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Education

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Professional Publications

Peer-Reviewed Journal Articles

1. Roesch M, Singh T, Brown P, **Mullins SE**, Schoenbaum G. Decision-related neural activity in the ventral striatum integrates cue value and response, *Journal of Neuroscience*, 29.42 (2009): 13365-76.
2. Markham JA, **Mullins SE**, Koenig JI. Peri-adolescent maturation of the prefrontal cortex is sex-specific and disrupted by prenatal stress, *Journal of Comparative Neurology*, 521.8 (2013): 1828-43.
3. **Raver SM**, Haughwout SP, Keller A. Chronic adolescent exposure to cannabinoids permanently suppresses cortical oscillations in adult mice, *Neuropharmacology*, 38.12 (2013): 2338:47.

Abstracts

1. **Mullins S**, Davis C, Franklin L, Schrott L. Prenatal oxycodone exposure does not affect spatial learning in adult rats. LeARN Internship Research Conference, 2006
2. Davis CP, **Mullins S**, Franklin L, Schrott L. Prenatal oxycodone induced deficit in spatial memory retention: Role of PKM ζ and GluR2. Society for Neuroscience Meeting 2007, poster 816.8
3. Gabel LA, **Mullins SE**. Effects of environmental enrichment on learning and memory in Fmr-KO mice. Society for Neuroscience Meeting 2007, poster 696.6
4. Singh T, Brown PL, **Mullins SE**, Schoenbaum G, Roesch MR. Decision-related activity in ventral striatum reflects value and direction. Society for Neuroscience Meeting 2008, poster 98.11
5. **Mullins S**, Li Y, Keller A. Adolescent cannabinoid exposure to cannabinoid agonist results in abnormal oscillations in adult mice. Society for Neuroscience Meeting 2010, poster 553.1

6. **Mullins S**, Li Y, Keller A. Adolescent cannabinoid exposure to cannabinoid agonist results in abnormal oscillations in adult mice. Baltimore Chapter of the Society for Neuroscience Meeting 2011, third place in chapter-wide poster competition
7. **Mullins SE**, Haughwout S, Keller A. Chronic adolescent, but not adult cannabinoid exposure permanently attenuates neocortical oscillations in adult mice. Society for Neuroscience Meeting 2012, poster 639.13
8. **Raver SM**. Adolescent cannabinoid exposure impairs cortical oscillations and behavior in adult mice, University of Maryland Program in Neuroscience Retreat, June 2013
9. **Raver SM**, Haughwout SP, Keller A. Chronic adolescent cannabinoid exposure permanently alters cortical oscillations and cognitive behavioral performance in adult mice, Society for Neuroscience Meeting 2013, poster 129.04

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- April 2013: Schoenbaum Lab, National Institute on Drug Abuse Intramural Research Program, Baltimore, MD: “Adolescent cannabinoid exposure permanently alters cortical oscillations in adult mice.”
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- May 2013: Brown Lab, Johns Hopkins School of Medicine, Program in Neuroscience, Baltimore, MD: “Adolescent cannabinoid exposure permanently alters cortical oscillations in adult mice.”
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- 7/1/2011 – 6/30/2014: Sylvina M. Raver (PI, 100%)
“Adolescent Marijuana Use and Maturation of Cortical Functions”
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Abstract

Title of Dissertation: Teenage Brains on Pot: Adolescent Cannabinoid Exposure to Mice and Maturation of Cortical Functions

Sylvina Mullins Raver, Doctor of Philosophy, 2014

Dissertation Directed by: Asaf Keller, Professor, Program in Neuroscience

Regular use of marijuana during adolescence — but not adulthood — permanently impairs cognitive functions, and significantly elevates the risk for developing severe psychiatric diseases, such as schizophrenia, in some users. This vulnerable adolescent period coincides with the emergence of synchronous, network activity in the neocortex, termed cortical oscillations, as well as the anatomical and physiological maturation of the neural networks, neurotransmitter systems, and the endocannabinoid (eCB) system that shape oscillations. Cortical oscillations are implicated in cognitive and sensory processing, and are abnormal in patients with schizophrenia, in which these functions are impaired. We therefore proposed a link between adolescent use of marijuana, and abnormal cortical network activity in adulthood. Specifically, we hypothesized that repeated cannabinoid administration to adolescent mice would permanently alter cortical oscillations and related cognitive behaviors in adult animals. We tested this hypothesis by administering cannabinoid receptor ligands to adolescent mice, and recording oscillations both *in vitro* from isolated cortical preparations, and *in vivo* from intact, behaving mice once they reached adulthood. We find that chronic cannabinoid exposure to adolescent, but not adult animals, persistently suppresses pharmacologically-evoked cortical oscillations *in vitro*, preferentially in rostral neocortical areas that are less developed at the time of drug exposure. In awake,

behaving adult mice, chronic exposure to cannabinoids in adolescence attenuates pharmacologically-evoked cortical oscillations, impairs working memory, and alters oscillations associated with cognitive behaviors. We reveal that attenuation of cortical oscillations in adulthood by a shorter-period of cannabinoid exposure during early adolescence, or by chronic exposure to the primary active ingredient in marijuana, Δ^9 THC, is mediated by the cannabinoid-1 receptor (CB1R), and can be reversed with a CB1R antagonist. However, chronic exposure to a more potent cannabinoid receptor agonist cannot be reversed by chronic exposure to cannabinoid receptor antagonists, as antagonists also persistently attenuate oscillations in adulthood when administered alone. These data support the hypothesis that marijuana use in adolescence persistently alters synchronous activity in cortical networks in adulthood, and serve as a novel link between early cannabis use and alterations in cortical network activity implicated in cognitive processing and psychiatric disease.

Teenage Brains on Pot: Adolescent Cannabinoid Exposure to Mice and Maturation of
Cortical Functions

By: Sylvina Mullins Raver

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
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Doctor of Philosophy
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Dedication

My Dissertation is dedicated to my maternal grandparents, Thomas and Sylvina Church. Their generosity allowed me to attend an academic summer program in high school, during which I was first exposed to neuroscience in a course called “The Brain, Mind, and Human Behavior.” That short summer class ignited a spark that would grow into my steady, and ongoing interest in the biological, chemical, and physical workings of the brain, and would send me to Lafayette College for a B.S. in neuroscience, and eventually to the University of Maryland, Baltimore for a Ph.D. Syl died before she could see me fulfill any of these accomplishments. Tom developed Parkinson’s Disease, and later in his life, while I was a student at UMB, we often discussed what I was learning in school, in the context of his disease. He expressed the faith that he placed in the research that I, and other scientists like me, were conducting to work towards a cure for the disease that stole so much from him, and for other neurological and psychiatric diseases that afflicted so many.

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General Introduction

I. Consequences of Heavy Marijuana Use: Adolescence as a Vulnerable Period

Marijuana is the most commonly used illicit drug in the United States (Substance Abuse and Mental Health Services Administration (SAMHSA), 2013). In 2012, 18.9 million Americans ages 12 and older reported using marijuana at least once in the past month. Forty percent of these 18.9 million people are characterized as “heavy” users, as they report having used marijuana 20 or more days within the past month, while 17% of marijuana users report using the drug at least 300 of the past 365 days. These figures translate into 5.4 million people using marijuana daily, or almost daily, within the past year in the United States (SAMHSA, 2013), a figure that has steadily increased over the past 5 years, and may grow in the foreseeable future as marijuana legalization efforts, and social acceptability of cannabis use, continue to gain traction in the US. Long-term, heavy use of marijuana has been associated with impairments in cognitive abilities, including memory, attention, inhibitory control, and executive functioning, with the greatest deficits seen in the heaviest users (Bolla *et al.*, 2002; Solowij and Pesa, 2010). These conclusions remain controversial though, as other studies find no consistent long-term cognitive impairment after heavy marijuana use when use is initiated in adulthood (Pope *et al.*, 2001; Meier *et al.*, 2012).

A. Adolescent Marijuana Use in Humans: Persistent Cognitive Effects

However, there is near unanimous consensus that early use of cannabis—before age 18—has deleterious consequences for some users (Pope Jr and Yurgelun-Todd, 1996). Among those Americans who report using marijuana, use of the drug is concentrated within the young adult and adolescent populations (< 25 years old), with approximately

7% of adolescents ages 12-17, and 19% of young-adults between the ages of 18 and 25 reporting marijuana use in the past month (SAMHSA, 2013). Regularly smoking marijuana before adulthood can impair cognitive functioning in some users (Pope *et al.*, 2003), and these impairments persist for longer than in those who start using marijuana later in life (Jacobus *et al.*, 2009). These cognitive deficits typically manifest as impairments in the broad category of “executive functioning”, and include difficulties in problem solving, focusing attention, inhibiting impulsive responses, demonstrating behavioral flexibility, and working memory (Fontes *et al.*, 2011; Gruber *et al.*, 2012). Alarming, cognitive decline induced by early-onset marijuana use is not reversed by prolonged periods of abstinence. In a recently published longitudinal study (Meier *et al.*, 2012), subjects who persistently used marijuana before the age of 18 exhibited significant cognitive decline between ages 13 and 38 years, equivalent to a drop of 6 IQ points. Moreover, this decline was not reversed with cessation of marijuana use, even after the authors controlled for potentially confounding variables in this human population, such as socioeconomic status, and differences in personality types (Moffitt *et al.*, 2013). These deleterious effects are not apparent in subjects who initiated cannabis use after the age of 18, regardless of current abstinence of marijuana use (Meier *et al.*, 2012). These reports highlight accumulating evidence that implicate adolescence as a developmental stage during which marijuana use can lead to persistent, and severe, cognitive consequences.

B. Adolescent Marijuana Use in Humans: Persistent Psychiatric Effects

In addition to the long-term cognitive impairments associated with marijuana use in adolescence, cannabis consumption before adulthood can substantially increase the risk for developing severe psychiatric illnesses, such as schizophrenia (Arseneault *et al.*,

2004), bipolar disorder (van Laar *et al.*, 2007), depression (Fergusson *et al.*, 2002), or anxiety disorders in vulnerable individuals (Arseneault *et al.*, 2002; Moore *et al.*, 2007). Longitudinal studies have found significantly higher incidences of anxiety, depression, and suicidal ideation in subjects that regularly used marijuana before the age of 16 (Patton *et al.*, 2002; Hayatbakhsh *et al.*, 2007). The relationship between cannabis and mental illness is dose-dependent, with the heaviest users showing the greatest subsequent incidence of illness, and does not appear to be confounded by a self-medication effect, as depression or anxiety during adolescence does not predict subsequent cannabis use (Patton *et al.*, 2002).

A meta-analysis of many of the reported links between marijuana use in adolescence and subsequent psychiatric disease confirms an association between affective disorders and cannabis use (Moore *et al.*, 2007). However, these authors conclude that the greatest risk posed by frequent, early marijuana use is in the subsequent development of psychosis. Any past use of marijuana is associated with a 40% increase in the prevalence of psychotic symptoms, with heavy users demonstrating anywhere between a 50 to 200% increase in risk of psychosis. Potential links between cannabis use and psychosis have been reported for decades. In healthy subjects, acute marijuana intoxication can replicate many of the symptoms of schizophrenia, such as thought disorder, working memory impairments, and distorted visual perception (Ames, 1958), and in subjects with schizophrenia, the drug can exacerbate many of these same symptoms (Hall *et al.*, 2004). Again, as with the relationship between marijuana and cognitive impairments, a greater risk for the development of schizophrenia, or closely related disorders, is seen in those who used the drug during adolescence. Repeated,

independent analyses of a population of Swedish conscripts have reached the same conclusions: frequent marijuana use before the age of 18 significantly increases the prevalence of schizophrenia, as assessed in a 15 year follow-up, after controlling for a prior diagnoses of psychiatric disease and other potentially confounding environmental factors (Andreasson *et al.*, 1987; Zammit *et al.*, 2002). Of the 1,648 subjects in these studies with a history of any cannabis use by the age of 18, 1.1% of them subsequently developed schizophrenia, compared to 0.6% of study participants who had never used the drug at the time of conscription (Zammit *et al.*, 2002). The prevalence of schizophrenia was significantly associated with the frequency of marijuana use, as 5.7% of individuals who reported using marijuana more than 50 times before age 18 subsequently developed schizophrenia (Zammit *et al.*, 2002). Interestingly, an increased risk for schizophrenia was found to be specific to early marijuana use, as no association between use of other drugs and the disease was determined (Andreasson *et al.*, 1987; Zammit *et al.*, 2002). Multiple analyses of different populations—including in the Netherlands: (van Os *et al.*, 2002); New Zealand: (Arseneault *et al.*, 2002; Fergusson *et al.*, 2002); Greece (Stefanis *et al.*, 2004); and Germany: (Henquet *et al.*, 2005))—have reached the same conclusions, and emerged at the overall consensus that marijuana use before the age of 18 or 21 poses a 2 to 7-fold increased risk of subsequently developing schizophrenia or schizophreniform disorder later in life. Importantly, however, adolescent cannabis use *alone* is neither a necessary or sufficient cause of psychosis (Arseneault *et al.*, 2004; Moore *et al.*, 2007), but rather contributes to psychotic disease in conjunction with other factors, such as an underlying genetic susceptibility (Caspi *et al.*, 2005).

II. Animal Models of Adolescent Cannabinoid Exposure: Cognitive Effects and Behaviors Relevant to Psychiatric Disease

Marijuana plants (*Cannabis sativa* and *Cannabis indica*) contain an array of active compounds, known as cannabinoids (Joy *et al.*, 1999) that exert a multitude of physiological and psychotropic effects when the drug is consumed. Identification of the primary psychoactive cannabinoid in the plant, Δ -⁹ tetrahydrocannabinol (THC) (Gaoni and Mechoulam, 1964), has facilitated the study of the effects of marijuana using animal models. However, because marijuana contains more than 80 cannabinoids that interact to produce a complex phenotype, administration of THC, or synthetic cannabinoids alone, can mimic some of these biological effects, but does not recreate marijuana intoxication. Interestingly, administration of synthetic forms of THC (e.g. dronabinol) to human subjects fails to recapitulate many of the subjectively pleasant effects of smoking marijuana. For example, patients who orally consume dronabinol often report unpleasant feelings of dizziness, anxiety, and distorted perception, which can be attributed to the potent psychoactive activity of THC (Physician's Desk Reference, 2014). These adverse effects of THC alone, compared to pleasant feelings of "highness" induced by marijuana itself, may be due to potent antagonism of THC's actions by other cannabinoids released when the plant is burned or ingested (Bhattacharyya *et al.*, 2010). Nonetheless, experiments conducted in animals that are intended to mimic human adolescent marijuana exposure have reproduced many of the cognitive features seen in adult humans who regularly consume marijuana during adolescence, suggesting that the primary active ingredients in marijuana, or their chemical analogues, are responsible for the drug's lasting effects on cognition.

In these models, administration of THC or synthetic cannabinoids alone, to adolescent animals can reproduce many of the cognitive deficits and emotional abnormalities seen in human marijuana users (O'Shea *et al.*, 2006; Schneider, 2008; Rubino and Parolaro, 2008). One of the most common findings in adult rodents exposed to THC or synthetic cannabinoids during the pubertal period is impaired working memory performance, as assessed by tests of object recognition (Schneider and Koch, 2003; O'Shea *et al.*, 2004; Schneider and Koch, 2007; Quinn *et al.*, 2008). Typically, animals that are repeatedly exposed to cannabinoids (THC or synthetic analogues) during the adolescent period display impaired object recognition ability when assessed 15-30 days following the final day of exposure, once animals reach adulthood (Schneider and Koch, 2003; O'Shea *et al.*, 2004; Quinn *et al.*, 2008; Realini *et al.*, 2011). Importantly, consistent with the human literature, these reported cognitive impairments are not evident if animals are administered cannabinoids once they are fully mature (Schneider and Koch, 2003; O'Shea *et al.*, 2004; Quinn *et al.*, 2008; Schneider *et al.*, 2008). Object recognition impairments demonstrated in rodents following repeated cannabinoid exposure in adolescence mimic many of those seen in patients with schizophrenia (Heckers *et al.*, 2000; Doniger *et al.*, 2002), and may reflect working memory aspects of the cognitive impairments that have emerged as a core feature of the disease (Goldman-Rakic, 1994). Non-cognitive symptoms of schizophrenia, such as sensorimotor gating impairments, and anhedonia-like behavior, are also reproduced by adolescent (Schneider and Koch, 2003; Rubino *et al.*, 2008; Wegener and Koch, 2009), but not, adult cannabinoid exposure in rodents (Bortolato *et al.*, 2005), and can be reversed by antipsychotic treatment (Schneider and Koch, 2007). These findings further support the presence of a link

between cannabis use in adolescence, and the development of schizophrenia, or related disorders, in adulthood.

III. *The Endocannabinoid (eCB) System*

The active ingredients in marijuana, as well as other structurally similar cannabinoids, exert their psychotropic effects by acting on the endogenous cannabinoid, or endocannabinoid (eCB) system [for review: (Alger, 2002; Freund *et al.*, 2003; Mackie, 2008; Kano *et al.*, 2009; Katona and Freund, 2012)]. This system consists of the eCBs themselves, their synthetic and degradative enzymes, and the receptors to which they bind. The two primary eCBs in the brain are anandamide and 2-arachidonylglycerol (2AG), both of which are synthesized from membrane phospholipid precursors in response to a rise in the intracellular Ca^{2+} concentration alone, or in combination with neurotransmitter activation of other G-protein coupled receptors (GPCRs) (Varma *et al.*, 2001; Kim *et al.*, 2002). eCBs are remarkable in that they function as retrograde messengers, meaning that they are synthesized and released from postsynaptic neurons and travel backwards across the synapse to act at cannabinoid-1 (CB1R) receptors on presynaptic terminals, or at cannabinoid-2 (CB2R) receptors, which may be localized to neuronal and glial processes (Ohno-Shosaku *et al.*, 2001; Wilson and Nicoll, 2001). The CB1R the most abundantly expressed GPCR in the brain, with widespread distribution throughout the brain and brainstem (Devane *et al.*, 1988; Herkenham *et al.*, 1990). Expression of the CB2R is higher in the periphery (Murno *et al.*, 1993), although it can also be minimally expressed in the central nervous system (Gong *et al.*, 2006; Onaivi *et al.*, 2006). To date, the localization, development, and role of the CB2R in synaptic transmission is less clear than that of CB1Rs. CB1Rs and CB2Rs are GPCRs that

typically couple to $G\alpha_{i/o}$ subunits, thereby inhibiting the activity of adenylyl cyclase, activating intracellular signaling cascades, inhibiting certain voltage-sensitive Ca^{2+} channels, and activating G-protein modulated K^+ channels (Howlett *et al.*, 2002). CB1Rs are abundantly expressed in the cerebral cortex and hippocampus on inhibitory interneurons that co-express the neuropeptide cholecystokinin (CCK) (Marsicano and Lutz, 1999; Bodor *et al.*, 2005), as well as on the terminals of excitatory pyramidal terminals (Domenici *et al.*, 2006). CB1R activation typically induces the inhibition of neurotransmitter release, and subsequent attenuation of inhibitory or excitatory neurotransmission. CB1Rs and eCBs mediate multiple forms of synaptic plasticity, ranging from short-term depolarization-induced suppression of inhibition (DSI) (Pitler and Alger, 1992; Wilson and Nicoll, 2001; Ohno-Shosaku *et al.*, 2001) or excitation (DSE) (Kreitzer and Regehr, 2001; Fortin and Levine, 2007), to synchronization of presynaptic and postsynaptic activity and subsequent strengthening of coordinated synaptic responses (Hashimotodani *et al.*, 2007), to long-term depression (Gerdeman *et al.*, 2002). The eCB system, therefore, acts as a potent and pervasive neuromodulatory system throughout the brain, capable of modulating a variety of brain functions.

IV. Development of the eCB, Glutamatergic, and GABAergic Systems in Adolescence

As the eCB system serves as the target for cannabinoids, it is interesting to note that components of this system in the cerebral cortex undergo substantial maturation during adolescence. This maturation coincides with the critical period during which marijuana use can induce profound, and persistent cognitive and psychiatric consequences (Bossong and Niesink, 2010). All components of the eCB system mature during adolescence. For example, the synthesis, tissue levels, and degradation of the

primary eCBs, 2AG and anandamide, change throughout the adolescent period, which are manifested as an overall reduction in eCB activity in adult humans and rodents compared to that seen in children or juvenile animals (Ellgren *et al.*, 2008; Long *et al.*, 2012; Lee *et al.*, 2013). In parallel, the density and distribution of the CB1R decreases during adolescence (Ellgren *et al.*, 2008; Eggan *et al.*, 2010; Heng *et al.*, 2011), with the most dramatic reduction apparent in frontal, higher-order cognitive areas than in more caudal, primary sensory cortical regions (Heng *et al.*, 2011).

The GABAergic and glutamatergic neurotransmitter systems, which are potently modulated by the eCB system, also demonstrate considerable adolescent maturation. For example, changes in GABA-A receptor subunit distribution (Yu *et al.*, 2006), the subcortical innervation of GABAergic cell targets in the prefrontal cortex (PFC) (Cunningham *et al.*, 2002; Cunningham *et al.*, 2008), and changes in pre and post-synaptic markers of GABAergic synapses on cortical pyramidal neurons (Cruz *et al.*, 2003) have all been reported throughout adolescence. The cortical glutamatergic system also matures at this time, and exhibits changes in kainate (Bahn *et al.*, 1994) and NMDA receptor expression (Cao *et al.*, 2000), as well as excitatory synaptogenesis and synapse elimination (Bourgeois *et al.*, 1994).

V: Anatomical and Physiological Maturation of the Adolescent Neocortex

Changes in the adolescent cortical eCB, excitatory, and inhibitory neurotransmitter systems are representative of the ongoing and dramatic development within the entire cerebral cortex throughout this period. The dynamic nature of the actively maturing adolescent neocortex makes it vulnerable to exogenous factors, such as marijuana use (Spear, 2000; Andersen, 2003; Dahl, 2004; Tau and Peterson, 2010). Adolescence,

broadly defined as the period between non-reproductive (childhood) and reproductive ages (adulthood)(Spear, 2000), has been described as a critical period for the emergence of many higher-order cognitive functions that are governed by frontal cortical regions (Luna and Sweeney, 2004; Nelson, 2004) and are late to mature (Gogtay *et al.*, 2004). This period of cognitive development is distinct from childhood in that it is characterized by improvements in existing abilities, rather than in the acquisition of new skills (Luna and Sweeney, 2004). Throughout childhood, an overabundance of synapses is formed as newly born neurons make connections with each other. During adolescence, the developmental strategy shifts from one that focuses simply on building synapses to one that favors refining existing synapses to increase the efficiency of neuronal computations. To achieve this goal, many synapses that are created earlier in development are pruned and eliminated, while others are strengthened and stabilized (Luo and O'Leary, 2005; Whitford *et al.*, 2007; Luna, 2009). This process critically depends on the synchronization of pre and postsynaptic activity (Segal, 2005), whereby synchronization stabilizes synaptic connections, and desynchronization can lead to synapse elimination (Nagerl *et al.*, 2004). The eCB system thus plays a pivotal role in the organization of developing cortical networks (Berghuis *et al.*, 2007; Harkany *et al.*, 2007), including establishing and refining synaptic contacts (Mulder *et al.*, 2008). Indeed, activation of CB1Rs, such as by cannabinoids in marijuana, can prevent activity-dependent pruning of synapses (Kim *et al.*, 2008), and cannabis exposure at early prenatal or postnatal stages can permanently alter the development of maturing neural substrates (Berghuis *et al.*, 2007; Harkany *et al.*, 2007).

Normal synapse elimination during adolescence results in a reduction in cortical

gray matter along a gradient that progresses from caudal-to-rostral regions (Huttenlocher and Dabholkar, 1997; Gogtay *et al.*, 2004; Tau and Peterson, 2010). The cortical areas that subserve higher-order cognitive processes, such as working memory, response inhibition, decision making, and the organization and planning of goal-directed behaviors, are the last to mature (Yurgelun-Todd, 2007), and do not reach a stable maturational state until adulthood (Tau and Peterson, 2010). In parallel, these executive functions do not fully emerge until the period spanning from late adolescence to early adulthood, whereas basic perceptual and sensorimotor functions that rely on more caudally located cortical areas are apparent in childhood (Luciana and Nelson, 1998; Luna *et al.*, 2004; Luna, 2009). Coincident with selective synapse elimination, myelination of existing synaptic pathways increases, which becomes manifest as an increase in white matter that advances along the same developmental gradient (Huttenlocher and Dabholkar, 1997; Giedd *et al.*, 1999; Bartzokis *et al.*, 2001). Together with synaptic pruning, myelination of existing axons enhances the efficiency and speed of synaptic transmission and neuronal computation. Studies of human subjects with a history of marijuana use during adolescence consistently show abnormalities in brain structure that may alter neural network communication. For example, adults in their early 30's who started using marijuana before age 17 have reduced whole brain volumes, and thinner gray matter than control subjects who did not use marijuana (Wilson *et al.*, 2000). Changes in both gray and white matter have been demonstrated in the brains of adolescent marijuana users (Matochik *et al.*, 2005; Bava *et al.*, 2009). Furthermore, impairment in the structural integrity of white matter tracts, including the corpus callosum, have been documented in former marijuana users, with a positive association between the degree of integrity

impairment, the duration of marijuana use, and an earlier age of onset (Arnone *et al.*, 2008). These reports suggest that marijuana use in adolescence may interfere with the normal maturation of cortical structures, and subsequently interfere with the speed of neuronal communication, and the coordinated activity of distributed neuronal networks.

VI: Cortical Oscillations

Coincident with the anatomical and physiological maturation of the adolescent neocortex is the emergence of synchronous patterns of network activity that result from these maturational processes, termed cortical oscillations (Uhlhaas *et al.*, 2009). Although the membrane voltages of individual neurons can oscillate, rhythmic fluctuations in extracellularly recorded electrical activity, such as in local field potentials (LFPs), electrocorticograms (ECoGs), or electroencephalograms (EEGs), reflect the summed activity of multiple cells (Buzsaki *et al.*, 2012). Therefore, these oscillations represent the temporally coordinated activity of entire neural networks, and are a candidate mechanism by which distinct cellular ensembles communicate with each other over short, or long distances (Buzsaki and Draguhn, 2004; Buzsaki, 2006). Synchronous, rhythmic cortical network activity is generated by an interplay between the coordinated and collective action of inhibitory interneuronal networks, and elevated excitability of excitatory pyramidal neurons, at specific intervals (Cardin *et al.*, 2009; Wang, 2010; Buzsaki *et al.*, 2012). Thus, this oscillatory activity is shaped by both glutamatergic and GABAergic activity, and neuromodulatory factors, such as the eCB system, that regulate inhibitory and excitatory neurotransmission. Indeed, acute exposure to cannabinoid receptor agonists attenuates the strength of neural oscillations (Hajos *et al.*, 2000; Robbe *et al.*, 2006; Hajos *et al.*, 2008; Kucewicz *et al.*, 2011; Sales-Carbonell *et al.*, 2013).

Oscillations can be decomposed into the frequencies that contribute to the continuous electrical signal, and are subsequently classified into distinct frequency bandwidths, while the strength of the signal carried at a certain frequency can be indicated by the metric “power.” Oscillation frequency bandwidths and their associated functions include, but are not limited to: gamma (~30-80 or 100 Hz) oscillations, which may underlie attention, sensory integration, and neural computations engaged by cognitive processes; beta (~13-30 Hz) activity, described to play a role in motor planning; alpha (~8-12 Hz) oscillations, that predominate during environmental exploration; theta (~4-7 Hz) activity, present in the hippocampus during spatial navigation and working memory tasks; and delta (~1-3 Hz) oscillations, that may play a role in memory consolidation, and predominate during slow wave sleep [reviewed in (Buzsaki, 2006; Wang, 2010; Traub and Whittington, 2010)]. Neural oscillations are thought to be integral to sensory processing (Gray *et al.*, 1989), working memory (Roux *et al.*, 2012), attention (Tiitinen *et al.*, 1993), and nearly all cognitive functions (Fries, 2005; Wang, 2010), and are abnormal in diseases in which these functions are impaired, such as schizophrenia (Gonzalez-Burgos and Lewis, 2008; Uhlhaas and Singer, 2010).

VII: *Cortical Oscillation Abnormalities in Schizophrenia*

Abnormal oscillations in the gamma bandwidth have been particularly well-documented in patients with schizophrenia, and include both enhanced gamma activity during baseline, resting-state conditions (Spencer, 2011), as well impaired gamma activity evoked during sensory and cognitive tasks (Krishnan *et al.*, 2005; Krishnan *et al.*, 2009; Cho *et al.*, 2006; Uhlhaas and Singer, 2010). These gamma abnormalities are thought to contribute to the profound cognitive impairment (Spencer *et al.*, 2004), and

sensory hallucinations (Spencer *et al.*, 2009) that are hallmarks of schizophrenia. Furthermore, abnormal cortical oscillations are a conserved physiological endophenotype, as they exist in first-degree relatives of patients with the disease (Hong *et al.*, 2008; Leicht *et al.*, 2010), and in animal models of schizophrenia (Lodge *et al.*, 2009; Featherstone *et al.*, 2012; Phillips *et al.*, 2012). Cortical oscillations underlie efficient neural network processing (Buzsaki and Draguhn, 2004), and are dependent upon the anatomical and physiological processes that mature throughout adolescence (Uhlhaas *et al.*, 2009; Uhlhaas *et al.*, 2010). Perturbation of these processes by exposure to exogenous factors, such as marijuana, during critical developmental windows may have profound consequences on the coordinated activity within neural networks, and therefore may alter cortical oscillations. Because of the cognitive impairments and increased risk of schizophrenia reported following early marijuana use, the integral role that cortical oscillations play in cognitive and sensory processing, and the abnormalities in these functions seen in individuals with schizophrenia, we therefore propose that a link exists between these phenomena.

VIII: Hypothesis and Predictions

Specifically, we hypothesize that marijuana use during adolescence persistently alters cortical oscillations and related cognitive behavior in adulthood. From this hypothesis arise several predictions that we test in the four experimental chapters of this Dissertation.

- **Chaper 1: Specific Aim I:** Cannabinoid exposure during adolescence suppresses pharmacologically-evoked cortical oscillations in adult mice.
 - **Prediction 1:** Chronic adolescent exposure to a CB1R agonist persistently

suppresses pharmacologically-evoked cortical oscillations *in vitro*.

