed disrupted synaptic adhesion molecules like MDGA2 in schizophrenia and autism cases versus controls (Parikshak et al., 2013). Large collaborative gene sequencing efforts continue providing insights into genetic convergence. Leveraging transcriptomic datasets to nominate novel synaptic pruning candidates could elucidate interacting pathways (Liu et al., 2019). These unbiased genetic approaches may uncover unanticipated regulators beyond specialized GABAergic signaling. Integrating with cellular and pharmacological techniques enables testing causal roles in remodeling. Elucidating the complex genetic choreography guiding adolescent brain development represents an ongoing endeavor enabling novel insights into psychiatric vulnerabilities.

Limitations

While this thesis research significantly advances understanding of α4βδ GABAergic regulation of pubertal synaptic pruning, limitations provide opportunities for ongoing refinement. Findings are predominantly based on morphological spine density quantification in female mice, necessitating additional electrophysiology to strengthen functional and behavioral inferences. The lack of input-, cell-type-, and projection-specificity inherent in global knockout models remains a constraint for detailed circuit-level mechanistic comprehension. Furthermore, focusing on changes within layer 5 prelimbic cortex provides a limited vantage into broader coordination across brain regions and cell classes guiding system-wide remodeling. Finally, potential molecular and neuroimmune interacting partners beyond α4βδ receptors are acknowledged but not comprehensively delineated, underscoring open questions surrounding points of convergence.

The experimental approaches emphasize acute homeostatic changes surrounding puberty rather than developmental trajectories, limiting longitudinal insights. For instance, detecting exactly when pruning deficits in α4 knockout mice arise developmentally could help disambiguate between delayed onset versus entirely disrupted pruning. Does spine density show early overproduction but delayed initiation of elimination? Or is adolescent-specific pruning prevented with maintenance of juvenile-like density into adulthood? Defining critical period deviations requires chronic in vivo imaging not employed here.

Additionally, the studies center on a specific layer 5 subpopulation of thick-tufted pyramidal neurons. Other excitatory neuron types exhibit distinct connectivity and plasticity that warrant equal consideration (Hattox & Nelson, 2007). For instance, slender-tufted layer 5 neurons show greater local projections within medial prefrontal cortex compared to basolateral amygdala-projecting thick-tufted counterparts (Dembrow et al., 2010). Thin-tufted layer 2/3 neurons also exhibit earlier adolescent spine pruning than thick-tufted layer 5 cells (Drzewiecki et al., 2016). Pruning regulation may differ across these specialized subnetworks. Even within thick-tufted neurons, compartment-specificity remains unaddressed, as projections from basal tufts versus apical dendrites show distinct synaptic integration suited for unique computation (Poirazi et al., 2003).

Regarding inhibitory signaling, extra-synaptic α4βδ receptors likely contribute to pruning effects, but phasic synaptic inhibition sculpting pyramidal neuron activity could also play important roles. For instance, fast-spiking parvalbumin interneurons coordinate gamma oscillations implicated in cognition (Kim et al., 2016). Disinhibition of projection neurons could arise through defective GABA release from specific interneuron subtypes. Examining coordinated excitatory-inhibitory transformations remains imperative for comprehension but intractable with global manipulations.

Furthermore, the research analyzes only a single region within a vastly interconnected anxiety regulation circuit. Future work should investigate complementary changes in other cortical areas, subcortical structures, and white matter pathways. For example, optogenetics could clarify if dysregulated pruning in basolateral amygdala corresponds with behavioral changes even following selective manipulation of the prelimbic cortex. This input-output systems neuroscience approach enables mapping multi-region effects underlying emerges functions like anxiety.

Finally, potential interacting partners spanning immune, endocrine, metabolic, and epigenetic pathways require deeper investigation. Molecular candidates like microglia, estradiol, BDNF, and C1q were noted but not comprehensively delineated or tested. Exploring multi-dimensional regulatory networks will be essential for situated comprehension. Overall, the limitation of existing findings underscores ripe opportunities and need for ongoing research delineating behavioral, temporal, regional, cell-type, sex-specific, and molecular nuances guiding adolescent brain remodeling.

Future Directions

The limitations identified underscore key areas needed to expand this impactful work and address open questions. Consistent with the author's aim assertions, additional electrophysiology, calcium imaging, and local field potential characterizations will strengthen the functional implications of morphological spine changes resulting from dysregulated α4βδ signaling during puberty.

Examining compartment- and projection-specific effects represents an important avenue leveraging advances in optogenetics, chemogenetics, and pathway-targeted viral manipulations (Beier et al., 2011; Tervo et al., 2016). For instance, selectively knocking down α4 subunits in layer 5 pyramidal neurons projecting to the basolateral amygdala could help isolate remodeling effects on this key anxiety-related pathway. Interrogating input-defined ensembles will also be informative, like optogenetically activating hippocampal terminals while monitoring synaptic responses and neuronal firing in layer 5 pyramidal neurons following pubertal α4βδ disruption.

Incorporating in vivo two-photon imaging to longitudinally track spine dynamics across periods spanning early postnatal development through adulthood will provide further insights, especially regarding critical period deviations (Zuo et al., 2005). Imaging in head-fixed animals also enables combining spine visualization with behavioral assessments like reward learning or anxiety tests (Vanni et al., 2017). This could help relate real-time structural plasticity to emerging functions as circuits mature. Chronic windows for imaging over months to years remains an ongoing technical challenge.

To comprehensively map multi-region circuit alterations, techniques like CLARITY and BLOCK-FACE serial electron microscopy enable whole-brain nanoscale imaging (Ye et al., 2016). This can elucidate changes in axonal boutons across projection pathways relevant for anxiety behaviors, like altered prelimbic cortical innervation of amygdala areas. Genome-editing to insert fluorescent reporters into projection-defined neurons also facilitates comprehensive anatomical mapping of afferent and efferent connections (Dougherty et al., 2010).

Profiling approaches like mass spectrometry, RNA sequencing, and ATAC sequencing will help delineate molecular pathways underlying cytoskeletal remodeling and phagocytic engulfment that converge with α4βδ signaling (Azadi & Heiss, 2009; Preissl et al., 2018). This multi-omics integration across cell types can nominate novel candidates guiding adolescent transitions. Perturbing these pathways using CRISPR interference or overexpression approaches during puberty could test roles in spine pruning and behavioral effects.

Finally, the promising preclinical efficacy of α4/δ-positive allosteric modulators as novel anxiolytics supports their potential clinical development (Fischer et al., 2010). Pharmacologically enhancing tonic inhibition represents a promising translational opportunity, as these compounds exhibit anxiolytic efficacy comparable to benzodiazepines but reduced sedation and improved side effect profiles in animal studies. Advancing one of these lead modulators through IND-enabling studies and early phase human trials could provide exciting avenues for therapeutic modulation of GABAergic signaling underlying anxiety disorders. Overall, the breadth of technical innovation across molecular profiling, structural imaging, optogenetics, and drug development promises to rapidly advance comprehension of adolescent brain development in relation to mental health.

Conclusion

In conclusion, this thesis research significantly expands understanding of α4βδ GABAA receptor regulation of dendritic spine pruning in layer 5 prelimbic cortex during female pubertal development. The findings substantiate a model wherein transiently increased α4βδ signaling suppresses NMDAR-Kalirin7 pathways otherwise supporting the dendritic cytoskeleton, leading to selective synapse disassembly. Preventing this remodeling through knockdown of α4-containing receptors inhibits typical spine pruning, maintaining excess juvenile connectivity theorized to potentiate anxiety-like avoidance behavior following aversive stimuli.

While providing compelling support for this overall hypothesis, limitations involving the cursory behavioral characterization, lack of specificity, and focus on a single brain region underscore key frontiers for ongoing investigation. The Discussion identified numerous rich opportunities leveraging cutting-edge techniques to address these gaps through sophisticated electrophysiology, in vivo imaging, cell-type-specific targeting, multi-regional analysis, and molecular profiling. Advancing our comprehension of adolescent neurodevelopment will require tackling the field’s inherent complexity through diverse, convergent approaches.

Elucidating the elaborate choreography guiding maturation of prefrontal cortical circuits remains imperative for elucidating executive function and the roots of mental illness. The present work implicates a specialized role for α4βδ GABAA receptors in consolidating adolescent neural networks through experience-dependent pruning rendered necessary for curtailing anxiety pathways. While extensive future research is needed decrypting nuances in the precise signaling, cellular populations, brain regions, and molecular

interactions involved, this thesis provides a seminal foundation edifying our comprehension of the dynamic adolescent mind in health and disease.

VII. References

Adhikari, A., Topiwala, M. A. & Gordon, J. A. (2010). Synchronized activity between the ventral hippocampus and the medial prefrontal cortex during anxiety. Neuron, 65(2), 257-269. https://doi.org/10.1016/j.neuron.2009.12.002

Afroz, S., Parato, J., Shen, H. & Smith, S. S. (2016). Synaptic pruning in the female hippocampus is triggered at puberty by extrasynaptic GABAA receptors on dendritic spines. eLife, 5. https://doi.org/10.7554/eLife.15106

Afroz, S., Shen, H., & Smith, S. S. (2017). α4βδ GABAA receptors reduce dendritic spine density in CA1 hippocampus and impair relearning ability of adolescent female mice: effects of a GABA agonist and a stress steroid. Neuroscience, 347, 22-35.

Akimova, E., Lanzenberger, R., & Kasper, S. (2009). The serotonin-1A receptor in anxiety disorders. Biological psychiatry, 66(7), 627-635.

Almada, R., Coimbra, N., & Brandão, M. (2015). Medial prefrontal cortex serotonergic and GABAergic mechanisms modulate the expression of contextual fear: Intratelencephalic pathways and differential involvement of cortical subregions. Neuroscience, 284, 988-997. https://doi.org/10.1016/j.neuroscience.2014.11.001.

Alvarez, V. A., Ridenour, D. A. & Sabatini, B. L. (2007). Distinct structural and ionotropic roles of NMDA receptors in controlling spine and synapse stability. Journal of Neuroscience, 27(28), 7365-7376. https://doi.org/10.1523/JNEUROSCI.0956-07.2007

Amateau, S. K. & McCarthy, M. M. (2002). A novel mechanism of dendritic spine plasticity involving estradiol induction of prostaglandin-E2. Journal of Neuroscience, 22(18), 8586-8596.

Aoki, C., Chen, Y. W., Chowdhury, T. G., & Piper, W. (2018). α4βδ-GABAA receptors in dorsal hippocampal CA1 of adolescent female rats traffic to the plasma membrane of dendritic spines following voluntary exercise and contribute to protection of animals from activity-based anorexia through localization at excitatory synapses. Journal of neuroscience research, 96(9), 1450-1466.

Armand, E. J., Li, J., Xie, F., Luo, C., & Mukamel, E. A. (2021). Single-cell sequencing of brain cell transcriptomes and epigenomes. Neuron, 109(1), 11-26. https://doi.org/10.1016/j.neuron.2020.12.010

Arruda-Carvalho, M., Wu, W. C., Cummings, K. A., & Clem, R. L. (2017). Optogenetic Examination of Prefrontal-Amygdala Synaptic Development. The Journal of neuroscience : the official journal of the Society for Neuroscience, 37(11), 2976–2985. https://doi.org/10.1523/JNEUROSCI.3097-16.2017

Asselmann, E., & Beesdo-Baum, K. (2015). Predictors of the Course of Anxiety Disorders in Adolescents and Young Adults. Current Psychiatry Reports, 17, 1-8. https://doi.org/10.1007/s11920-014-0543-z.

Azadi, P., & Heiss, C. (2009). Mass spectrometry of N-linked glycans. Methods in molecular biology (Clifton, N.J.), 534, 37–51. https://doi.org/10.1007/978-1-59745-022-5_3

Baldwin, D. S., Anderson, I. M., Nutt, D. J., Bandelow, B., Bond, A., Davidson, J. R., ... & Wittchen, H. U. (2005). Evidence-based guidelines for the pharmacological treatment of anxiety disorders: recommendations from the British Association for Psychopharmacology. Journal of Psychopharmacology, 19(6), 567-596.

Bandelow, B., & Michaelis, S. (2015). Epidemiology of anxiety disorders in the 21st century. Dialogues in clinical neuroscience, 17(3), 327–335. https://doi.org/10.31887/DCNS.2015.17.3/bbandelow

Barker, J. M., Taylor, J. R., & Chandler, L. J. (2014). A unifying model of the role of the infralimbic cortex in extinction and habits. Learning &Amp; Memory, 21(9), 441-448. https://doi.org/10.1101/lm.035501.114

Basu, J., & Siegelbaum, S. A. (2015). The Corticohippocampal Circuit, Synaptic Plasticity, and Memory. Cold Spring Harbor perspectives in biology, 7(11), a021733. https://doi.org/10.1101/cshperspect.a021733

Beesdo, K., Knappe, S., & Pine, D. S. (2009). Anxiety and anxiety disorders in children and adolescents: developmental issues and implications for DSM-V. Psychiatric Clinics, 32(3), 483-524.

Beesdo, K., Pine, D. S., Lieb, R. & Wittchen, H. U. (2010). Incidence and risk patterns of anxiety and depressive disorders and categorization of generalized anxiety disorder. Archives of General Psychiatry, 67(1), 47-57. https://doi.org/10.1001/archgenpsychiatry.2009.177

Beier, K. T., Saunders, A., Oldenburg, I. A., Miyamichi, K., Akhtar, N., Luo, L., Whelan, S. P., Sabatini, B., & Cepko, C. L. (2011). Anterograde or retrograde transsynaptic labeling of CNS neurons with vesicular stomatitis virus vectors. Proceedings of the National Academy of Sciences of the United States of America, 108(37), 15414–15419. https://doi.org/10.1073/pnas.1110854108

Belardi, B., Watson, B. O., Tatavarty, V., & Svoboda, K. (2014). Wave-like spreading of complex action potentials in hippocampal pyramidal neuron dendrites. Nature Communications, 5, 4657.