- **Prediction 2:** Effects of chronic adolescent CB1R agonist exposure are more pronounced in rostral cortical areas that are less developmentally mature at the time of drug exposure than in caudal, more mature cortical regions.
 - **Prediction 3:** Chronic exposure to a CB1R agonist in adulthood does not produce persistent effects on oscillations.
 - **Prediction 4:** Chronic adolescent exposure to the primary psychoactive ingredient in marijuana, THC, persistently suppresses pharmacologically-evoked oscillations *in vitro*.
 - **Prediction 5:** Chronic adolescent exposure a CB1R agonist persistently suppresses pharmacologically-evoked cortical oscillations *in vivo* in intact, behaving adult mice.
- **Chapter 2: Specific Aim II:** Cannabinoid exposure during adolescence suppresses pharmacologically-evoked cortical oscillations via CB1Rs.
 - **Prediction 1:** Repeated CB1R antagonist administration in adolescence prevents suppression of pharmacologically-evoked oscillations by chronic adolescent cannabinoid exposure.
 - **Prediction 2:** Repeated CB2R antagonist administration in adolescence does not alter suppression of pharmacologically-evoked oscillations by chronic adolescent cannabinoid exposure.
 - **Prediction 3:** Repeated adolescent administration of a CB1R inactive compound does not persistently suppress pharmacologically-evoked oscillations.

- **Prediction 4:** Repeated adolescent cannabinoid administration does not alter pharmacologically-evoked oscillations in CB1R knockout mice.
- **Chapter 3: Specific Aim III:** Cannabinoid exposure during adolescence persistently alters cognitive behavior in adult mice.
 - **Prediction 1:** Adolescent exposure to a CB1R agonist impairs working memory performance in the Novel Object Recognition Task.
 - **Prediction 2:** Adolescent exposure to a CB1R agonist impairs working memory performance in the Object Recency Task.
 - **Prediction 3:** Adolescent exposure to a CB1R agonist persistently alters sleep behavior in adult mice.
- **Chapter 4: Specific Aim IV:** Cannabinoid exposure during adolescence alters behaviorally-evoked oscillations *in vivo* in adult mice.
 - **Prediction 1:** The Novel Object Recognition (NOR) task modulates behaviorally-evoked frontal ECoG oscillations.
 - **Prediction 2:** Adolescent exposure to a CB1R agonist impairs modulation of behaviorally-evoked ECoG oscillations in the NOR task.
 - **Prediction 3:** The NOR task modulates behaviorally-evoked LFP oscillations in mPFC.
 - **Prediction 4:** Adolescent exposure to CB1R agonists suppresses modulation of behaviorally-evoked LFP oscillations in mPFC during the NOR task.
 - **Prediction 5:** Adolescent exposure to a CB1R agonist suppresses modulation of behaviorally-evoked ECoG oscillations in the Object Recency task.
 - **Prediction 6:** Auditory stimuli evoke robust sensory stimulus-generated

ECoG oscillations in the prefrontal cortex.

- **Prediction 7:** Adolescent exposure to a CB1R agonist suppresses spontaneously-generated resting-state ECoG oscillations.

Chapter 1: Cannabinoid Exposure During Adolescence Suppresses Pharmacologically-Evoked Cortical Oscillations in Adult Mice

I. Introduction

Marijuana is the most commonly used illicit drug among adolescents and young adults in the United States (SAMHSA, 2013). Persistent marijuana use before adulthood may permanently impair cognitive functioning (Solowij *et al.*, 2002; Meier *et al.*, 2012) and confer a higher risk of developing psychiatric diseases, such as schizophrenia, in susceptible individuals (Arseneault *et al.*, 2004). Chronic adolescent, but not adult cannabinoid exposure produces lasting working memory impairments and recapitulates other schizophrenia endophenotypes in rodents, including impaired sensorimotor gating, social avoidance, and anhedonia/avolition (Schneider and Koch, 2003; O'Shea *et al.*, 2004; Quinn *et al.*, 2008).

Adolescents are especially sensitive to repeated marijuana use, presumably because marijuana interferes with ongoing anatomical and physiological maturation of the brain, particularly the cerebral cortex (Andersen, 2003). The cortex matures along a caudal-to-rostral gradient, with primary sensory cortical areas maturing earlier, and prefrontal regions developing into early adulthood (Gogtay *et al.*, 2004). The cortical endogenous cannabinoid (eCB) system, upon which marijuana acts, develops along the same gradient, such that cannabinoid-1 receptor (CB1R) density and distribution (Heng *et al.*, 2011) and eCB metabolism (Long *et al.*, 2012) mature first in more caudal regions.

Coincident with eCB development is the maturation of cortical oscillations (Uhlhaas *et al.*, 2009). Oscillations reflect the synchronous activity of neural networks (Buzsaki and Draguhn, 2004) and are integral to sensory processing, working memory,

and nearly all cognitive functions, as they may provide a mechanism by which the brain “binds” the activity of distributed neural ensembles into a coherent representation of cognitive or sensory content (Singer and Gray, 1995; Fries, 2005; Buzsaki, 2006). Cortical oscillations are abnormal in diseases in which these functions are impaired, such as schizophrenia (Gonzalez-Burgos and Lewis, 2008; Uhlhaas and Singer, 2010). Furthermore, oscillations underlie efficient cortical network processing (Buzsaki and Draguhn, 2004) and are dependent on the anatomical and physiological processes that mature throughout adolescence (Uhlhaas *et al.*, 2009; Uhlhaas *et al.*, 2010).

Robust oscillatory activity can be evoked *in vitro* in reduced preparations and *in vivo* from intact animals using several pharmacological methods (Plourde *et al.*, 1997; Buhl *et al.*, 1998; Fisahn *et al.*, 1998; Hakami *et al.*, 2009; Oke *et al.*, 2010). We used the glutamatergic kainate receptor agonist kainic acid (KA; 400 nM) and the cholinergic muscarinic agonist carbachol (CCh; 20 μ M) (Buhl *et al.*, 1998) to reliably produce beta (13-29 Hz) and gamma (30-80 Hz) oscillations in cortical slices that resemble network synchrony in the intact neocortex (Steriade *et al.*, 1996). KA and CCh promote robust local field potential (LFP) oscillations *in vitro* by enhancing excitatory drive (Buhl *et al.*, 1998; Cunningham *et al.*, 2003) and activating cholinergic receptors, primarily on GABAergic interneurons (Fisahn *et al.*, 1998; Gulyas *et al.*, 2010), although in certain regions CCh can generate certain rhythms by acting solely on inhibitory interneurons when excitatory transmission is blocked (Nagode *et al.*, 2011; Nagode *et al.*, 2014). In intact awake mice, we administered the non-competitive NMDA receptor antagonist ketamine, which evokes robust oscillations in both rodents and humans (Plourde *et al.*, 1997; Hakami *et al.*, 2009). Importantly, these oscillations are independent of animals’

behavioral states (Hakami *et al.*, 2009), which reduces between-subject variability. The mechanism of oscillation generation by KA + CCh and ketamine have been relatively well-studied (Buhl *et al.*, 1998; Fisahn *et al.*, 1998; Homayoun and Moghaddam, 2007), and therefore changes in these oscillations may provide mechanistic insight into how cannabis exposure during adolescence affects the developing adolescent cortex.

Previous studies have used similar pharmacological methods to examine the effects of *acute* cannabinoid exposure on network oscillations in the hippocampal CA3 region (Hajos *et al.*, 2000; Hajos *et al.*, 2008; Holderith *et al.*, 2011). While CB1R activation suppresses both GABA and glutamate release (Wilson and Nicoll, 2001; Kreitzer and Regehr, 2001), acute cannabinoid administration attenuates the power of beta and gamma oscillations (Hajos *et al.*, 2000; Robbe *et al.*, 2006; Hajos *et al.*, 2008; Kucewicz *et al.*, 2011; Sales-Carbonell *et al.*, 2013) by acting at CB1Rs on excitatory terminals and suppressing glutamate release from pyramidal neurons (Holderith *et al.*, 2011). Although CB1R activation potently suppresses GABA release from inhibitory interneurons (Wilson and Nicoll, 2001), this action is not thought to contribute to acute CB1R-mediated suppression of pharmacologically-evoked beta and gamma oscillations (Holderith *et al.*, 2011), as GABA release from CB1 expressing terminals may be silenced by CCh (Fukudome *et al.*, 2004; Gulyas *et al.*, 2010). Cortical oscillations are suppressed in chronic marijuana users, particularly in those with an earlier age of onset of use (Skosnik *et al.*, 2012). The maturation of cortical oscillations in adolescence, and the ongoing development of the eCB system that modulates this network activity, suggest that oscillations may be vulnerable to adolescent marijuana use. In Chapter 1, we test the prediction that chronic adolescent cannabinoid exposure suppresses pharmacologically-

evoked oscillations both *in vitro* and *in vivo* in adult mice. We predict that these effects will be more robust in rostral cortical areas that are less developed in adolescence, and will be absent following chronic adult cannabinoid administration.

II. Materials and Methods

A. Animals

All experiments were performed according to University of Maryland School of Medicine Institutional Animal Use and Care Committee protocols. Data were collected and analyzed by an experimenter “blind” to animals’ treatment condition. Male CD-1 mice (Harlan Laboratories, Inc., Frederick, MD, USA) were obtained at postnatal day 30 (P30) (adolescent treatment) or P65 (adult treatment) and injected once daily intraperitoneally (i.p.) from P35-P55 (adolescence) or P70-P90 (adulthood). Mice were group housed with cage-mates exposed to both vehicle and WIN55-212,2 (WIN) or Δ^9 -tetrahydrocannabinol (THC). After the last injection, adolescent-treated mice were left undisturbed until adulthood (Figure 1A); adult-treated mice were left undisturbed for at least 10 days after the final injection to allow cannabinoids to wash out. For *in vivo* electrocorticogram (ECoG) experiments, mice were injected once per day i.p. for 3 days with saline, 10 or 20 mg/kg ketamine; the order of injections was alternated between animals.

B. Drugs

WIN (0.25 or 1 mg/kg; Sigma Aldrich, St. Louis, MO, USA) and THC (5 mg/kg; National Institute on Drug Abuse Drug Supply Program, Bethesda, MD, USA) were dissolved in ethanol and administered in a 1:1:18 solution of ethanol: Emulphor (Alkamuls EL-620, Rhodia Chemicals, USA): 0.9 % saline (1 mL/kg). Control animals

were injected vehicle (1:1:18 ethanol:Emulphor:saline). Ketamine (10 or 20 mg/kg; Bioniche Pharma, IL, USA) was dissolved 1:5 in 0.9% saline and injected i.p. (0.1 mL). Kainic acid (KA) was dissolved in normal artificial cerebrospinal fluid (ACSF; recipe described below) at a concentration of 1 mM. The dissolved KA solution was brought to a neutral pH (7.4) by adding 1M NaOH, 1 μ L at a time. Carbachol (CCh) was dissolved in MQ pure water at a concentration of 10 mM.

C. In vitro Slice Preparation and LFP Recordings

Adult mice (>P100) were anesthetized with ketamine (100 mg/kg) and decapitated. Two or three 400- μ m-thick coronal sections were cut from each cortical region: medial prefrontal cortex (mPFC: \sim 1.3 – 2.3 mm anterior to bregma) and primary somatosensory cortex (SCx: \sim 1.0 – 2.1 mm posterior to bregma) from either the right or left hemisphere using a microtome (Integraslice 7550MM, Campden Instruments, IN, USA). During brain extraction and cutting, tissue was immersed in ice-cold or 4°C thermoregulated artificial cerebrospinal fluid (ACSF), respectively, containing (in mM): 26 NaHCO₃, 5 BES, 15 glucose, 200 sucrose, 3 KCl, 1.5 MgSO₄, 1 CaCl₂. Next, slices were incubated for 30 minutes at 36°C followed by 30 minutes at 22°C in ACSF containing (in mM): 120 NaCl, 25 NaHCO₃, 5 BES, 15 glucose, 3 KCl, 1.3 MgSO₄, 2 CaCl₂ (normal ACSF). Slices were continually saturated with 95% O₂ 5% CO₂. Slices were maintained at 36°C in an interface-type recording chamber and were perfused at 0.7 mL/min with ACSF containing (in mM): 120 NaCl, 25 NaHCO₃, 5 BES, 15 glucose, 5 KCl, 1.3 MgSO₄, 2 CaCl₂ (High K⁺ ACSF). Kainic acid (KA; 400 nM) and carbachol (CCh; 20 μ M) were added to High K⁺ ACSF to evoke robust beta and gamma oscillations. Local field potential (LFP) recordings were made through a glass pipette filled with normal ACSF

(impedance ~ 0.5 -1 M Ω) with an ER-1 amplifier (Cygnus Technology, USA), sampled at 5 kHz, filtered between 0.1 Hz and 1 KHz, and stored on a Mac computer using Igor Pro (Version 6.1, Wavemetrics, Portland, OR, USA). LFPs were recorded from layers II/III of different regions of mPFC [prelimbic area (PL), infralimbic area (IL), and anterior cingulate (AC)] and in the barrel field of SCx from layers II/III, LIV, and LV/VI. Recordings sites were combined for analysis such that each mPFC slice yielded 3 LFPs (PL, IL, AC) and each SCx slice yielded 3 LFPs (LII/III, LIV, LV/VI).

D. In vitro Data Analysis

We analyzed *in vitro* LFP data with custom-written Igor Pro scripts and the Time-Frequency Toolkit (<http://www.igorexchange.com/project/TFPlot>). Discrete fast Fourier transforms (FFTs) were performed on 10 seconds of LFP data and oscillation power (area under the curve) was integrated at different frequencies [theta (4-7 Hz); alpha (8-12 Hz); beta (13-29 Hz); gamma (30-80 Hz)]. Designation of frequency bandwidths is based on previous reports (Buhl *et al.*, 1998; Uhlhaas and Singer, 2010). Statistical analyses were performed with STATA (Version 12, StataCorp, College Station, Texas, USA). Data were tested for normality and extreme outliers were identified using the fourth-spread outlier detection test and removed (Hoaglin *et al.*, 1986). Kruskal-Wallis (KW) or Mann-Whitney U (MWU) tests were used to determine a significant main effect of treatment (significant $p < 0.05$). Pair-wise comparisons were performed with MWU tests (significant $p < 0.05$).

E. In vivo Surgical Procedures and ECoG Recordings

Adult mice (>P100) were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg). An F20-EET radio-telemetric transmitter (Data Sciences International,

Minneapolis, MN, USA) was implanted subcutaneously and its leads implanted over the dura above the frontal cortex (1.7 mm anterior to bregma) and the cerebellum (6.4 mm posterior to bregma). Both bipotential leads contain a reference lead that is internalized within the silastic insulation surrounding the leads, therefore eliminating the requirement for a separate reference signal. Animals were individually housed and recovered from surgery for at least 48 hours before recording. Mice were acclimated to the behavior testing room for 1 hour before ECoG recordings. Frontal ECoGs were recorded with the Dataquest A.R.T. acquisition system (Data Sciences International). Baseline ECoG (10 minute) recordings were followed by an i.p. injection of saline, 10 or 20 mg/kg ketamine and 40 minutes of post-injection recordings.

F. In vivo Data Analysis

We analyzed *in vivo* frontal ECoGs with custom-written MATLAB scripts (Version 2012a, Mathworks, MA, USA) and the `mtspecgramc` routine in the Chronux Toolbox (<http://chronux.org>) (Mitra and Bokil, 2006). Oscillation power in each bandwidth [δ (1-3 Hz); theta (4-7 Hz); alpha (8-12 Hz); beta (13-29 Hz); gamma (30-80 Hz)] was computed in 10-second bins from spectrograms for each animal, and averaged into 10-minute bins. Post-injection power was compared to the baseline period with MWU tests (significant $p < 0.05$) using STATA.

III. Results

A. Adolescent WIN Exposure Persistently Suppresses Pharmacologically-Evoked Cortical Oscillations in vitro

We first tested the prediction that adolescent cannabinoid exposure persistently suppresses pharmacologically-evoked cortical oscillations *in vitro*. Coronal brain slices

containing either medial prefrontal cortex (mPFC) or primary somatosensory cortex (SCx) were prepared from adult mice chronically administered the CB1 receptor agonist WIN55,212-2 (WIN; 0.25 or 1 mg/kg) or vehicle during adolescence (from P35-P55) (Figure 1A). We used kainic acid (KA; 400 μ M) and carbachol (CCh; 20 μ M) to reliably produce beta and gamma oscillations in local field potentials (LFPs) in cortical slices (Buhl et al., 1998; Oke et al., 2010).

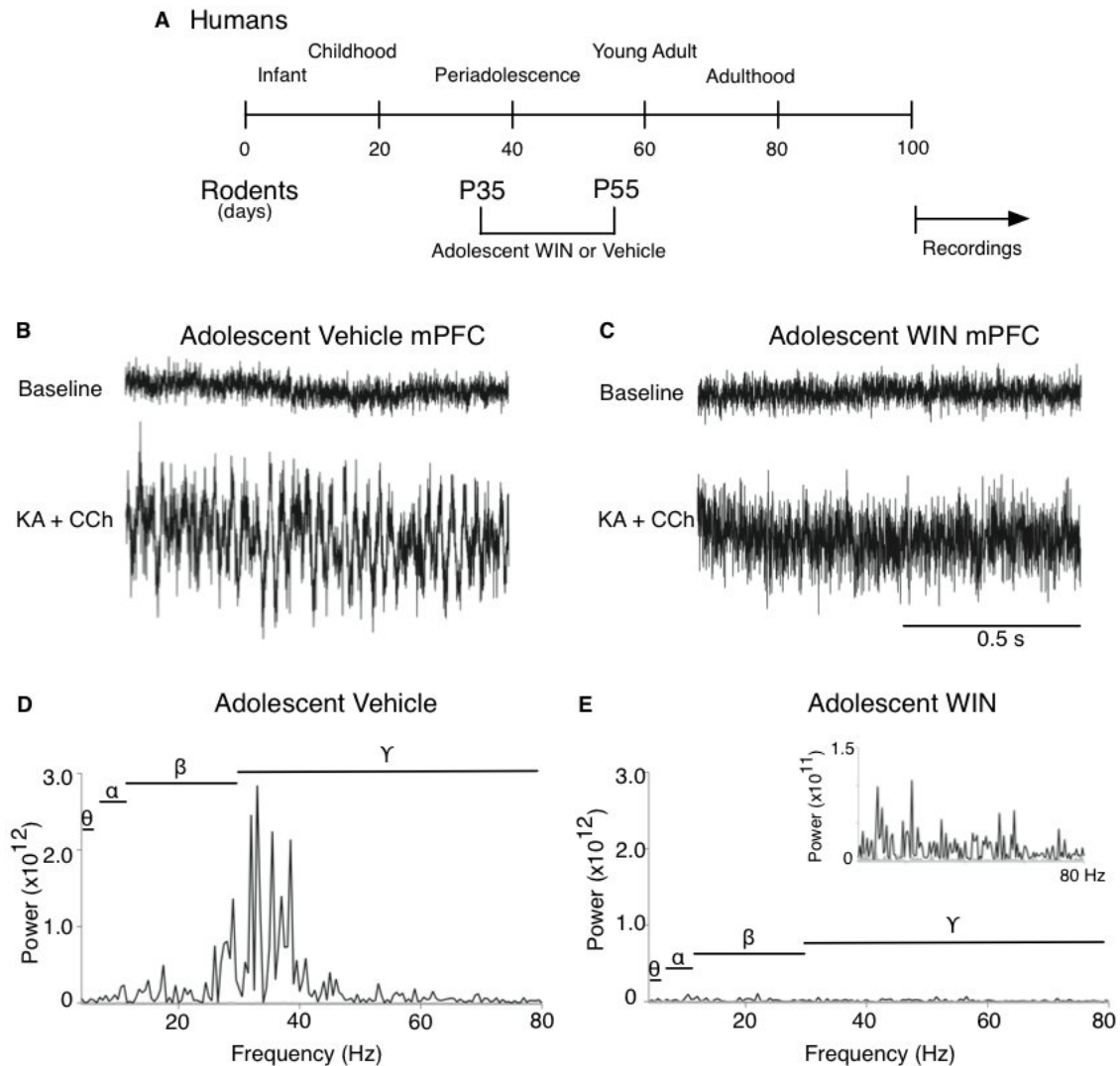


Figure 1: Robust oscillations are pharmacologically-evoked *in vitro* in adult mouse neocortex in mice treated with vehicle, but not WIN, in adolescence. (A) Experimental time course: comparisons of human and rodent development are modified from (Andersen, 2003). The CB1R agonist WIN55,212-2 (WIN; 0.25

or 1 mg/kg) or vehicle was administered to adolescent (P35–P55) mice once daily for 20 days. LFPs were recorded in brain slices from adult mice (P100). (B,C) 1-second epoch of *in vitro* LFP from an mPFC slice from an adult mouse administered vehicle (B) or 1 mg/kg WIN (C) during adolescence. LFPs were recorded before (baseline) and during kainic acid (KA; 400 nM) and carbachol (CCh; 20 μ M) perfusion (KA + CCh). (D,E) Fourier transform of 10-second LFP recordings in (B) and (C), respectively. KA + CCh (black trace) markedly increases power at all frequencies compared with baseline conditions (grey trace). Frequency ranges: theta (θ) = 4–7 Hz; alpha (α) = 8–12 Hz; beta (β) = 13–29 Hz; gamma (γ) = 30–80 Hz. Inset in (E) shows a magnified view of the FFT in (E).

An example LFP recorded *in vitro* from mPFC of an adult mouse treated during adolescence with repeated vehicle injections is depicted in Figure 1B. KA + CCh perfusion resulted in robust beta and gamma oscillations and increased power in theta and alpha bandwidths (theta: 4-7 Hz; alpha: 8-12 Hz; beta: 13-29 Hz; gamma: 30-80 Hz). This is quantified in the accompanying FFTs of the LFPs (Figure 1D) and in the spectrogram and power spectral density (PSD) in Figure 2A.

In adult mPFC slices from adolescent WIN-treated mice, pharmacologically-evoked oscillations were profoundly suppressed. Figure 1C shows a representative recording from mPFC of an adult mouse treated in adolescence with 1 mg/kg WIN. Note the markedly attenuated power of these oscillations as quantified in the FFT (Figure 1E), and spectrogram and PSD (Figure 2B), compared with oscillation power in an adolescent vehicle-treated mouse (Figure 1B,D, Figure 2A).

In slices of adult mPFC, power in gamma, beta, and alpha bandwidths was significantly and dose-dependently attenuated by adolescent WIN exposure (Figures 2C-E, Table 1A). Although it approached statistical significance, there was no significant main effect of WIN on theta power (Figure 2F, Table 1A).

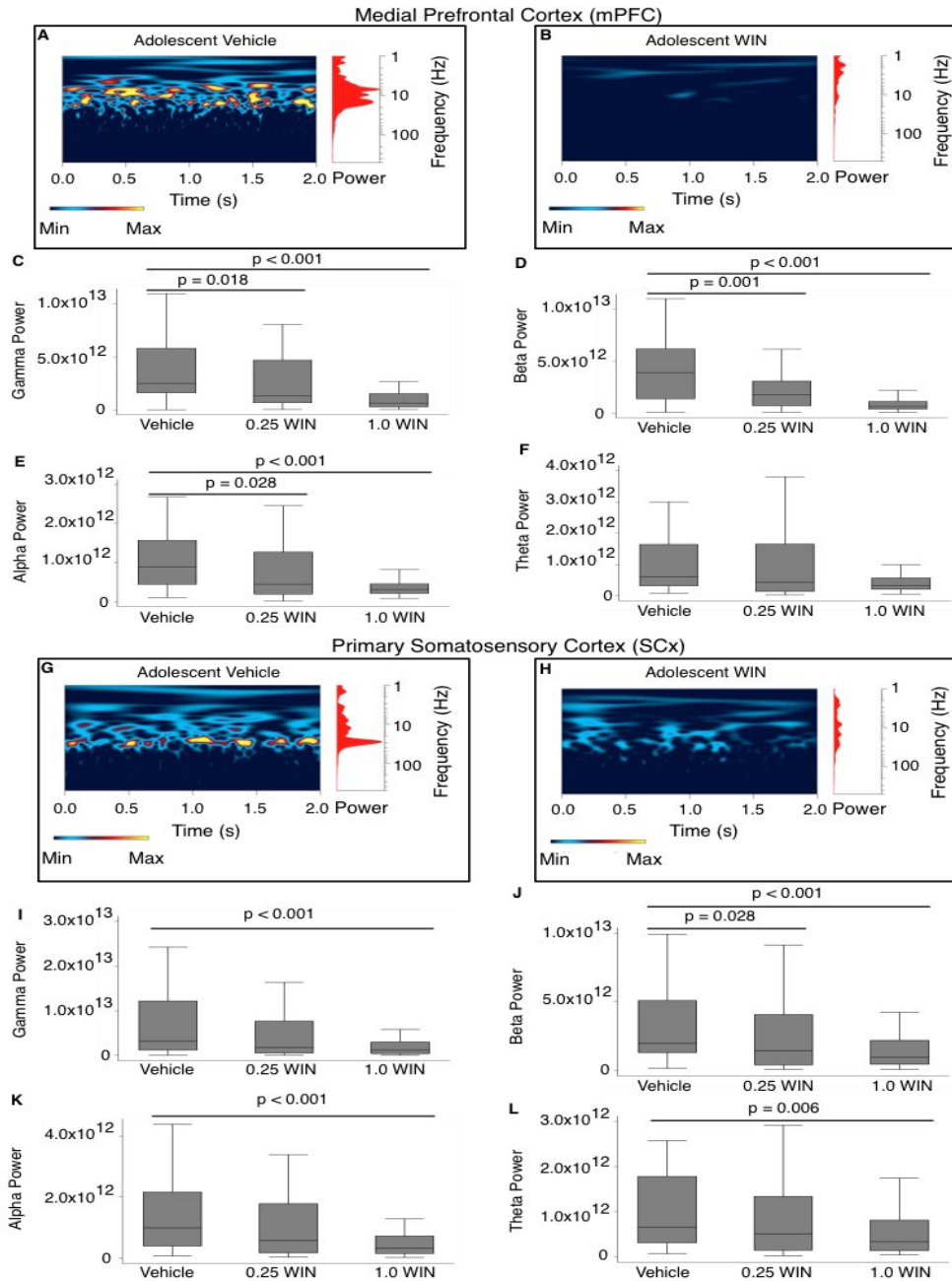


Figure 2: Chronic adolescent WIN administration attenuates pharmacologically-evoked oscillations *in vitro* in mPFC and SCx. (A,B) Spectrogram (left) and power spectral density (right) of a 1-second representative KA + CCh LFP in mPFC of an adult mouse administered vehicle (A) or 1 mg/kg WIN (B) during adolescence. Min–max scales are the same in A and B. (C-F) Box-and-whisker plots (box: 25th percentile, median, 75th percentile; whiskers: adjacent value to 25% or 75% values) of power from FFTs of LFPs in mPFC of adult mice with KA + CCh perfusion. Mice were administered WIN (0.25 or 1 mg/kg) or vehicle during adolescence (Figure 1A). KW tests determined a significant main effect of WIN and pairwise comparisons were performed using MWU tests (significant $p < 0.05$). (G,H) Spectrograms and power spectral densities of 1-second example KA + CCh LFP recorded in SCx of adult mouse administered vehicle (G) or 1 mg/kg WIN (H) during adolescence. Minimum–maximum scales are the same in G and H. (I–L) Box-and-whisker plots of power from FFTs of LFPs in SCx of adult mice in the presence of KA + CCh. Mice were treated and statistical analyses were performed as described for (C-F).

A

Medial Prefrontal Cortex (mPFC)

| | Gamma (30-80 Hz) | Beta (13-29 Hz) | Alpha (8-12 Hz) | Theta (4-7 Hz) |
|---|----------------------|----------------------|----------------------|----------------------|
| Chronic Adolescent WIN Treatment | $p < 0.001$ | $p < 0.001$ | $p < 0.001$ | $p = 0.071$ |
| Vehicle (n=12 slices/8 mice) vs. WIN (0.25 mg/kg) (n = 16 slices/10 mice) | $p = 0.018$, 52% | $p = 0.001$, 46% | $p = 0.028$, 51% | |
| Vehicle vs. WIN (1 mg/kg) (n = 14 slices/10 mice) | $p < 0.001$, 26% | $p < 0.001$, 17% | $p < 0.001$, 35% | |
| Chronic Adult WIN Treatment | | | | |
| Vehicle (n=8 slices/3 mice) vs. WIN (1 mg/kg) (n = 12 slices/5 mice) | $p = 0.543$ | $p = 0.918$ | $p = 0.662$ | $p = 0.264$ |
| Chronic Adolescent THC Treatment | | | | |
| Vehicle (n=12 slices/8 mice) vs. THC (5 mg/kg) (n = 21slices/8 mice) | $p < 0.001$, 35% | $p < 0.001$, 14% | $p < 0.001$, 18% | $p < 0.001$, 24% |

B

Primary Somatosensory Cortex (SCx)

| | Gamma (30-80 Hz) | Beta (13-29 Hz) | Alpha (8-12 Hz) | Theta (4-7 Hz) |
|--|----------------------|----------------------|----------------------|----------------------|
| Chronic Adolescent WIN Treatment | $p = 0.002$ | $p < 0.001$ | $p < 0.001$ | $p = 0.006$ |
| Vehicle (n=21 slices/12 mice) vs. WIN (0.25 mg/kg) (n = 26 slices/12 mice) | $p = 0.051$ | $p = 0.028$, 72% | $p = 0.066$, 51% | $p = 0.103$ |
| Vehicle vs. WIN (1 mg/kg) (n = 24 slices/12 mice) | $p < 0.001$, 37% | $p < 0.001$, 49% | $p < 0.001$, 33% | $p < 0.001$, 49% |
| Chronic Adult WIN Treatment | | | | |
| Vehicle (n=8 slices/3 mice) vs. WIN (1 mg/kg) (n = 13 slices/5 mice) | $p = 0.523$ | $p = 0.305$ | $p = 0.294$ | $p = 0.225$ |
| Chronic Adolescent THC Treatment | | | | |
| Vehicle (n=21 slices/12 mice) vs. THC (5 mg/kg) (n = 24slices/12 mice) | $p = 0.131$ | $p = 0.901$ | $p = 0.129$ | $p = 0.025$, 75% |

Table 1: (A) Summary of statistical analyses of cannabinoid effects on the power of pharmacologically-evoked LFP oscillations recorded *in vitro* in mPFC of adult mice. Kruskal–Wallis (KW) tests determined significant differences among three treatment conditions, and pairwise comparisons were tested with Mann–Whitney U-tests (MWU; significant $p < 0.05$). No pairwise comparisons were performed if KW $p > 0.05$. MWU tests compared two treatment conditions. Percentages reported are relative to median LFP power recorded in vehicle-treated mice. (B) Summary of statistical analysis of LFP oscillations recorded *in vitro* in SCx of adult mice. Analysis was performed as in (A)

B. Adolescent WIN Exposure Preferentially Affects the Rostral mPFC, Compared to the Caudal SCx

We next tested the prediction that the caudally located SCx would be less sensitive to chronic adolescent cannabinoid exposure than the rostral mPFC. In SCx of a

vehicle-treated mouse, KA + CCh evoked robust gamma oscillations (Figure 2G), in contrast to oscillations recorded from a mouse treated in adolescence with 1 mg/kg WIN (Figure 2H). Adolescent WIN administration significantly suppressed gamma, beta, alpha, and theta oscillations in adult SCx (Figures 2I-L, Table 1B). However, SCx was less sensitive than mPFC to chronic adolescent WIN exposure, as evidenced by the relatively weak effects of adolescent 0.25 mg/kg WIN, and the markedly smaller percent reduction in oscillation power by 1 mg/kg in SCx compared to mPFC (Table 1B).