Belelli, D., Harrison, N. L., Maguire, J., Macdonald, R. L., Walker, M. C., & Cope, D. W. (2009). Extrasynaptic gabaareceptors: form, pharmacology, and function. The Journal of Neuroscience, 29(41), 12757-12763. https://doi.org/10.1523/jneurosci.3340-09.2009

Biedermann, S. V., Fuss, J., Steinle, J., Auer, M. K., Dormann, C., Falfán-Melgoza, C., Ende, G., Gass, P., & Weber-Fahr, W. (2017). An elevated plus-maze in mixed reality for studying human anxiety-related behavior. BMC Biology, 15(1), 1-12. https://doi.org/10.1186/s12915-017-0463-6

Blaeser, A. S., Connors, B. W. & Nurmikko, A. V. (2017). Spontaneous dynamics of neural networks in deep layers of prefrontal cortex. Journal of Neurophysiology, 117(4), 1581-1594. https://doi.org/10.1152/jn.00295.2016

Blagburn-Blanco, S. V., Chappell, M. S., Biase, L. M. D., & DeNardo, L. A. (2022). Synapse-specific roles for microglia in development: new horizons in the prefrontal cortex. Frontiers in Molecular Neuroscience, 15. https://doi.org/10.3389/fnmol.2022.965756

Bloodgood, B. L., Sharma, N., Browne, H. A., Trepman, A. Z., & Greenberg, M. E. (2013). The activity-dependent transcription factor NPAS4 regulates domain-specific inhibition. Nature, 503(7474), 121–125. https://doi.org/10.1038/nature12743

Blurton-Jones, M., & Tuszynski, M. H. (2002). Estrogen receptor-beta colocalizes extensively with parvalbumin-labeled inhibitory neurons in the cortex, amygdala, basal forebrain, and hippocampal formation of intact and ovariectomized adult rats. The Journal of comparative neurology, 452(3), 276–287. https://doi.org/10.1002/cne.10393

Bonin, R. P., Martin, L. J., MacDonald, J. F., & Orser, B. A. (2007). α5GABAA receptors regulate the intrinsic excitability of mouse hippocampal pyramidal neurons. Journal of neurophysiology, 98(4), 2244-2254.

Botta, P., Demmou, L., Kasugai, Y., Markovic, M., Xu, C., Fadok, J. P., ... & Lüthi, A. (2015). Regulating anxiety with extrasynaptic inhibition. Nature neuroscience, 18(10), 1493-1500.

Boulanger L. M. (2009). Immune proteins in brain development and synaptic plasticity. Neuron, 64(1), 93–109. https://doi.org/10.1016/j.neuron.2009.09.001

Bourne, J. & Harris, K. M. (2007). Do thin spines learn to be mushroom spines that remember? Current Opinion in Neurobiology, 17(3), 381-386. https://doi.org/10.1016/j.conb.2007.04.009

Bourne, J. N., & Harris, K. M. (2008). Balancing structure and function at hippocampal dendritic spines. Annual Review of Neuroscience, 31, 47-67.

Bracht, T., Tüscher, O., Schnell, S., Kreher, B., Rüsch, N., Glauche, V., ... & van Elst, L. T. (2009). Extraction of prefronto-amygdalar pathways by combining probability maps. Psychiatry Research: Neuroimaging, 174(3), 217-222. https://doi.org/10.1016/j.pscychresns.2009.05.001

Bradshaw, N. J., & Porteous, D. J. (2012). DISC1-binding proteins in neural development, signalling and schizophrenia. Neuropharmacology, 62(3), 1230–1241. https://doi.org/10.1016/j.neuropharm.2010.12.027

Bresink, I., Danysz, W., Parsons, C. G. & Mutschler, E. (1995). Different binding affinities of NMDA receptor channel blockers in various brain regions--indication of NMDA receptor heterogeneity. Neuropharmacology, 34(5), 533-540. https://doi.org/10.1016/0028-3908(95)00017-z

Breslau, N., Schultz, L., & Peterson, E. (1995). Sex differences in depression: a role for preexisting anxiety. Psychiatry research, 58(1), 1-12.

Brickley, S. G. and Módy, I. (2012). Extrasynaptic gabaa receptors: their function in the cns and implications for disease. Neuron, 73(1), 23-34. https://doi.org/10.1016/j.neuron.2011.12.012

Bright, D. P., Aller, M. I., & Brickley, S. G. (2007). Synaptic release generates a tonic gabaareceptor-mediated conductance that modulates burst precision in thalamic relay neurons. The Journal of Neuroscience, 27(10), 2560-2569. https://doi.org/10.1523/jneurosci.5100-06.2007

Brooks-Kayal, A. R., Shumate, M. D., Jin, H., Lin, D. D., Rikhter, T. Y., Holloway, K. L., & Coulter, D. A. (1999). Human neuronal γ-aminobutyric acidA receptors: coordinated subunit mRNA expression and functional correlates in individual dentate granule cells. Journal of Neuroscience, 19(19), 8312-8318.

Brown, N., Kerby, J., Bonnert, T. P., Whiting, P. J. & Wafford, K. A. (2002). Pharmacological characterization of a novel cell line expressing human α4β3δ GABA(A) receptors. British Journal of Pharmacology, 136(7), 965-974. https://doi.org/10.1038/sj.bjp.0704796

Buchmann, A., Ringli, M., Kurth, S., Schaerer, M., Geiger, A., Jenni, O. G., & Huber, R. (2011). EEG sleep slow-wave activity as a mirror of cortical maturation. Cerebral cortex, 21(3), 607-615.

Burgos-Robles, A., Vidal-Gonzalez, I., & Quirk, G. J. (2009). Sustained conditioned responses in prelimbic prefrontal neurons are correlated with fear expression and extinction failure. The Journal of

neuroscience : the official journal of the Society for Neuroscience, 29(26), 8474–8482. https://doi.org/10.1523/JNEUROSCI.0378-09.2009

Butz, M., Wörgötter, F., & van Ooyen, A. (2009). Activity-dependent structural plasticity. Brain research reviews, 60(2), 287–305. https://doi.org/10.1016/j.brainresrev.2008.12.023

Buzsaki, G. & Kandel, A. (1998). Somadendritic backpropagation of action potentials in cortical pyramidal cells of the awake rat. Journal of Neurophysiology, 79(3), 1587-1591. https://doi.org/10.1152/jn.1998.79.3.1587

Caballero, A., Granberg, R., & Tseng, K. Y. (2016). Mechanisms contributing to prefrontal cortex maturation during adolescence. Neuroscience & Biobehavioral Reviews, 70, 4-12.

Caceres, A., Payne, M. R., Binder, L. I. & Steward, O. (1983). Immunocytochemical localization of actin and microtubule-associated protein MAP2 in dendritic spines. Proceedings of the National Academy of Sciences, 80(6), 1738-1742. https://doi.org/10.1073/pnas.80.6.1738

Campbell, I. G., Grimm, K. J., de Bie, E. & Feinberg, I. (2012). Sex, puberty, and the timing of sleep EEG measured adolescent brain maturation. Proceedings of the National Academy of Sciences, 109(15), 5740-5743. https://doi.org/10.1073/pnas.1120860109

Cardinal, R. N., Parkinson, J. A., Hall, J., & Everitt, B. J. (2003, October). The contribution of the amygdala, nucleus accumbens, and prefrontal cortex to emotion and motivated behaviour. In International Congress Series (Vol. 1250, pp. 347-370). Elsevier.

Carlisle, H. J. & Kennedy, M. B. (2005). Spine architecture and synaptic plasticity. Trends in Neurosciences, 28(4), 182-187. https://doi.org/10.1016/j.tins.2005.01.008

Casey, B. J., Heller, A. S., Gee, D. G., & Cohen, A. O. (2019). Development of the emotional brain. Neuroscience Letters, 693, 29-34. https://doi.org/10.1016/j.neulet.2017.11.055

Casey, B. J., Tottenham, N., Liston, C., & Durston, S. (2005). Imaging the developing brain: what have we learned about cognitive development?. Trends in cognitive sciences, 9(3), 104–110. https://doi-org.newproxy.downstate.edu/10.1016/j.tics.2005.01.011

Ċastrén, E. and Antila, H. (2017). Neuronal plasticity and neurotrophic factors in drug responses. Molecular Psychiatry, 22(8), 1085-1095. https://doi.org/10.1038/mp.2017.61

Casula, E. P., Pellicciari, M. C., Picazio, S., Caltagirone, C., & Koch, G. (2016). Spike-timing-dependent plasticity in the human dorso-lateral prefrontal cortex. Neuroimage, 143, 204-213.

Chandra, D. (2008). Extrasynaptic GABA type A receptors in the mechanism of action of ethanol (Doctoral dissertation, University of Pittsburgh).

Chandra, D., Jia, F., Liang, J., Peng, Z., Suryanarayanan, A., Werner, D. F., Spigelman, I., Houser, C. R., Olsen, R. W., Harrison, N. L., & Homanics, G. E. (2006). GABAA receptor alpha 4 subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. Proceedings of the National Academy of Sciences of the United States of America, 103(41), 15230–15235. https://doi.org/10.1073/pnas.0604304103

Cheriyan, J., Kaushik, M. K., Ferreira, A. N. & Sheets, P. L. (2016). Specific targeting of the basolateral amygdala to projectionally defined pyramidal neurons in prelimbic and infralimbic cortex. eNeuro, 3(4). https://doi.org/10.1523/ENEURO.0002-16.2016

Chozinski, T. J., Halpern, A. R., Okawa, H., Kim, H. J., Tremel, G. J., Wong, R. O., & Vaughan, J. C. (2016). Expansion microscopy with conventional antibodies and fluorescent proteins. Nature Methods, 13(6), 485-488.

Chung, W. S., Clarke, L. E., Wang, G. X., Stafford, B. K., Sher, A., Chakraborty, C., Joung, J., Foo, L. C., Thompson, A., Chen, C., Smith, S. J., & Barres, B. A. (2013). Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. Nature, 504(7480), 394–400. https://doi.org/10.1038/nature12776

Chung, W., Allen, N. J., & Eroğlu, Ç. (2015). Astrocytes control synapse formation, function, and elimination. Cold Spring Harbor Perspectives in Biology, 7(9), a020370. https://doi.org/10.1101/cshperspect.a020370

Clifton, N. E., Trent, S., Thomas, K. L., & Hall, J. (2019). Regulation and function of activity-dependent homer in synaptic plasticity. Complex Psychiatry, 5(3), 147-161. https://doi.org/10.1159/000500267

Cole, P. D., Olney, J. W., Weitzman, M. D., Smith, M. T., Kamen, B. A. & Moody, T. W. (2013). Memantine protects rats treated with intrathecal methotrexate from developing spatial memory deficits. Clinical Cancer Research, 19(16), 4446-4454. https://doi.org/10.1158/1078-0432.CCR-13-1179

Colgin, L. L. (2013). Mechanisms and functions of theta rhythms. Annual Review of Neuroscience, 36, 295-312.

Comer, J., Blanco, C., Hasin, D., Liu, S., Grant, B., Turner, J., & Olfson, M. (2011). Health-related quality of life across the anxiety disorders: results from the national epidemiologic survey on alcohol and related conditions (NESARC).. The Journal of clinical psychiatry, 72 1, 43-50 . https://doi.org/10.4088/JCP.09m05094blu.

Corre, C., Shinoda, G., Zhu, H., Cousminer, D. L., Crossman, C., Bellissimo, C., ... & Palmert, M. R. (2016). Sex-specific regulation of weight and puberty by the Lin28/let-7 axis. The Journal of Endocrinology, 228(3), 179-191. https://doi.org/10.1530/JOE-15-0360

Corrigan, N. M., Yarnykh, V. L., Hippe, D. S., Owen, J. P., Huber, E., Zhao, T. C., … & Kuhl, P. K. (2021). Myelin development in cerebral gray and white matter during adolescence and late childhood. NeuroImage, 227, 117678. https://doi.org/10.1016/j.neuroimage.2020.117678

Cremers, H. R., Demenescu, L. R., Aleman, A., Renken, R., van Tol, M. J., van der Wee, N. J., ... & Roelofs, K. (2010). Neuroticism modulates amygdala—prefrontal connectivity in response to negative emotional facial expressions. Neuroimage, 49(1), 963-970. https://doi.org/10.1016/j.neuroimage.2009.08.023

Cui, C. P., Wong, C. C., Kai, A. K., Ho, D. W., Lau, E. Y., Tsui, Y. M., Chan, L. K., Cheung, T. T., Chok, K. S., Chan, A. C. Y., Lo, R. C., Lee, J. M., Lee, T. K., & Ng, I. O. L. (2017). SENP1 promotes hypoxia-induced cancer stemness by HIF-1α deSUMOylation and SENP1/HIF-1α positive feedback loop. Gut, 66(12), 2149–2159. https://doi.org/10.1136/gutjnl-2016-313264

Dabiri, M., Firouzabadi, F. D., Yang, K., Barker, P. B., Lee, R. R., & Yousem, D. M. (2022). Neuroimaging in schizophrenia: a review article. Frontiers in Neuroscience, 16. https://doi.org/10.3389/fnins.2022.1042814

Dalby, N. O., Falk-Petersen, C. B., Leurs, U., Scholze, P., Krall, J., Frølund, B., … & Wellendorph, P. (2020). Silencing of spontaneous activity at α4β1/3δ gabaa receptors in hippocampal granule cells reveals different ligand pharmacology. British Journal of Pharmacology, 177(17), 3975-3990. https://doi.org/10.1111/bph.15146

Dani, A., & Huang, B. (2010). New frontiers in super-resolution microscopy. Annual Review of Biochemistry, 79, 285-307.

Delaney, A. J., & Sah, P. (1999). GABA receptors inhibited by benzodiazepines mediate fast inhibitory transmission in the central amygdala. Journal of Neuroscience, 19(22), 9698-9704.

Dembrow, N. C., Chitwood, R. A., & Johnston, D. (2010). Projection-specific neuromodulation of medial prefrontal cortex neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience, 30(50), 16922–16937. https://doi.org/10.1523/JNEUROSCI.3644-10.2010

Dembrow, N. C., Zemelman, B. V. & Johnston, D. (2015). Temporal dynamics of L5 dendrites in medial prefrontal cortex regulate integration versus coincidence detection of afferent inputs. Journal of Neuroscience, 35(12), 4501-4514. https://doi.org/10.1523/JNEUROSCI.4673-14.2015

Desai, N. S., Rutherford, L. C., & Turrigiano, G. G. (2002). Plasticity in the intrinsic excitability of cortical pyramidal neurons. Nature Neuroscience, 5(5), 515-520.

Desmond, N. L., & Weinberg, R. J. (1998). Synaptic plasticity in the hippocampus: A fine-structural perspective. Hippocampus, 8(6), 621-635.

Deutsch, S. I., Luyo, Z. N., & Burket, J. A. (2022). Targeted nmda receptor interventions for autism: developmentally determined expression of glun2b and glun2a-containing receptors and balanced allosteric modulatory approaches. Biomolecules, 12(2), 181. https://doi.org/10.3390/biom12020181

Dixon, M. A., Greferath, U., Fletcher, E. L., & Jobling, A. I. (2021). The contribution of microglia to the development and maturation of the visual system. Frontiers in Cellular Neuroscience, 15, 659843.

Dobrunz, L. E., & Stevens, C. F. (1997). Heterogeneity of release probability, facilitation, and depletion at central synapses. Neuron, 18(6), 995-1008.

Dougherty, J. D., Schmidt, E. F., Nakajima, M., & Heintz, N. (2010). Analytical approaches to RNA profiling data for the identification of genes enriched in specific cells. Nucleic acids research, 38(13), 4218–4230. https://doi.org/10.1093/nar/gkq130

Drasbek, K. R., & Jensen, K. (2006). THIP, a hypnotic and antinociceptive drug, enhances an extrasynaptic GABAA receptor-mediated conductance in mouse neocortex. Cerebral cortex, 16(8), 1134-1141.

Drevets, W. C., Price, J. L. & Furey, M. L. (2008). Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. Brain Structure and Function, 213(1), 93-118. https://doi.org/10.1007/s00429-008-0189-x

Drzewiecki, C. M., Willing, J., & Juraska, J. M. (2016). Synaptic number changes in the medial prefrontal cortex across adolescence in male and female rats: a role for pubertal onset. Synapse, 70(9), 361-368.

Duncan, B. W., Murphy, K. E. & Maness, P. F. (2021). Molecular mechanisms of L1 and NCAM adhesion molecules in synaptic pruning, plasticity, and stabilization. Frontiers in Cellular and Developmental Biology, 9, 625340. https://doi.org/10.3389/fcell.2021.625340

Engin, E., Treit, D., & Dickson, C. T. (2009). Anxiolytic- and antidepressant-like properties of ketamine in behavioral and neurophysiological animal models. Neuroscience, 161(2), 359–369. https://doi.org/10.1016/j.neuroscience.2009.03.038

Engmann, O., Hortobágyi, T., Pidsley, R., Troakes, C., Bernstein, H. G., Kreutz, M. R., Mill, J., Nikolic, M., & Giese, K. P. (2011). Schizophrenia is associated with dysregulation of a Cdk5 activator that regulates synaptic protein expression and cognition. Brain : a journal of neurology, 134(Pt 8), 2408–2421. https://doi.org/10.1093/brain/awr155

Errington, A. C., Cope, D. W., & Crunelli, V. (2011). Augmentation of tonic gabaainhibition in absence epilepsy: therapeutic value of inverse agonists at extrasynaptic gabaareceptors. Advances in Pharmacological Sciences, 2011, 1-12. https://doi.org/10.1155/2011/790590

Etkin, A., Egner, T., & Kalisch, R. (2011). Emotional processing in anterior cingulate and medial prefrontal cortex. Trends in cognitive sciences, 15(2), 85-93.

Etkin, A., Egner, T., Peraza, D. M., Kandel, E. R., & Hirsch, J. (2006). Resolving emotional conflict: a role for the rostral anterior cingulate cortex in modulating activity in the amygdala. Neuron, 51(6), 871–882. https://doi.org/10.1016/j.neuron.2006.07.029

Etkin, A., Prater, K. E., Schatzberg, A. F., Menon, V., & Greicius, M. D. (2009). Disrupted amygdalar subregion functional connectivity and evidence of a compensatory network in generalized anxiety disorder. Archives of general psychiatry, 66(12), 1361–1372. https://doi.org/10.1001/archgenpsychiatry.2009.104

Farrant, M., & Kaila, K. (2007). The cellular, molecular and ionic basis of GABAA receptor signalling. Progress in brain research, 160, 59-87.

Faust, T. E., Gunner, G., & Schafer, D. P. (2021). Mechanisms governing activity-dependent synaptic pruning in the developing mammalian cns. Nature Reviews Neuroscience, 22(11), 657-673. https://doi.org/10.1038/s41583-021-00507-y

Featherstone, D. E. (2009). Intercellular glutamate signaling in the nervous system and beyond. ACS Chemical Neuroscience, 1(1), 4-12. https://doi.org/10.1021/cn900006n

Feng, X., Shaw, D. S., & Silk, J. S. (2008). Developmental trajectories of anxiety symptoms among boys across early and middle childhood. Journal of abnormal psychology, 117(1), 32.

Feng, Y., Wang, X., Li, L., Wang, Q., Li, J., Zhao, J., ... & Xing, H. (2010). Association of the GABRD gene and childhood-onset mood disorders. Genes, Brain and Behavior, 9(6), 668-672. https://doi.org/10.1111/j.1601-183X.2010.00598.x

Fenton, G. E., Pollard, A. K., Halliday, D. M., Mason, R., Bredy, T. W. & Stevenson, C. W. (2014). Persistent prelimbic cortex activity contributes to enhanced learned fear expression in females. Learning & Memory, 21(2), 55-60. https://doi.org/10.1101/lm.033514.113

Fiebig, F. and Lansner, A. (2016). A spiking working memory model based on hebbian short-term potentiation. The Journal of Neuroscience, 37(1), 83-96. https://doi.org/10.1523/jneurosci.1989-16.2016

Fischer, B. D., Licata, S. C., Edwankar, R. V., Wang, Z. J., Huang, S., He, X., Yu, J., Zhou, H., Johnson, E. M., Jr, Cook, J. M., Furtmüller, R., Ramerstorfer, J., Sieghart, W., Roth, B. L., Majumder, S., & Rowlett, J. K. (2010). Anxiolytic-like effects of 8-acetylene imidazobenzodiazepines in a rhesus monkey conflict procedure. Neuropharmacology, 59(7-8), 612–618. https://doi.org/10.1016/j.neuropharm.2010.08.011

Fitzgerald, J. M., Phan, K. L., Kennedy, D. N., Shankman, S. A., Langenecker, S. A., Klumpp, H., & Monk, C. S. (2017). Prefrontal and amygdala engagement during emotional reactivity and regulation in generalized anxiety disorder. Journal of Affective Disorders, 218, 398-406. https://doi.org/10.1016/j.jad.2017.05.013

Foley, D. L., Goldston, D. B., Costello, E. J. & Angold, A. (2006). Proximal psychiatric risk factors for suicidality in youth: the Great Smoky Mountains Study. Archives of General Psychiatry, 63(9), 1017-1024. https://doi.org/10.1001/archpsyc.63.9.1017

Follesa, P., Porcu, P., Sogliano, C., Cinus, M., Biggio, F., Mancuso, L., Mostallino, M. C., Paoletti, A. M., Purdy, R. H., Biggio, G., & Concas, A. (2002). Changes in GABAA receptor gamma 2 subunit gene expression induced by long-term administration of oral contraceptives in rats. Neuropharmacology, 42(3), 325–336. https://doi.org/10.1016/s0028-3908(01)00187-3

Forman, S. A. and Stewart, D. S. (2011). Mutations in the gabaa receptor that mimic the allosteric ligand etomidate. Methods in Molecular Biology, 317-333. https://doi.org/10.1007/978-1-61779-334-9_17

Friedman, N. P. and Robbins, T. W. (2021). The role of prefrontal cortex in cognitive control and executive function. Neuropsychopharmacology, 47(1), 72-89. https://doi.org/10.1038/s41386-021-01132-0

Gabbott, P. L., Warner, T. A., Jays, P. R., Salway, P., & Busby, S. J. (2005). Prefrontal cortex in the rat: projections to subcortical autonomic, motor, and limbic centers. Journal of Comparative Neurology, 492(2), 145-177.

Gee, D. G., Humphreys, K. L., Flannery, J., Goff, B., Telzer, E. H., Shapiro, M., … & Tottenham, N. (2013). A developmental shift from positive to negative connectivity in human amygdala–prefrontal circuitry. The Journal of Neuroscience, 33(10), 4584-4593. https://doi.org/10.1523/jneurosci.3446-12.2013

Gerfen, C. R., Paletzki, R., & Heintz, N. (2013). GENSAT BAC Cre-recombinase driver lines to study the functional organization of cerebral cortical and basal ganglia circuits. Neuron, 80(6), 1368-1383.

Germann, M., Brederoo, S., & Sommer, I. (2021). Abnormal synaptic pruning during adolescence underlying the development of psychotic disorders. Current Opinion in Psychiatry, 34(3), 222-227. https://doi.org/10.1097/yco.0000000000000696

Ghit, A., Assal, D., Al-Shami, A. S., & Hussein, D. E. E. (2021). GABAA receptors: structure, function, pharmacology, and related disorders. Journal, genetic engineering & biotechnology, 19(1), 123. https://doi.org/10.1186/s43141-021-00224-0

Ginger, M., Haberl, M., Conzelmann, K. K., Schwarz, M. K., & Frick, A. (2013). Revealing the secrets of neuronal circuits with recombinant rabies virus technology. Frontiers in neural circuits, 7, 2. https://doi.org/10.3389/fncir.2013.00002

Ginsberg, D. (2004). Women and Anxiety Disorders: Implications for Diagnosis and Treatment. CNS Spectrums, 9, 1 - 3. https://doi.org/10.1017/S1092852900001978.

Globus, A., & Scheibel, A. B. (1967). Synaptic loci on visual cortical neurons of the rabbit: the specific afferent radiation. Experimental neurology, 18(1), 116-131.

Gonzalez-Burgos, G., & Lewis, D. A. (2012). NMDA receptor hypofunction, parvalbumin-positive neurons, and cortical gamma oscillations in schizophrenia. Schizophrenia Bulletin, 38(5), 950-957.

Goodwin, R. D., Weinberger, A. H., Kim, J. H., Wu, M. & Galea, S. (2020). Trends in anxiety among adults in the United States, 2008-2018: Rapid increases among young adults. Journal of psychiatric research, 130, 441-446. https://doi.org/10.1016/j.jpsychires.2020.08.014

Grillon, C., Baas, J. M., Cornwell, B. & Johnson, L. (2006). Context conditioning and behavioral avoidance in a virtual reality environment: effect of predictability. Biological psychiatry, 60(7), 752-759. https://doi.org/10.1016/j.biopsych.2006.03.077

Grupe, D. W. & Nitschke, J. B. (2013). Uncertainty and anticipation in anxiety: an integrated neurobiological and psychological perspective. Nature Reviews Neuroscience, 14(7), 488-501. https://doi.org/10.1038/nrn3524

Grupe, D. W., Oathes, D. J. & Nitschke, J. B. (2013). Dissecting the anticipation of aversion reveals dissociable neural networks. Cerebral cortex, 23(8), 1874-1883. https://doi.org/10.1093/cercor/bhs175

Gunn, B. G., Cunningham, L., Cooper, M. A., Corteen, N. L., Seifi, M., Swinny, J. D., Lambert, J. J., & Belelli, D. (2013). Dysfunctional astrocytic and synaptic regulation of hypothalamic glutamatergic transmission in a mouse model of early-life adversity: relevance to neurosteroids and programming of the stress response. The Journal of neuroscience : the official journal of the Society for Neuroscience, 33(50), 19534–19554. https://doi.org/10.1523/JNEUROSCI.1337-13.2013

Guo, Z. V., Li, N., Huber, D., Ophir, E., Gutnisky, D., Ting, J. T., Feng, G., & Svoboda, K. (2014). Flow of cortical activity underlying a tactile decision in mice. Neuron, 81(1), 179–194. https://doi.org/10.1016/j.neuron.2013.10.020

Hakobyan, S., Vazirian, S., Lee-Cheong, S., Krausz, M., Honer, W. G., & Schütz, C. (2020). Concurrent disorder management guidelines. systematic review. Journal of Clinical Medicine, 9(8), 2406. https://doi.org/10.3390/jcm9082406

Halvagal, M. S. and Zenke, F. (2023). The combination of hebbian and predictive plasticity learns invariant object representations in deep sensory networks. Nature Neuroscience, 26(11), 1906-1915. https://doi.org/10.1038/s41593-023-01460-y

Hanamsagar, R., Alter, M. D., Block, C. S., Sullivan, H., Bolton, J. L., & Bilbo, S. D. (2017). Generation of a microglial developmental index in mice and in humans reveals a sex difference in maturation and immune reactivity. Glia, 65(9), 1504–1520. https://doi.org/10.1002/glia.23176

Hantsoo, L. & Epperson, C. N. (2017). Anxiety disorders among women: A female lifespan approach. FOCUS, 15(2), 162-172. https://doi.org/10.1176/appi.focus.20160042

Hara, Y., Waters, E. M., McEwen, B. S., & Morrison, J. H. (2015). Estrogen Effects on Cognitive and Synaptic Health Over the Lifecourse. Physiological reviews, 95(3), 785–807. https://doi.org/10.1152/physrev.00036.2014

Harrison, P. J. (2000). Postmortem studies in schizophrenia. Dialogues in Clinical Neuroscience, 2(4), 349-357. https://doi.org/10.31887/dcns.2000.2.4/pharrison

Harvey, C. D., & Svoboda, K. (2007). Locally dynamic synaptic learning rules in pyramidal neuron dendrites. Nature, 450(7173), 1195-1200.