C. Adult WIN Exposure Does Not Alter Pharmacologically-Evoked Cortical Oscillations in vitro

Long-term cognitive impairments and elevated risk of psychiatric disorders in regular marijuana users are less pronounced or non-existent when use is initiated in adulthood, compared to adolescence (Arseneault *et al.*, 2004; Meier *et al.*, 2012). Our hypothesis predicts that the long-term effects of WIN on cortical oscillations would be restricted to adolescent exposure and would not be seen after chronic WIN exposure in adulthood. To test this prediction, adult mice were chronically administered WIN (1 mg/kg) or vehicle for 20 days (P70-P90) (Figure 3A) and oscillations were evoked *in vitro* with KA + CCh, as described before (Figure 3B,C). Adult treatment had no significant effect on oscillation power in any frequency examined in mPFC (Figures 3D-G; Table 1A) or SCx (Figures 3H-K; Table 1B). The persistent suppression of pharmacologically evoked oscillations recorded in mPFC and SCx of adult mice after chronic WIN exposure during adolescence, but not adulthood, supports our hypothesis that cortical oscillations are markedly sensitive to repeated adolescent cannabinoid exposure.

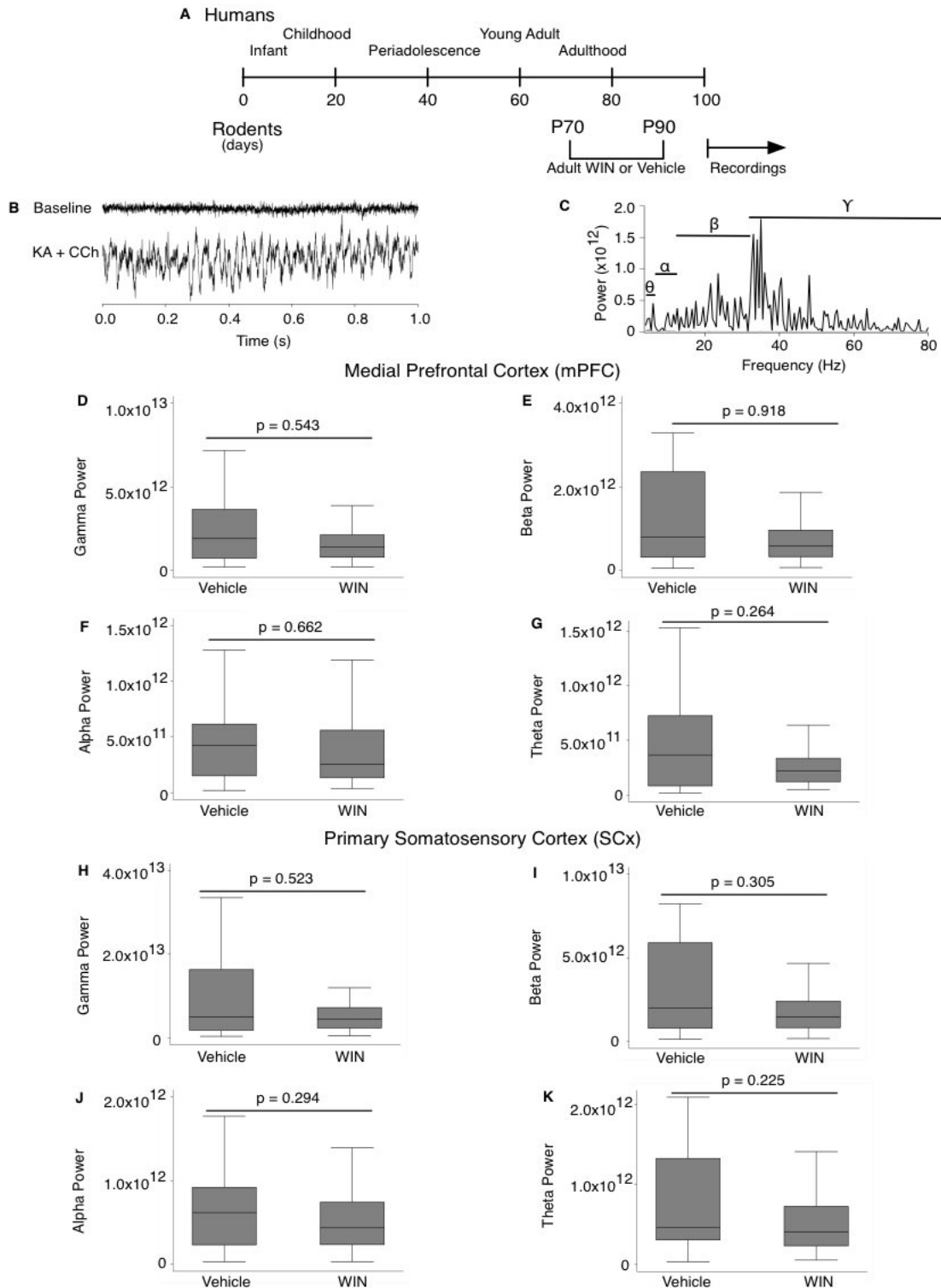


Figure 3: Robust oscillations are pharmacologically-evoked *in vitro* in adult mouse neocortex in adult mice treated with vehicle or WIN in adulthood. (A) Experimental time course as in Figure 1A. WIN (1 mg/kg) or vehicle was administered to adult (P70-P90) mice once daily for 20 days. LFPs were recorded in brain slices from adult mice (P100). (B) 1-second epoch of *in vitro* LFP and (C) FFT from an mPFC slice from an adult mouse administered vehicle as an adult. LFPs were recorded before (baseline) and during KA

+ CCh. KA + CCh (black trace) markedly increases power at all frequencies compare with baseline conditions (grey trace). (KA; 400 nM) and carbachol (CCh; 20 μ M) perfusion (KA + CCh). (D) Box-and-whisker plots of power from FFTs of LFPs in mPFC of adult mice with KA + CCh perfusion. Mice were administered WIN (1 mg/kg) or vehicle as adults (A). MWU tests used (significant $p < 0.05$).

D. Adolescent THC Exposure Persistently Suppresses Pharmacologically-Evoked

Cortical Oscillations in vitro

Although WIN is used experimentally to induce cannabinoid-mediated effects, treating mice with the primary active ingredient in marijuana, Δ^9 tetrahydrocannabinol (THC), permitted a more direct test of our hypothesis that repeated adolescent marijuana use persistently disrupts cortical oscillations, and more closely mimics human marijuana consumption. We chronically administered THC (5 mg/kg) to adolescent mice and recorded oscillations *in vitro* as above (Figure 4A).

In slices of adult mPFC, power in gamma, beta, alpha and theta bandwidths was significantly attenuated by repeated adolescent THC administration (Figures 4B-E; Table 1A). SCx was less sensitive than mPFC to adolescent THC exposure (Figures 4F-I; Table 1B) as evidenced by a lack of significant effect on gamma, beta, or alpha oscillations, and the mild attenuation of theta power by chronic adolescent THC. This suppression of oscillations in mPFC and SCx by adolescent THC exposure supports our hypothesis that persistent marijuana use during adolescence permanently alters cortical oscillations in adulthood.

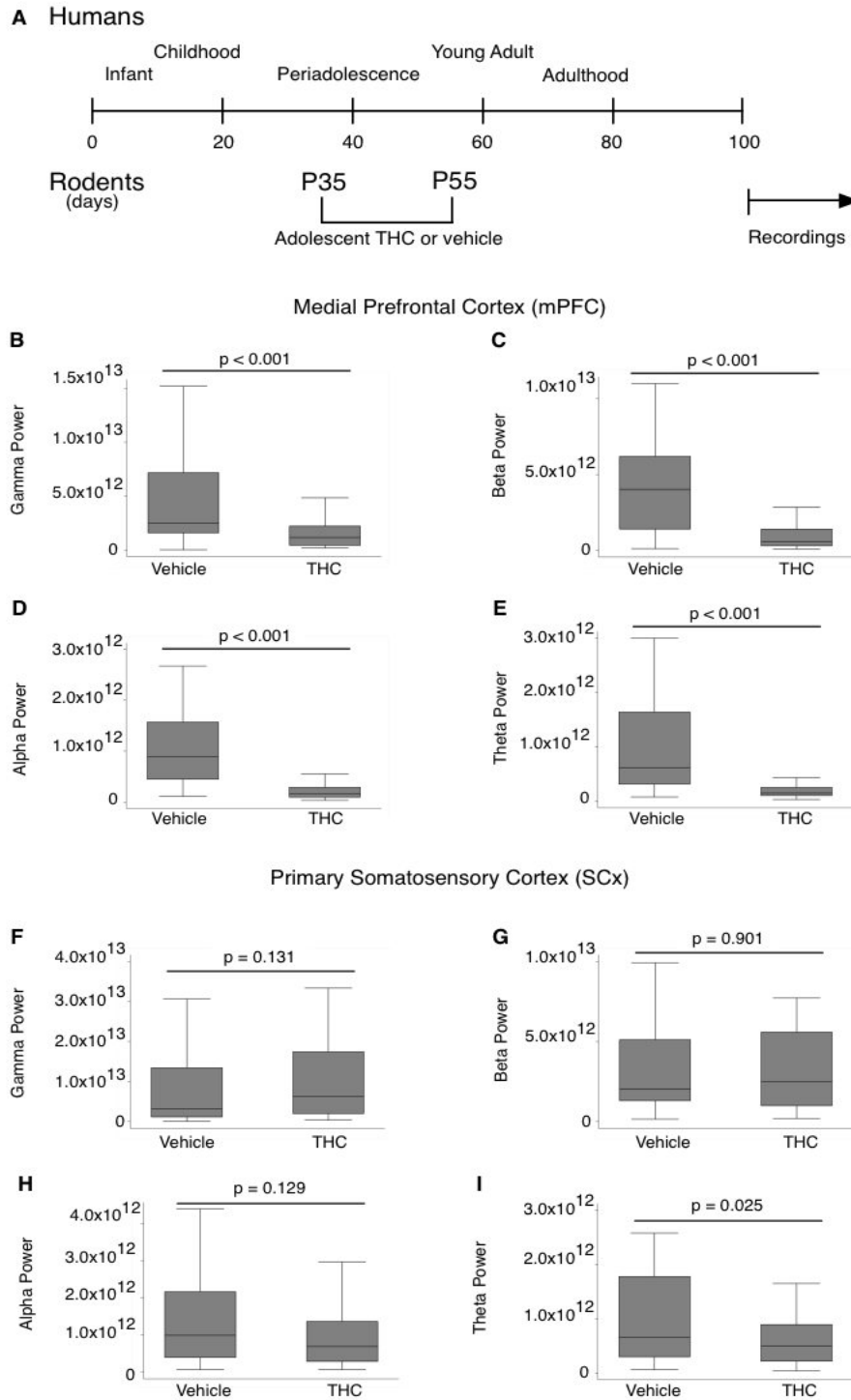


Figure 4: Chronic adolescent THC administration suppresses oscillations *in vitro* preferentially in mouse mPFC versus SCx. (A) THC (5mg/kg) or vehicle was administered to adolescent mice (P35–P55) and LFPs were recorded in slices from adult mice (>P100). (B-E) Box-and-whisker plots of power from FFTs of LFPs in mPFC of adult mice with KA + CCh perfusion. Power from adolescent THC or vehicle-treated mice were compared using MWU tests (significant $p < 0.05$). (F-I) Box-and-whisker plots of power extracted from FFTs of LFPs in SCx of adult mice in the presence of KA + CCh. Mice were treated and statistical analyses were performed as in (B-E).

E. Adolescent WIN Exposure Persistently Suppresses Pharmacologically-Evoked Oscillations in vivo

To test whether adolescent cannabinoid exposure suppresses cortical oscillations in the *intact* adult brain, we recorded ECoGs *in vivo* from freely moving adult mice chronically administered WIN (1 mg/kg) or vehicle during adolescence. Sub-anesthetic doses of the non-competitive NMDA receptor antagonist ketamine evoke robust cortical gamma oscillations in rodents and humans (Plourde *et al.*, 1997; Hakami *et al.*, 2009) that are independent of subjects' behavioral states (Hakami *et al.*, 2009), and we used this pharmacological method to evoke gamma oscillations in ECoGs *in vivo*.

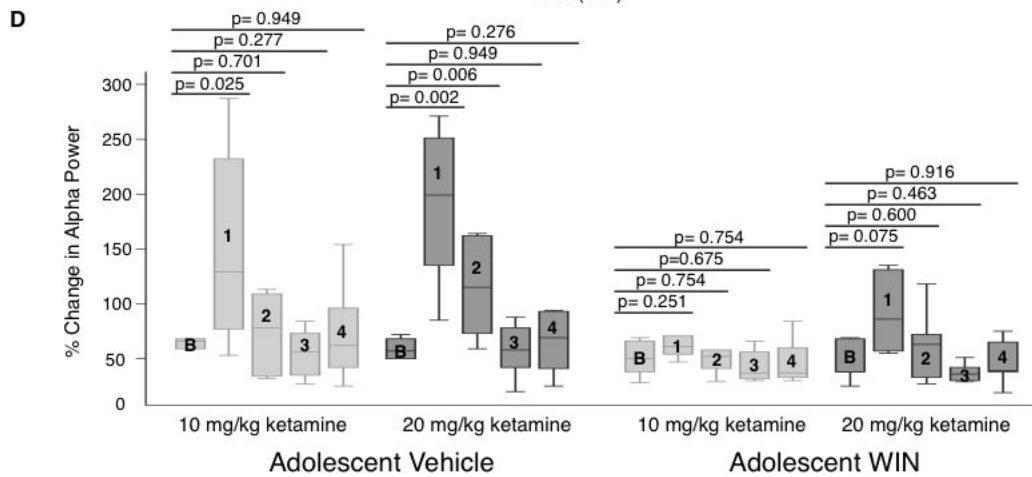
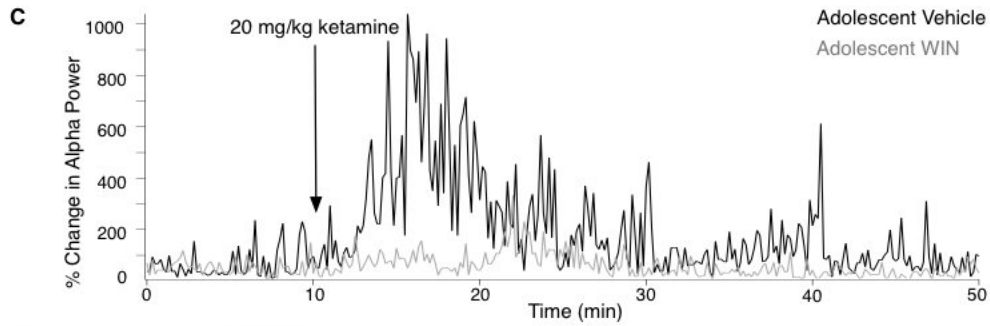
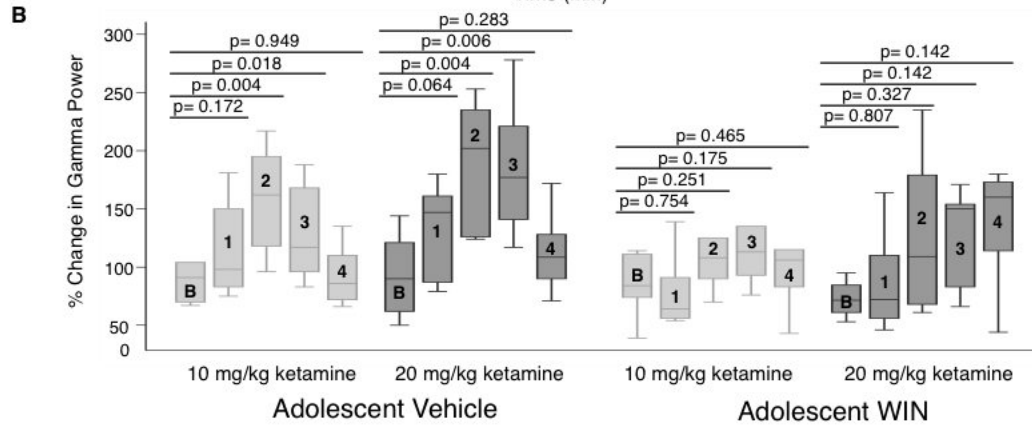
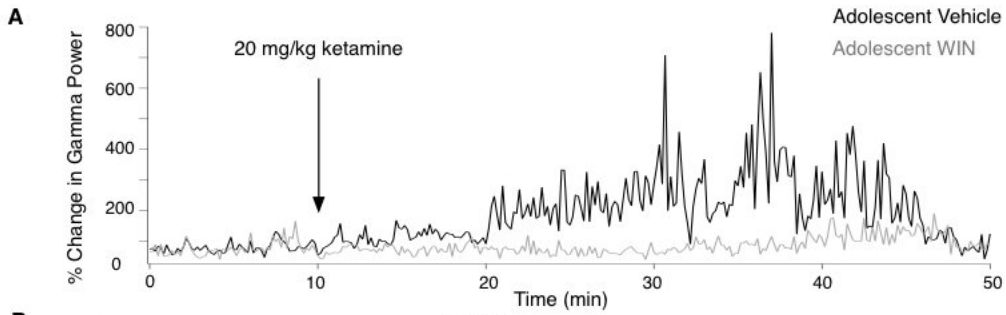
Ten-minute baseline frontal ECoG recordings were followed by an i.p. injection of saline, 10 or 20 mg/kg ketamine, and 40 minutes of post-injection recording (Figure 5A). Immediately after ketamine injection, all mice displayed characteristic movement abnormalities (tottering, falling over, and turning in circles). In vehicle-treated mice, ketamine (10 or 20 mg/kg) markedly increased gamma and alpha power (Figures 5A,B) but did not increase power at other frequencies (data not shown). To analyze the effects of ketamine, evoked gamma (Figures 5A,B) and alpha power (Figures 5C,D) were normalized to power before and after a saline injection, which alone did not produce changes in power at either frequency (data not shown).

1. Gamma Oscillations: An example time-course from an adolescent vehicle-treated mouse demonstrates that gamma power increased approximately 10 minutes after 20 mg/kg ketamine and peaked at nearly 800% of saline levels from 20 to 30 minutes before it returned to baseline (Figure 5A). Group data analysis (Figure 5B) revealed that 10 or 20 mg/kg ketamine significantly increased gamma power above baseline from 10 to

30 minutes after injection in adolescent vehicle-treated mice. In contrast, neither 10 nor 20 mg/kg had a significant effect on gamma power in frontal ECoGs of chronic adolescent WIN-treated mice, as shown in the example time-course of gamma power (Figure 5A) and in group data (Figure 5B).

2. Alpha Oscillations: Acute administration of 10 or 20 mg/kg ketamine to mice treated in adolescence with vehicle also dose-dependently increased alpha power, as shown in the example time-course (Figure 5C), and in group data (Figure 5D). In adolescent vehicle-treated mice, ketamine failed to elevate alpha power above baseline levels (Figures 5C&D). Therefore, while both 10 and 20 mg/kg ketamine significantly increased gamma and alpha power in frontal ECoGs of freely moving adolescent vehicle-treated mice, chronic adolescent WIN treatment prevented this ketamine-induced increase in oscillatory power.

Figure 5: Chronic adolescent administration of WIN attenuates cortical oscillations *in vivo*. (A) Representative time course of gamma power in frontal ECoG before and after injection of 20 mg/kg ketamine. Mice were treated with 1 mg/kg WIN (grey trace) or vehicle (black trace) from P35 to P55 and ECoGs were recorded from adults (>P100). Gamma power after ketamine injection was normalized to gamma power after saline injection. (B) Box-and-whisker plots of ECoG gamma power in adolescent vehicle (n = 5) or WIN-treated (n = 7) mice after injection of 10 mg/kg (light grey) or 20 mg/kg (dark grey) ketamine, normalized as above. B = minutes 0–10 of recording; 1 = minutes 10–20 of recording; 2 = minutes 20–30 of recording; 3 = minutes 30–40 of recording; 4 = minutes 40–50 of recording. Post-injection power was compared with baseline power with MWU tests (significant $p < 0.05$). (C) Representative time course of alpha power in frontal ECoG before and after injection of 20 mg/kg ketamine in an adolescent vehicle (black trace) or WIN-treated (grey trace) adult mouse. Data are presented as in (A). (D) Box-and-whisker plots of ECoG alpha power in adolescent vehicle (n = 5) or WIN-treated (n = 7) mice after injection of 10 mg/kg (light grey) or 20 mg/kg (dark grey) ketamine, normalized to power before and after saline injection. Time segments are indicated and statistical analyses were performed as in (B).



IV. Discussion

In Chapter 1, we present evidence that 20-day cannabinoid administration to adolescent, but not adult mice, persistently attenuates pharmacologically-evoked cortical oscillations in adults, and does so predominately in cortical areas that are less developed in adolescence. These findings are consistent with reports that regular marijuana users have suppressed evoked beta and gamma oscillations, with those who initiated use earliest showing the greatest subsequent deleterious effects (Skosnik *et al.*, 2012). However, as these studies were conducted in adults, most of whom continued to regularly use marijuana, a link between marijuana use during adolescence and suppressed oscillations in adulthood cannot be determined. Our findings now establish such a link in an animal model, and are also consistent with a recent report that the modulation of mPFC LFP amplitudes evoked by 20 or 40 Hz ventral hippocampus stimulation is impaired in adult animals repeatedly exposed to a CB1R agonist in adolescence (Cass *et al.*, 2014). Our data parallel reports of impaired ketamine-evoked gamma synchrony in animal models of psychiatric illnesses (Featherstone *et al.*, 2012; Phillips *et al.*, 2012) that are characterized by abnormal network activity (Uhlhaas and Singer, 2010) and more likely in humans that regularly used marijuana as adolescents (Arseneault *et al.*, 2004; Bossong and Niesink, 2010).

The heightened sensitivity of the adolescent cortex is likely attributable to anatomical and neurochemical development that occurs during this period (Andersen, 2003). Postnatal maturation of cortical grey and white matter progresses along a caudal-to-rostral gradient (Giedd *et al.*, 1999; Gogtay *et al.*, 2004) with primary sensory areas developing earliest, and rostral cortical areas responsible for executive functioning and

“higher order” cognitive processes not reaching stable levels until early adulthood (Gogtay *et al.*, 2004). Because sensory cortical areas develop earlier than prefrontal regions (Luna, 2009), prefrontal cortical circuitry is particularly vulnerable to adolescent drug use (Andersen, 2003). Our data confirm this vulnerability, as the magnitude of oscillation suppression seen after adolescent cannabinoid exposure is greater in the rostral mPFC than the caudal SCx.

Neocortical glutamatergic and GABAergic synapses and receptors also undergo substantial adolescent reorganization (Bahn *et al.*, 1994; Huttenlocher and Dabholkar, 1997; Cao *et al.*, 2000; Yu *et al.*, 2006; Gonzalez-Burgos *et al.*, 2008; Hashimoto *et al.*, 2009). These networks synchronize the firing of pyramidal neurons and sculpt the temporal profile of cortical oscillations (Whittington *et al.*, 2000; Cardin *et al.*, 2009; Sohal *et al.*, 2009). Coincident adolescent development of the cortical eCB system includes changes in the synthesis, tissue levels, and degradation of the primary endocannabinoids, 2AG and anandamide (Ellgren *et al.*, 2008; Long *et al.*, 2012; Lee *et al.*, 2013) and reduced CB1R expression (Deshmukh *et al.*, 2007; Heng *et al.*, 2011), especially in rostral cortical regions (Heng *et al.*, 2011) (K. Y. Tseng, personal communication). The highly dynamic nature of glutamatergic, GABAergic and eCB system development parallels the maturation of cortical oscillations in the gamma and beta bandwidths that emerge during adolescence (Uhlhaas *et al.*, 2009), and reflect the increased temporal and spatial precision of neuronal interactions in the adolescent neocortex.

Pharmacological methods are useful for evoking oscillations in LFPs or ECoGs as they provide a uniform stimulus to all animals, and generate oscillations through

relatively well-understood mechanisms. KA and CCh enhance excitatory drive (Buhl *et al.*, 1998; Cunningham *et al.*, 2003) and activate cholinergic receptors, preferentially on GABAergic interneurons (Fisahn *et al.*, 1998; Gulyas *et al.*, 2010; Nagode *et al.*, 2011). This combined action promotes robust beta and gamma oscillations *in vitro* in LFPs through a recurrent feedback loop consisting of excitatory pyramidal neurons and inhibitory interneurons (Buhl *et al.*, 1998; Fisahn *et al.*, 1998; Mann *et al.*, 2005), in which the discharge of pyramidal cells is governed by the peri-somatic inhibition provided by interneurons that are, in turn, driven by excitatory input (Mann *et al.*, 2005; Gulyas *et al.*, 2010; Holderith *et al.*, 2011).

Acute sub-anesthetic doses of ketamine also produce sustained increases in gamma oscillation power (Plourde *et al.*, 1997; Hakami *et al.*, 2009). Less is known concerning the mechanism of ketamine-induced oscillation generation, but evidence shows a preferential action of ketamine at NMDA receptors located on inhibitory interneurons (Grunze *et al.*, 1996; Homayoun and Moghaddam, 2007). Low dose ketamine administration, therefore, produces the net effect of dis-inhibition, by inhibiting NMDA currents preferentially on these GABAergic interneurons and increasing the excitability of the neural network (Homayoun and Moghaddam, 2007), reminiscent of the mechanism underlying KA and CCh-driven oscillatory activity.

Acute cannabinoid exposure attenuates the power of neural oscillations recorded *in vitro* (Hajos *et al.*, 2000; Hajos *et al.*, 2008; Holderith *et al.*, 2011) and *in vivo*, in freely moving animals (Robbe *et al.*, 2006; Hajos *et al.*, 2008; Kucewicz *et al.*, 2011; Sales-Carbonell *et al.*, 2013) similar to what we observe after chronic adolescent administration. This suppression has been shown to be mediated by the CB1R (Robbe *et al.*, 2006; Hajos

et al., 2008; Holderith *et al.*, 2011; Sales-Carbonell *et al.*, 2013). Subsequent experiments will address whether a similar CB1R mechanism is responsible for the persistent suppression of cortical network activity following adolescent WIN and THC exposure.

Chapter 2: Treatment with Cannabinoid Receptor Ligands in Adolescence **Suppresses Pharmacologically-Evoked Oscillations in Adulthood**

I. Introduction

The *in vitro* and *in vivo* findings, described in Chapter 1, are consistent with our hypothesis that adolescent cannabinoid exposure persistently alters cortical oscillations. Previous studies have shown a similar attenuation of oscillatory power after *acute* administration of CB1R agonists such as WIN and THC, the two cannabinoid receptor ligands that we tested (Hajos *et al.*, 2000; Robbe *et al.*, 2006; Hajos *et al.*, 2008; Holderith *et al.*, 2011; Sales-Carbonell *et al.*, 2013). These acute suppressive effects are mediated by CB1Rs, as they can be antagonized by pre-treatment or co-administration of CB1R antagonists, such as AM251 or SR141516 (Robbe *et al.*, 2006; Hajos *et al.*, 2008; Holderith *et al.*, 2011; Sales-Carbonell *et al.*, 2013), and are absent in mice lacking the CB1R receptor (Holderith *et al.*, 2011).

As we have discovered similar suppression of oscillations after chronic exposure to either WIN or THC—two structurally different cannabinoids that both act as CB1R agonists—we predict that CB1Rs may be responsible for these chronic effects as well. However, as WIN and THC have additional targets including CB2Rs (Showalter *et al.*, 1996) and other G-protein coupled receptors (GPCRs)(Breivogel *et al.*, 2001; Ryberg *et al.*, 2007), it is possible that non-CB1R receptors may underlie the suppression of oscillations that we observe in adult mice after chronic adolescent WIN or THC administration. We predict that persistent suppression of adult cortical oscillations *in vitro* by chronic exposure to cannabinoid receptor ligands during adolescence is mediated by CB1Rs and will be prevented by co-administration of CB1R antagonists.

II. Materials and Methods

A. Animals

Adolescent male CD-1 mice were treated once daily with several cannabinoid compounds or a vehicle solution. Treatment was administered chronically during adolescence (20 days; from P35-P55), or sub-chronically (6 days) during early (P35-P40) or late (P47-P52) adolescence. Animals were treated as described in Chapter 1. CB1R knockout (CB1R KO) mice were a generous gift from Dr. Eliot Gardner (National Institute on Drug Abuse Intramural Research Program, NIDA, Baltimore, MD), and were generated as previously described (Zimmer *et al.*, 1999). We received 4 breeding pairs of adult mice heterozygous for the CB1R (CB1R HETs) and established a breeding colony in our animal facilities at the University of Maryland School of Medicine. Offspring were genotyped to confirm the presence (CB1R WT) or absence of the CB1R (CB1R KO), and mice of both sexes were administered WIN (1 mg/kg), THC (5 mg/kg), or vehicle chronically during adolescence (P35-P55).

B. Genotyping

Mice were genotyped by Shana Howarth of the University of Maryland Genotyping Core using a protocol from Dr. Gardner's lab that was modified by Shana Howarth and Dr. Frank Margolis. Tail samples were obtained from mice at weaning age (P21), and tail tip DNA was digested according to the Sigma Red Extract-N-Amp Tissue PCR Kit (Sigma Aldrich). DNA was amplified using primers to CB1R DNA in a thermocycler, and products were separated with electrophoresis on a 1.5% agarose gel. Distinct bands were present for the CB1R KO (515 bp), CB1R WT (284 bp) and CB1R HET (both bands) genotypes. Mice determined to be heterozygous for the CB1R were

ethanized, and CB1R WT and CB1R KO mice of both sexes were used for experiments.

C. Drugs

The CB1R/CB2R agonist WIN55-212,2 (1 or 2 mg/kg; Sigma Aldrich, St. Louis, MO, USA), the CB1R inverse agonist/antagonist AM251 (0.3, 0.5, 1 or 2 mg/kg; Sigma Aldrich), the CB1R/CB2R agonist THC (5 mg/kg; National Institute on Drug Abuse Drug Supply Program, Bethesda, MD), and the putative CB1R-inactive enantiomer WIN55-212,3 (1 mg/kg; Tocris Bioscience, Bristol, UK) were dissolved in 100% ethanol and injected in a 1:1:18 solution of 100% ethanol: Emulphor (Alkamuls EL-620, Rhodia Chemicals): 0.9% saline (1 mL/kg). The CB1R neutral antagonist AM4113 (1 mg/kg; a generous gift from Dr. Alexandros Makriyannis, Northeastern University, Boston, MA) and the CB2R inverse agonist/antagonist AM630 (1 mg/kg; Tocris Bioscience, Bristol, UK) were dissolved in 1:1 dimethyl sulfoxide (DMSO): 100% ethanol with sonication and were injected in a 0.5:0.5:1:18 solution of DMSO solution: 100% ethanol: Emulphor: 0.9% saline. Control animals were injected vehicle (1:1:18 ethanol: Emulphor: saline). A separate cohort of vehicle animals were injected with the DMSO-containing vehicle (0.5:0.5:1:18 DMSO: ethanol: Emulphor: saline) and were compared to the 1:1:18 vehicle animals to ensure that DMSO had no effect on its own, as it was a component of the prepared AM4113 and AM630 injection solutions. We found no significant differences in cortical oscillations recorded from DMSO-containing vehicle-administered mice compared to those exposed to the 1:1:18 vehicle solution (data not shown), and therefore used only 1:1:18 (ethanol: Emulphor: saline) administered animals for our vehicle control population. Animals that were assigned to groups in which the effects of antagonist + agonist administration were tested were given 2 separate injections, spaced

20-30 minutes apart, with the antagonist administered first. In antagonist alone groups, these injections were a) antagonist + vehicle; in agonist alone groups, they were b) vehicle + agonist; and in antagonist-agonist groups, animals received injections of c) antagonist + agonist. All injections were titrated such that total injection volume was less than or equal to 1 mL/kg. Please note that throughout the Methods and Results sections, AM251 (a CB1R inverse agonist/antagonist), AM4113 (a neutral CB1R antagonist) and AM630 (a CB2R inverse agonist/antagonist) will be classified as “antagonists”, and their respective mechanisms of antagonism will be discussed later.

D. Determining the Dose of Antagonists

To test whether CB1Rs are responsible for WIN-induced changes in oscillatory power in adulthood, we co-administered AM251 with WIN to try to prevent WIN’s acute effects. Because the phenotype of interest (suppressed oscillations after chronic adolescent exposure) has not previously been investigated, the dose of AM251 that would antagonize this phenotype was unknown. We therefore performed behavioral testing to determine a dose of AM251 that would prevent *acute* behavioral effects of WIN in adolescent mice. Administration of CB1R ligands produces a characteristic tetrad of behavioral effects that can be prevented by CB1R antagonists: hypothermia, catalepsy, hypolocomotion, and analgesia (McMahon and Koek, 2007). Consultation with Dr. Roger Cachope (then at the University of Maryland School of Medicine, Baltimore, MD) prompted us to pursue tests of analgesia and hypolocomotion.

1. Analgesia: With assistance from Charles Raver, we measured mechanical withdrawal thresholds in naïve adolescent (~P35) male CD-1 mice using a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). This procedure involves placing mice on a wire mesh

platform and targeting a filament to the plantar surface of the mouse's hind-paw with increasing force until the animal withdraws its paw. The force at which the animal withdraws is considered its withdrawal threshold. Analgesic compounds, such as CB1R agonists, lower these thresholds. We compared mechanical withdrawal thresholds in mice administered vehicle or 1 mg/kg WIN and found no change after WIN injection (data not shown), prompting us to increase the dose to 2.5 mg/kg WIN. Similarly, we found no effect of this higher dose of WIN on plantar mechanical thresholds, and, due to high within-animal threshold variability, we decided to pursue a different behavioral metric of cannabinoid action.

2. *Hypolocomotion:* In conjunction with Sean Piantadosi and Dr. Todd Gould (University of Maryland School of Medicine, Baltimore, MD), we measured open field locomotion in naïve adolescent (~ P35) male CD-1 mice to test for hypolocomotive effects of WIN and antagonism by AM251. Previous work has shown that low doses of WIN—such as those that we chronically administered (0.25 and 1 mg/kg)—do not produce behavioral changes that can be measured with the cannabinoid tetrad of tests (Fox *et al.*, 2001; McMahon and Koek, 2007; Abalo *et al.*, 2009), and we therefore increased the dose to 5 mg/kg WIN to test for acute hypolocomotion. Mice were habituated to Dr. Gould's behavioral testing room for 2 hours in low light conditions in the presence of white background noise. Animals were videotaped during exploration of an open field testing arena (49 cm x 49 cm) with a black floor that was cleaned with MB-10 solution after each experiment. Mice were assigned 1 of 4 treatment conditions, n = 5 mice for each condition: vehicle + vehicle (vehicle condition), vehicle + WIN (5 mg/kg) (WIN condition), AM251 (1 mg/kg) + vehicle (AM251 condition), AM251 (1 mg/kg) +

WIN (5 mg/kg) (AM251 + WIN condition). Drugs were prepared as previously described, and animals were injected i.p. Mice were given their first injection and returned to their home-cages for 30 minutes, after which they were given their second injection and immediately placed into the open field arena where their behavior was recorded for 1 hour after the final injection. This time course is in accordance with previous reports of WIN's peak hypolocomotive activity 20-30 minutes after administration (McMahon and Koek, 2007). Total distance traveled during the hour was measured with assistance from Sean Piantadosi using the CleverSys Top Scan software package (CleverSys Inc., VA, USA) and was calculated for each animal in 10-minute bins during the 60-minute behavioral recoding session. Because all mice reduced locomotion after 30 minutes in the open field, we analyzed locomotion during the first 30 minutes in the arena for predicted effects of cannabinoids. Data were normally distributed and analyzed with a one-way ANOVA. Although there was an observable reduction in total distance traveled in the WIN condition, we found no significant main effect of treatment when all 4 conditions were compared ($p = 0.131$) (Figure 6A). Because we did not expect to see a change in locomotion with the low dose of AM251 tested (1 mg/kg) according to previous reports (McMahon and Koek, 2007) — and indeed locomotion in animals administered AM251 alone was equivalent to that in vehicle-treated mice [vehicle ($n = 5$): $183,483 \pm 46,620$ mm; AM251 ($n = 5$): $99,622 \pm 15,292$ mm; 2-tailed t-test $p = 0.886$] — we excluded AM251 data from analysis to determine whether WIN produced a significant locomotion suppression, relative to vehicle-treatment, that could be reversed by AM251 treatment. Hypolocomotion in WIN animals approached, but did not reach significance ($p = 0.078$) when AM251 data was excluded from ANOVA analysis, likely do the mild effects of this

relatively low dose of WIN and the small sample size ($n = 5$ mice for each condition). We concluded from this experiment that 5 mg/kg WIN slightly, but non-significantly, attenuates locomotion, and that these effects may be ameliorated by pre-treatment with 1 mg/kg AM251 (Figure 6A).

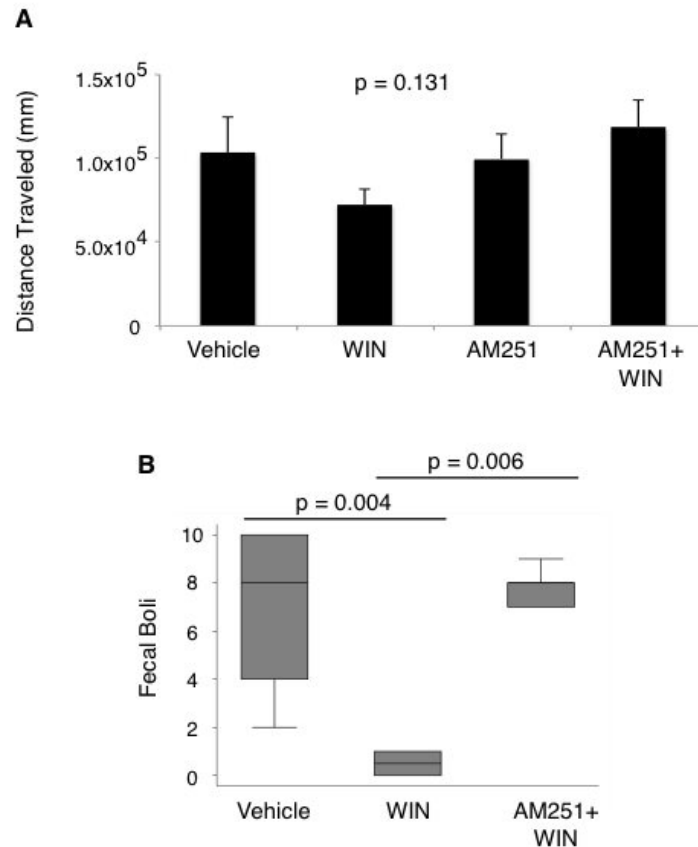


Figure 6: AM251 antagonism of behavioral effects by acute adolescent WIN administration. (A) Total open-field locomotion for 30 minutes post injection with vehicle ($n = 5$ mice), WIN (5 mg/kg; $n = 5$ mice), AM251 (1 mg/kg; $n = 5$ mice), or AM251 + WIN (1 mg/kg + 5 mg/kg; $n = 5$ mice). Total distance traveled was analyzed with a one-way ANOVA ($p = 0.131$). (B) Total number of fecal boli deposited in the open-field arena by the same animals as in (A). Data were analyzed with MWU tests, significant $p < 0.05$.

3. Fecal Boli Test: During the open field locomotion test, we observed that WIN-treated mice deposited noticeably fewer fecal boli, compared to mice treated with vehicle, AM251, or AM251 + WIN. Defecation is a reliable indication of anxiety behavior in

rodents (Green *et al.*, 2010), and acute CB1R activation has been shown to be anxiolytic (Haller *et al.*, 2004). During the 1-hour open field session (Figure 6B), WIN-treated mice deposited significantly fewer boli than vehicle-treated animals (vehicle: median = 8 boli; WIN: median = 0.5 boli, 99.9% fewer fecal boli under the influence of WIN than vehicle) that was completely reversed by AM251 treatment (WIN: median = 0.5 boli; AM251 + WIN = 8 boli; 99.9% more boli under the influence of AM251 + WIN than WIN). Again, we removed AM251 data from our comparisons as we saw no effect of AM251 alone on locomotion behavior or fecal boli relative to vehicle, and the purpose of the experiment was to test whether WIN produced a significant behavioral effect that could be reversed by AM251 in adolescent mice. Data were non-normally distributed and were analyzed with the non-parametric Kruskal-Wallis test, which revealed a significant main effect of treatment ($p = 0.010$). Post-hoc analysis, adjusted for multiple comparisons, revealed a significant reduction of fecal boli by WIN, compared to vehicle ($p = 0.004$), with a reversal by AM251 treatment (WIN vs. AM251 + WIN: $p = 0.006$). AM251 + WIN did not differ from vehicle ($p = 0.500$).

We therefore concluded that 1 mg/kg of the CB1R antagonist AM251 can reverse the behavioral effects of a dose of WIN five times higher than the dose that we chronically administered to adolescent mice. We felt confident that 1 mg/kg AM251 should be a sufficient dose to antagonize the oscillation suppressing effects of chronic adolescent 1 mg/kg WIN.

In subsequent experiments, we chose doses of antagonists from the literature. We administered 1 mg/kg of the CB1R antagonist AM4113 chronically to adolescent animals, a dose that does not produce behavioral effects when administered acutely on its own, but

antagonizes the acute effects of up to 3 mg/kg WIN (Jarbe *et al.*, 2008). Similarly, we administered 1 mg/kg of the CB2R antagonist AM630, which does not produce behavioral effects on its own when acutely administered (Guindon *et al.*, 2007). To determine lower doses of AM251 that would not be expected to produce a phenotype on their own, we consulted with Dr. Aron Lichtman (Virginia Commonwealth University, Richmond, VA) who recommended doses of 0.3 and 0.5 mg/kg AM251 to be administered in studies with THC. Doses of WIN and AM251 were increased (2 mg/kg vs. 1 mg/kg in chronic studies) for our sub-chronic adolescent studies and were determined in consultation with Dr. Kuei-Yuan Tseng (Rosalind Franklin University, Chicago, IL).

E. In vitro Slice Preparation and LFP Recordings

Slices containing either medial prefrontal cortex (mPFC) or primary somatosensory cortex (SCx) were prepared from adult male mice (> P100) as described in Chapter 1. Briefly, kainic acid (400 nM; KA) and carbachol (20 μ M; CCh) were perfused over slices in ACSF containing a slightly elevated K^+ concentration (5 mM). LFPs were recorded from layers II/III of the prelimbic region of mPFC (PL) and layers II/III of the barrel cortex region of primary somatosensory cortex (SCx) after at least 45 minutes of KA + CCh perfusion.

F. In vitro Data Analysis

LFPs were analyzed with custom-written Igor Pro scripts. Discrete fast Fourier transforms (FFTs) were performed on 10-seconds of LFP data and oscillation power (area under the curve of the FFT) was integrated at the following frequencies: gamma (30-80 Hz), beta (13-29 Hz), alpha (8-12 Hz) and theta (4-7 Hz). Average power in each

frequency was calculated for vehicle-administered mice and was used to normalize all other oscillation power values in the different treatment conditions. Two or three slices of mPFC and SCx were collected for each animal. Oscillation power in each frequency was normalized to the average oscillation power in that frequency for vehicle-treated mice. Four normalized values (theta, alpha, beta, gamma) were obtained from each LFP (2-3 LFPs per animal, one from each slice) and were combined for each treatment condition. This allowed us to recreate the entire oscillation frequency spectrum, rather than examining individual frequencies to test whether antagonists altered normalized oscillation power compared to vehicle or WIN/THC. These normalized oscillation power values were plotted with cumulative probability distributions and analyzed with non-parametric Kolmogorov-Smirnov (KS) tests (significant $p < 0.05$) to examine the effect of adolescent cannabinoid exposure on cortical oscillations.

III. Results

A. Sub-Chronic WIN Administration During Early, but not Late Adolescence

Persistently Suppresses Pharmacologically-Evoked Cortical Oscillations in vitro Through CB1Rs

We tested the prediction that adolescent cannabinoid exposure suppresses pharmacologically-evoked oscillations in the adult neocortex by acting on CB1Rs. Recent evidence indicates that 5 days of WIN administration to rats early in adolescence impairs the modulation of oscillatory LFP activity in mPFC evoked by ventral hippocampus stimulation, and is mediated by the CB1R (Cass *et al.*, 2014). We predicted that sub-chronic (6 day) WIN exposure during early adolescence (P35-P40) would similarly act through CB1Rs, and therefore be antagonized by the CB1R antagonist

AM251. We also predicted that these effects would be more pronounced after early adolescent WIN administration than after similar exposure during late adolescence (P47-P52), due to more advanced development of the cortical eCB system earlier in adolescent development (Heng *et al.*, 2011; Cass *et al.*, 2014).

To test these predictions, mice were treated with vehicle, WIN (2 mg/kg), AM251 (2 mg/kg), or AM251 + WIN for 6 days from P35-P40 and *in vitro* oscillations were pharmacologically evoked with KA + CCh in adulthood (Figure 7A). We computed total oscillation power in each bandwidth (gamma, beta, alpha, and theta), as described in Chapter 1. We then normalized these values to the average power of corresponding bandwidths recorded from vehicle-treated animals. We performed this normalization separately for data from mPFC (LII/III of the prelimbic area) and SCx (LII/III of the barrel field). Finally, we combined these normalized values (% of vehicle control) for each bandwidth and analyzed all normalized power values with non-parametric Kolmogorov-Smirnov statistical tests.

In agreement with our hypothesis, early adolescent sub-chronic WIN exposure significantly suppressed oscillations in mPFC relative to vehicle control levels (Figure 7B, Table 2A). This is apparent in the significant leftward shift of the normalized distribution by WIN from that of vehicle (Figure 7B). We found no effect of AM251 administration alone (Figure 7C, Table 2A). Oscillation power in animals exposed to AM251 + WIN in early adolescence was significantly higher than WIN alone and equal to vehicle levels (Figure 7C, Table 2A), indicating that AM251 prevented the effects of WIN in mPFC. These data suggest that sub-chronic early adolescent WIN suppresses oscillations in mPFC via CB1Rs. Statistical summaries of sub-chronic adolescent pharmacology results

are shown in Table 2.

SCx proved to be less sensitive to the effects of adolescent cannabinoid exposure, as neither WIN nor AM251 altered adult cortical oscillations when administered sub-chronically during early adolescence (Figures 7E&F, Table 2B).

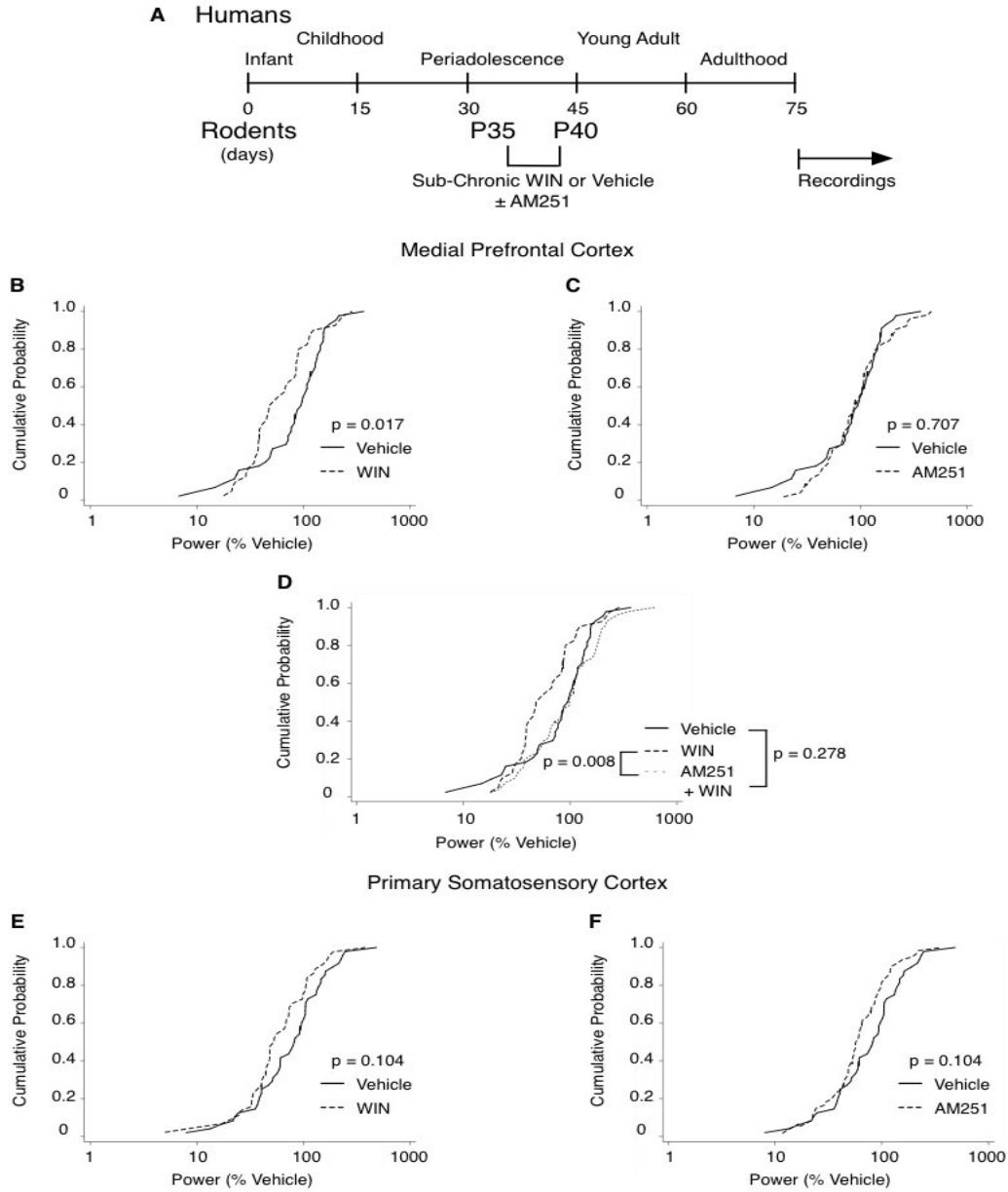


Figure 7: Sub-chronic administration of WIN in early adolescence suppresses pharmacologically-evoked oscillations *in vitro* in mPFC, but not SCx, via CB1Rs. (A) Experimental time course. The CB1R agonist WIN (2 mg/kg) was administered with vehicle or the CB1R inverse agonist/antagonist AM251 (2 mg/kg) to early adolescent mice (P35-P40) once daily for 6 days. LFPs were recorded in brain slices from adult mice (>P100). (B,C) Cumulative probability distributions of normalized oscillation power plotted on a logarithmic scale for early adolescent vehicle (solid line) or (B) WIN (dashed line) or (C) AM251 (dashed line) treated LFPs recorded from mPFC *in vitro*. Kolmogorov-Smirnoff (KS) tests were used to compare the effect of adolescent treatment on normalized oscillation power (significant $p < 0.05$). (D) Cumulative probability distributions of normalized oscillation power plotted on a logarithmic scale for early adolescent vehicle (solid line) or WIN (dashed line) or AM251 + WIN (dotted line). WIN data was compared to AM251 + WIN, and AM251 + WIN was compared to vehicle data with KS tests (significant $p < 0.05$). (E,F) Cumulative probability distributions of normalized oscillation power plotted on a logarithmic scale for early adolescent vehicle (solid line) or (E) WIN (dashed line) or (F) AM251 (dashed line) treated LFPs recorded from SCx *in vitro*.

A

| Sub-Chronic Adolescent Cannabinoid Treatment | mPFC |
|--|-----------------|
| Early Adolescent (P35-P40) WIN Treatment Vehicle (n = 11 slices/4 mice) vs. WIN (2 mg/kg) (n = 10 slices/4 mice) | p = 0.017, 52% |
| Early Adolescent (P35-P40) AM251 Treatment Vehicle (n = 11 slices/4 mice) vs. AM251 (2 mg/kg) (n = 13 slices/5 mice) | p = 0.707 |
| Early Adolescent (P35-P40) AM251 + WIN Treatment WIN (2 mg/kg) (n = 10 slices/4 mice) vs. AM251 (2 mg/kg) + WIN (2 mg/kg) (n = 13 slices/5 mice) | p = 0.008, 194% |
| Vehicle (n = 11 slices/4 mice) vs. AM251 (2 mg/kg) + WIN (2 mg/kg) (n = 13 slices/5 mice) | p = 0.278 |
| Late Adolescent (P47-P52) WIN Treatment Vehicle (n = 14 slices/5 mice) vs. WIN (2 mg/kg) (n = 13 slices/5 mice) | p = 0.057 |
| Late Adolescent (P47-P52) AM251 Treatment Vehicle (n = 14 slices/5 mice) vs. AM251 (2 mg/kg) (n = 15 slices/5 mice) | p = 0.137 |

B

| Sub-Chronic Adolescent Cannabinoid Treatment | SCx |
|--|-----------|
| Early Adolescent (P35-P40) WIN Treatment Vehicle (n = 12 slices/4 mice) vs. WIN (2 mg/kg) (n = 11 slices/4 mice) | p = 0.104 |
| Early Adolescent (P35-P40) AM251 Treatment Vehicle (n = 12 slices/4 mice) vs. AM251 (2 mg/kg) (n = 15 slices/5 mice) | p = 0.104 |
| Late Adolescent (P47-P52) WIN Treatment Vehicle (n = 15 slices/5 mice) vs. WIN (2 mg/kg) (n = 15 slices/5 mice) | p = 0.064 |
| Late Adolescent (P47-P52) AM251 Treatment Vehicle (n = 15 slices/5 mice) vs. AM251 (2 mg/kg) (n = 15 slices/5 mice) | p = 0.160 |

Table 2: (A) Summary of statistical analysis of sub-chronic early adolescent (P35-P40) or late adolescent (P47-P52) cannabinoid effects on the normalized power of pharmacologically-evoked LFP oscillations recorded *in vitro* in LII/III of the prelimbic area of mPFC in adult mice. Kolmogorov-Smirnoff tests determined significant differences between treatment conditions (significant $p < 0.05$). Percentages reported are the median normalized LFP power in the treatment after “vs” in the statistical comparison, relative to the median normalized LFP power in the treatment before “vs.” (B) Summary of statistical analysis of sub-chronic adolescent cannabinoid effects recorded in LII/III of the barrel field in SCx of adult mice. Statistical analyses were performed as in (A).

We further tested the temporal window of cortical oscillation sensitivity to adolescent cannabinoid exposure by restricting WIN exposure to 6 days during late adolescence, from P47-P52 (Figure 8A). In mPFC, neither sub-chronic WIN nor AM251 administration in the late adolescent period significantly altered oscillations relative to vehicle levels (Figures 8B,C, Table 2A). Similarly, late adolescent sub-chronic WIN or AM251 exposure had no effect on oscillations in SCx (Figures 8D,E, Table 2B). These data further support a gradient of cannabinoid sensitivity that parallels the adolescent development of the cortical eCB system (Heng *et al.*, 2011).

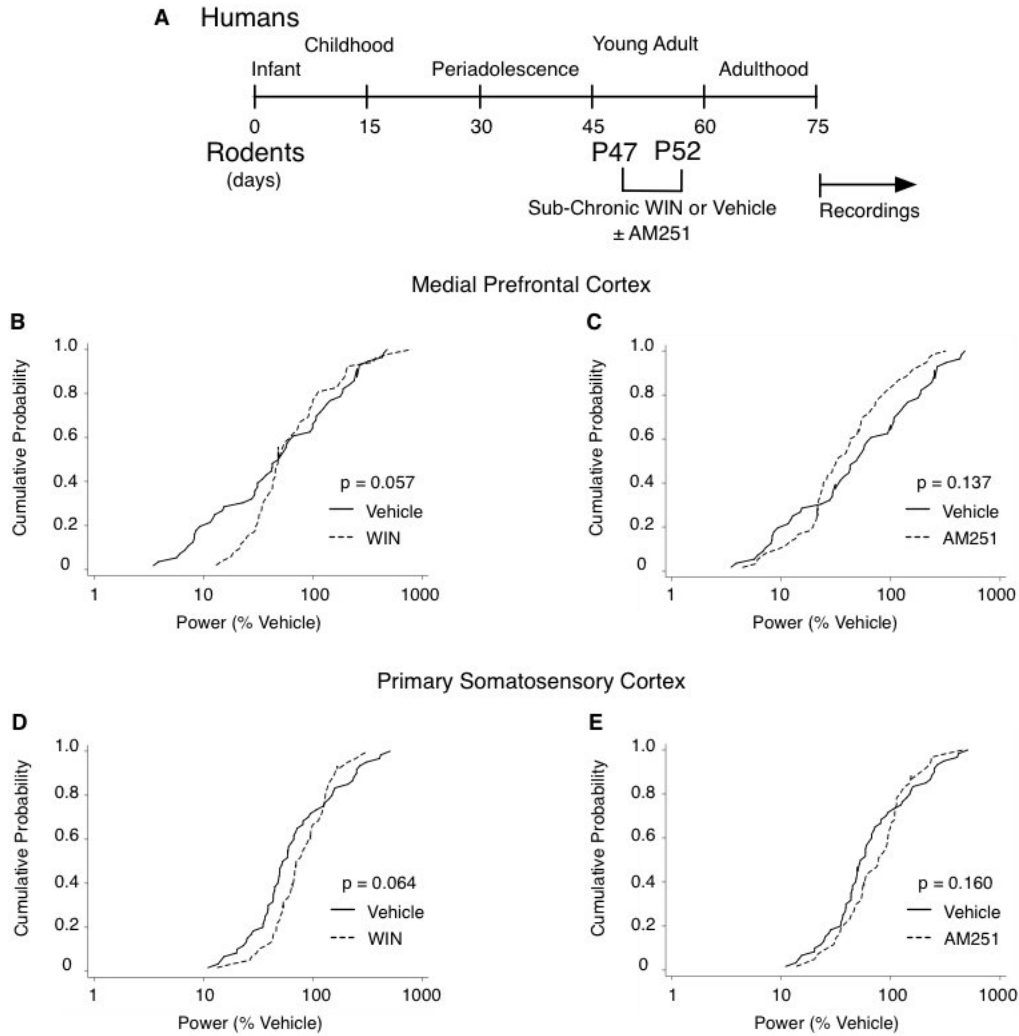


Figure 8: Sub-chronic administration of WIN in late adolescence does not suppress pharmacologically-evoked oscillations *in vitro* in mPFC or SCx. (A) Experimental time course. The CB1R agonist WIN (2 mg/kg) or the CB1R inverse agonist/antagonist AM251 (2 mg/kg) or vehicle were administered to late adolescent mice (P47-P52) once daily for 6 days. LFPs were recorded in brain slices from adult mice (>P100). (B,C) Cumulative probability distributions of normalized oscillation power plotted on a logarithmic scale for late adolescent vehicle (solid line) or (B) WIN (dashed line) or (C) AM251 (dashed line) treated LFPs recorded from mPFC *in vitro*. KS tests were used to compare the effect of adolescent treatment on normalized oscillation power (significant $p < 0.05$). (D,E) Cumulative probability distributions of normalized oscillation power plotted on a logarithmic scale for late adolescent vehicle (solid line) or (D) WIN (dashed line) or (E) AM251 (dashed line) treated LFPs recorded from SCx *in vitro*. Statistical analyses were performed as in (B,C).

B. Chronic Adolescent THC Exposure Persistently Suppresses Pharmacologically-Evoked Cortical Oscillations in vitro in SCx, but not in mPFC, Through CB1Rs

A published report from our lab indicates that chronic, 20-day exposure to THC and WIN significantly suppresses pharmacologically evoked oscillations in adult mPFC and SCx (Raver *et al.*, 2013). We tested the prediction that these chronic effects were mediated by CB1R activation, similar to sub-chronic adolescent (Cass *et al.*, 2014) and acute WIN or THC suppression of oscillations (Hajos *et al.*, 2000; Robbe *et al.*, 2006; Holderith *et al.*, 2011). Animals were exposed to THC (5 mg/kg), AM251 (0.3 or 0.5 mg/kg), AM251 + THC, or vehicle chronically during adolescence (P35-P55), and cortical oscillations were evoked in adulthood as previously described (Figure 9A). Statistical summaries of chronic adolescent pharmacology results from mPFC are presented in Table 3, and summaries of results from SCx are presented in Table 4. Chronic adolescent exposure to THC significantly suppressed the power of pharmacologically evoked oscillations in both adult mPFC (Figure 9B, Table 3) and SCx (Figure 9D, Table 4). To test whether the CB1R antagonist AM251 would prevent these effects, we first analyzed oscillations in animals administered AM251 alone, as previous reports have demonstrated no effect of acute or sub-chronic adolescent CB1R inverse agonist/antagonist exposure on cortical oscillations (Robbe *et al.*, 2006; Holderith *et al.*, 2011; Sales-Carbonell *et al.*, 2013; Cass *et al.*, 2014). Surprisingly, in mPFC, both 0.3 and 0.5 mg/kg AM251 significantly suppressed oscillations, relative to vehicle levels (Figure 9C, Table 3). We therefore reasoned that we could not use these doses of AM251 to try to prevent the chronic effects of THC in mPFC, as they produced the same phenotype as THC exposure.