Hattox, A. M., & Nelson, S. B. (2007). Layer V neurons in mouse cortex projecting to different targets have distinct physiological properties. Journal of neurophysiology, 98(6), 3330–3340. https://doi.org/10.1152/jn.00397.2007

Hawkins, J., Ahmad, S., & Cui, Y. (2017). A theory of how columns in the neocortex enable learning the structure of the world. Frontiers in Neural Circuits, 11. https://doi.org/10.3389/fncir.2017.00081

Hayashi-Takagi, A., Takaki, M., Graziane, N., Seshadri, S., Murdoch, H., Dunlop, A. J., Makino, Y., Seshadri, A. J., Ishizuka, K., Srivastava, D. P., Xie, Z., Baraban, J. M., Houslay, M. D., Tomoda, T., Brandon, N. J., Kamiya, A., Yan, Z., Penzes, P., & Sawa, A. (2010). Disrupted-in-Schizophrenia 1 (DISC1) regulates spines of the glutamate synapse via Rac1. Nature neuroscience, 13(3), 327–332. https://doi.org/10.1038/nn.2487

Hayward, C. & Sanborn, K. (2002). Puberty and the emergence of gender differences in psychopathology. Journal of Adolescent Health, 30(4), 49-58. https://doi.org/10.1016/S1054-139X(02)00336-1

Herd, M. B., Belelli, D., & Lambert, J. J. (2007). Neurosteroid modulation of synaptic and extrasynaptic GABAA receptors. Pharmacology & therapeutics, 116(1), 20-34.

Hesse, S., Stengler, K., Regenthal, R., Patt, M., Becker, G. A., Franke, A., ... & Sabri, O. (2011). The serotonin transporter availability in untreated early-onset and late-onset patients with obsessive–compulsive disorder. International Journal of Neuropsychopharmacology, 14(5), 606-617.

Hettema, J. M., Neale, M. C., & Kendler, K. S. (2001). A review and meta-analysis of the genetic epidemiology of anxiety disorders. American journal of Psychiatry, 158(10), 1568-1578.

Hettema, J. M., Prescott, C. A., Myers, J. M., Neale, M. C., & Kendler, K. S. (2005). The structure of genetic and environmental risk factors for anxiety disorders in men and women. Archives of general psychiatry, 62(2), 182-189.

Hodes, G. E. & Shors, T. J. (2005). Distinctive stress effects on learning during puberty. Hormones and behavior, 48(2), 163-171. https://doi.org/10.1016/j.yhbeh.2005.02.004

Hoehn-Saric, R. (2007). Physiologic responses in anxiety. Current Psychiatry Reviews, 3(3), 196-204.

Holm, M. M., Nieto-González, J. L., Vardya, I., Henningsen, K., Jayatissa, M. N., Wiborg, O., … & Jensen, K. (2011). Hippocampal gabaergic dysfunction in a rat chronic mild stress model of depression. Hippocampus, 21(4), 422-433. https://doi.org/10.1002/hipo.20758

Holtmaat, A., & Caroni, P. (2016). Functional and structural underpinnings of neuronal assembly formation in learning. Nature neuroscience, 19(12), 1553-1562.

Hong, S., Dissing-Olesen, L., & Stevens, B. (2016). New insights on the role of microglia in synaptic pruning in health and disease. Current opinion in neurobiology, 36, 128–134. https://doi.org/10.1016/j.conb.2015.12.004

Hoover, W. B. & Vertes, R. P. (2007). Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. Brain Structure and Function, 212(2), 149-179. https://doi.org/10.1007/s00429-007-0150-4

Hou, B., Zhang, D., Zhao, S., Wei, M., Yang, Z., Wang, S., ... & Zhang, Z. J. (2015). Scalable and DiI-compatible optical clearance of the mammalian brain. Frontiers in Neuroanatomy, 9, 19.

Hutchison, S. M., Mâsse, L. C., Pawluski, J. L., & Oberlander, T. F. (2021). Perinatal selective serotonin reuptake inhibitor (SSRI) and other antidepressant exposure effects on anxiety and depressive behaviors in offspring: a review of findings in humans and rodent models. Reproductive Toxicology, 99, 80-95.

Huttenlocher, P. R. (1979). Synaptic density in human frontal cortex - developmental changes and effects of aging. Brain research, 163(2), 195-205. https://doi.org/10.1016/0006-8993(79)90349-4

Huttenlocher, P. R., & Dabholkar, A. S. (1997). Regional differences in synaptogenesis in human cerebral cortex. Journal of Comparative Neurology, 387(2), 167-178.

Ibañez, S., Luebke, J. I., Chang, W., Dragulјić, D., & Weaver, C. M. (2020). Network Models Predict That Pyramidal Neuron Hyperexcitability and Synapse Loss in the dlPFC Lead to Age-Related Spatial Working Memory Impairment in Rhesus Monkeys. Frontiers in computational neuroscience, 13, 89. https://doi.org/10.3389/fncom.2019.00089

Indovina, I., Robbins, T. W., Núñez-Elizalde, A. O., Dunn, B. D. & Bishop, S. J. (2011). Fear-conditioning mechanisms associated with trait vulnerability to anxiety in humans. Neuron, 69(3), 563-571. https://doi.org/10.1016/j.neuron.2010.12.034

Irfan, M., Evonuk, K. S., & DeSilva, T. M. (2022). Microglia phagocytose oligodendrocyte progenitor cells and synapses during early postnatal development: implications for white versus gray matter maturation. The FEBS Journal, 289(8), 2110-2127.

Ishikawa, A., & Nakamura, S. (2006). Ventral hippocampal neurons project axons simultaneously to the medial prefrontal cortex and amygdala in the rat.. Journal of neurophysiology, 96 4, 2134-8 . https://doi.org/10.1152/JN.00069.2006.

Jalnapurkar, I., Allen, M., & Pigott, T. (2018). Sex differences in anxiety disorders: A review. J Psychiatry Depress Anxiety, 4(12), 3-16.

Jeevakumar, V., Kroener, S., Chandra, R., Cortez, I., Vargas, A., & Pogorelov, V. (2018). Ketamine administration during the second week of life induces permanent neuronal deficits in adulthood. Psychopharmacology, 235(3), 793-805.

Jia, F., Pignataro, L., Schofield, C. M., Yue, M., Harrison, N. L., & Goldstein, P. A. (2005). An extrasynaptic GABAA receptor mediates tonic inhibition in thalamic VB neurons. Journal of neurophysiology, 94(6), 4491-4501.

Jobson, D. D., Hase, Y., Clarkson, A. N., & Kalaria, R. N. (2021). The role of the medial prefrontal cortex in cognition, ageing and dementia. Brain Communications, 3(3). https://doi.org/10.1093/braincomms/fcab125

Joshi, S., & Kapur, J. (2016). Neurosteroid Regulation of Seizures: Role of GABA A Receptor Plasticity. Antiepileptic drug discovery: novel approaches, 127-146.

Kaczkurkin, A. N., Moore, T. M., Ruparel, K., Ciric, R., Calkins, M. E., Shinohara, R. T., Elliott, M. A., Hopson, R., Roalf, D. R., Vandekar, S. N., Gennatas, E. D., Wolf, D. H., Scott, J. C., Pine, D. S., Leibenluft, E., Detre, J. A., Foa, E. B., Gur, R. E., Gur, R. C., & Satterthwaite, T. D. (2016). Elevated Amygdala Perfusion Mediates Developmental Sex Differences in Trait Anxiety. Biological psychiatry, 80(10), 775–785. https://doi.org/10.1016/j.biopsych.2016.04.021

Kalra, H., Kamath, P., Trivedi, J. K., & Janca, A. (2008). Caregiver burden in anxiety disorders. Current opinion in psychiatry, 21(1), 70-73.

Kawaguchi, Y. & Kubota, Y. (1997). GABAergic cell subtypes and their synaptic connections in rat frontal cortex. Cerebral cortex, 7(6), 476-486. https://doi.org/10.1093/cercor/7.6.476

Kellner, Y., Gödecke, N., Dierkes, T., Thieme, N., Zagrebelsky, M., & Korte, M. (2014). The BDNF effects on dendritic spines of mature hippocampal neurons depend on neuronal activity. Frontiers in synaptic neuroscience, 6, 5. https://doi.org/10.3389/fnsyn.2014.00005

Kennedy, S. H., Giacobbe, P., Rizvi, S. J., Placenza, F. M., Nishikawa, Y., Mayberg, H. S. & Lozano, A. M. (2011). Deep brain stimulation for treatment-resistant depression: follow-up after 3 to 6 years. American Journal of Psychiatry, 168(5), 502-510. https://doi.org/10.1176/appi.ajp.2010.10081187

Kenwood, M. M., Kalin, N. H., & Barbas, H. (2021). The prefrontal cortex, pathological anxiety, and anxiety disorders. Neuropsychopharmacology, 47(1), 260-275. https://doi.org/10.1038/s41386-021-01109-z

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R. & Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the national comorbidity survey replication. Archives of general psychiatry, 62(6), 593-602. https://doi.org/10.1001/archpsyc.62.6.593

Kessler, R. C., Chiu, W. T., Demler, O., & Walters, E. E. (2005). Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Archives of general psychiatry, 62(6), 617-627.

Kessler, R. C., McGonagle, K. A., Zhao, S., Nelson, C. B., Hughes, M., Eshleman, S., ... & Kendler, K. S. (1994). Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States: results from the National Comorbidity Survey. Archives of general psychiatry, 51(1), 8-19.

Kim, H., Ährlund-Richter, S., Wang, X., Deisseroth, K., & Carlén, M. (2016). Prefrontal Parvalbumin Neurons in Control of Attention. Cell, 164(1-2), 208–218. https://doi.org/10.1016/j.cell.2015.11.038

Kim, J. J., & Hibbs, R. E. (2021). Direct structural insights into GABAA receptor pharmacology. Trends in biochemical sciences, 46(6), 502-517.

Kim, M. J., Loucks, R. A., Palmer, A., Brown, A. C., Solomon, K. M., Marchante, A. N., … & Whalen, P. J. (2011). The structural and functional connectivity of the amygdala: from normal emotion to pathological anxiety. Behavioural Brain Research, 223(2), 403-410. https://doi.org/10.1016/j.bbr.2011.04.025

Kim, W. B., & Cho, J. H. (2017). Synaptic targeting of double-projecting ventral CA1 hippocampal neurons to the medial prefrontal cortex and basal amygdala. Journal of Neuroscience, 37(19), 4868-4882.

Kim, Y., Choi, H., Lee, W., Park, H., Kam, M., Lee, D., ... & Lee, S. (2020). Microtubule-associated protein 2 mediates induction of long-term potentiation in hippocampal neurons. The FASEB Journal, 34(8), 6965-6983. https://doi.org/10.1096/fj.201902122RR

Klune, C. B., Jin, B., & DeNardo, L. A. (2021). Linking mpfc circuit maturation to the developmental regulation of emotional meJobson, D. D., Hase, Y., Clarkson, A. N., & Kalaria, R. N. (2021). The role of the medial prefrontal cortex in cognition, ageing and dementia. Brain Communications, 3(3). https://doi.org/10.1093/braincomms/fcab125mory and cognitive flexibility. eLife, 10. https://doi.org/10.7554/elife.64567

Koh, W., Kwak, H., Cheong, E., & Lee, C. J. (2023). Gaba tone regulation and its cognitive functions in the brain. Nature Reviews Neuroscience, 24(9), 523-539. https://doi.org/10.1038/s41583-023-00724-7

Kolb, B., Mychasiuk, R., Muhammad, A., & Li, Y. (2012). Experience and the developing prefrontal cortex. Proceedings of the National Academy of Sciences, 109(Supplement 2), 17186-17193.

Kolk, S. M. and Rakić, P. (2021). Development of prefrontal cortex. Neuropsychopharmacology, 47(1), 41-57. https://doi.org/10.1038/s41386-021-01137-9

Konstantoudaki, X., Chalkiadaki, K., Vasileiou, E., Kalemaki, K., Karagogeos, D., & Sidiropoulou, K. (2018). Prefrontal cortical-specific differences in behavior and synaptic plasticity between adolescent and adult mice. Journal of neurophysiology, 119(3), 822-833.