Oscillations in SCx were insensitive to either 0.3 or 0.5 mg/kg AM251 (Figure 9E, Table 4), which allowed us to test the prediction that these doses of AM251 could attenuate suppression of oscillation power by THC. AM251 (0.3 mg/kg) paired with THC prevented oscillation suppression by adolescent THC exposure (Figure 9F, Table 4). Oscillation power in animals treated with 0.3 mg/kg AM251 + THC was significantly higher than in mice exposed to THC alone, and was equal to vehicle power (Figure 9F, Table 4), indicating a full attenuation of oscillation suppression by THC. AM251 (0.5 mg/kg) + THC partially attenuated the effects of THC. Oscillation power in SCx of animals treated with 0.5 AM251 + THC fell between that of animals exposed to THC or vehicle, and was therefore statistically indistinguishable from either THC or vehicle control power (Figure 9G, Table 4). The full attenuation of THC's oscillation suppression by 0.3 mg/kg AM251, and partial reversal by 0.5 mg/kg AM251, indicate that chronic adolescent THC suppresses oscillations in SCx via CB1Rs.

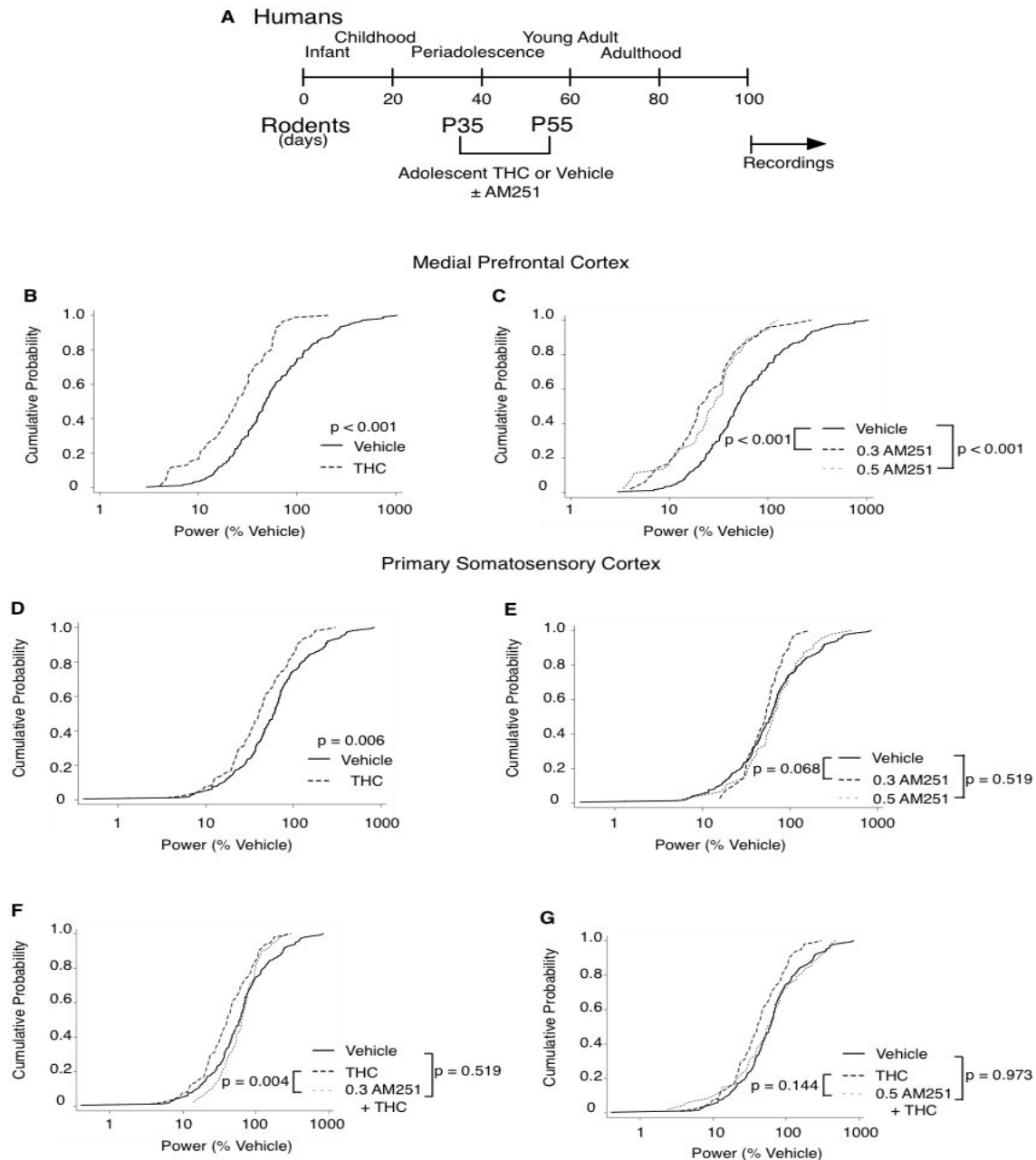


Figure 9: Chronic administration of THC in adolescence suppresses pharmacologically-evoked oscillations *in vitro* in mPFC and in SCx via CB1Rs. (A) Experimental time course. The CB1R agonist THC was administered with vehicle or the CB1R inverse agonist/antagonist AM251 (0.3 or 0.5 mg/kg) to adolescent (P35-P55) mice once daily for 20 days. LFPs were recorded in brain slices from adult mice (>P100). (B,C) Cumulative probability distributions of normalized oscillation power plotted on a logarithmic scale for adolescent vehicle (solid line) or (B) THC (dashed line) or (C) AM251 [(0.3 mg/kg; dashed line) or (0.5 mg/kg; dotted line)] treated LFPs recorded from mPFC *in vitro*. KS tests were used to compare the effect of adolescent treatment on normalized oscillation power (significant $p < 0.05$). (D,E) Cumulative probability distributions of normalized oscillation power plotted on a logarithmic scale for adolescent vehicle (solid line) or (D) THC (dashed line) or (E) AM251 [(0.3 mg/kg; dashed line) or (0.5 mg/kg; dotted line)] treated LFPs recorded from SCx *in vitro*. KS tests were used to compare the effect of

adolescent treatment on normalized oscillation power (significant $p < 0.05$). (F,G) Cumulative probability distributions of normalized oscillation power plotted on a logarithmic scale for adolescent vehicle (solid line) or (F) THC (dashed line) or 0.3 mg/kg AM251 + THC (dotted line) or (G) THC (dashed line) or 0.5 mg/kg AM251 + THC (dotted line). THC data was compared to AM251 (0.3 or 0.5 mg/kg) + THC, and AM251 (0.3 or 0.5 mg/kg) + THC was compared to vehicle data with KS tests (significant $p < 0.05$).

| Chronic Adolescent Cannabinoid Treatment | mPFC |
|--|--|
| Adolescent THC Treatment Vehicle (n = 60 slices/26 mice) vs. THC (5 mg/kg) (n = 21 slices/7 mice) | p < 0.001, 51% |
| Adolescent AM251 Treatment Vehicle (n = 60 slices/26 mice) vs. AM251 (0.3 mg/kg) (n = 12 slices/4 mice) Vehicle (n = 60 slices/26 mice) vs. AM251 (0.5 mg/kg) (n = 11 slices/4 mice) Vehicle (n = 60 slices/26 mice) vs. AM251 (1.0 mg/kg) (n = 7 slices/3 mice) | p < 0.001, 43% p < 0.001, 58% p = 0.010, 63% |
| Adolescent WIN Treatment Vehicle (n = 60 slices/26 mice) vs. WIN (1 mg/kg) (n = 50 slices/20 mice) | p < 0.001, 56% |
| Adolescent AM4113 Treatment Vehicle (n = 60 slices/26 mice) vs. AM4113 (1 mg/kg) (n = 19 slices/8 mice) | p = 0.031, 78% |
| Adolescent AM630 Treatment Vehicle (n = 60 slices/26 mice) vs. AM630 (1 mg/kg) (n = 15 slices/6 mice) | p < 0.001, 53% |
| Adolescent WIN-3 Treatment Vehicle (n = 60 slices/26 mice) vs. WIN-3 (1 mg/kg) (n = 18 slices/7 mice) | p < 0.001, 49% |

Table 3: Summary of statistical analysis of chronic adolescent (P35-P55) cannabinoid effects on the normalized power of pharmacologically-evoked LFP oscillations recorded *in vitro* in LII/III of the prelimbic area of mPFC in adult mice. Kolmogorov-Smirnoff tests determined significant differences between treatment conditions (significant $p < 0.05$). Percentages reported are the median normalized LFP power in the treatment after “vs” in the statistical comparison, relative to the median normalized LFP power in the treatment before “vs.”

| Chronic Adolescent Cannabinoid Treatment | SCx |
|---|--|
| <p style="text-align: center;">Adolescent THC Treatment</p> <p>Vehicle (n = 61 slices/24 mice) vs. THC (5 mg/kg) (n = 24 slices/7 mice)</p> | p = 0.006, 66% |
| <p style="text-align: center;">Adolescent AM251 Treatment</p> <p>Vehicle (n = 61 slices/24 mice) vs. AM251 (0.3 mg/kg) (n = 10 slices/4 mice)</p> <p>Vehicle (n = 61 slices/24 mice) vs. AM251 (0.5 mg/kg) (n = 12 slices/4 mice)</p> <p>Vehicle (n = 61 slices/24 mice) vs. AM251 (1.0 mg/kg) (n = 8 slices/3 mice)</p> | <p>p = 0.068</p> <p>p = 0.519</p> <p>p < 0.010, 59%</p> |
| <p style="text-align: center;">Adolescent THC + AM251 Treatment</p> <p>THC (5 mg/kg) (n = 24 slices/7 mice) vs. + AM251 (0.3 mg/kg) + THC (5 mg/kg) (n = 12 slices/4 mice)</p> <p>Vehicle (n = 61 slices/24 mice) vs. + AM251 (0.3 mg/kg) + THC (5 mg/kg) (n = 12 slices/4 mice)</p> | <p>p = 0.004, 160%</p> <p>p = 0.519</p> |
| <p style="text-align: center;">Adolescent THC + AM251 Treatment</p> <p>THC (5 mg/kg) (n = 24 slices/7 mice) vs. + AM251 (0.5 mg/kg) + THC (5 mg/kg) (n = 12 slices/4 mice)</p> <p>Vehicle (n = 61 slices/24 mice) vs. + AM251 (0.5 mg/kg) + THC (5 mg/kg) (n = 12 slices/4 mice)</p> | <p>p = 0.973</p> <p>p = 0.144</p> |
| <p style="text-align: center;">Adolescent WIN Treatment</p> <p>Vehicle (n = 61 slices/24 mice) vs. WIN (1 mg/kg) (n = 55 slices/21 mice)</p> | p = 0.003, 74% |
| <p style="text-align: center;">Adolescent AM4113 Treatment</p> <p>Vehicle (n = 61 slices/24 mice) vs. AM4113 (1 mg/kg) (n = 22 slices/8 mice)</p> | p = 0.621 |
| <p style="text-align: center;">Adolescent AM4113 + WIN Treatment</p> <p>WIN (1 mg/kg) (n = 55 slices/21 mice) vs. AM4113 (1 mg/kg) + WIN (1 mg/kg) (n = 20 slices/7 mice)</p> <p>Vehicle (n = 61 slices/24 mice) vs. AM4113 (1 mg/kg) + WIN (1 mg/kg) (n = 20 slices/7 mice)</p> | <p>p = 0.021, 63%</p> <p>p < 0.001, 46%</p> |
| <p style="text-align: center;">Adolescent AM630 Treatment</p> <p>Vehicle (n = 61 slices/24 mice) vs. AM630 (1 mg/kg) (n = 19 slices/6 mice)</p> | p = 0.009, 67% |
| <p style="text-align: center;">Adolescent WIN-3 Treatment</p> <p>Vehicle (n = 61 slices/24 mice) vs. WIN-3 (1 mg/kg) (n = 21 slices/7 mice)</p> | p = 0.312 |

Table 4: Summary of statistical analysis of chronic adolescent (P35-P55) cannabinoid effects on the normalized power of pharmacologically-evoked LFP oscillations recorded *in vitro* in LII/III of the barrel field of SCx in adult mice. Kolmogorov-Smirnoff tests determined significant differences between treatment conditions (significant $p < 0.05$). Percentages reported are the median normalized LFP power in the treatment after “vs” in the statistical comparison, relative to the median normalized LFP power in the treatment before “vs.”

C. Chronic Adolescent CB1R Antagonist (AM251) Exposure Persistently Suppresses Pharmacologically-Evoked Cortical Oscillations *in vitro* in mPFC and SCx

We tested the prediction that chronic adolescent WIN exposure suppresses pharmacologically-evoked oscillations in the adult neocortex by acting on CB1Rs. We exposed adolescent (P35-P55) male CD-1 mice to WIN alone (Figure 10A) or in combination with AM251 (Figure 11A) and evoked oscillations in adult neocortical slices containing mPFC or SCx, as previously described.

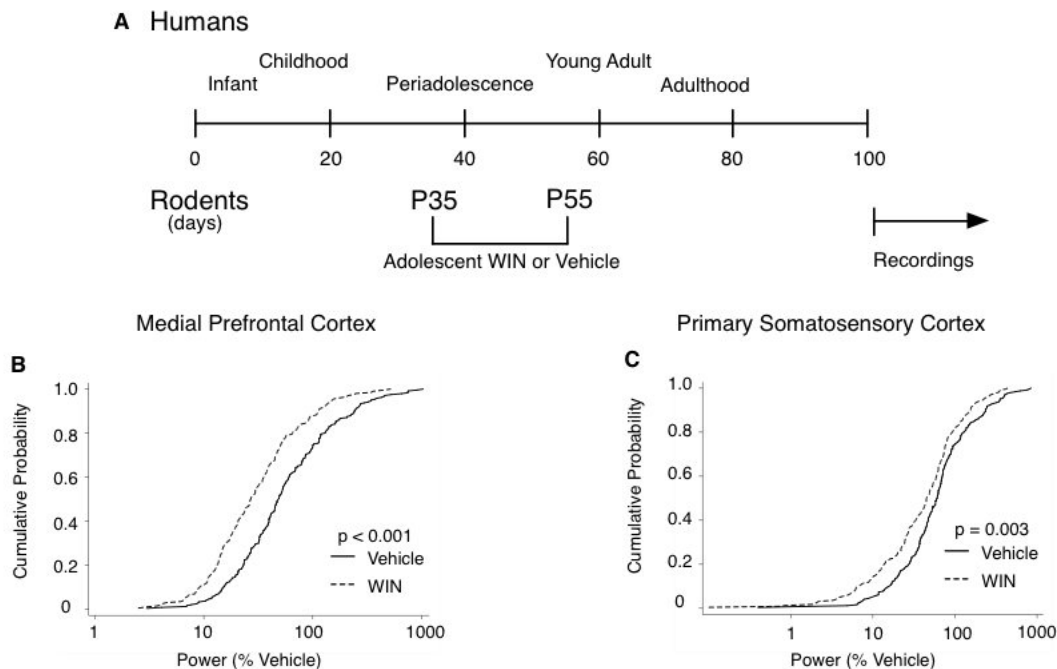


Figure 10: Chronic administration of WIN during adolescence suppresses pharmacologically-evoked oscillations *in vitro* in mPFC and SCx. (A) Experimental time course. The CB1R agonist WIN55,212-2 (WIN; 1 mg/kg) or vehicle was administered to adolescent (P35–P55) mice once daily for 20 days. LFPs were recorded in brain slices from adult mice (>P100). (B) Cumulative probability distributions of normalized oscillation power (% vehicle mean) were plotted on a logarithmic scale for adolescent vehicle (solid line) or WIN (dashed line) treated LFPs recorded from mPFC *in vitro*. Kolmogorov-Smirnoff (KS) tests were used to compare the effect of adolescent treatment on normalized oscillation power (significant $p < 0.05$). (C) Cumulative probability distributions of normalized oscillation power plotted on a logarithmic scale for adolescent vehicle (solid line) or WIN (dashed line) treated LFPs recorded from SCx *in vitro*. Statistical analyses were performed as in (B).

Chronic adolescent exposure to WIN (1 mg/kg) significantly suppressed the power of pharmacologically evoked oscillations in both mPFC (Figure 10B, Table 3) and

SCx (Figure 10C, Table 4). We analyzed oscillations in animals exposed to AM251 (1 mg/kg) (Figure 11A) to test the prediction that AM251 has no effect on its own. Animals were exposed to a slightly higher dose of AM251 in this study than what was used in the THC study (1 mg/kg vs. 0.3 or 0.5 mg/kg) because WIN is a full CB1 agonist, whereas THC displays partial agonist activity at the CB1R (Pertwee, 2008; Pertwee, 2010). Therefore, we predicted that a higher dose of AM251 would be necessary to antagonize WIN's effects. Unexpectedly, oscillations in mice chronically exposed to 1 mg/kg AM251 during adolescence were significantly suppressed in both mPFC (Figure 11B, Table 3) and SCx (Figure 11E, Table 4). We therefore reasoned that we could not use AM251 at this dose to try to antagonize the effects of chronic WIN exposure, as exposure to the antagonist alone mimicked the effects seen with WIN.

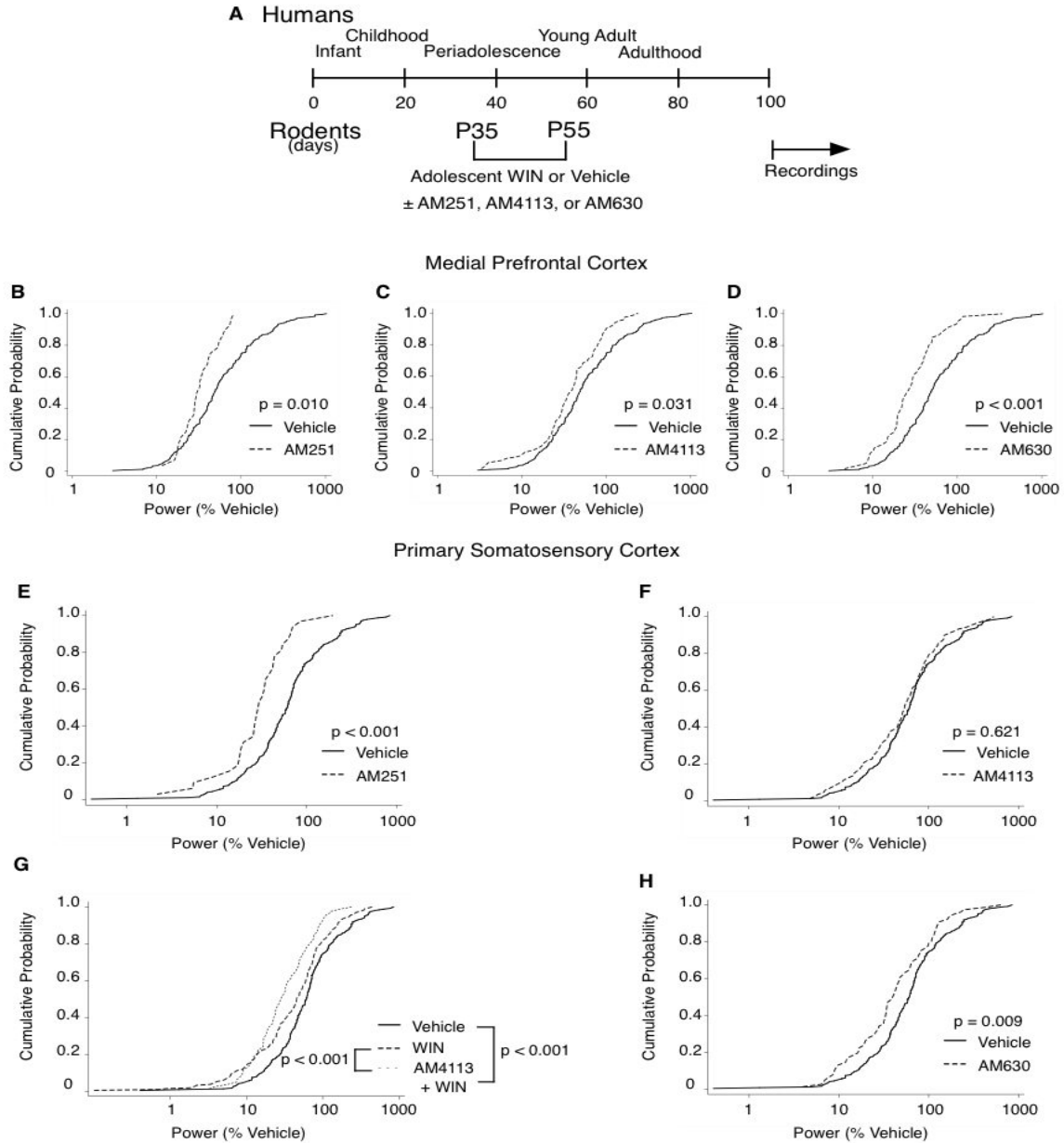


Figure 11: Chronic administration of CB1R and CB2R antagonists during adolescence suppresses pharmacologically-evoked oscillations *in vitro* in mPFC and SCx. (A) Experimental time course. The CB1R inverse agonist/antagonist AM251 (1 mg/kg), the CB1R neutral antagonist AM4113 (1 mg/kg), or the CB2R inverse agonist/antagonist AM630 (1 mg/kg) were administered either with vehicle or WIN (1 mg/kg) to adolescent (P35-P55) mice once daily for 20 days. LFPs were recorded in brains slices from adult mice (>P100). (B-D) Cumulative probability distributions of normalized oscillation power were plotted on a logarithmic scale for adolescent vehicle (solid line) or (B) AM251 (dashed line), (C) AM4113 (dashed line), or (D) AM630 (dashed line) treated LFPs recorded from mPFC *in vitro*. KS tests were used to compare the effect of adolescent treatment on normalized oscillation power (significant $p < 0.05$). (E,F) Cumulative probability distributions of normalized oscillation power were plotted on a logarithmic scale for adolescent vehicle (solid line) or (E) AM251 (dashed line) or (F) AM4113 treated LFPs recorded from SCx *in vitro*. Statistical analyses were performed as in (B-D). (G) Cumulative probability distributions of normalized oscillation power were plotted on a logarithmic scale for adolescent vehicle (solid line), WIN (dashed line), or AM4113 + WIN (dotted line) treated LFPs recorded from SCx *in vitro*. WIN data was

compared to AM4113 + WIN, and AM4113 + WIN was compared to vehicle data with KS tests (significant $p < 0.05$). (H) Cumulative probability distributions of normalized oscillation power were plotted on a logarithmic scale for adolescent vehicle (solid line) or AM630 (dashed line) treated LFPs recorded from SCx *in vitro*. Statistical analyses were performed as in (B-D).

D. Chronic Adolescent CB1R Antagonist (AM4113) Exposure Persistently Suppresses Pharmacologically-Evoked Cortical Oscillations in vitro in mPFC, but not SCx, and Does Not Reverse WIN's Effects in SCx

AM251 can antagonize CB1R mediated effects of cannabinoid agonists through its action as an inverse agonist. Acting as such, AM251 both blocks the activity of endogenous and exogenous CB1R agonists and decreases the constitutive activity of CB1Rs (Pertwee, 2005). Therefore, we next tested the prediction that the neutral CB1R antagonist AM4113 would antagonize WIN's effects without altering oscillations on its own, since a neutral antagonist should not affect constitutive CB1R activity, but only prevent agonist activity at the CB1R (Jarbe *et al.*, 2008).

However, as was the case with AM251, chronic adolescent administration of the neutral CB1R antagonist AM4113 alone (1 mg/kg) (Figure 11A) significantly suppressed oscillations in mPFC, relative to vehicle-treated animals (Figure 11C, Table 3). These unexpected effects of the CB1R neutral antagonist AM4113 may be due to chronic antagonism of eCBs at the CB1R, and will be further addressed in the Discussion. We again reasoned that we could not use AM4113 to try to attenuate chronic WIN's oscillation suppression in mPFC, as it produces the same phenotype as WIN. In SCx, AM4113 (1 mg/kg) did not alter oscillation power when administered alone (Figure 11F, Table 4), allowing us to test the prediction that AM4113 would attenuate WIN's suppression. This was not the case. Chronic adolescent administration of AM4113 did not attenuate the significant oscillation suppression induced by WIN in SCx, but,

rather, further suppressed power relative to both vehicle and WIN levels (Figure 11G).

E. Chronic Adolescent CB2R Antagonist (AM630) Exposure Persistently Suppresses Pharmacologically-Evoked Cortical Oscillations in vitro in mPFC and SCx

While WIN acts as a full agonist at the CB1R, it also has high affinity for CB2Rs (Showalter *et al.*, 1996; Pertwee, 2008). Although CB2R expression has historically been thought to be confined to peripheral tissues, mounting evidence indicates the presence of neuronal and glial CB2Rs in the CNS (Gong *et al.*, 2006; Onaivi *et al.*, 2006; Atwood and Mackie, 2010). Thus far, we have been unable to test whether chronic adolescent WIN exposure acts via CB1Rs to suppress oscillations in adult mPFC, as CB1R antagonists exert similar effects on their own. Therefore, we next tested the prediction that the CB2R antagonist, AM630, would not alter the response to WIN, if WIN suppressed oscillations exclusively via CB1Rs (Figure 11A).

As above, the requisite experiment is predicated on AM630 alone not having lasting effects on cortical oscillations. However, chronic adolescent AM630 exposure alone significantly suppressed oscillations in adult mPFC (Figure 11D, Table 3) and SCx (Figure 11H, Table 4). We were therefore unable to test whether AM630 can antagonize WIN's effects, as AM630 produced similar results to those seen with WIN. We were also unable to exclude the possibility that CB2Rs may contribute to the effects of WIN, as a CB2R antagonist mimicked WIN's effects.

F. Chronic Adolescent Exposure to Compound with Putative CB1R Inactivity (WIN-3) Persistently Suppresses Pharmacologically-Evoked Cortical Oscillations in vitro in mPFC, but not SCx

Thus far, we have shown that the CB1R/CB2R agonists WIN and THC, a CB1R

inverse agonist/antagonist and neutral antagonist, and a CB2R inverse agonist/antagonist suppress oscillations in adult neocortex when chronically administered during adolescence, with milder effects in SCx than mPFC. To further test whether these effects were specific to compounds with CB1R or CB2R activity, we exposed adolescent animals to a drug that is presumed to be inactive at cannabinoid receptors. The chemical enantiomer of WIN55,212-2 (WIN) — WIN55, 212-3 (WIN-3) (Figure 12A) — has been described to be inactive at the CB1R at low concentrations (Pacheco *et al.*, 1991; Felder *et al.*, 1992). If the oscillation suppressing effects of chronic adolescent WIN are due to its action at cannabinoid receptors, then we predict that exposure to WIN-3 would have no effect on cortical oscillations.

Unexpectedly, chronic exposure to WIN-3 during adolescence significantly suppressed oscillations in mPFC, relative to those recorded in vehicle-treated adult mice (Figure 12B, Table 3). In agreement with our prediction, we found no effect of adolescent WIN-3 on oscillations recorded in adult SCx (Figure 12C, Table 4), indicating that WIN-3's lack of effect was due to its predicted mechanism as an inactive enantiomer of WIN55,212-2 in SCx, but that it may be exerting non-CB1R effects in mPFC. Therefore, while sub-chronic and acute WIN exposure suppresses oscillations through CB1Rs, it appears that chronically administered WIN may exert these suppressive effects through CB2Rs or via non-cannabinoid targets.

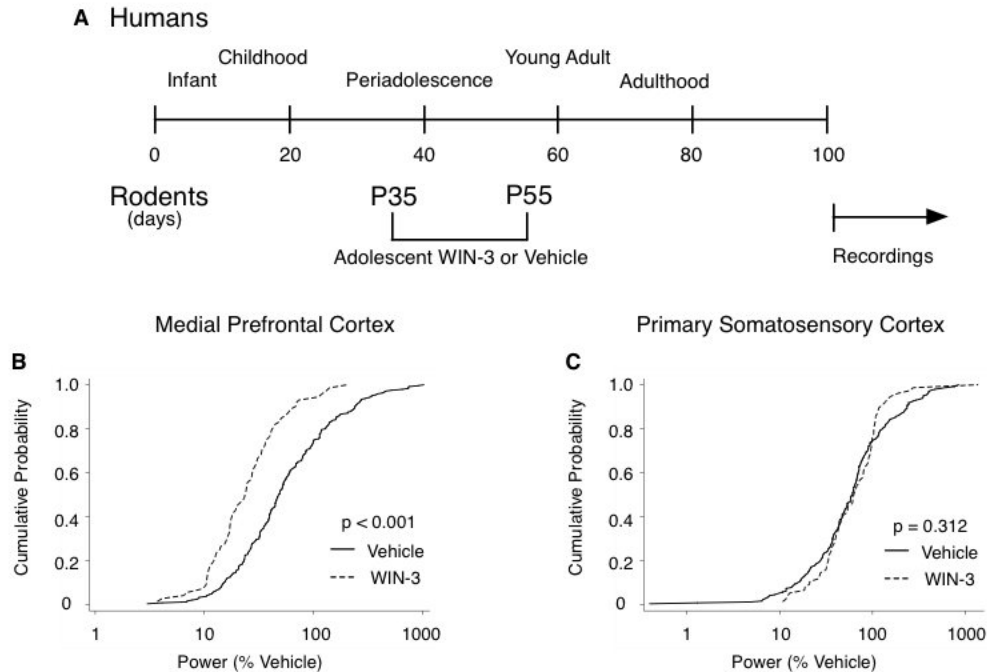


Figure 12: Chronic administration of the putative inactive enantiomer WIN55,212-3 (WIN-3) in adolescence suppresses pharmacologically-evoked oscillations *in vitro* in mPFC but not SCx. (A) Experimental time course. WIN-3 (1 mg/kg) was administered to adolescent mice (P35-P55) once daily for 20 days. LFPs were recorded in brain slices from adult mice (>P100). (B,C) Cumulative probability distributions of normalized oscillation power were plotted on a logarithmic scale for adolescent vehicle (solid line) or WIN-3 (dashed line) treated LFPs recorded from (B) mPFC or (C) SCx *in vitro*. KS tests were used to compare the effect of adolescent treatment on normalized oscillation power (significant $p < 0.05$).

G. Chronic Adolescent WIN and THC Exposure Have Mixed Effects in Mice

Genetically Lacking the CB1R

As a final test of our hypothesis that permanent suppression of pharmacologically-evoked cortical oscillations by chronic adolescent WIN or THC exposure is acting via CB1Rs, we tested the effects of WIN and THC (P35-P55) in mice lacking these receptors from birth (CB1R KO mice). We predicted that, if WIN and THC were acting exclusively via CB1Rs, then oscillations recorded in adult CB1R KO mice exposed to WIN or THC in adolescence would not differ from those in vehicle-administered animals. Male and female CB1R KO and WT mice were generated from

mating mice heterozygous for the CB1R (a generous gift from Dr. Eliot Gardner at NIDA) as described previously (Zimmer *et al.*, 1999; Xi *et al.*, 2011) and were genotyped at weaning age. Male and female litter-mates homozygous for the CB1R (CB1R WT) were used as control animals. Mice were injected with WIN (1 mg/kg), THC (5 mg/kg) or vehicle from P35-P55 (Figure 13A). Statistical summaries of pharmacological effects in CB1R WT and KO mice are presented in Table 5A (mPFC data) and Table 5B (SCx data).

In the limited number of CB1R WT mice that were generated in our laboratory during the time available for these experiments, we were unable to replicate our previous findings of robust WIN and THC-induced oscillation suppression (Figures 13B,C, Table 5A,B). In mPFC of CB1R WT mice, oscillation power after chronic adolescent WIN exposure did not significantly differ from that of vehicle administered controls (Figure 13B, Table 5A). Oscillation power in mPFC of animals exposed to THC was unexpectedly and significantly higher than that seen in vehicle-administered CB1R WT mice (Figure 13B, Table 5A). In SCx, WIN significantly suppressed oscillations in CB1R WT mice as expected, but THC had no effect (Figure 13C, Table 5B).

Chronic adolescent WIN and THC exposure had mixed effects in CB1R KO mice. In CB1R KO mice exposed to THC during adolescence, oscillations in mPFC had significantly higher power relative to vehicle-treated mice (Figure 13D, Table 5A), while oscillation power in WIN-treated CB1R KO mice approached but did not reach statistical significance, with WIN power being higher than vehicle (Figure 13D, Table 5A). Similar oscillation power enhancement after chronic adolescent cannabinoid exposure was recorded in SCx of CB1R KO mice (Figure 13E, Table 5B). WIN significantly increased

oscillation power relative to vehicle levels (Figure 13E, Table 5B), while THC had no significant effect on oscillations (Figure 13E, Table 5B). Because we were unable to replicate our previously reported effects of WIN and THC in the control CB1R WT animal population, we cannot draw definitive conclusions about the effects of these cannabinoids in CB1R KO mice.

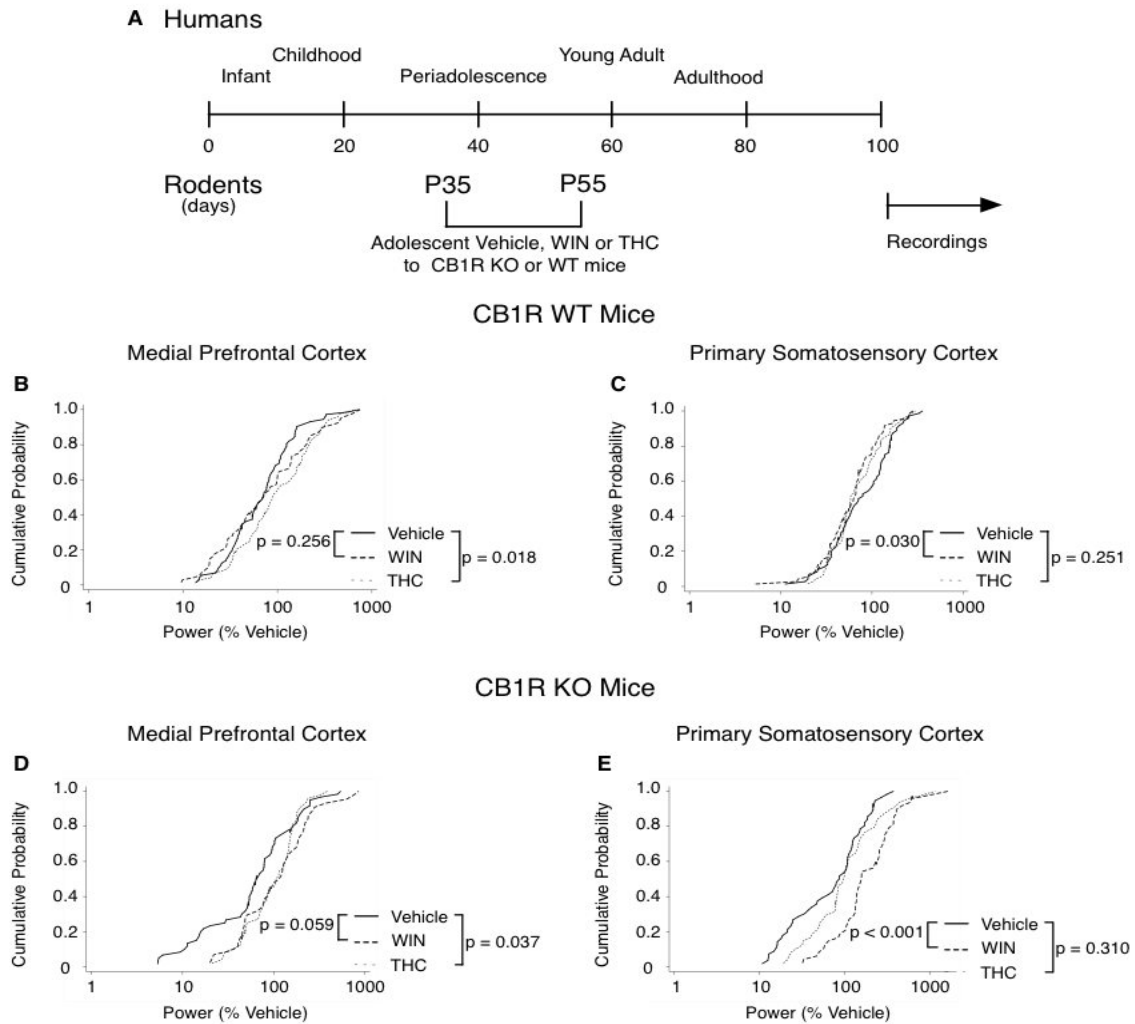


Figure 13: Administration of WIN or THC in adolescence has mixed effects on pharmacologically-evoked oscillations *in vitro* in mPFC and SCx of CB1R wild-type (CB1R WT) and knockout (CB1R KO) mice. (A) Experimental time course. The CB1R agonists WIN (1 mg/kg) or THC (5 mg/kg) or vehicle were administered to adolescent CB1R WT or CB1R KO (P35-P55) once daily for 20 days. LFPs were recorded in brain slices from adult mice (>P100). (B,C) Cumulative probability distributions of normalized oscillation power were plotted on a logarithmic scale for adolescent vehicle (solid line) or WIN (dashed line) or THC (dotted line) treated LFPs from (B) mPFC or (C) SCx of CB1R WT mice. WIN and THC data were compared to vehicle data with KS tests (significant $p < 0.05$). (D,E) Cumulative probability

distributions of normalized oscillation power were plotted on a logarithmic scale for adolescent vehicle (solid line) or WIN (dashed line) or THC (dotted line) treated LFPs from (D) mPFC or (E) SCx of CB1R KO mice. Statistical analyses were performed as in (B,C).

A

| | |
|---|-----------------|
| Chronic Adolescent Cannabinoid Treatment in CB1R WT Mice | mPFC |
| Chronic Adolescent WIN Treatment Vehicle (n = 18 slices/6 mice) vs. WIN (1 mg/kg) (n = 17 slices/6 mice) | p = 0.256 |
| Chronic Adolescent THC Treatment Vehicle (n = 18 slices/6 mice) vs. THC (5 mg/kg) (n = 18 slices/6 mice) | p = 0.018, 126% |
| Chronic Adolescent Cannabinoid Treatment in CB1R KO Mice | mPFC |
| Chronic Adolescent WIN Treatment Vehicle (n = 15 slices/5 mice) vs. WIN (1 mg/kg) (n = 11 slices/4 mice) | p = 0.059 |
| Chronic Adolescent THC Treatment Vehicle (n = 15 slices/5 mice) vs. THC (5 mg/kg) (n = 12 slices/4 mice) | p = 0.037, 169% |

B

| | |
|---|-----------------|
| Chronic Adolescent Cannabinoid Treatment in CB1R WT Mice | SCx |
| Chronic Adolescent WIN Treatment Vehicle (n = 18 slices/6 mice) vs. WIN (1 mg/kg) (n = 18 slices/6 mice) | p = 0.030, 85% |
| Chronic Adolescent THC Treatment Vehicle (n = 18 slices/6 mice) vs. THC (5 mg/kg) (n = 17 slices/6 mice) | p = 0.251 |
| Chronic Adolescent Cannabinoid Treatment in CB1R KO Mice | SCx |
| Chronic Adolescent WIN Treatment Vehicle (n = 14 slices/5 mice) vs. WIN (1 mg/kg) (n = 12 slices/4 mice) | p = 0.001, 182% |
| Chronic Adolescent THC Treatment Vehicle (n = 15 slices/5 mice) vs. THC (5 mg/kg) (n = 12 slices/4 mice) | p = 0.310 |

Table 5: (A) Summary of statistical analysis of chronic adolescent (P35-P55) cannabinoid effects on the normalized power of pharmacologically-evoked LFP oscillations recorded *in vitro* in LII/III of the prelimbic area of mPFC in adult CB1R WT and CB1R KO mice. Kolmogorov-Smirnoff tests determined significant differences between treatment conditions (significant $p < 0.05$). Percentages reported are the median normalized LFP power in the treatment after “vs” in the statistical comparison, relative to the

median normalized LFP power in the treatment before “vs.” (B) Summary of statistical analysis of chronic adolescent cannabinoid effects recorded in LII/III of the barrel field in SCx. Analyses were performed as in (A).

IV: Discussion

In Chapter 2 we test the hypothesis that repeated cannabinoid exposure during adolescence permanently suppresses oscillations in adult neocortex by acting at CB1Rs. A short period of WIN exposure during the first 6 days of adolescence suppresses oscillations in mPFC, but not SCx, and this effect is mediated by the CB1R. The same administration paradigm late in adolescence does not alter oscillations in either cortical region. The early adolescent sensitivity of mPFC to CB1R activation that we describe parallels recent work demonstrating a selective impairment of LFP modulation in the mPFC that is induced by short-term WIN exposure during early and mid, but not late, adolescence, and is attenuated by AM251 (Cass *et al.*, 2014). Data from SCx also supports the hypothesis of a CB1R-mediated effect, as oscillation suppression by chronic adolescent THC exposure is fully reversed to vehicle levels by low doses of AM251 that do not produce effects on their own. Multiple attempts to test the prediction that mPFC exhibits similar CB1R-mediated suppression of oscillations were inconclusive, as we were unable to find a dose of CB1R or CB2R antagonist that does not suppress oscillations in mPFC when administered alone.

A. CB1R Modulation of Cortical Oscillations

Experiments performed in the CA3 region of hippocampus suggest that *acute* cannabinoid exposure suppresses pharmacologically evoked gamma oscillations *in vitro* by acting at CB1Rs expressed on glutamatergic terminals, to suppress excitatory postsynaptic currents (Holderith *et al.*, 2011). This action may preferentially attenuate the frequency and firing precision of fast-spiking GABAergic interneurons that are crucial

for sculpting gamma oscillations (Cardin *et al.*, 2009), resulting in smaller and less synchronized field potential activity (Holderith *et al.*, 2011) that manifests as reduced oscillatory power. A similar mechanism may underlie the CB1R mediated suppression of oscillation power that we find in SCx after chronic THC administration, and in mPFC after sub-chronic early adolescent WIN exposure. However, the mechanisms by which acute cannabinoid exposure suppresses high-frequency oscillations recorded in the neocortex are less clear. The contributions of CB1Rs expressed on different neural populations to the effects of acute cannabinoid exposure on cortical ECoG oscillations were recently examined (Sales-Carbonell *et al.*, 2013). Acute CB1R agonist exposure suppresses oscillations faster than 12 Hz, and this effect is reversed by CB1R antagonist administration. Previous work (Holderith *et al.*, 2011) suggests that knocking out CB1Rs from excitatory neurons should fully prevent the suppressive effects of acute cannabinoid administration on ECoG activity faster than 12 Hz, if cortical oscillation suppression is similarly mediated by CB1Rs on excitatory neurons. Interestingly, this cannabinoid-induced suppression of ECoG power was attenuated, but not completely prevented by removing CB1Rs from either glutamatergic cortical neurons, or from all principal forebrain neurons. Furthermore, while activation of CB1Rs on inhibitory interneurons may not contribute to oscillation suppression by CB1R agonists in the CA3 region of the hippocampus *in vitro* (Holderith *et al.*, 2011), removal of CB1Rs from all GABAergic forebrain neurons *increases* cannabinoid agonist-mediated suppression of cortical oscillatory activity (Sales-Carbonell *et al.*, 2013) *in vivo*, suggesting a potential bidirectional regulation of oscillatory activity by CB1Rs localized to glutamatergic and GABAergic neurons. Interestingly, in the CA1 region of the hippocampus, CB1R-

positive interneurons can generate theta rhythms when driven by muscarinic acetylcholine receptor (mAChR) agonists when excitatory transmission is blocked, and these rhythms can be completely abolished by exogenous and endogenous CB1R agonists (Nagode *et al.*, 2011; Nagode *et al.*, 2014), revealing a role for inhibitory-localized CB1Rs in pharmacologically-evoked oscillation suppression. Further tests of the contribution of CB1Rs expressed by these different neuronal populations to adolescent cannabinoid-induced suppression of cortical oscillations could be examined using transgenic mouse strains lacking CB1Rs in specific neuronal populations to clarify whether cannabinoids attenuate oscillations in the cortex through different mechanisms than found in the CA3 or CA1 hippocampal networks, or whether there are other alternatives.

B. Preferential Sensitivity of Rostral mPFC vs. Caudal SCx

In agreement with data presented in Chapter 1, we find that the adolescent mPFC is highly sensitive to manipulation of the eCB system. Chronic exposure to CB1/CB2R agonists, CB1R antagonists, a CB2R antagonist and a putative inactive enantiomer of WIN (WIN-3) all significantly attenuate pharmacologically evoked oscillations *in vitro* in mPFC. The SCx is less sensitive to adolescent cannabinoid exposure, in parallel with the caudal-to-rostral gradient of cortical development (Gogtay *et al.*, 2004; Heng *et al.*, 2011). Oscillations in adult SCx, unlike in mPFC, are unaffected by either lower doses of a CB1R inverse agonist/antagonist or by a CB1R neutral antagonist. Furthermore, while a shorter period of cannabinoid exposure during early adolescence significantly suppresses oscillations in mPFC, oscillations in SCx are unaffected by such exposure. Although this caudal-to-rostral gradient of cortical sensitivity is probably due to the prolonged

adolescent and early adult maturation of prefrontal cortical circuitry (Gogtay *et al.*, 2004) and network oscillations (Uhlhaas and Singer, 2011), it may be somewhat specific to cannabinoid exposure. The density of CB1Rs declines more dramatically from juvenile to adult ages in rostral, associative cortical areas than in caudal, primary sensory cortical regions (Heng *et al.*, 2011), and expression of the receptor is significantly higher in mPFC than SCx throughout this period (Heng *et al.*, 2011) (Dr. K.Y. Tseng, personal communication). This gradient of CB1R expression may underlie the sensitivity of the mPFC to chronic adolescent cannabinoid exposure that we report, as well as the preferential suppression of oscillations in mPFC by early adolescent sub-chronic WIN treatment.

C. CB1R Antagonism and Cortical Oscillations

We are surprised to discover that chronic adolescent exposure to AM251 or AM4113, a CB1R inverse agonist/antagonist and neutral antagonist, respectively, significantly attenuates the power of oscillations in adult mPFC. Although several previous studies have shown that oscillation suppression by acute CB1R agonist exposure is reversed with co-administration of a CB1R antagonist (Hajos *et al.*, 2000; Robbe *et al.*, 2006; Hajos *et al.*, 2008; Holderith *et al.*, 2011; Sales-Carbonell *et al.*, 2013), there have been fewer tests of whether these agents modulate oscillatory activity by themselves. Such studies often show that administration of AM251 or SR141716, both CB1R antagonists, do not affect oscillation power (Robbe *et al.*, 2006; Holderith *et al.*, 2011; Sales-Carbonell *et al.*, 2013), leading to the conclusion that tonic eCB activity does not contribute to the generation of oscillations (Gulyas *et al.*, 2010). However, there exists evidence to the contrary (Kim *et al.*, 2002; Fortin *et al.*, 2004; Nagode *et al.*, 2014), and

this discrepancy will be discussed further in the General Discussion. Our data suggest a role for tonic eCB activity during adolescence, as chronic adolescent blockade of the CB1R with both AM251 and AM4113 lead to a persistent suppression of oscillatory power in the adult cortex. While we find it surprising that exposure to cannabinoid receptor antagonists recapitulate the effects seen with agonist administration, these effects are not unprecedented (Manzanedo *et al.*, 2010) as chronic administration of CB1R antagonists can cause lasting changes in cannabinoid receptor distribution and eCB levels (Guidali *et al.*, 2011; Castelli *et al.*, 2007), reminiscent of those seen after repeated CB1R agonist administration (Hunter and Burstein, 1997; Romero *et al.*, 1997; Sim-Selley and Martin, 2002; Rubino and Parolaro, 2008).

Through its action as a CB1 inverse agonist, AM251 modulates tonic CB1R activity by both reducing the receptor's constitutive activity in the absence of an agonist, and by occluding the action of endogenous CB1R agonists, such as the eCBs (Pertwee, 2005). AM41113 also interferes with endogenous CB1R agonist activity, but it lacks the ability to modulate constitutive CB1R signaling (Chambers *et al.*, 2007; Sink *et al.*, 2008). Therefore, similar results produced by both AM251 and AM4113 in mPFC suggest that chronic interference with ongoing CB1R activity during adolescence, such as antagonism or displacement of eCBs at the CB1R, impairs the ability of the adult mPFC to generate pharmacologically evoked oscillations. Further tests of this hypothesis could be performed by chronically administering indirect cannabinoid agonists that prevent the synthesis or breakdown of eCBs and would therefore interfere with tonic eCB signaling in adolescence. If chronic antagonism of eCBs at CB1Rs during adolescence contributes to persistent suppression of oscillations in adulthood, we would predict that inhibiting the

synthesis of the eCBs 2AG and anandamide by interfering with diacylglycerol (DAG) lipase or NAPE-PLD, respectively, would produce similar oscillation suppression as seen following CB1R/CB2R antagonist administration. Alternatively, if adolescent CB1R antagonist exposure suppresses oscillations by displacing eCBs from their intended receptors, subsequently allowing them to act at unoccupied CB1Rs or at non-CB1R targets, preventing the breakdown of 2AG or anandamide with inhibitors of monoglyceride lipase (MGL) or fatty acid amide hydrolase (FAAH), respectively, would enhance eCB tone and potentially mimic the persistent oscillation suppression induced by chronic CB1R antagonist administration. Interestingly, sustained inactivation of MGL activity, but not FAAH activity, induces functional antagonism of the eCB system (Schlosburg *et al.*, 2010), suggesting nuanced and potentially independent functions of these two eCBs.

D. Non-CB1R Contribution to Oscillation Suppression

In SCx, we find that chronic adolescent exposure to the neutral CB1R antagonist AM4113 alone does not alter oscillations in adults, allowing us to test for the predicted CB1R mechanism underlying WIN's effects. Unexpectedly, AM4113 paired with WIN suppresses oscillatory activity further than WIN alone, suggesting that AM4113 and WIN may act synergistically and suppress oscillations, or act at different targets. Because WIN has potent CB2R agonist activity in addition to its action at CB1Rs (Showalter *et al.*, 1996; Atwood *et al.*, 2012), we posit that CB2Rs may contribute to oscillation suppression by WIN. The CB2R is centrally expressed in cortical areas that include the prefrontal and somatosensory cortices (Gong *et al.*, 2006; Onaivi *et al.*, 2006), positioning it to contribute to the phenotype that we observe. This hypothesis of a CB2R

contribution is supported by data showing a significant suppression of oscillations in adult SCx and mPFC by the CB2R inverse agonist/antagonist AM630 alone, although we are unable to test whether AM630 ameliorates the effects of WIN due to oscillation suppression by actions at CB2Rs. Although centrally expressed CB2Rs are increasingly implicated in diverse functions, including cannabinoid-mediated memory impairments and drug reward and reinforcement (Atwood and Mackie, 2010; Xi *et al.*, 2011; Garcia-Gutierrez *et al.*, 2013), to date, little information exists regarding a possible role of the CB2R in network activity or oscillations. Interestingly, in entorhinal cortex, activation of CB2Rs suppresses GABAergic inhibition that is not occluded by CB1R antagonism (Morgan *et al.*, 2009), revealing the functional expression of CB2Rs at central inhibitory synapses that could play a role in oscillation generation. Further tests of a possible contribution of CB2Rs to oscillations are warranted, and could be accomplished by administering CB2R selective agonists, lowering the dose of AM630 to find one that produces no effect on its own, or by testing the effects of WIN in CB2R knockout mice. However, all results obtained from experiments with cannabinoid compounds must be interpreted with caution, as AM630, AM251, WIN and many other pharmaceutical agents used by the cannabinoid field and presumed to be specific for individual receptors, exert effects at targets in addition to the ones advertised (Ross *et al.*, 1999; Ryberg *et al.*, 2007; Pertwee, 2010). As we have neither independently verified the CB1R or CB2R specificity of these agonists and antagonists in our laboratory, nor performed complete dose-response curves, we cannot be sure of the precise mechanisms by which these drugs act in our experiments.

Further evidence that non-CB1Rs may underlie oscillation suppression in mPFC

by chronic adolescent cannabinoid exposure comes from tests with the putative inactive enantiomer of WIN, WIN-3. We find that WIN-3 unexpectedly and significantly attenuates oscillations in mPFC, but has no effect in SCx. Although converging evidence of mPFC oscillation suppression by chronic adolescent exposure to CB1R/CB2R agonists, CB1R antagonists and a CB2R antagonist suggests the involvement of CB1R and/or CB2Rs in this suppression, similar suppression by WIN-3 suggests that the effects of WIN-2 may be, at least in part, cannabinoid independent. Previous reports reveal that WIN-3 can produce identical effects as WIN-2 (Price *et al.*, 2004; Price *et al.*, 2007; Nemeth *et al.*, 2008) that are not mediated by CB1Rs. These data raise the possibility that chronic adolescent WIN-2 may act via a non-CB1R/CB2R mechanism (Pertwee, 2010), such as at the non-CB1R/CB2R putative cannabinoid receptor GPR55 (Ryberg *et al.* 2007), at TRPV-1 like receptors (Pertwee, 2006) to modulate glutamate release, by allosteric modulation of receptors for other neurotransmitters (Barann *et al.*, 2002), or by direct modulation of cation permeable channels (Shen and Thayer, 1998). Further experiments are clearly needed to address these potential non-cannabinoid effects of chronic adolescent WIN exposure, and must include multiple doses to create a full dose-response curve. Finally, the inactivity of WIN-3 at cannabinoid receptors is questionable, as previous reports show that it can act as a neutral CB2R antagonist and an inverse agonist at the CB1R (Savinainen *et al.*, 2005), although we have not independently verified these potential actions of WIN-3 in our studies. Nevertheless, we must entertain the alternative hypothesis that WIN-3 may act in mPFC in a cannabinoid-dependent manner by modulating constitutive CB1R signaling similar to the CB1R inverse agonist AM251, or may prevent the action of eCBs at CB2Rs, similar to AM630.

E. Cortical Oscillations in CB1R Knockout Mice

Our attempts to further test the specificity of WIN and THC's actions in mPFC and SCx in a CB1R KO mouse model were inconclusive. We had limited time to complete these studies, and after establishing a breeding colony at UMB, we were unable to generate sufficient numbers of animals to achieve adequate statistical power.

Particularly troubling was our inability to replicate our previously reported oscillation suppressing effects of chronic adolescent WIN and THC in CB1R WT mice. There are a number of possible reasons that may account for the lack of effect in these animals.

The CB1R KO mouse model used for these experiments was generated on a different genetic background (C57BL/6J inbred strain) than what we used for all other experiments (CD-1 outbred strain). While different responses to cannabinoids by different genetic mouse strains would be a surprising explanation for these results, this is not unprecedented (Hoffman *et al.*, 2005; Avdesch *et al.*, 2013). Secondly, we used mice of both sexes for these studies, whereas we used all male animals in other experiments. We did not have a large enough sample size of treated CB1R WT males and females to appropriately test for sex differences in oscillation power. However, as males and females have been shown to respond differently to repeated cannabinoid exposure (Cha *et al.*, 2007; Burston *et al.*, 2010; Higuera-Matas *et al.*, 2012), sex differences may contribute to the lack of effect of WIN and THC in these CB1R WT mice. Although all offspring were genotyped to determine the presence or absence of the CB1R, the presence or absence of cortical CB1R expression should be confirmed by immunohistochemistry to ensure the integrity of the CB1R WT and KO animals. Finally, the samples sizes for all of treatments in both CB1R WT and KO mice were small and

prevented us from achieving desired statistical power. Because of these many mitigating circumstances, further tests of adolescent WIN and THC in CB1R WT and KO mice are warranted.

Chapter 3: Cannabinoid Exposure During Adolescence Persistently Alters Cognitive Behavior in Adult Mice

I. Introduction

Our hypothesis predicts that adolescent cannabinoid exposure permanently alters cortical oscillations recorded *in vivo* from awake, behaving mice. To detect abnormalities in behaviorally-evoked oscillations in mice treated with cannabinoids in adolescence, we first sought to identify behaviors that were associated with increases in cortical oscillation power, and that are altered by adolescent cannabinoid exposure. Several cognitive tasks are associated with distinct changes in oscillations at particular bandwidths (Gruber *et al.*, 2008; Martinovic *et al.*, 2008). In Chapter 3 we focus on the following tasks and cognitive states: (1) Novel object recognition task; (2) Object recency task; (3) Sleep states. We present data from experiments designed to test the prediction that chronic adolescent cannabinoid exposure alters the behavior of adult mice in these tasks and states.

Heavy marijuana use before adulthood is associated with impaired cognitive functioning in humans (Solowij *et al.*, 2002; Meier *et al.*, 2012; Smith *et al.*, 2014). Adolescent cannabinoid administration to rodents impairs performance in cognitive tasks, such as tests of object recognition memory (Schneider and Koch, 2003; O’Shea *et al.*, 2004; Quinn *et al.*, 2008; Realini *et al.*, 2011). We aimed to replicate these working memory deficits in adult animals chronically treated with WIN during adolescence (P35-P55), with the subsequent goal of recording cortical oscillations during these behavioral tasks and testing for predicted abnormal oscillatory activity in mice exposed to cannabinoids in adolescence.

A. The Novel Object Recognition Task

The novel object recognition (NOR) task is one of the most widely used behavioral tests of working memory, and capitalizes on a rodent's innate preference for novelty (Ennaceur and Delacour, 1988; Dere *et al.*, 2007; Lyon *et al.*, 2011). In this test, animals are initially presented with two identical objects that they freely investigate during a sample session. They then undergo a delay period of variable length, and are reintroduced to the testing chamber where they encounter one of the objects that they previously investigated (familiar object) and an unfamiliar object (novel object). During this test session, a preference for the novel versus the familiar object indicates that the animal recognizes and remembers the familiar object (Figure 14A). The NOR task is commonly used as a measure of cognitive ability, and is considered a test of "pure" working memory as it does not require additional reinforcers or punishments, nor does the rodent need to learn a series of rules (Lyon *et al.*, 2011). Adolescent, but not adult, cannabinoid exposure impairs performance in the NOR task (Schneider and Koch, 2003; O'Shea *et al.*, 2004; Quinn *et al.*, 2008). Although object recognition relies on a network of cortical areas (Barker *et al.*, 2007), the NOR task engages the medial prefrontal cortex (Watson *et al.*, 2012). In light of previous evidence of impaired NOR behavior following adolescent cannabinoid exposure, and our own data showing abnormal network activity in mPFC in mice treated in adolescence with cannabinoids, we predict that chronic WIN administration in adolescence will impair performance in the NOR task.

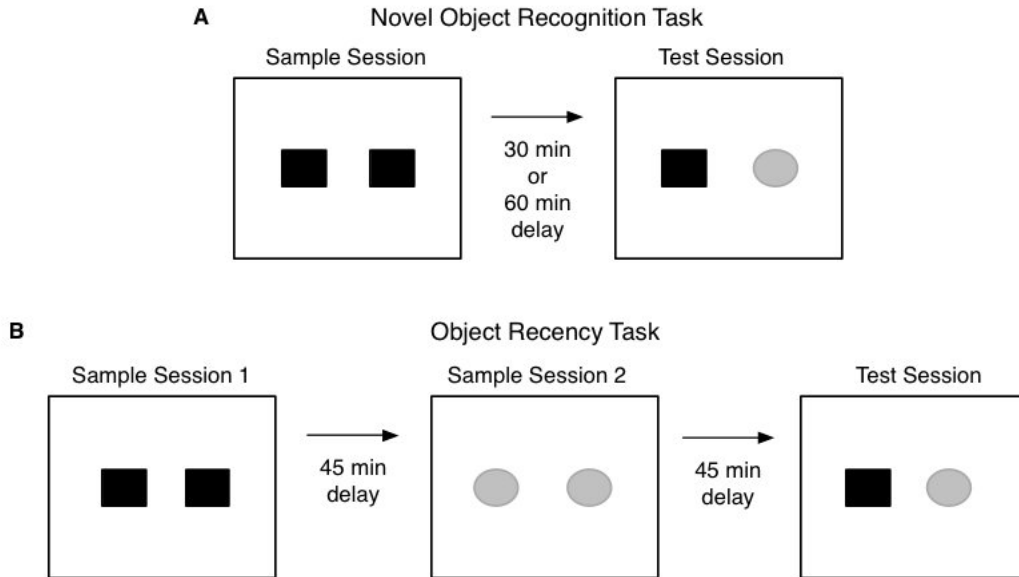


Figure 14: Schematic of the Novel Object Recognition and Object Recency Behavioral Tasks. (A) Schematic of the Novel Object Recognition (NOR) task. The sample session consists of exposure to two identical objects, one of which is reintroduced to the testing arena during the test session after a 30 or 60-minute delay. (B) Schematic of the Object Recency (OR) task. The first sample session consists of exposure to two identical objects and is followed by a 45-minute delay. Two different objects are presented during the second sample session, which is followed by a second 45-minute delay. During the test session, one object from each sample session is presented.

B. The Object Recency Task

A variation on the NOR task, the object recency or temporal order task, may identify specific impairments in cognitive function mediated by the mPFC. The object recency (OR) task is similar to the NOR task in that it is also a “pure” test of working memory that exploits spontaneous object exploration. However, the OR task detects an animal’s ability to differentiate between two familiar objects encountered at different times, and to discriminate between their relative recency (Mitchell and Laiacina, 1998). In the OR test, mice are presented with two identical copies of an object during the first sample session, followed by a delay period. Two new objects are presented during a second sample session, after which animals undergo a second delay period. During the test session, the animal can freely investigate one copy of each object previously

encountered [sample session 1 (S1) and sample session 2 (S2) objects] (Figure 14B). Again, due to their preference for novelty, animals that correctly recognize the temporal order of the object presentation will spend significantly more time with the S1 than the S2 object, because they encountered it less recently. To our knowledge, there are no reports of impaired temporal order memory following adolescent cannabinoid exposure. Nonetheless, as the OR test has been repeatedly shown to both actively engage and require the mPFC (Hannesson *et al.*, 2004; Barker *et al.*, 2007; Nelson *et al.*, 2011), we predict that adolescent cannabinoid exposure will impair OR task performance in adult mice.