Korgaonkar, M. S., Grieve, S. M., Etkin, A., Koslow, S. H., & Williams, L. M. (2012). Using standardized fmri protocols to identify patterns of prefrontal circuit dysregulation that are common and specific to cognitive and emotional tasks in major depressive disorder: first wave results from the ispot-d study. Neuropsychopharmacology, 38(5), 863-871. https://doi.org/10.1038/npp.2012.252

Korte, S. M., Prins, J., Vinkers, C. H., & Olivier, B. (2009). On the origin of allostasis and stress-induced pathology in farm animals: celebrating Darwin's legacy. Veterinary journal (London, England : 1997), 182(3), 378–383. https://doi.org/10.1016/j.tvjl.2009.08.023

Koss, W. A., Belden, C. E., Hristov, A. D. & Juraska, J. M. (2014). Dendritic remodeling in the adolescent medial prefrontal cortex and the basolateral amygdala of male and female rats. Synapse, 68(2), 61-72. https://doi.org/10.1002/syn.21716

Kredlow, M. A., Fenster, R. J., Laurent, E., Ressler, K. J., & Phelps, E. A. (2021). Prefrontal cortex, amygdala, and threat processing: implications for ptsd. Neuropsychopharmacology, 47(1), 247-259. https://doi.org/10.1038/s41386-021-01155-7

Krook-Magnuson, E., Armstrong, C., Oijala, M., & Soltesz, I. (2014). On-demand optogenetic control of spontaneous seizures in temporal lobe epilepsy. Nature Communications, 5, 3676.

Kucken, A. M., Wagner, D. A., Ward, P. R., Teissére, J. A., Boileau, A. J., & Czajkowski, C. (2000). Identification of benzodiazepine binding site residues in the γ2 subunit of the γ-aminobutyric acidA receptor. Molecular pharmacology, 57(5), 932-939.

Kuroda, M., Yokofujita, J., & Murakami, K. (1998). An ultrastructural study of the neural circuit between the prefrontal cortex and the mediodorsal nucleus of the thalamus. Progress in neurobiology, 54(4), 417–458. https://doi.org/10.1016/s0301-0082(97)00070-1

Kwan, A. C., & Dan, Y. (2012). Dissection of cortical microcircuits by single-neuron stimulation in vivo. Current biology : CB, 22(16), 1459–1467. https://doi.org/10.1016/j.cub.2012.06.007

Ladouceur, C. D., Peper, J. S., Crone, E. A., & Dahl, R. E. (2012). White matter development in adolescence: the influence of puberty and implications for affective disorders. Developmental cognitive neuroscience, 2(1), 36-54.

Lanzenberger, R. R., Mitterhauser, M., Spindelegger, C., Wadsak, W., Klein, N., Mien, L. K., ... & Tauscher, J. (2007). Reduced serotonin-1A receptor binding in social anxiety disorder. Biological psychiatry, 61(9), 1081-1089.

Laube, C., van den Bos, W., & Fandakova, Y. (2020). The relationship between pubertal hormones and brain plasticity: Implications for cognitive training in adolescence. Developmental cognitive neuroscience, 42, 100753.

Lenz, K. M., & McCarthy, M. M. (2015). A starring role for microglia in brain sex differences. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry, 21(3), 306–321. https://doi.org/10.1177/1073858414536468

Lewis, D., Melchitzky, D., & Burgos, G. (2002). Specificity in the functional architecture of primate prefrontal cortex. Journal of Neurocytology, 31, 265-276. https://doi.org/10.1023/A:1024174026286.

Li, Y., Wang, D., Wang, L., Yu, J., Du, G., & Zhang, L. (2019). Single-nucleus sequencing identifies Huntington disease astrocyte states. Cell Reports, 26(11), 2993-3008.e6.

Likhtik, E., & Paz, R. (2020). Maladaptive learning and the amygdala—prefrontal circuit. In Stress Resilience (pp. 323-348). Academic Press.

Little, J. P., & Carter, A. G. (2012). Subcellular synaptic connectivity of layer 2 pyramidal neurons in the medial prefrontal cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience, 32(37), 12808–12819. https://doi.org/10.1523/JNEUROSCI.1616-12.2012

Liu, S. Q., & Zukin, R. S. (2007). Ca2+-permeable AMPA receptors in synaptic plasticity and neuronal death. Trends in Neurosciences, 30(3), 126-134.

Liu, S., Li, A., Zhu, M., Li, J., & Liu, B. (2019). Genetic influences on cortical myelination in the human brain. Genes, brain, and behavior, 18(4), e12537. https://doi.org/10.1111/gbb.12537

Lloyd-Lewis, B., Davis, F. M., Harris, O. B., Hitchcock, J. R., & Watson, C. J. (2016). Imaging the mammary gland and mammary tumours in 3D: Optical tissue clearing and immunofluorescence methods. Breast Cancer Research, 18(1), 127.

Loades, M. E., Chatburn, E., Higson-Sweeney, N., Reynolds, S., Shafran, R., Brigden, A., Linney, C., McManus, M. N., Borwick, C. & Crawley, E. (2020). Rapid systematic review: The impact of social isolation and loneliness on the mental health of children and adolescents in the context of COVID-19. Journal of the American Academy of Child & Adolescent Psychiatry, 59(11), 1218-1239. https://doi.org/10.1016/j.jaac.2020.05.009

López-Muñoz, F., Álamo, C., & García-García, P. (2011). The discovery of chlordiazepoxide and the clinical introduction of benzodiazepines: half a century of anxiolytic drugs.. Journal of anxiety disorders, 25 4, 554-62 . https://doi.org/10.1016/j.janxdis.2011.01.002.

Lu, W., Bushong, E. A., Shih, T. P., Ellisman, M. H., & Nicoll, R. A. (2013). The cell-autonomous role of excitatory synaptic transmission in the regulation of neuronal structure and function. Neuron, 78(3), 433–439. https://doi.org/10.1016/j.neuron.2013.02.030

Luscher B, Fuchs T, Kilpatrick CL. GABAA receptor trafficking-mediated plasticity of inhibitory synapses. Neuron. 2011 May 12;70(3):385-409. doi: 10.1016/j.neuron.2011.03.024. PMID: 21555068; PMCID: PMC3093971.

Lydiard, R. B. (2003). The role of GABA in anxiety disorders. Journal of Clinical Psychiatry, 64, 21-27.

Ma, X. M., Huang, J., Wang, Y., Eipper, B. A. & Mains, R. E. (2003). Kalirin, a multifunctional Rho guanine nucleotide exchange factor, is necessary for maintenance of hippocampal pyramidal neuron dendrites and dendritic spines. The Journal of neuroscience, 23(33), 10593-10603. https://doi.org/10.1523/JNEUROSCI.23-33-10593.2003

Ma, X. M., Kiraly, D. D., Gaier, E. D., Wang, Y., Kim, E. J., Levine, E. S., Eipper, B. A., & Mains, R. E. (2008). Kalirin-7 is required for synaptic structure and function. The Journal of neuroscience : the official journal of the Society for Neuroscience, 28(47), 12368–12382. https://doi.org/10.1523/JNEUROSCI.4269-08.2008

Ma, X. M., Wang, Y., Ferraro, F., Mains, R. E. & Eipper, B. A. (2014). Nonenzymatic domains of Kalirin7 contribute to spine morphogenesis through interactions with phosphoinositides and Abl. Molecular Biology of the Cell, 25(8), 1458-1471. https://doi.org/10.1091/mbc.E13-04-0215

Maguire, E. P., Macpherson, T., Swinny, J. D., Dixon, C. I., Herd, M. B., Belelli, D., ... & Lambert, J. J. (2014). Tonic inhibition of accumbal spiny neurons by extrasynaptic α4βδ GABAA receptors modulates the actions of psychostimulants. Journal of Neuroscience, 34(3), 823-838.

Maguire, J. L., Stell, B. M., Rafizadeh, M., & Mody, I. (2005). Ovarian cycle-linked changes in GABA(A) receptors mediating tonic inhibition alter seizure susceptibility and anxiety. Nature neuroscience, 8(6), 797–804. https://doi.org/10.1038/nn1469

Maguire, J., & Mody, I. (2007). Neurosteroid synthesis-mediated regulation of GABA(A) receptors: relevance to the ovarian cycle and stress. The Journal of neuroscience : the official journal of the Society for Neuroscience, 27(9), 2155–2162. https://doi.org/10.1523/JNEUROSCI.4945-06.2007

Mahler, S. V., Moorman, D. E., Smith, R. J., James, M. H., & Aston-Jones, G. (2014). Motivational activation: a unifying hypothesis of orexin/hypocretin function. Nature neuroscience, 17(10), 1298–1303. https://doi.org/10.1038/nn.3810

Mallya, A. P., Wang, H., Lee, H. N. R., & Deutch, A. Y. (2018). Microglial pruning of synapses in the prefrontal cortex during adolescence. Cerebral Cortex, 29(4), 1634-1643. https://doi.org/10.1093/cercor/bhy061

Markham, J. A., Morris, J. R., & Juraska, J. M. (2007). Neuron number decreases in the rat ventral, but not dorsal, medial prefrontal cortex between adolescence and adulthood. Neuroscience, 144(3), 961–968. https://doi-org.newproxy.downstate.edu/10.1016/j.neuroscience.2006.10.015

Marowsky, A., Yanagawa, Y., Obata, K., & Vogt, K. E. (2005). A specialized subclass of interneurons mediates dopaminergic facilitation of amygdala function. Neuron, 48(6), 1025–1037. https://doi.org/10.1016/j.neuron.2005.10.029

Martinelli, D. C., Chew, K. S., Rohlmann, A., Lum, M. Y., Ressl, S., Hattar, S., ... & Südhof, T. C. (2016). Expression of C1ql3 in discrete neuronal populations controls efferent synapse numbers and diverse behaviors. Neuron, 91(5), 1034-1051.

Matsumoto, M. & Hikosaka, O. (2009). Two types of dopamine neuron distinctly convey positive and negative motivational signals. Nature, 459(7248), 837-841. https://doi.org/10.1038/nature08028

Matsuzaki, M., Honkura, N., Ellis-Davies, G. C. & Kasai, H. (2004). Structural basis of long-term potentiation in single dendritic spines. Nature, 429(6993), 761-766. https://doi.org/10.1038/nature02617

Mayberg, H. S., Lozano, A. M., Voon, V., McNeely, H. E., Seminowicz, D., Hamani, C., Schwalb, J. M. & Kennedy, S. H. (2005). Deep brain stimulation for treatment-resistant depression. Neuron, 45(5), 651-660. https://doi.org/10.1016/j.neuron.2005.02.014

Mazzanti, C. M., Lappalainen, J., Long, J. C., Bengel, D., Naukkarinen, H., Eggert, M., ... & Goldman, D. (1998). Role of the serotonin transporter promoter polymorphism in anxiety-related traits. Archives of General Psychiatry, 55(10), 936-940.

McClure, E. B., Monk, C. S., Nelson, E. E., Parrish, J. M., Adler, A., Blair, R. J., Fromm, S., Charney, D. S., Leibenluft, E., Ernst, M., & Pine, D. S. (2007). Abnormal attention modulation of fear circuit function in pediatric generalized anxiety disorder. Archives of general psychiatry, 64(1), 97–106. https://doi.org/10.1001/archpsyc.64.1.97

McCutcheon, R., Keefe, R. S., & McGuire, P. (2023). Cognitive impairment in schizophrenia: aetiology, pathophysiology, and treatment. Molecular Psychiatry, 28(5), 1902-1918. https://doi.org/10.1038/s41380-023-01949-9

McEwen B. (2002). Estrogen actions throughout the brain. Recent progress in hormone research, 57, 357–384. https://doi.org/10.1210/rp.57.1.357

McLean, C. P., Asnaani, A., Litz, B. T., & Hofmann, S. G. (2011). Gender differences in anxiety disorders: prevalence, course of illness, comorbidity and burden of illness. Journal of psychiatric research, 45(8), 1027–1035. https://doi.org/10.1016/j.jpsychires.2011.03.006

McLean, C., Asnaani, A., Litz, B., & Hofmann, S. (2011). Gender differences in anxiety disorders: prevalence, course of illness, comorbidity and burden of illness.. Journal of psychiatric research, 45 8, 1027-35 . https://doi.org/10.1016/j.jpsychires.2011.03.006.

Meera, P., Wallner, M., & Otis, T. S. (2011). Molecular basis for the high THIP/gaboxadol sensitivity of extrasynaptic GABA(A) receptors. Journal of neurophysiology, 106(4), 2057–2064. https://doi.org/10.1152/jn.00450.2011

Meisner, O., Nair, A., & Chang, S. (2022). Amygdala connectivity and implications for social cognition and disorders. Handbook of Clinical Neurology, 381-403. https://doi.org/10.1016/b978-0-12-823493-8.00017-1

Menon, V. and D'Esposito, M. (2021). The role of pfc networks in cognitive control and executive function. Neuropsychopharmacology, 47(1), 90-103. https://doi.org/10.1038/s41386-021-01152-w

Merali, Z., Du, L., Hrdina, P., Palkovits, M., Faludi, G., Poulter, M. O. & Anisman, H. (2004). Dysregulation in the suicide brain: mRNA expression of corticotropin-releasing hormone receptors and GABAA receptor subunits in frontal cortical brain region. Journal of Neuroscience, 24(6), 1478-1485. https://doi.org/10.1523/JNEUROSCI.4734-03.2004

Milad, M. R. & Quirk, G. J. (2002). Neurons in medial prefrontal cortex signal memory for fear extinction. Nature, 420(6911), 70-74. https://doi.org/10.1038/nature01138

Millan, M. J. (2009). Dual-and triple-acting agents for treating core and co-morbid symptoms of major depression: novel concepts, new drugs. Neurotherapeutics, 6(1), 53-77.