C. Marijuana Use and Sleep Disturbances

We also tested for non-cognitive behavioral abnormalities induced by adolescent cannabinoid exposure that may contribute to the working memory impairments that we predicted in these adult animals by analyzing sleep behavior. Abnormalities in sleep behavior have consistently been associated with both acute marijuana intoxication (Nicholson *et al.*, 2004) and prolonged use of the drug (Jacobus *et al.*, 2009). Acute marijuana intoxication alters sleep in adolescents and young adults by increasing the time to fall sleep and increasing wakefulness after sleep onset (Nicholson *et al.*, 2004). Sleep abnormalities are also present in adolescent subjects who persistently use marijuana (Jacobus *et al.*, 2009) and are not restored after short periods of abstinence. These chronic adolescent users experience insufficient sleep, and the sleep that they do receive may be less restful due to increased fragmentation (Jacobus *et al.*, 2009). Heavy marijuana use has been linked to reductions in both major types of sleep activity: rapid eye movement (REM) and slow wave sleep (SWS) (Bolla *et al.*, 2008). Furthermore, poor quality of

sleep impairs working memory performance and other cognitive abilities (Palchykova *et al.*, 2006; Tucker *et al.*, 2010; Shekleton *et al.*, 2014), and may contribute to cognitive impairments following adolescent cannabinoid exposure. We analyzed sleep activity and architecture to test the prediction that chronic adolescent cannabinoid administration alters sleep behavior in adult mice.

II. Materials and Methods

A. Animals

Animals were obtained and treated as previously described in Chapter 1. Male CD-1 mice (Harlan Laboratories, Inc., Frederick, MD, USA) were obtained at P30 and injected with either vehicle or WIN (1 mg/kg) during adolescence (P35-P55). Mice were group housed with cage-mates exposed to both treatments and were left undisturbed after the last injection until adulthood (>P100).

B. Drugs

WIN55-212,2 (1 mg/kg; Sigma Aldrich, St. Louis, MO, USA) was dissolved in ethanol and administered in a 1:1:18 solution of ethanol: Emulphor (Alkamuls EL-620, Rhodia Chemicals, USA): 0.9 % saline (1 mL/kg). Control animals were injected vehicle (1:1:18 ethanol:Emulphor:saline). All injections were delivered i.p.

C. Novel Object Recognition (NOR) Task

Adult mice (>P100) were habituated to a black, plastic behavioral arena (12''x 9''x 11'') for 1 hour in low light conditions. Twenty-four hours after habituation, mice were introduced to two identical objects (2 shot glasses, 1.5'' wide x 3'' tall, or 2 white glass vases, 3'' wide x 4'' tall) for 10 minutes in the arena (sample session). They were then returned to their home-cage for 30 or 60 minutes and then exposed to one object from the

first session (vase or shot glass) and one novel object (shot glass or vase) for 10 minutes (test session) (Figure 14A). The objects and the arena were cleaned thoroughly with 70% ethanol between sessions to eliminate olfactory cues. Object identity (shot glass or vase) and the location of novel/familiar objects (left or right) were counterbalanced between animals. Behavior was videotaped during these sessions and the time spent investigating each object during the first 3 minutes of the test session was manually scored offline by an experimenter blind to animals' treatment condition and to the novel/familiar nature of the objects. It has been reported that the first 3 minutes of test session investigative behavior in the NOR task is the most sensitive to animals' object preferences (Barker *et al.*, 2007) (Dr. T. Gould, personal communication).

Object investigation was said to occur when the animal investigated the object directly with its snout or forepaws, as well as when the animals' snout was oriented toward the object within 2 cm of its edge. Time spent investigating novel and familiar objects was normalized to each animal's total time investigating both objects ($\% \text{ novel} = \frac{\text{time (s) with novel}}{\text{time (s) with novel} + \text{time (s) with familiar objects}}$) to account for individual differences in animals' total interaction times, as previously described (O'Shea *et al.*, 2004; Quinn *et al.*, 2008; Realini *et al.*, 2011). All object investigation data were tested for normality. To test for an effect of adolescent WIN treatment on NOR task behavior, novel object preferences ($\% \text{ time with novel object}$) and total time investigating both objects were compared between vehicle and WIN-treated animals with 2-tailed Student's *t*-tests (significant $p < 0.05$). Potential side biases were tested for with chi-square tests (significant $p < 0.05$), in which each animals' time spent with the object on the left or right side of the testing arena was compared to an "expected" value if no side bias were

present, calculated as 50% of total object investigation time. As convention, normally distributed data that are analyzed with statistical tests of means and variance are presented in bar graphs of means \pm SEM.

D. Object Recency (OR) Task

Adult mice (>P100) were tested in the same arena and with the same objects as were used for the NOR task. The habituation procedure was the same, object location (left vs. right) was similarly counterbalanced, and the arena and objects were cleaned as previously described. Twenty-four hours after habituation, mice were introduced to two identical objects (2 shot glasses or 2 white glass vases) for 10 minutes in the arena (sample session 1) and were returned to their home-cage for 45 minutes. They were then exposed to two new identical objects that differed from those seen during sample session 1 for 10 minutes (sample session 2), and returned to their home-cages for a 45-minute delay. During the test session, mice were exposed to one object from the first sample session (S1 object) and one from the second (S2 object) for 10 minutes (Figure 15A). Behavior was videotaped during these sessions, and the time spent investigating each object during the first 1 or 3 minutes of the test session was manually scored offline by an experimenter blind to animals' treatment condition and to the novel/familiar nature of the objects. Object investigation during the first 3 minutes of the test session was analyzed, similar to NOR behavior analysis. However, previous reports have claimed that the first 1 minute of test session investigative behavior in the Object Recency Task is the most sensitive to animals' preferences (Dix and Aggleton, 1999; Barker *et al.*, 2007); therefore, we analyzed the first 1 minute of behavior as well. Object investigation was said to occur when the animal investigated the object directly with its snout or forepaws, as well as

when the animals' snout was oriented toward the object within 2 cm of its edge. Time spent investigating S1 and S2 objects was normalized to each animal's total time investigating both objects ($\% S1 = \text{time (s) with S1} / \text{time (s) with S1 + S2 objects}$) to account for individual differences in animals' total interaction times, as described for NOR data. All object investigation data were tested for normality. To test for an effect of adolescent WIN treatment on OR task behavior, S1 object preferences ($\% \text{ time with S1 object}$) and total time investigating both objects were compared between vehicle and WIN-treated animals with 2-tailed Students' t-tests (significant $p < 0.05$).

E. Sleep Behavior

Adult mice (>P100) were implanted with F20-EET radio-telemetric transmitters (Data Sciences International, Minneapolis, MN, USA) as described in Chapter 1. However, for these sleep behavior experiments, one lead was implanted over the dura of the frontal cortex (1.7 mm anterior to bregma) to record an ECoG signal and the other lead was implanted through the cervical trapezius muscles in the dorsal region of the neck to record an EMG signal. Both bipotential leads contain a reference lead that is internalized within the silastic insulation surrounding the leads, therefore eliminating the requirement for a separate reference signal. Animals were individually housed and recovered from surgery for at least 48 hours. Mice were acclimated to the behavior testing room for at least 1 hour before recordings started. The experimenter entered the room to initiate the recording session, but did so as quietly as possible so as to not startle the animals. After initiating the recordings, animals were left undisturbed either overnight (~6 pm to 8 am) or during the daytime (~8 am to 5 pm). The recording room was kept dark for overnight recordings, and left illuminated with overhead lighting during daytime

recordings to best approximate the dark/light cycles to which animals were accustomed. ECoG, EMG, and activity data (horizontal movement of the animal across the home cage which sat on top of DSI telemetric receivers) were collected with the Dataquest A.R.T. Acquisition system (Data Sciences International) and analyzed using DSI's neural visualization and analysis program Neuroscore (Data Sciences International). The entire recording session was analyzed in 10 second bins using Neuroscore's sleep analysis toolkit which detects wakefulness from slow wave sleep (SWS) and paradoxical, or rapid eye movement (REM) sleep. Recordings were visually inspected following automated analysis to confirm appropriate detection parameters were being used by Neuroscore, and these parameters were adjusted for individual animals' recordings as needed. Parameters describing sleep behavior were tested for normality and analyzed with non-parametric Mann-Whitney U tests (MWU) between adolescent vehicle and WIN-treated mice (significant $p < 0.05$).

III. Results

A. Chronic Adolescent WIN Exposure Impairs NOR Performance After a Long, But Not a Short Delay Period

To test the prediction that chronic adolescent cannabinoid exposure produces lasting cognitive impairments, we tested adult mice that had been treated in adolescence with WIN (1 mg/kg) or vehicle in the novel object recognition task (NOR) of working memory. Mice were exposed to two identical objects for 3 minutes during a sample session (Figure 14A), followed by a delay period of either 30 or 60 minutes. Next, during the test session, mice were introduced to one of the previously encountered objects from the sample session (familiar object) as well as a novel object that they had not previously

seen (Figure 14A). Animals that recognize the familiar object will devote more of their total object investigation time toward exploring the novel object. Because of animals' individual variability in total object investigation, NOR data is consistently presented in the literature as the normalized metric "Percent Time with Novel." This novel object preference is lower in adolescent cannabinoid-treated animals than in vehicle-treated controls (O'Shea *et al.*, 2004; Quinn *et al.*, 2008; Realini *et al.*, 2011), which is considered representative of a working memory impairment. To be consistent with other published reports, we have analyzed our data using the same approach. We analyzed behavior during the first 3 minutes of the task, as this segment has been reported to be most sensitive to animals' object preference in the NOR task (Barker *et al.*, 2007) (Dr. T. Gould, personal communication).

During the test session following a 30-minute delay period, working memory performance, as indicated by novel object preference, was equivalent in vehicle and WIN-treated animals [vehicle (n = 14 mice): novel preference = $75.8 \pm 3.2\%$; WIN (n = 12 mice): novel preference = $69.3 \pm 4.3\%$; $p = 0.225$] (Figure 15A). During this test session, vehicle and WIN mice spent the same total time exploring both objects (vehicle: 22.7 ± 3.2 sec; WIN: 19.7 ± 3.8 sec; $p = 0.536$) (Figure 15B). These data indicate that chronic adolescent WIN exposure, at the dose tested, does not impair NOR task performance after a 30-minute delay.

We next increased the challenge of the NOR task by introducing a 60-minute delay period between the sample and test sessions (Figure 14A). During this test session, WIN-treated mice demonstrated a significantly lower preference for the novel object than did vehicle mice [vehicle (n = 7 mice): novel preference = $70.4 \pm 5.1\%$; WIN (n = 12

mice): novel preference = $51.2 \pm 4.5\%$; $p = 0.014$] (Figure 15C). This suggests that WIN treated animals were impaired in their ability to recognize a previously encountered object in the NOR task after a 60-minute, but not a 30-minute delay. Total object investigation time did not significantly differ between vehicle and WIN-treated mice (vehicle: 15.4 ± 3.9 sec; WIN: 23.5 ± 4.4 sec; $p = 0.198$) (Figure 15D), suggesting that the behavioral impairment seen in WIN animals is not biased by differences in locomotion or interest in exploring the objects during the NOR test session.

We analyzed object investigation behavior during the sample session of the NOR task prior to the 60-minute delay to determine whether WIN-treated animals' NOR test session impairment might be due to differences in animals' investigative behavior during their first encounter with the objects. Interestingly, during the sample session, WIN-treated animals spent significantly *more* total time investigating both objects than did vehicle animals (vehicle: 14.5 ± 4.7 sec; WIN: 26.2 ± 2.5 sec; $p = 0.044$) (Figure 15E). This suggests that insufficient investigation of the objects when they were first presented does not account for WIN animals' apparent inability to recognize the familiar object during the test session. Furthermore, while vehicle animals do not display a significant directional bias for either the left or right object during the sample session (chi-square test, $p = 0.48$), individual WIN-treated animals demonstrated a significant preference for either the left or right object (chi-square test, $p = 0.01$), with the WIN population seeming to prefer the object located on the left side of the testing arena (Figure 15F). This directional bias in WIN animals should not have affected their object preference during the test session because neither WIN nor vehicle animals demonstrated a significant directional preference during the NOR test session after a 60-minute delay (vehicle: chi-

square test, $p = 0.18$; WIN: chi-square test, $p = 0.51$) (Figure 15G). In addition, the location (left or right) of the familiar and novel objects was counterbalanced, further reducing a potential bias introduced by a side preference. It is unclear whether this directional bias in WIN-treated animals during the sample session, but not the test session, represents a separate consequence of adolescent cannabinoid treatment (addressed in the Discussion). Overall, the data indicate that WIN-treated mice perform the NOR test differently than vehicle-treated mice, as indicated by their significantly greater object investigation and left/right bias during the sample session, and significantly lower novel object preference during the test session.

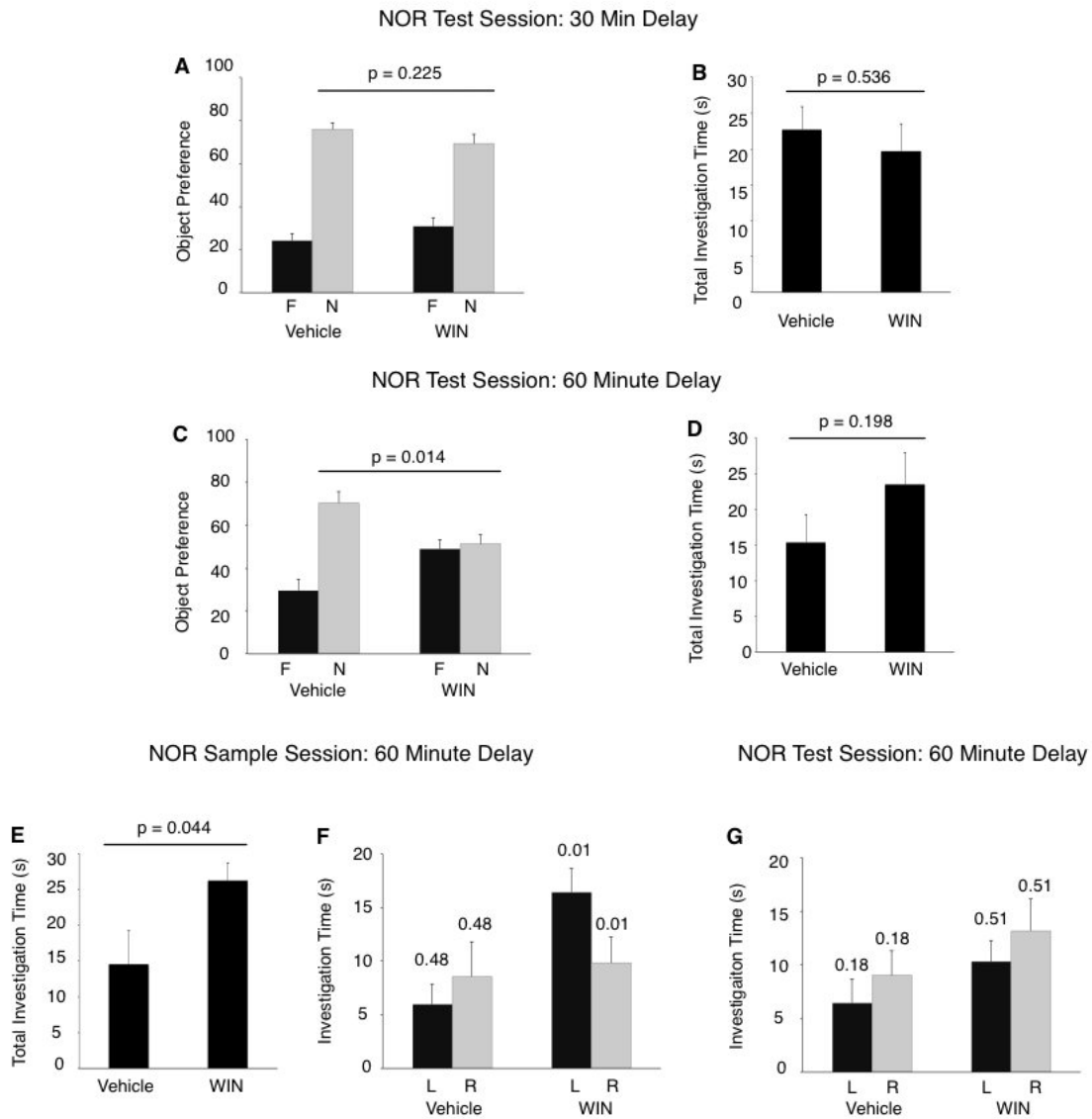


Figure 15: Chronic adolescent WIN exposure impairs novel object recognition (NOR) performance after a 60, but not a 30-minute delay. (A) Mean preference for the familiar (F) or novel object (N) object (% preference = time investigating F or N objects/total time investigating both objects) during the first 3 minutes of the test session after a 30-minute delay by vehicle (n = 7) and adolescent (P35-P55) WIN (1 mg/kg; n = 8) treated adult mice. Novel object preference is compared between vehicle and WIN animals with 2-tailed Student's t-tests (significant $p < 0.05$) (B) Mean total object investigation time in the first 3 minutes of the test session after a 30-minute delay compared between vehicle and WIN animals with 2-tailed Student's t-tests. (C) Mean preference for the familiar or novel object during the first 3 minutes of the test session following a 60-minute delay by vehicle (n = 7) and WIN (n = 8) treated mice. Statistical analysis was performed as in (A) (D) Mean total object investigation time in the first 3 minutes of the test session after a 60-minute delay. Statistical analysis was performed as in (B) (E) Mean total object investigation time in the first 3 minutes of the sample session before a 60-minute delay. Statistical analysis was performed as in (B) and (D). (F) Mean time investigating the object located on the left (L) and right (R) side of the behavioral arena during the sample session prior to a 60-minute delay. *P* values from chi-square tests are presented from comparisons of the actual time animals' spent with L and R objects

compared expected time (50% of total time spent with each object) if animals' investigated each object equally. (G) Mean time investigation the L and R objects during the test session after a 60-minute delay. Statistical analyses were performed as in (F). Throughout, all error bars represent the standard error of the mean (SEM).

B. Chronic Adolescent WIN Exposure Does Not Alter Behavior in the Object Recency

Task After Delay Periods of Moderate Length

We tested adult mice in the Object Recency (OR) Task to further test the hypothesis that adolescent cannabinoid administration impairs cognitive processes and working memory abilities that are dependent upon the mPFC (Mitchell and Laiacona, 1998; Hannesson *et al.*, 2004; Barker *et al.*, 2007; Nelson *et al.*, 2011). As shown in Figure 14B, mice were presented with 2 identical objects in a sample session (sample session 1) and then returned to their home cage for a 45-minute delay. They were then presented with 2 different identical objects (sample session 2) followed by a second 45 - minute delay, and then exposed to one object from each sample session in a test session. The amount of time investigating objects from the first (S1) and second (S2) sessions was scored and normalized to each individual animal's total interaction time with both objects. OR data are presented the same as NOR data, except that S1 preference (% Time with S1 Object) in the OR task represents recognition of the temporal order in which objects were presented, akin to % Time with Novel in the NOR task. We analyzed object investigation behavior in the first 3 minutes of the sessions, similar to NOR behavioral analysis. During the first 3 minutes of the OR test session, vehicle and WIN animals behaved identically, as indicated by the percentage of time that they investigated the S1 object [vehicle (n = 7 mice): S1 preference = $61.7 \pm 16.2\%$; WIN (n = 9 mice): S1 preference = $71.7 \pm 24.8\%$; $p = 0.374$) (Figure 16A). Interestingly, during this 3-minute epoch, WIN-treated mice investigated both objects for significantly *less* time than did vehicle animals

(vehicle: 21.9 ± 7.8 sec; WIN: 13.8 ± 5.8 sec; $p = 0.039$) (Figure 16B). This reduction in total investigation time by WIN animals may indicate a reduced interest in investigating the objects, and more time engaged in other behaviors (for example, exploring the arena or grooming), although it is interesting that these mice correctly recognize the temporal order of objects in less time than vehicle-treated mice.

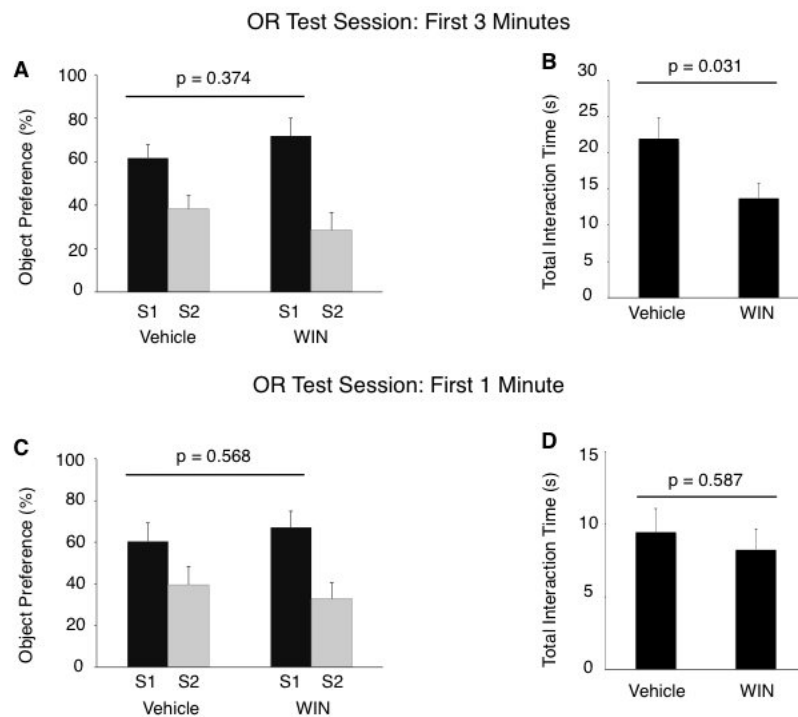


Figure 16: Chronic adolescent WIN exposure does not alter object recency (OR) performance. (A) Mean preference for the S1 or S2 objects (% preference = time investigating S1 or S2 objects/total time investigating both objects) during the first 3 minutes of the test session by adolescent vehicle ($n = 7$) and WIN (1 mg/kg; $n = 9$) treated adult mice. S1 object preference is compared between vehicle and WIN animals with 2-tailed Student's t -tests (significant $p < 0.05$). (B) Mean total object investigation time during the first 3 minutes of the test session. (C) Mean preference for the S1 or S2 objects during the first 1 minute of the test session by adolescent vehicle and WIN-treated adult mice. Statistical analysis was performed as in (A). (D) Mean total object investigation time during the first 1 minute of the test session. Statistical analysis was performed as in (B). Throughout, all error bars represent the standard error of the mean (SEM).

Previous reports suggest that the first minute of the OR test session may be the most sensitive and reflective of animals' preferences (Dix and Aggleton, 1999; Barker *et al.*, 2007). We therefore analyzed vehicle and WIN-treated animals' behavior during the

first minute of the test session, to determine whether adolescent WIN exposure impaired temporal order memory during this time segment. Again, vehicle and WIN-treated mice demonstrate equivalent S1 object preferences [vehicle (n = 7 mice): S1 preference = $60.4 \pm 23.3\%$; WIN (n = 9 mice): S1 preference = $67.2 \pm 22.9\%$; $p = 0.568$] (Figure 16C).

Interestingly, unlike data collected from the first 3 minutes, during the first minute of the OR test session, vehicle and WIN animals investigated both objects for equal amounts of time (vehicle: 9.4 ± 4.4 sec; WIN: 8.3 ± 4.1 sec; $p = 0.711$) (Figure 16D). These data further suggest that WIN animals may quickly lose interest in the objects by the second and third minutes of the OR test session and spend their time engaged in other behaviors. We conclude from these experiments that adolescent WIN exposure at the tested dose does not impair discrimination of object recency, as assessed by the OR task with moderate delay periods, at the concentration of WIN tested here.

C. Chronic Adolescent WIN Exposure Does Not Persistently Alter Sleep Behavior

We tested the prediction that adolescent WIN exposure alters sleep behavior in adult mice by analyzing sleep behavior during both the animals' dark and light cycles. Both acute and chronic marijuana use has been associated with sleep abnormalities (Nicholson *et al.*, 2004; Jacobus *et al.*, 2009). Poor sleep quality impairs working memory performance and other cognitive abilities (Palchykova *et al.*, 2006; Tucker *et al.*, 2010; Shekleton *et al.*, 2014), and may contribute to the NOR working memory deficit that we have found in adult mice exposed to WIN in adolescence. We used radio-telemetric transmitters and implanted electrode leads into the frontal cortex to record ECoGs and the neck muscle to record EMGs. Recordings continued interrupted in animals' home cages overnight (approximately 14 hours) or during the day

(approximately 9 hours). The entire recording session was analyzed in 10-second bins with Neuroscore's sleep analysis toolkit. This analysis method detects wakefulness from slow wave sleep (SWS) and paradoxical, or rapid eye movement (REM) sleep.

Wakefulness is characterized by lower amplitude/ higher frequency ECoG activity and high amplitude EMG activity. ECoG activity during REM sleep looks similar to wake, however the EMG signal is flat, while SWS consists of high amplitude/low frequency ECoG activity and a flat EMG signal (Hermansteyne *et al.*, 2013).

We compared a variety of sleep parameters between adolescent vehicle and WIN-treated mice during both dark (Table 6) and light cycles (Table 7). None of these parameters were significantly affected by adolescent WIN exposure at the administered dose. We found no significant difference in the percentage of time that vehicle or WIN animals spent in wake or sleep, nor in the amount of time spent in REM or SWS (Tables 6,7).

Similarly unaffected by adolescent 1 mg/kg WIN exposure were measures of the latency to the first sleep bout, as well as the amount of time that animals spent awake once they had first fallen asleep. The number of individual measurements (10 second bins or "counts") of each sleep stage (wake, REM, SWS) were not significantly different between the treatment conditions, nor were the number of transitions between the sleep stages, indicating similar sleep fragmentation in adolescent vehicle and WIN-treated mice (Tables 6,7). These data indicate that chronic adolescent WIN exposure at the dose administered does not persistently alter sleep behavior in adult mice.

| Overnight Sleep Statistic | Chronic Adolescent Vehicle (n = 10 mice) | Chronic Adolescent WIN (1 mg/kg) (n = 11 mice) | MWU Test Results (<i>p</i> value) |
|-----------------------------------|---|--|---------------------------------------|
| % Total Time Spent in Wake | Mean: 29.6 ± 13.9 Median: 26.6 | Mean: 18.9 ± 8.7 Median: 20.0 | 0.067 |
| % Total Time Spent Sleeping | Mean: 70.4 ± 13.9 Median: 73.2 | Mean: 81.0 ± 8.7 Median: 80.0 | 0.067 |
| % Total Time Spent in SWS | Mean: 57.0 ± 21.4 Median: 60.3 | Mean: 70.5 ± 14.5 Median: 76.9 | 0.189 |
| % Total Time Spent in REM Sleep | Mean: 13.4 ± 9.5 Median: 13.2 | Mean: 10.5 ± 9.3 Median: 10.0 | 0.481 |
| Wake time after sleep onset (min) | Mean: 264.0 ± 127.5 Median: 219.1 | Mean: 176.0 ± 80.8 Median: 191.3 | 0.159 |
| Time to sleep onset (min) | Mean: 2.99 ± 5.0 Median: 0.3 | Mean: 1.43 ± 3.5 Median: 0.0 | 0.100 |
| Time to SWS onset (min) | Mean: 2.99 ± 5.0 Median: 0.3 | Mean: 1.43 ± 3.5 Median: 0.0 | 0.100 |
| Time to REM sleep onset (min) | Mean: 16.6 ± 19.2 Median: 10.8 | Mean: 16.4 ± 16.9 Median: 9.0 | 0.860 |
| Wake count | Mean: 195.0 ± 73.9 Median: 179.0 | Mean: 246.3 ± 96.3 Median: 275.0 | 0.193 |
| REM count | Mean: 147.5 ± 89.5 Median: 133.5 | Mean: 152.4 ± 125.8 Median: 122.0 | 0.860 |
| SWS count | Mean: 309.8 ± 77.9 Median: 305.5 | Mean: 369.8 ± 139.9 Median: 369.0 | 0.260 |
| Total Transitions | Mean: 650.5 ± 155.7 Median: 612.5 | Mean: 763.1 ± 284.4 Median: 744.0 | 0.231 |
| Wake to REM Sleep Transitions | Mean: 0 ± 0 Median: 0 | Mean: 0 ± 0 Median: 0 | N/A |
| Wake to SWS Transitions | Mean: 194.1 ± 73.9 Median: 179.0 | Mean: 243.3 ± 90.3 Median: 274.0 | 0.181 |
| REM Sleep to Wake Transitions | Mean: 32.2 ± 19.2 Median: 34.5 | Mean: 27.4 ± 22.1 Median: 24.0 | 0.526 |
| REM Sleep to SWS Transitions | Mean: 115.0 ± 74.2 Median: 92.5 | Mean: 124.5 ± 109.9 Median: 80.0 | 0.888 |
| SWS to Wake Transitions | Mean: 161.7 ± 90.7 Median: 143.5 | Mean: 215.6 ± 94.9 Median: 222.0 | 0.181 |
| SWS to REM Sleep Transitions | Mean: 147.5 ± 89.5 Median: 133.5 | Mean: 152.4 ± 125.8 Median: 122.0 | 0.860 |

Table 6: Summary of parameters describing the sleep of adult mice exposed to either vehicle or WIN (1 mg/kg) in adolescence during an approximately 14-hour overnight recording session. Parameters were extracted with a sleep analysis toolkit in Neuroscore and were compared between adolescent vehicle (n = 10) and WIN (n = 11) treated mice using MWU tests (significant $p < 0.05$).

| Daytime Sleep Statistic | Chronic Adolescent Vehicle (n = 3 mice) | Chronic Adolescent WIN (1 mg/kg) (n = 6 mice) | MWU Test Results (p value) |
|-----------------------------------|--|---|-------------------------------|
| % Total Time Spent in Wake | Mean: 22.0 ± 5.0 Median: 21.5 | Mean: 13.3 ± 7.8 Median: 10.4 | 0.121 |
| % Total Time Spent Sleeping | Mean: 77.0 ± 5.1 Median: 75.6 | Mean: 86.8 ± 7.7 Median: 89.6 | 0.121 |
| % Total Time Spent in SWS | Mean: 56.5 ± 23.8 Median: 64.4 | Mean: 68.1 ± 24.0 Median: 78.2 | 0.364 |
| % Total Time Spent in REM Sleep | Mean: 20.5 ± 19.5 Median: 11.2 | Mean: 18.6 ± 19.6 Median: 9.1 | 0.796 |
| Wake time after sleep onset (min) | Mean: 114.8 ± 28.4 Median: 123.8 | Mean: 62.6 ± 32.8 Median: 51.5 | 0.071 |
| Time to sleep onset (min) | Mean: 0.6 ± 0.7 Median: 0.5 | Mean: 0.9 ± 2.0 Median: 0.0 | 0.396 |
| Time to SWS onset (min) | Mean: 0.6 ± 0.7 Median: 0.5 | Mean: 0.9 ± 2.0 Median: 0.0 | 0.396 |
| Time to REM sleep onset (min) | Mean: 10.1 ± 11.2 Median: 5.2 | Mean: 45.4 ± 63.9 Median: 22.7 | 0.302 |
| Wake count | Mean: 97.7 ± 41.5 Median: 96.0 | Mean: 94.3 ± 41.4 Median: 88.5 | 1.000 |
| REM count | Mean: 135.0 ± 43.6 Median: 115.0 | Mean: 124.8 ± 95.5 Median: 102.0 | 0.606 |
| SWS count | Mean: 214.0 ± 15.0 Median: 214.0 | Mean: 210.2 ± 57.6 Median: 189.0 | 0.606 |
| Total Transitions | Mean: 458.3 ± 16.3 Median: 455.0 | Mean: 428.2 ± 122.4 Median: 385.0 | 0.439 |
| Wake to REM Sleep Transitions | Mean: 0 ± 0 Median: 0 | Mean: 0 ± 0 Median: 0 | N/A |
| Wake to SWS Transitions | Mean: 95.3 ± 38.0 Median: 96.0 | Mean: 94.2 ± 41.6 Median: 88.5 | 1.000 |
| REM Sleep to Wake Transitions | Mean: 16.7 ± 13.1 Median: 21.0 | Mean: 9.7 ± 7.1 Median: 9.5 | 0.302 |
| REM Sleep to SWS Transitions | Mean: 118.0 ± 34.1 Median: 103.0 | Mean: 115.2 ± 88.7 Median: 92.5 | 0.606 |
| SWS to Wake Transitions | Mean: 78.3 ± 43.9 Median: 93.0 | Mean: 84.3 ± 47.2 Median: 83.5 | 1.000 |
| SWS to REM Sleep Transitions | Mean: 150.0 ± 35.0 Median: 150.0 | Mean: 124.8 ± 95.5 Median: 102.0 | 0.439 |

Table 7: Summary of parameters describing the sleep of adult animal exposed to either vehicle or WIN (1 mg/kg) during an approximately 9-hour daytime recording session. Parameters were extracted with a sleep analysis toolkit in Neuroscore and were compared between adolescent vehicle (n = 3) and WIN (n = 6) treated mice using MWU tests (significant $p < 0.05$).