Miller, P. S., & Aricescu, A. R. (2014). Crystal structure of a human GABAA receptor. Nature, 512(7514), 270-275.

Mo, A., Mukamel, E. A., Davis, F. P., Luo, C., Henry, G. L., Picard, S., Urich, M. A., Nery, J. R., Sejnowski, T. J., Lister, R., Eddy, S. R., Ecker, J. R., & Nathans, J. (2015). Epigenomic Signatures of Neuronal Diversity in the Mammalian Brain. Neuron, 86(6), 1369–1384. https://doi.org/10.1016/j.neuron.2015.05.018

Morales, M. & Fifková, E. (1989). Distribution of MAP2 in dendritic spines and its colocalization with actin. An immunogold electron-microscope study. Cell and Tissue Research, 256(2), 447-456. https://doi.org/10.1007/BF00225592

Motzkin, J. C., Philippi, C. L., Wolf, R. C., Baskaya, M. K. & Koenigs, M. (2015). Ventromedial prefrontal cortex is critical for the regulation of amygdala activity in humans. Biological psychiatry, 77(3), 276-284. https://doi.org/10.1016/j.biopsych.2014.02.014

Motzkin, J., Philippi, C., Wolf, R., Başkaya, M., & Koenigs, M. (2015). Ventromedial Prefrontal Cortex Is Critical for the Regulation of Amygdala Activity in Humans. Biological Psychiatry, 77, 276-284. https://doi.org/10.1016/j.biopsych.2014.02.014.

Moujalled, D., Strasser, A., & Liddell, J. R. (2021). Molecular mechanisms of cell death in neurological diseases. Cell Death &Amp; Differentiation, 28(7), 2029-2044. https://doi.org/10.1038/s41418-021-00814-y

Moyer, C. E., Shelton, M. A., & Sweet, R. A. (2015). Dendritic spine alterations in schizophrenia. Neuroscience Letters, 601, 46-53. https://doi.org/10.1016/j.neulet.2014.11.042

Mueller, E. M., Panitz, C., Hermann, C. & Pizzagalli, D. A. (2014). Prefrontal oscillations during recall of conditioned and extinguished fear in humans. The Journal of neuroscience, 34(24), 7059-7066. https://doi.org/10.1523/JNEUROSCI.3427-13.2014

Nasif, F. J., Hu, X. T., Ramirez, O. A., & Perez, M. F. (2011). Inhibition of neuronal nitric oxide synthase prevents alterations in medial prefrontal cortex excitability induced by repeated cocaine administration. Psychopharmacology, 218, 323-330.

National College Health Assessment. (2008-2019). Publications and reports. American College Health Association. https://www.acha.org/NCHA/ACHANCHA_Data/Publications_and_Reports

Negrón-Oyarzo, I., Aboitiz, F., & Fuentealba, P. (2016). Impaired functional connectivity in the prefrontal cortex: a mechanism for chronic stress-induced neuropsychiatric disorders. Neural Plasticity, 2016, 1-16. https://doi.org/10.1155/2016/7539065

Nelson, J., & Chouinard, G. (1999). Guidelines for the clinical use of benzodiazepines: pharmacokinetics, dependency, rebound and withdrawal. Canadian Society for Clinical Pharmacology.. The Canadian journal of clinical pharmacology = Journal canadien de pharmacologie clinique, 6 2, 69-83 .

Nett, K. E., & LaLumiere, R. T. (2021). Infralimbic cortex functioning across motivated behaviors: Can the differences be reconciled?. Neuroscience and biobehavioral reviews, 131, 704–721. https://doi-org.newproxy.downstate.edu/10.1016/j.neubiorev.2021.10.002

Nicholson, D. A., & Geinisman, Y. (2009). Axospinous synaptic remodeling in the aging rat hippocampus. Neurobiology of Aging, 30(2), 255-268.

Nollet, M., Wisden, W., & Franks, N. P. (2020). Sleep deprivation and stress: a reciprocal relationship. Interface focus, 10(3), 20190092. https://doi.org/10.1098/rsfs.2019.0092

Olsen, R. W., & Sieghart, W. (2009). GABAA receptors: subtypes provide diversity of function and pharmacology. Neuropharmacology, 56(1), 141-148.

Pan, F., & Gan, W. B. (2008). Two-photon imaging of dendritic spine development in the mouse cortex. Developmental neurobiology, 68(6), 771-778.

Panzanelli, P., Gunn, B. G., Schlatter, M. C., Benke, D., Tyagarajan, S. K., Scheiffele, P., ... & Fritschy, J. M. (2011). Distinct mechanisms regulate GABAA receptor and gephyrin clustering at perisomatic and axo-axonic synapses on CA1 pyramidal cells. The Journal of physiology, 589(20), 4959-4980.

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T. A., Guiducci, E., Dumas, L., Ragozzino, D., & Gross, C. T. (2011). Synaptic pruning by microglia is necessary for normal brain development. Science (New York, N.Y.), 333(6048), 1456–1458. https://doi.org/10.1126/science.1202529

Parato, J., Shen, H., & Smith, S. S. (2019). α4βδ GABAA receptors trigger synaptic pruning and reduce dendritic length of female mouse CA3 hippocampal pyramidal cells at puberty. Neuroscience, 398, 23-36.

Parikshak, N. N., Luo, R., Zhang, A., Won, H., Lowe, J. K., Chandran, V., Horvath, S., & Geschwind, D. H. (2013). Integrative functional genomic analyses implicate specific molecular pathways and circuits in autism. Cell, 155(5), 1008–1021. https://doi.org/10.1016/j.cell.2013.10.031

Pattwell, S. S., Duhoux, S., Hartley, C. A., Johnson, D. C., Jing, D., Elliott, M. D., ... & Casey, B. J. (2016). Altered fear learning across development in both mouse and human. Proceedings of the National Academy of Sciences, 113(40), E5749-E5758.

Pattwell, S. S., Liston, C., Jing, D., Ninan, I., Yang, R. R., Witztum, J., Murdock, M. H., Dincheva, I., Bath, K. G., Casey, B. J., Deisseroth, K. & Lee, F. S. (2016). Dynamic changes in neural circuitry during adolescence are associated with persistent attenuation of fear memories. Nature communications, 7(1), 1-13. https://doi.org/10.1038/ncomms11475

Paul, A., Crow, M., Raudales, R., He, M., Gillis, J., & Huang, Z. J. (2017). Transcriptional Architecture of Synaptic Communication Delineates GABAergic Neuron Identity. Cell, 171(3), 522–539.e20. https://doi.org/10.1016/j.cell.2017.08.032

Paus, T., Keshavan, M. & Giedd, J. N. (2008). Why do many psychiatric disorders emerge during adolescence?. Nature Reviews Neuroscience, 9(12), 947-957. https://doi.org/10.1038/nrn2513

Paus, T., Keshavan, M. S., & Giedd, J. N. (2008). Why do many psychiatric disorders emerge during adolescence?. Nature Reviews Neuroscience, 9(12), 947-957. https://doi.org/10.1038/nrn2513

Payne, H. L., Donoghue, P. S., Connelly, W. M., Hinterreiter, S., Tiwari, P., Ives, J. H., ... & Thompson, C. L. (2006). Aberrant GABAA receptor expression in the dentate gyrus of the epileptic mutant mouse stargazer. Journal of Neuroscience, 26(33), 8600-8608.

Pellow, S., Chopin, P., File, S. E. & Briley, M. (1985). Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. Journal of neuroscience methods, 14(3), 149-167. https://doi.org/10.1016/0165-0270(85)90031-7

Peng, H., Chen, G., Lai, C., Hsieh, M., & Lin, T. (2013). Spinal Serum-Inducible and Glucocorticoid-Inducible Kinase 1 Mediates Neuropathic Pain via Kalirin and Downstream PSD-95-Dependent NR2B Phosphorylation in Rats. The Journal of Neuroscience, 33, 5227 - 5240. https://doi.org/10.1523/JNEUROSCI.4452-12.2013.

Peng, Z., Hauer, B., Mihalek, R. M., Homanics, G. E., Sieghart, W., Olsen, R. W., & Houser, C. R. (2002). GABAA receptor changes in δ subunit-deficient mice: altered expression of α4 and γ2 subunits in the forebrain. Journal of Comparative Neurology, 446(2), 179-197.

Peng, Z., Huang, C. S., Stell, B. M., Mody, I., & Houser, C. R. (2004). Altered expression of the delta subunit of the GABAA receptor in a mouse model of temporal lobe epilepsy. The Journal of neuroscience : the official journal of the Society for Neuroscience, 24(39), 8629–8639. https://doi.org/10.1523/JNEUROSCI.2877-04.2004

Penzes, P., Johnson, R. C., Sattler, R., Zhang, X., Huganir, R. L., Kambampati, V., Mains, R. E. & Eipper, B. A. (2001). The neuronal Rho-GEF Kalirin-7 interacts with PDZ domain-containing proteins and regulates dendritic morphogenesis. Neuron, 29(1), 229-242. https://doi.org/10.1016/S0896-6273(01)00193-3

Petanjek, Z., Judaš, M., Šimic, G., Rasin, M. R., Uylings, H. B., Rakic, P., & Kostovic, I. (2011). Extraordinary neoteny of synaptic spines in the human prefrontal cortex. Proceedings of the National Academy of Sciences of the United States of America, 108(32), 13281–13286. https://doi.org/10.1073/pnas.1105108108

Peters, J., Kalivas, P. W., & Quirk, G. J. (2009). Extinction circuits for fear and addiction overlap in prefrontal cortex. Learning & memory, 16(5), 279-288.

Pine, D. S., Cohen, P., Gurley, D., Brook, J. & Ma, Y. (1998). The risk for early-adulthood anxiety and depressive disorders in adolescents with anxiety and depressive disorders. Archives of general psychiatry, 55(1), 56-64. https://doi.org/10.1001/archpsyc.55.1.56

Poirazi, P., Brannon, T., & Mel, B. W. (2003). Pyramidal neuron as two-layer neural network. Neuron, 37(6), 989–999. https://doi.org/10.1016/s0896-6273(03)00149-1

Porcher, C., Medina, I., & Gaïarsa, J. (2018). Mechanism of bdnf modulation in gabaergic synaptic transmission in healthy and disease brains. Frontiers in Cellular Neuroscience, 12. https://doi.org/10.3389/fncel.2018.00273

Poulter, M. O., Du, L., Zhurov, V., Palkovits, M., Faludi, G., Merali, Z. & Anisman, H. (2010). Altered organization of GABA(A) receptor mRNA expression in the depressed suicide brain. Frontiers in molecular neuroscience, 3, 3. https://doi.org/10.3389/neuro.02.003.2010

Preissl, S., Fang, R., Huang, H., Zhao, Y., Raviram, R., Gorkin, D. U., … & Ren, B. (2018). Single-nucleus analysis of accessible chromatin in developing mouse forebrain reveals cell-type-specific transcriptional regulation. Nature Neuroscience, 21(3), 432-439. https://doi.org/10.1038/s41593-018-0079-3

Pribiag, H., & Stellwagen, D. (2013). TNF-α downregulates inhibitory neurotransmission through protein phosphatase 1-dependent trafficking of GABA(A) receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience, 33(40), 15879–15893. https://doi-org.newproxy.downstate.edu/10.1523/JNEUROSCI.0530-13.2013

Purves, K. L., Coleman, J. R., Meier, S. M., Rayner, C., Davis, K. A., Cheesman, R., ... & Eley, T. C. (2020). A major role for common genetic variation in anxiety disorders. Molecular psychiatry, 25(12), 3292-3303.

Qi, L., Ma, X. C., Zhou, D. D., Guan, S., Gao, F. S., & Cong, P. X. (2020). Wavelet and principal component analysis of electromyographic activity and slow component of oxygen uptake during heavy and

severe cycling exercise. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme, 45(2), 187–192. https://doi.org/10.1139/apnm-2019-0037

Quiquempoix, M., Fayad, S. L., Boutourlinsky, K., Leresche, N., Lambert, R. C., & Bessaih, T. (2018). Layer 2/3 Pyramidal Neurons Control the Gain of Cortical Output. Cell reports, 24(11), 2799–2807.e4. https://doi-org.newproxy.downstate.edu/10.1016/j.celrep.2018.08.038

Raghuram, H., Scheefhals, N., & Smit, A. B. (2019). Mechanisms underlying synapse-specific clustering and local synaptic changes after long-term potentiation. Journal of Neuroscience, 39(44), 8719-8725.

Ramaswamy, S., & Markram, H. (2015). Anatomy and physiology of the thick-tufted layer 5 pyramidal neuron. Frontiers in cellular neuroscience, 9, 233. https://doi-org.newproxy.downstate.edu/10.3389/fncel.2015.00233

Reinert, S., Hübener, M., Bonhoeffer, T., & Goltstein, P. M. (2021). Mouse prefrontal cortex represents learned rules for categorization. Nature, 593(7859), 411-417. https://doi.org/10.1038/s41586-021-03452-z

Remes, O., Brayne, C., van der Linde, R., & Lafortune, L. (2016). A systematic review of reviews on the prevalence of anxiety disorders in adult populations. Brain and behavior, 6(7), e00497. https://doi.org/10.1002/brb3.497

Rempel-Clower, N. L., & Barbas, H. (1998). Topographic organization of connections between the hypothalamus and prefrontal cortex in the rhesus monkey. Journal of Comparative Neurology, 398(3), 393-419.

Ressler, K., & Nemeroff, C. (2000). Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders. Depression and Anxiety, 12. https://doi.org/10.1002/1520-6394(2000)12:1+<2::AID-DA2>3.0.CO;2-4.

Resstel, L. B., Souza, R. F. & Guimarães, F. S. (2008). Anxiolytic-like effects induced by medial prefrontal cortex inhibition in rats submitted to the Vogel conflict test. Physiology & behavior, 93(1-2), 200-205. https://doi.org/10.1016/j.physbeh.2007.08.009

Riga, D., Matos, M. R., Glas, A., Smit, A. B., Spijker, S. & Van den Oever, M. C. (2014). Optogenetic dissection of medial prefrontal cortex circuitry. Frontiers in systems neuroscience, 8, 230. https://doi.org/10.3389/fnsys.2014.00230

Risher, W. C., Ustunkaya, T., Singh Alvarado, J. & Eroglu, C. (2014). Rapid Golgi analysis method for efficient and unbiased classification of dendritic spines. PloS one, 9(9), e107591. https://doi.org/10.1371/journal.pone.0107591

Rosenkranz, J. A. & Grace, A. A. (2002). Cellular mechanisms of infralimbic and prelimbic prefrontal cortical inhibition and dopaminergic modulation of basolateral amygdala neurons in vivo. Journal of Neuroscience, 22(1), 324-337. https://doi.org/10.1523/JNEUROSCI.22-01-00324.2002

Roth, T. L., Puig-Saus, C., Yu, R., Shifrut, E., Carnevale, J., Li, P. J., ... & Marson, A. (2021). Reprogramming human T cell function and specificity with non-viral genome targeting. Nature, 559(7714), 405-409.

Roussignol, G., Ango, F., Romorini, S., Tu, J. C., Sala, C., Worley, P. F., Bockaert, J. & Fagni, L. (2005). Shank expression is sufficient to induce functional dendritic spine synapses in aspiny neurons. Journal of Neuroscience, 25(14), 3560-3570. https://doi.org/10.1523/JNEUROSCI.4354-04.2005

Sabaliauskas, N., Shen, H., Molla, J., Alvarado, J. S., Aoki, C. & Smith, S. S. (2014). Neurosteroid effects at α4βδ GABAA receptors alter spatial learning and synaptic plasticity in CA1 hippocampus across the estrous cycle of the mouse. Brain research. https://doi.org/10.1016/j.brainres.2014.12.026

Sabaliauskas, N., Shen, H., Yu, T. S., Aoki, C. & Smith, S. S. (2012). Knockout of the γ-aminobutyric acid receptor subunit α4 reduces functional δ-containing extrasynaptic GABAA receptors in hippocampal pyramidal cells at the onset of puberty. Brain research, 1450, 11-23.

Saitoh, A., Yamada, M., Yamada, M., Kubota, Y., Kobayashi, K., Song, L., ... & Tanaka, T. (2014). Activation of the prelimbic medial prefrontal cortex induces anxiety-like behaviors via N-Methyl-D-aspartate receptor-mediated glutamatergic neurotransmission in mice. Journal of neuroscience research, 92(8), 1044-1053. https://doi.org/10.1002/jnr.23391

Sakai, J. (2020). How synaptic pruning shapes neural wiring during development and, possibly, in disease. Proceedings of the National Academy of Sciences, 117(28), 16096-16099. https://doi.org/10.1073/pnas.2010281117

Santomauro, D. F., Herrera, A. M. M., Shadid, J., Zheng, P., Ashbaugh, C., Pigott, D. M., ... & Ferrari, A. J. (2021). Global prevalence and burden of depressive and anxiety disorders in 204 countries and territories in 2020 due to the COVID-19 pandemic. The Lancet, 398(10312), 1700-1712.

Scala, F., Kobak, D., Shen, S., Bernaerts, Y., Laturnus, S., Cadwell, C. R., … & Tolias, A. S. (2019). Layer 4 of mouse neocortex differs in cell types and circuit organization between sensory areas. Nature Communications, 10(1). https://doi.org/10.1038/s41467-019-12058-z

Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., Ransohoff, R. M., Greenberg, M. E., Barres, B. A., & Stevens, B. (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron, 74(4), 691–705. https://doi.org/10.1016/j.neuron.2012.03.026

Sekar, A., Bialas, A. R., de Rivera, H., Davis, A., Hammond, T. R., Kamitaki, N., Tooley, K., Presumey, J., Baum, M., Van Doren, V., Genovese, G., Rose, S. A., Handsaker, R. E., Schizophrenia Working Group of the Psychiatric Genomics Consortium, Daly, M. J., Carroll, M. C., Stevens, B., & McCarroll, S. A. (2016). Schizophrenia risk from complex variation of complement component 4. Nature, 530(7589), 177–183. https://doi.org/10.1038/nature16549

Selemon, L. D. (2013). A role for synaptic plasticity in the adolescent development of executive function. Translational Psychiatry, 3(3), e238-e238. https://doi.org/10.1038/tp.2013.7

Sequeira, A., Mamdani, F., Ernst, C., Vawter, M. P., Bunney, W. E., Lebel, V., ... & Klempan, T. (2009). Global brain gene expression analysis links glutamatergic and GABAergic alterations to suicide and major depression. PloS one, 4(8), e6585. https://doi.org/10.1371/journal.pone.0006585

Sexton, C. A., Penzinger, R., Mortensen, M., Bright, D. P., & Smart, T. G. (2021). Structural determinants and regulation of spontaneous activity in gabaa receptors. Nature Communications, 12(1). https://doi.org/10.1038/s41467-021-25633-0

Shader, R., & Greenblatt, D. (1993). Use of benzodiazepines in anxiety disorders.. The New England journal of medicine, 328 19, 1398-405 . https://doi.org/10.1056/NEJM199305133281907.

Shah, A. A., Sjovold, T. & Treit, D. (2004). Inactivation of the medial prefrontal cortex with the GABAA receptor agonist muscimol increases open-arm activity in the elevated plus-maze and attenuates shock-probe burying in rats. Brain research, 1028(1), 112-115. https://doi.org/10.1016/j.brainres.2004.08.061

Sharp, B. (2017). Basolateral amygdala and stress-induced hyperexcitability affect motivated behaviors and addiction. Translational Psychiatry, 7. https://doi.org/10.1038/tp.2017.161.

Sharpe, M. J. & Killcross, S. (2014). The prelimbic cortex uses higher-order cues to modulate both the acquisition and expression of conditioned fear. Frontiers in systems neuroscience, 8, 235. https://doi.org/10.3389/fnsys.2014.00235

Shen, H., Gong, Q. H., Aoki, C., Yuan, M., Ruderman, Y., Dattilo, M., Williams, K., & Smith, S. S. (2007). Reversal of neurosteroid effects at alpha4beta2delta GABAA receptors triggers anxiety at puberty. Nature neuroscience, 10(4), 469–477. https://doi.org/10.1038/nn1868

Shen, W., Flajolet, M., Greengard, P., & Surmeier, D. J. (2008). Dichotomous dopaminergic control of striatal synaptic plasticity. Science (New York, N.Y.), 321(5890), 848–851. https://doi.org/10.1126/science.1160575

Shu, H., Bracamontes, J., Taylor, A., Wu, K., Eaton, M., Akk, G., Manion, B., Evers, A., Krishnan, K., Covey, D., Zorumski, C., Steinbach, J., & Mennerick, S. (2012). Characteristics of concatemeric GABAA receptors containing α4/δ subunits expressed in Xenopus oocytes. British Journal of Pharmacology, 165. https://doi.org/10.1111/j.1476-5381.2011.01690.x.

Sieghart, W. (2006). Structure, pharmacology, and function of GABAA receptor subtypes. Advances in pharmacology, 54, 231-263.

Sigel, E. & Lüscher, B. P. (2011). A closer look at the high affinity benzodiazepine binding site on GABAA receptors. Current topics in medicinal chemistry, 11(2), 241-246. https://doi.org/10.2174/156802611794858953

Sigel, E., & Ernst, M. (2018). The Benzodiazepine Binding Sites of GABAA Receptors.. Trends in pharmacological sciences, 39 7, 659-671 . https://doi.org/10.1016/j.tips.2018.03.006.

Sigel, E., & Steinmann, M. E. (2012). Structure, function, and modulation of GABAA receptors. Journal of Biological Chemistry, 287(48), 40224-40231.

Simpson, J. R., Drevets, W. C., Snyder, A. Z., Gusnard, D. A. & Raichle, M. E. (2001). Emotion-induced changes in human medial prefrontal cortex: II. During anticipatory anxiety. Proceedings of the National Academy of Sciences, 98(2), 688-693. https://doi.org/10.1073/pnas.98.2.688

Sipe, G. O., Lowery, R. L., Tremblay, M., Kelly, E. A., Lamantia, C. E., & Majewska, A. K. (2016). Microglial p2y12 is necessary for synaptic plasticity in mouse visual cortex. Nature Communications, 7(1). https://doi.org/10.1038/ncomms10905

Sisk, C. L., & Zehr, J. L. (2005). Pubertal hormones organize the adolescent brain and behavior. Frontiers in neuroendocrinology, 26(3-4), 163–174. https://doi.org/10.1016/j.yfrne.2005.10.003

Smith S. S. (2013). α4βδ GABAA receptors and tonic inhibitory current during adolescence: effects on mood and synaptic plasticity. Frontiers in neural circuits, 7, 135. https://doi.org/10.3389/fncir.2013.00135

Smith, S. S., Ruderman, Y., Frye, C., Homanics, G. & Yuan, M. (2006). Steroid withdrawal in the mouse results in anxiogenic effects of 3α, 5β-THP: a possible model of premenstrual dysphoric disorder. Psychopharmacology, 186(3), 323-333. https://doi.org/10.1007/s00213-006-0363-2

Somel, M., Franz, H., Yan, Z., Lorenc, A., Guo, S., Giger, T., Kelso, J., Nickel, B., Dannemann, M., Bahn, S., Webster, M. J., Weickert, C. S., Lachmann, M., Pääbo, S., & Khaitovich, P. (2009). Transcriptional neoteny in the human brain. Proceedings of the National Academy of Sciences of the United States of America, 106(14), 5743–5748. https://doi.org/10.1073/pnas.0900544106

Song, D., Opris, I., Chan, R. H., Marmarelis, V. Z., Hampson, R. E., Deadwyler, S. A., & Berger, T. W. (2012). Functional connectivity between Layer 2/3 and Layer 5 neurons in prefrontal cortex of nonhuman primates during a delayed match-to-sample task. Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Annual International Conference, 2012, 2555–2558. https://doi-org.newproxy.downstate.edu/10.1109/EMBC.2012.6346485

Spear, L. P. (2013). Adolescent neurodevelopment. Journal of Adolescent Health, 52(2), S7-S13. https://doi.org/10.1016/j.jadohealth.2012.05.006

Spellman, T., Rigotti, M., Ahmari, S. E., Fusi, S., Gogos, J. A., & Gordon, J. A. (2015). Hippocampal-prefrontal input supports spatial encoding in working memory. Nature, 522(7556), 309-314.

Stevens, B., Allen, N. J., Vazquez, L. E., Howell, G. R., Christopherson, K. S., Nouri, N., Micheva, K. D., Mehalow, A. K., Huberman, A. D., Stafford, B., Sher, A., Litke, A. M., Lambris, J. D., Smith, S. J., John, S. W., & Barres, B. A. (2007). The classical complement cascade mediates CNS synapse elimination. Cell, 131(6), 1164–1178. https://doi.org/10.1016/j.cell.2007.10.036

Ströhle, A., Gensichen, J., & Domschke, K. (2018). The diagnosis and treatment of anxiety disorders. Deutsches Ärzteblatt International. https://doi.org/10.3238/arztebl.2018.0611

Stuart, G., Spruston, N., Sakmann, B. & Häusser, M. (1997). Action potential initiation and backpropagation in neurons of the mammalian CNS. Trends in neurosciences, 20(3), 125-131. https://doi.org/10.1016/S0166-2236(96)10075-8

Surmeier, D. J., Shen, W., Day, M., Gertler, T., Chan, S., Tian, X., & Plotkin, J. L. (2010). The role of dopamine in modulating the structure and function of striatal circuits. Progress in brain research, 183, 149–167. https://doi.org/10.1016/S0079-6123(10)83008-0

Suzuki, S., Brown, C. M. & Wise, P. M. (2006). Mechanisms of neuroprotection by estrogen. Endocrine, 29(2), 209-215. https://doi.org/10.1385/ENDO:29:2:209

Tamnes, C. K., Ostby, Y., Fjell, A. M., Westlye, L. T., Due-Tønnessen, P., & Walhovd, K. B. (2010). Brain maturation in adolescence and young adulthood: regional age-related changes in cortical thickness and white matter volume and microstructure. Cerebral cortex (New York, N.Y. : 1991), 20(3), 534–548. https://doi-org.newproxy.downstate.edu/10.1093/cercor/bhp118

Tang, G., Gudsnuk, K., Kuo, S. H., Cotrina, M. L., Rosoklija, G., Sosunov, A., ... & Tanter, M. (2014). Loss of mTOR-dependent macroautophagy causes autistic-like synaptic pruning deficits. Neuron, 83(5), 1131-1143. https://doi.org/10.1016/j.neuron.2014.07.040

Tang, X., Jaenisch, R., & Sur, M. (2021). The role of GABAergic signalling in neurodevelopmental disorders. Nature Reviews Neuroscience, 22(5), 290-307.

Tedesco, R., Thomas, J. A., Katzenellenbogen, B. S., & Katzenellenbogen, J. A. (2001). The estrogen receptor: a structure-based approach to the design of new specific hormone-receptor combinations. Chemistry & biology, 8(3), 277–287. https://doi.org/10.1016/s1074-5521(01)00006-0

ter Horst, J. P., de Kloet, E. R., Schächinger, H., & Oitzl, M. S. (2012). Relevance of stress and female sex hormones for emotion and cognition. Cellular and molecular neurobiology, 32(5), 725–735. https://doi.org/10.1007/s10571-011-9774-2

Tervo, D. G., Hwang, B. Y., Viswanathan, S., Gaj, T., Lavzin, M., Ritola, K. D., Lindo, S., Michael, S., Kuleshova, E., Ojala, D., Huang, C. C., Gerfen, C. R., Schiller, J., Dudman, J. T., Hantman, A. W., Looger, L. L., Schaffer, D. V., & Karpova, A. Y. (2016). A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons. Neuron, 92(2), 372–382. https://doi.org/10.1016/j.neuron.2016.09.021

Thapar, A. and Riglin, L. (2020). The importance of a developmental perspective in psychiatry: what do recent genetic-epidemiological findings show?. Molecular Psychiatry, 25(8), 1631-1639. https://doi.org/10.1038/s41380-020-0648-1

Tretter, V., Jacob, T. C., Mukherjee, J., Fritschy, J. M., Pangalos, M. N., & Moss, S. J. (2008). The clustering of GABA(A) receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor alpha 2 subunits to gephyrin. The Journal of neuroscience : the official journal of the Society for Neuroscience, 28(6), 1356–1365. https://doi.org/10.1523/JNEUROSCI.5050-07.2008

Turrigiano G. (2011). Too many cooks? Intrinsic and synaptic homeostatic mechanisms in cortical circuit refinement. Annual review of neuroscience, 34, 89–103. https://doi.org/10.1146/annurev-neuro-060909-153238

Tye, K. M., Prakash, R., Kim, S., Fenno, L. E., Grosenick, L., Zarabi, H., … & Deisseroth, K. (2011). Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature, 471(7338), 358-362. https://doi.org/10.1038/nature09820

Urban, D. J., & Roth, B. L. (2015). DREADDs (designer receptors exclusively activated by designer drugs): chemogenetic tools with therapeutic utility. Annual review of pharmacology and toxicology, 55, 399–417. https://doi.org/10.1146/annurev-pharmtox-010814-124803

Urbanski, H. F., Noriega, N., Lemos, D. R., & Kohama, S. G. (2009). Gene expression profiling in the rhesus macaque: experimental design considerations. Methods, 49(1), 26-31. https://doi.org/10.1016/j.ymeth.2009.05.009

Urrutia-Piñones, J., Morales-Moraga, C., Sanguinetti-González, N., Escobar, A. P., & Chiu, C. (2022). Long-range gabaergic projections of cortical origin in brain function. Frontiers in Systems Neuroscience, 16. https://doi.org/10.3389/fnsys.2022.841869

Uygun, D. S. and Basheer, R. (2022). Circuits and components of delta wave regulation. Brain Research Bulletin, 188, 223-232. https://doi.org/10.1016/j.brainresbull.2022.06.006

Vanes, L. D., Moutoussis, M., Ziegler, G., Goodyer, I. M., Fonagy, P., Jones, P. B., ... & Dolan, R. J. (2020). White matter tract myelin maturation and its association with general psychopathology in adolescence and early adulthood. Human brain mapping, 41(3), 827-839.

Vanni, M. P., Chan, A. S. W., Balbi, M., Silasi, G., & Murphy, T. H. (2017). Mesoscale mapping of mouse cortex reveals frequency-dependent cycling between distinct macroscale functional modules. The Journal of Neuroscience, 37(31), 7513-7533. https://doi.org/10.1523/jneurosci.3560-16.2017

Varodayan, F. P., Sidhu, H., Kreifeldt, M., Roberto, M., & Contet, C. (2018). Morphological and functional evidence of increased excitatory signaling in the prelimbic cortex during ethanol withdrawal. Neuropharmacology, 133, 470-480.

Velasco, E. M. F. d., Hearing, M., Xia, Z., Victoria, N. C., Luján, R., & Wickman, K. (2015). Sex differences in gababr-girk signaling in layer 5/6 pyramidal neurons of the mouse prelimbic cortex. Neuropharmacology, 95, 353-360. https://doi.org/10.1016/j.neuropharm.2015.03.029

Vertes, R. P. (2004). Differential projections of the infralimbic and prelimbic cortex in the rat. Synapse, 51(1), 32-58.

Vertes, R. P. (2006). Interactions among the medial prefrontal cortex, hippocampus and midline thalamus in emotional and cognitive processing in the rat. Neuroscience, 142(1), 1-20.

Vidal-Gonzalez, I., Vidal-Gonzalez, B., Rauch, S. L. & Quirk, G. J. (2006). Microstimulation reveals opposing influences of prelimbic and infralimbic cortex on the expression of conditioned fear. Learning & memory, 13(6), 728-733. https://doi.org/10.1101/lm.306106

Wallace, W., & Jones, A. (2008). Three-dimensional analysis of spine morphology in dendrites. Methods in Cell Biology, 89, 267-280.

Wang, H. X., & Gao, W. J. (2009). Cell type-specific development of NMDA receptors in the interneurons of rat prefrontal cortex. Neuropsychopharmacology, 34(8), 2028-2040.

Weisberg, R. B. (2007). Psychiatric treatment in primary care patients with anxiety disorders: a comparison of care received from primary care providers and psychiatrists. American Journal of Psychiatry, 164(2), 276. https://doi.org/10.1176/appi.ajp.164.2.276

Whissell, P. D., Tohyama, S., & Martin, L. J. (2016). The use of dreadds to deconstruct behavior. Frontiers in Genetics, 7. https://doi.org/10.3389/fgene.2016.00070

Wiegert, J. S., Mahn, M., Prigge, M., Printz, Y., & Yizhar, O. (2017). Silencing Neurons: Tools, Applications, and Experimental Constraints. Neuron, 95(3), 504–529. https://doi.org/10.1016/j.neuron.2017.06.050

Wiera, G. and Mozrzymas, J. W. (2021). Extracellular metalloproteinases in the plasticity of excitatory and inhibitory synapses. Cells, 10(8), 2055. https://doi.org/10.3390/cells10082055

Willard, S. S. and Koochekpour, S. (2013). Glutamate, glutamate receptors, and downstream signaling pathways. International Journal of Biological Sciences, 9(9), 948-959. https://doi.org/10.7150/ijbs.6426

Winnubst, J., Cheyne, J. E., Niculescu, D., & Lohmann, C. (2015). Spontaneous activity drives local synaptic plasticity in vivo. Neuron, 87(2), 399-410.

Wisden, W., Laurie, D. J., Monyer, H., & Seeburg, P. H. (1992). The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. Journal of Neuroscience, 12(3), 1040-1062.

Wood, M., Adil, O., Wallace, T., Fourman, S., Wilson, S. P., Herman, J. P., … & Myers, B. (2018). Infralimbic prefrontal cortex structural and functional connectivity with the limbic forebrain: a combined viral genetic and optogenetic analysis. Brain Structure and Function, 224(1), 73-97. https://doi.org/10.1007/s00429-018-1762-6

Woolley, C. S. & McEwen, B. S. (1992). Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. The Journal of neuroscience, 12(7), 2549-2554. https://doi.org/10.1523/JNEUROSCI.12-07-02549.1992

World Health Organization. (2022). Mental health and COVID-19: early evidence of the pandemic's impact: scientific brief, 2 March 2022 (No. WHO/2019-nCoV/Sci_Brief/Mental_health/2022.1). World Health Organization.

Wu, Y., Grebenyuk, S., McHugh, T. J., Rusakov, D. A. & Semyanov, A. (2012). Backpropagating action potentials enable detection of extrasynaptic glutamate by NMDA receptors. Cell reports, 1(5), 495-505. https://doi.org/10.1016/j.celrep.2012.03.007

Wu, Y., Wang, W. & Richerson, G. B. (2001). GABA transaminase inhibition induces spontaneous and enhances depolarization-evoked GABA efflux via reversal of the GABA transporter. Journal of Neuroscience, 21(8), 2630-2639. https://doi.org/10.1523/JNEUROSCI.21-08-02630.2001

Xi, D., Zhang, W., Wang, H. X., Stradtman, G. G. & Gao, W. J. (2009). Dizocilpine (MK-801) induces distinct changes of N-methyl-D-aspartic acid receptor subunits in parvalbumin-containing interneurons in young

adult rat prefrontal cortex. The international journal of neuropsychopharmacology, 12(10), 1395-1408. https://doi.org/10.1017/S146114570900042X

Xie, Z., Srivastava, D. P., Photowala, H., Kai, L., Cahill, M. E., Woolfrey, K. M., Shum, C. Y., Surmeier, D. J., & Penzes, P. (2007). Kalirin-7 controls activity-dependent structural and functional plasticity of dendritic spines. Neuron, 56(4), 640–656. https://doi.org/10.1016/j.neuron.2007.10.005

Xie, Z., Srivastava, D. P., Photowala, H., Li, K., Cahill, M. E., Woolfrey, K. M., … & Penzes, P. (2007). Kalirin-7 controls activity-dependent structural and functional plasticity of dendritic spines. Neuron, 56(4), 640-656. https://doi.org/10.1016/j.neuron.2007.10.005

Yang, X., Fang, Y., Chen, H., Zhang, T., Yin, X., Man, J., Yang, L., & Lu, M. (2021). Global, regional and national burden of anxiety disorders from 1990 to 2019: results from the Global Burden of Disease Study 2019. Epidemiology and psychiatric sciences, 30, e36. https://doi.org/10.1017/S2045796021000275

Yasumatsu, N., Matsuzaki, M., Miyazaki, T., Noguchi, J., & Kasai, H. (2008). Principles of long-term dynamics of dendritic spines. Journal of Neuroscience, 28(50), 13592-13608.

Ye, L., Allen, W. E., Thompson, K. R., Tian, Q., Hsueh, B., Ramakrishnan, C., Wang, A. C., Jennings, J. H., Adhikari, A., Halpern, C. H., Witten, I. B., Barth, A. L., Luo, L., McNab, J. A., & Deisseroth, K. (2016). Wiring and Molecular Features of Prefrontal Ensembles Representing Distinct Experiences. Cell, 165(7), 1776–1788. https://doi.org/10.1016/j.cell.2016.05.010

Yoshii, A., & Constantine-Paton, M. (2010). Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. Developmental neurobiology, 70(5), 304–322. https://doi.org/10.1002/dneu.20765

Yu, J., Anderson, C. T., Kiritani, T., Sheets, P. L., Wokosin, D. L., Wood, L., & Shepherd, G. M. (2008). Local-Circuit Phenotypes of Layer 5 Neurons in Motor-Frontal Cortex of YFP-H Mice. Frontiers in neural circuits, 2, 6. https://doi.org/10.3389/neuro.04.006.2008

Yu, X. (2022). Less is more: a critical role of synapse pruning in neural circuit wiring. Nature Reviews Neuroscience, 24(2), 61-61. https://doi.org/10.1038/s41583-022-00665-7

Zagrebelsky, M., & Korte, M. (2014). Form follows function: BDNF and its involvement in sculpting the function and structure of synapses. Neuropharmacology, 76 Pt C, 628–638. https://doi.org/10.1016/j.neuropharm.2013.05.029

Zarrindast, M., & Khakpai, F. (2015). The Modulatory Role of Dopamine in Anxiety-like Behavior.. Archives of Iranian medicine, 18 9, 591-603 .

Zhang, S., Xu, M., Chang, W. C., Ma, C., Hoang Do, J. P., Jeong, D., Lei, T., Fan, J. L., & Dan, Y. (2016). Organization of long-range inputs and outputs of frontal cortex for top-down control. Nature neuroscience, 19(12), 1733–1742. https://doi.org/10.1038/nn.4417

Zhang, X., Liu, H., Wu, J., Zhang, X., Liu, M., & Wang, Y. (2009). Metabonomic alterations in hippocampus, temporal and prefrontal cortex with age in rats. Neurochemistry International, 54(8), 481-487. https://doi.org/10.1016/j.neuint.2009.02.004

Zhang, Y., Yoshida, T., Katz, D. B., & Lisman, J. E. (2019). NMDAR antagonist action in thalamus imposes δ oscillations on the hippocampus. Journal of Neurophysiology, 121(3), 908-917.

Zhao, X., Tang, L., & Lu, J. (2017). Automatic dendritic spine analysis in two-photon laser scanning microscopy images. Cytometry Part A, 91(2), 160-172.

Zhou, Y., Bhatt, H., Mojica, C. A., Xin, H., Pessina, M. A., Rosene, D. L., … & Medalla, M. (2023). Mesenchymal-derived extracellular vesicles enhance microglia-mediated synapse remodeling after cortical injury in aging rhesus monkeys. Journal of Neuroinflammation, 20(1). https://doi.org/10.1186/s12974-023-02880-0

Zhu, S., Qing, T., Zheng, Y., Jin, L., & Shi, L. (2017). Advances in single-cell RNA sequencing and its applications in cancer research. Oncotarget, 8(32), 53763–53779. https://doi.org/10.18632/oncotarget.17893

Zimmermann, K. S., Richardson, R., & Baker, K. D. (2019). Maturational changes in prefrontal and amygdala circuits in adolescence: implications for understanding fear inhibition during a vulnerable period of development. Brain sciences, 9(3), 65.

Zuo, Y., Lin, A., Chang, P., & Gan, W. B. (2005). Development of long-term dendritic spine stability in diverse regions of cerebral cortex. Neuron, 46(2), 181–189. https://doi.org/10.1016/j.neuron.2005.04.001