IV. Discussion

In Chapter 3 we test the prediction that chronic adolescent cannabinoid exposure persistently alters behavior in adulthood. We find that chronic adolescent 1 mg/kg WIN administration impairs cognitive performance in the novel object recognition test of working memory after a long, but not a short, delay. We find no impairment in temporal

order object discrimination by chronic adolescent WIN administration after moderate delays, as assessed by the object recency task. We also find that chronic exposure to WIN in adolescence does not measurably alter sleep behavior that could contribute to the working memory deficits found in adolescent WIN-treated animals.

A. Novel Object Recognition Task Behavior

Adolescent WIN-treated mice demonstrate impaired working memory as assessed by the NOR task, which is consistent with previous reports of cognitive impairments after persistent adolescent cannabis exposure in both rodents (Schneider and Koch, 2003; O'Shea *et al.*, 2004; Quinn *et al.*, 2008; Realini *et al.*, 2011) and humans (Solowij *et al.*, 2002; Meier *et al.*, 2012; Smith *et al.*, 2014). Impairments in working memory, processing speed, perceptual reasoning and other executive functions have been documented in long-term adolescent cannabis users (Solowij *et al.*, 2002; Bolla *et al.*, 2002; Fontes *et al.*, 2011; Meier *et al.*, 2012). Interestingly, we find that adolescent-WIN treated mice exhibit poor performance in the NOR task only when the task is made more challenging with a 60 minute delay period, and not when the demands of the task are simpler, such as after a 30 minute delay. These results of a delay-dependent working memory impairment are consistent with reports of impaired working memory following acute cannabinoid exposure in other cognitive tasks, including the hippocampus-dependent delayed non-match to sample test (Heyser *et al.*, 1993). The working memory deficit that we report is probably not due to impaired locomotion or limited engagement with objects by WIN animals, as total interaction time with both objects is equal between WIN and vehicle-treated animals during the NOR test session. We can therefore use the NOR task to assess differences in cortical network oscillations between vehicle and WIN-

treated animals *in vivo*, as will be discussed in Chapter 4.

It is interesting to note that adolescent WIN, but not vehicle-treated mice display a significant object location bias during the NOR test session prior to the 60 minute delay, but not during the NOR test session. The population mean reveals that WIN animals spend more time investigating the object located on the left side of the arena than the right side. To centrally position the recording camera during the NOR task, task lighting had to be offset to one side, potentially causing a gradient of illumination in the behavioral testing arena that, while undetectable to the experimenter, may have subtly influenced animals' behavior. During the sample session, WIN-treated animals preferred the side opposite to the location of the light. This observation may be in line with previous reports of increased anxiety-like behaviors in adult rodents repeatedly exposed to cannabinoids in adolescence (O'Shea *et al.*, 2006; Quinn *et al.*, 2008; Schneider *et al.*, 2008) and in humans that chronically smoke marijuana as teenagers (Patton *et al.*, 2002; Hayatbakhsh *et al.*, 2007). Also of note, WIN-treated mice spend significantly more time investigating both objects during the sample session than do vehicle-treated mice. In the absence of open-field locomotion data in these animals, object investigation time can serve as a proxy for animals' ambulation and may further support an anxiety-like phenotype in the WIN animals. However, the absence of a side-bias or significant differences in total object investigation time during the NOR test session would argue against this interpretation. Future experiments could directly test for anxiety-related behaviors in adult mice chronically exposed to cannabinoids in adolescence.

B. Object Recency Task Behavior

Although, to our knowledge, there exists no precedent of impaired object recency

discrimination in adult animals repeatedly administered cannabinoids in adolescence, the object recency task has been shown to both engage and require the mPFC (Mitchell and Laiacona, 1998; Hannesson *et al.*, 2004; Barker *et al.*, 2007; Nelson *et al.*, 2011). As we have discovered dramatically suppressed oscillations in the mPFC of WIN-treated mice, we predicted impaired OR task performance in these animals. Surprisingly, this is not the case. Regardless of the duration of object investigation time measured (the first 3 or 1 minute), adult mice treated with 1 mg/kg WIN in adolescence demonstrate the same preference for the least recently viewed object as do vehicle-treated mice. This lack of effect of adolescent WIN treatment — at least at the dose tested — may be due to the relatively short delay between the sample sessions. As we found for NOR behavior, impaired working memory may not be apparent in WIN-treated mice until the demands of the task are made more challenging, as could be accomplished by lengthening the delay between the sample sessions and/or between the sample and test sessions. Further experiments should be conducted to determine whether a longer delay reveals a cognitive impairment by adolescent-cannabinoid treatment mice in the OR task, and should include a full dose-response curve.

Previous reports of OR test behavior suggest that the data in the first 1 minute of the test session is more sensitive to animals' preferences (Dix and Aggleton, 1999) (Barker *et al.*, 2007). Interestingly, we found identical performance of this task in WIN and vehicle-treated animals during both the first 1 and 3 minutes of the OR test session, suggesting that the preference made by animals during the first minute of the session is conserved throughout the first 3 minutes. However, it is interesting to note that while WIN and vehicle animals investigated both objects equally during the first minute, WIN

animals' object engagement drops in minutes 2-3, as indicated by their significantly lower total object investigation time during the first 3 minutes. During the second and third minutes of the test session, WIN animals may lose interest in the objects and spend more of their time grooming, exploring the arena, or engaged in another behavior that is not apparent to the experimenter. Increased distractibility and impaired attention has been reported in adults who regularly used cannabis as adolescents (Meier *et al.*, 2012), and may contribute to this reduced object engagement in animals treated with WIN in adolescence. Subsequent behavioral experiments could test for increased distractibility or shorter attention spans in adult animals following chronic adolescent cannabinoid exposure.

C. Sleep Behavior

Abnormalities in sleep behavior have been reported after acute (Nicholson *et al.*, 2004) and persistent marijuana use (Jacobus *et al.*, 2009). Furthermore, as poor sleep quality adversely affects cognitive functioning and working memory tasks (Palchykova *et al.*, 2006; Tucker *et al.*, 2010) (Shekleton *et al.*, 2014), abnormal sleep behavior may contribute to impaired working memory performance in adolescent WIN-treated mice. We predicted that chronic exposure to WIN during adolescence will alter sleep behavior and sleep architecture, as chronic human marijuana users report greater sleep fragmentation and more time spent awake than non-using study control subjects (Bolla *et al.*, 2008; Jacobus *et al.*, 2009). Contrary to our prediction, we find no significant differences in sleep behavior after chronic adolescent WIN administration during either the dark or light phases of animals' circadian cycles. These negative findings may be partially due to the relatively small sample size in the daytime recording study ($n = 3$

vehicle and 6 WIN mice). Alternatively, these data might reflect a restoration of sleep behavior after the prolonged period of abstinence in adolescent WIN-treated mice, in parallel with previous reports of recovered sleep activity after 3 months of abstinence in heavy marijuana users (Jacobus *et al.*, 2009). Interestingly, equivalent sleep behavior in adolescent vehicle and WIN-treated mice suggest that the cognitive impairment demonstrated in the NOR task is not confounded by poor sleep quality or sleep deprivation in WIN animals, which have been shown to impair performance in assessments of working memory and other cognitive functions (Palchykova *et al.*, 2006; Tucker *et al.*, 2010; Shekleton *et al.*, 2014).

Chapter 4: Cannabinoid Exposure During Adolescence Alters Modulation of Behaviorally-Evoked Oscillations in Adult Mice

I. Introduction

In Chapter 3 we explored behavioral states that are associated with oscillations in prefrontal cortex, so that we could test our hypothesis that adolescent cannabinoid treatment affects oscillations in the adult, behaving animal. We presented data indicating that chronic adolescent exposure to cannabinoids impairs behavioral performance in the novel object recognition test of working memory, a task that engages the prefrontal cortex (Watson *et al.*, 2012). As we have also reported attenuated pharmacologically-evoked oscillations in mPFC LFPs and frontal cortex ECoGs in these mice, we predicted that cannabinoid exposure in adolescence will alter behaviorally-evoked oscillations recorded *in vivo* from intact, behaving mice. We employed multiple approaches to record *in vivo* oscillations and test this prediction in Chapter 4.

A. Induced Oscillations During Cognitive Behavioral Tasks

We recorded oscillations from intact adult mice while they performed cognitive tasks in which working memory is impaired in adolescent cannabinoid-treated mice. The novel object recognition (NOR) and object recency (OR) tasks have both been shown to engage the mPFC (Mitchell and Laiacona, 1998; Hannesson *et al.*, 2004; Barker *et al.*, 2007; Nelson *et al.*, 2011). Impaired cognitive ability, as assessed by the NOR task, has been demonstrated in adult animals chronically exposed to cannabinoids in adolescence (Schneider and Koch, 2003; O'Shea *et al.*, 2004; Quinn *et al.*, 2008; Realini *et al.*, 2011) and has been confirmed in our lab. To date, however, there are no published reports of electrophysiological abnormalities in adolescent cannabinoid-exposed animals *in vivo*

while they perform behavioral tasks that assess cognitive abilities.

Cortical oscillations underlie neural network processing (Buzsaki and Draguhn, 2004) and may be important for cognitive processes required for object familiarity discriminations and working memory. Indeed, in human subjects engaged in an object recognition task, gamma power in frontal cortical areas increases shortly after the presentation of familiar, but not unfamiliar stimuli (Gruber *et al.*, 2008) and other reports indicate that gamma-band activity coincides with the instant that human subjects report object identification (Martinovic *et al.*, 2008). Synchronous cortical neural activity plays a role in selective attention (Fries *et al.*, 2001), integration of sensory information (Singer and Gray, 1995), working memory (Roux *et al.*, 2012), and many other cognitive functions (Buzsaki, 2006; Wang, 2010). Furthermore, oscillations in distinct frequency bandwidths may underlie different aspects of working memory functions (Dipoppa and Gutkin, 2013). For example, gamma and beta activity may enable rapid memory access, while oscillations in the theta frequency have been shown to help maintain memories in the presence of distracting stimuli (Dipoppa and Gutkin, 2013). Synchronous activity emerges in multiple cortical areas during sensory encoding, memory formation and retrieval (Tiitinen *et al.*, 1993; Sederberg *et al.*, 2003; Roux *et al.*, 2012) and oscillations may represent a candidate mechanism by which the brain accomplishes these tasks. Oscillatory activity, such as described above, that is self-generated and not time-locked to the presentation of a sensory stimulus is called an induced oscillation. In diseases in which these cognitive processes are abnormal, such as schizophrenia, induced oscillations in frontal cortical regions in nearly all frequency bands (theta, alpha, beta, and gamma) are suppressed in parallel with impairments in working memory (Cho *et al.*, 2006;

Haenschel *et al.*, 2009).

Object recognition tasks presumably require animals to perform many of these cognitive processes that have been shown to evoke clear oscillatory activity: attention to sensory stimuli, integration and maintenance of sensory information, subsequent access to working memory processes, and recognition of a familiar object. Therefore, we predicted that object recognition tests of working memory modulate the power of frontal cortical oscillations, and that this modulation of synchronous network activity would be impaired in adult mice chronically exposed to cannabinoids during adolescence.

B. Evoked Oscillations During Auditory Stimuli Presentation

We used an alternative method of evoking robust oscillatory activity *in vivo*, by presenting trains of auditory stimuli at various frequencies. Frequency-dependent auditory steady-state evoked responses (ASSRs) can be elicited in cortical ECoGs by presenting trains of auditory stimuli at various frequencies. These ECoG ASSRs entrain to the frequency of the auditory stimuli (Franowicz and Barth, 1995; Brenner *et al.*, 2009) and are suppressed in human chronic marijuana users (Skosnik *et al.*, 2012) and in patients with schizophrenia (Kwon *et al.*, 1999; Brenner *et al.*, 2003). We therefore predicted that this method would elicit robust evoked oscillations, i.e. synchronous activity that is time-locked to the presentation of sensory stimuli. We further predicted that this evoked activity would be attenuated in animals exposed to chronic cannabinoid treatment during adolescence.

C. Resting-State Oscillations During Baseline Conditions

Finally, we analyzed a third class of *in vivo* ECoG oscillations that are distinct from those elicited during cognitive tasks (induced oscillations) or from oscillations that

are evoked by the presentation of sensory stimuli (evoked oscillations) (Uhlhaas and Singer, 2010). Resting state oscillatory activity recorded during “baseline” conditions has also been reported as abnormal in patients with schizophrenia. Specifically, previous reports suggest the presence of both reductions and increases in resting-state gamma activity (Rutter *et al.*, 2009; Kikuchi *et al.*, 2011) in patients with schizophrenia, as well as increased theta and alpha activity (Boutros *et al.*, 2008). This altered baseline activity may contribute to the decrease in evoked oscillation amplitude or coordination that is consistently reported in patients with the disease (Kwon *et al.*, 1999; Brenner *et al.*, 2003). We recorded resting state oscillations when mice were in two distinct behavioral states: alert and active, during exploration of a novel environment, or relaxed and undisturbed when mice were in their home-cages during overnight recording sessions. Because these “baseline” oscillations have been reported to be abnormal (both higher and lower) in patients with schizophrenia, we predicted similar abnormalities might be present in adult mice exposed to cannabinoids in adolescence.

II. *Materials and Methods*

A. *Animals*

Animals were obtained and treated as described in Chapter 1. Male CD-1 mice were obtained at P30 and injected with either vehicle or WIN (1 mg/kg) during adolescence (P35-P55). Mice were group housed with cage-mates exposed to both treatments and were left undisturbed after the last injection until adulthood (>P100).

B. *Drugs*

WIN55-212,2 (WIN) (0.25 or 1 mg/kg; Sigma Aldrich, St. Louis, MO, USA) or THC (5 mg/kg; National Institute on Drug Abuse Drug Supply Program, Bethesda, MD,

USA) were dissolved in ethanol and administered in a 1:1:18 solution of ethanol: Emulphor (Alkamuls EL-620, Rhodia Chemicals, USA): 0.9 % saline (1 mL/kg). Control animals were injected vehicle (1:1:18 ethanol:Emulphor:saline).

C. In vivo Surgical Procedures and ECoG Recordings

Mice were implanted with radio-telemetric transmitters as described in Chapter 1. Briefly, adult mice (>P100) were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg). A F20-EET radio-telemetric transmitter (Data Sciences International, Minneapolis, MN, USA) was implanted subcutaneously and its leads implanted over the dura above the frontal cortex (1.7 mm anterior to bregma) and the cerebellum (6.4 mm posterior to bregma). Both bipotential leads contain a reference lead that is internalized within the silastic insulation surrounding the leads, therefore eliminating the requirement for a separate reference signal. Animals were individually housed and recovered from surgery for at least 48 hours before recording.

D. Novel Object Recognition Task and in vivo ECoG Recording and Analysis

The Novel Object Recognition (NOR) task was performed as described in Chapter 3. Briefly, mice were habituated to the behavioral arena for 1 hour. Twenty-four hours later, they were exposed to two identical objects for 10 minutes (sample session) followed by either a 30-minute or 60-minute delay. During the test session, mice were introduced to object that they had previously encountered (familiar) as well as a novel object. Animal behavior was video recorded during both the sample and test sessions, and analyzed offline during the first 3 minutes of the behavioral sessions by an experimenter blind to animals' treatment condition and to the novel/familiar nature of the objects. The location (left or right) and the identity (vase or shot glass), was counterbalanced between

animals. Object investigation was considered to occur when the animal investigated the object directly with its snout or forepaws, as well as when the animals' snout was oriented toward the object within 2 cm from the edge. Frontal ECoGs were recorded with the Dataquest A.R.T. Acquisition system (Data Sciences International) during the sample and test sessions. We analyzed *in vivo* ECoGs with custom-written MATLAB scripts (Version 2012a, Mathworks, MA, USA) and the *mtspecgramc* routine in the Chronux Toolbox (<http://chronux.org>) (Mitra and Bokil, 2006). Oscillation power in each bandwidth [δ (1-3 Hz); θ (4-7 Hz); α (8-12 Hz); β (13-29 Hz); γ (30-80 Hz)] was computed in 10-second bins from spectrograms for each animal during the first 3 minutes of the sample and test sessions. Oscillation power in each bandwidth was averaged for each animal for the entire 3 minutes or within 30-second segments for the different behavioral sessions. Average power in the individual bandwidths from multiple animals of the same adolescent treatment condition were then combined, tested for normality, and analyzed to determine an effect of cannabinoid treatment with Mann-Whitney U tests (MWU) (significant $p < 0.05$). Oscillation power in the different frequency bandwidths was compared between the NOR sample and test sessions using the Wilcoxon matched-pairs signed rank test within each treatment condition (significant $p < 0.05$). All statistical analyses were performed in STATA.

E. In vivo Surgical Procedures and LFP Recordings

Adult mice (> P100) were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg). A microelectrode array (2x3 configuration including reference wire, 5 active channels, 25 μ m stainless steel micro-wire array with reference wire, 250 μ m between and within rows) (Plexon, Inc., Dallas, TX, USA) was implanted in the mPFC (1.7 mm

anterior to bregma, 0.4 mm lateral to the midline, 0.5 mm ventral to the cortical surface) and cemented to the skull using dental acrylic. Burr holes were drilled into the skull, and three stainless-steel screws were inserted to anchor the implant: one in the frontal bone contralateral to the electrode array, and the other two in each of the parietal bones. The stainless steel ground wire was wrapped around the screw implanted into the frontal bone. Animals were individually housed and recovered from surgery for at least 1 week. Mice were acclimated to the behavioral testing room for 1 hour before behavioral testing/LFP recordings. LFPs were recorded with Plexon's SortClient software package and animals' behavior was simultaneously recorded with Plexon's CinePlex software package (Plexon Inc.), a generous loan from Dr. Joseph Cheer.

F. Novel Object Recognition Task and in vivo LFP Recording and Analysis

The Novel Object Recognition (NOR) task was performed as described above, except as indicated below. LFPs were recorded with Plexon's SortClient software package and animals' behavior was simultaneously recorded with Plexon's CinePlex software package (Plexon Inc.). Behavior was videotaped using the Plexon Cineplex Capture behavior tracking system that simultaneously records behavior and LFP activity in conjunction with Plexon SortClient. Behavior was analyzed offline using Cineplex Markup by an experimenter blind to animals' treatment condition and to the novel/familiar nature of the objects. Object investigation was said to occur when the animal investigated the object directly with its snout or forepaws, as well as when the animals' snout was oriented toward the object within 2 cm from its edge. The time spent investigating objects was measured during the behavioral sessions. Time spent investigating novel and familiar objects was normalized to each animal's total time

investigating both objects (% novel = time (s) with novel/time (s) with novel + familiar objects) to account for individual differences in animals' total interaction times, as performed in Chapter 3. Sample and test sessions lasted for 3 minutes, rather than 10 minutes as in ECoG recording studies. We recognize the possibility that changing the duration of the sample and test sessions from 10 minutes (as above) to 3 minutes in this iteration of the NOR task may change the experience of the animals' undergoing the testing procedure. However, as object interaction sessions ranging from 3 – 10 minutes have been used to determine an effect of adolescent cannabinoid exposure on NOR performance (Schneider and Koch, 2003; O'Shea *et al.*, 2004; Quinn *et al.*, 2008; Realini *et al.*, 2011), we do not believe that shortening the object interaction sessions should confound our data interpretation.

All object investigation data were tested for normality. To test for an effect of adolescent WIN or THC treatment on NOR task behavior, novel object preferences (% time with novel object) and total time investigating both objects were compared between vehicle and either WIN or THC-treated animals with 2-tailed Students' t-tests (significant $p < 0.05$). Student's t-test can be reliably applied to normalized data, such as novel object preference, without inflating Type I or Type II errors, if data are normalized such that the variability of the population is conserved (Valcu and Valcu, 2011). We have normalized object investigation data using such an approach, by normalizing each animals' object preferences to that individual animals' total object investigation. Potential side biases were tested for with chi-square tests (significant $p < 0.05$) in which each animal's time spent with the object on the left or right side of the testing arena was compared to an "expected" value if no side bias were present, calculated as 50% of total object

investigation time. As convention, normally distributed data that are analyzed with statistical tests of means and variance are presented in bar graphs of means \pm SEM. Object interaction time-stamps and investigation epochs were exported from CinePlex Markup to Neuroexplorer (Version 4.0, NEX Technologies, Madison, AL, USA) where LFP activity was analyzed using peri-event spectrograms, spectrograms, and power spectral densities (PSDs). Oscillation PSDs during the entire test or sample session, as well as during the different behavioral epochs of object investigation or no object investigation, were exported from Neuroexplorer to STATA. Power within the different frequency bandwidths [δ (1-3 Hz); θ (4-7 Hz); α (8-12 Hz); β (13-29 Hz); γ (30-80 Hz)] was tested for normality.

Due to factors discussed later, the sample sizes in these NOR/LFP experiments were small [vehicle ($n = 4$), WIN ($n = 3$) and THC-treated mice ($n=3$)]. All NOR behavioral data variables were determined to be normally distributed and thus appropriate for analysis with Student's t -tests. We find evidence in the statistical literature that there are no objections to using the Student's t -test on data with sample sizes with n 's as low as 2 (de Winter, 2013). The same rationale cannot be applied to data that are non-normally distributed, such as LFP oscillation power. Similar to all LFP/ECOG oscillation data that we have collected, LFP oscillation power recorded during the NOR task from these 10 animals does not follow a normal distribution, therefore necessitating the use of the non-parametric rank-based statistical tests Mann-Whitney U (MWU) and the Wilcoxon matched-pairs signed rank test. However, sample sizes less than 5 cannot be reliably analyzed using rank-based tests (de Winter, 2013). Therefore, because the sample sizes for these LFP analysis ($n = 4$ and 3) violate this requirement, we present box-and-whisker

plots of LFP power from animals engaged in the NOR task, but are unable to conduct statistical analyses on these limited samples.

G. Object Recency Task and in vivo ECoG Recording and Analysis

The Object Recency (OR) task was performed as described in Chapter 3. Briefly, mice were habituated to the behavioral arena for 1 hour. Twenty-four hours later, they were exposed to two identical objects for 10 minutes (sample session 1) followed by a 45-minute delay session. Mice were then introduced to two different, identical objects for 10 minutes (sample session 2), followed by a second 45-minute delay. Animals were then introduced to one object from sample session 1 (S1 object) and one object from sample session 2 (S2 object) during the test session. Animal behavior was video recorded during the sample and test sessions, and analyzed offline by an experimenter blind to animals' treatment condition and to the novel/familiar nature of the objects. The analysis focused on the first minute of the sample and test sessions. Object investigation was said to occur when the animal investigated the object directly with its snout or forepaws, as well as when the animals' snout was oriented toward the object within 2 cm from its edge. Frontal ECoGs were recorded with the Dataquest A.R.T. Acquisition system (Data Sciences International) during the sample and test sessions. We analyzed *in vivo* ECoGs with custom-written Igor Pro scripts. Discrete fast Fourier transforms (FFTs) were performed on 1 minute of ECoG data and oscillation power (area under the curve) was integrated at different frequencies [delta (1-3 Hz); theta (4-7 Hz); alpha (8-12 Hz); beta (13-29 Hz); gamma (30-80 Hz)]. Data were tested for normality and analyzed for an effect of cannabinoid treatment with MWU tests (significant $p < 0.05$) using STATA.

H. In vivo Auditory Evoked Oscillation Recording and Analysis

Naïve adult C57B6 mice were used to record auditory evoked potentials in cortical ECoGs and LFPs. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) and placed in a stereotaxic frame. Burr holes were drilled through the skull above the auditory cortex (ACx) (3mm posterior to bregma, 3 mm lateral to the midline) and above the mPFC (1.7 mm anterior to bregma, 0.4 mm lateral to the midline) and stainless steel screws were inserted to make contact with cerebrospinal fluid.

Alligator clamps were attached to the screws and connected to the Plexon Omniplex system to record cortical ECoGs. Cortical LFPs were recorded with a glass tip electrode (impedance = 0.18 M Ω) that was similarly connected to the Omniplex system. Standard computer speakers were used to present auditory stimuli generated by a stimulator (AM Systems 2100 Stimulator, Carlsbog, WA, USA) according to the following parameters: 20 or 40 Hz pulses, 500 ms pulse duration, 1 sec between pulses, 80 dB pulse intensity. Speakers were initially positioned on either side of the anesthetized animal, but produced a saturating stimulus artifact with that orientation and were eventually positioned under the recording table and electrically shielded. ECoGs and LFPs were recorded from both ACx and PFC during presentation of 60-100 trains of auditory stimuli. Despite our many electrical grounding/shielding attempts, stimulus artifacts continued to contaminate LFP recordings and therefore only data from ECoG recordings are presented. ECoGs were analyzed in Neuroexplorer by averaging ECoG activity aligned to the onset of auditory stimuli. These signals were analyzed without filtering or were filtered using a 15 Hz high-pass filter (for analysis of 20 Hz stimuli) or 30 Hz high-pass filter (for analysis of 40 Hz stimuli).

I. In vivo Novel Environment ECoG Recording and Analysis

Animals were treated and implanted with radio-telemetric transmitters as described above. Mice were acclimated to the behavior testing room for 1 hour before ECoG recordings. Mice were then introduced to a black, plastic behavioral arena (12"x 9"x 11") for 1 hour in low light conditions that served as a novel environment. These novel environment recording sessions took place midway through the animals' light cycles (~11 am - 3 pm). Frontal ECoGs were recorded using the Dataquest A.R.T. acquisition system (Data Sciences International). We analyzed *in vivo* ECoGs with custom-written MATLAB scripts and the `mtspecgramc` routine in the Chronux Toolbox (<http://chronux.org>). Oscillation power in each bandwidth [delta (1-3 Hz); theta (4-7 Hz); alpha (8-12 Hz); beta (13-29 Hz); gamma (30-80 Hz)] was computed in 10-second bins from spectrograms for each animal during the first 50 minutes of the hour-long novel environment session. Not every animal had exactly 60 minutes of data, and therefore only the first 50 minutes of ECoG activity was analyzed. Oscillation power in each bandwidth was averaged for each animal for the entire 50 minutes or within 10-minute segments. Average power in each bandwidth from multiple animals of the same adolescent treatment condition were then combined, tested for normality, and analyzed with MWU tests (significant $p < 0.05$) using STATA.

J. In vivo Overnight ECoG Recording and Analysis

Animals were treated and implanted with radio-telemetric transmitters, as described above. Mice were acclimated to the behavior testing room for 1 hour before ECoG recordings. The experimenter entered the room to initiate the recording session, but did so as quietly as possible so as to not startle the animals. After initiating the recordings,

animals were left undisturbed overnight (~6 pm - 8 am). ECoG activity was collected with the Dataquest A.R.T. Acquisition system (Data Sciences International). We analyzed a sample segment (3 hours) of ECoG activity that occurred approximately midway through the overnight recording session (~ 12 am - 3 am). Oscillation power in each bandwidth [delta (1-3 Hz); theta (4-7 Hz); alpha (8-12 Hz); beta (13-29 Hz); gamma (30-80 Hz)] was computed in 10-second bins from spectrograms from each animal. Oscillation power in each bandwidth was averaged for each animal for the entire 3-hour segment or within 30-minute epochs. Average power in each bandwidth from multiple animals of the same adolescent treatment condition were then combined, tested for normality, and analyzed for an effect of cannabinoid treatment with MWU tests (significant $p < 0.05$) using STATA.

III. Results

A. Chronic Adolescent WIN Exposure Persistently Alters ECoG Oscillations in vivo During the Novel Object Recognition Task

To test the prediction that adolescent cannabinoid exposure alters *in vivo* cortical oscillations recorded from intact animals, we recorded ECoG activity during the novel object recognition (NOR) task. Male CD-1 mice were chronically treated during adolescence (P35-P55) with either vehicle or WIN (1 mg/kg) and were implanted with ECoG leads into the frontal cortex upon reaching adulthood (> P100). We recorded cortical ECoGs during the NOR task to test whether WIN-treated animals displayed abnormal cortical oscillations that might parallel their impaired object recognition behavior after a 60-minute delay between the sample and test session. ECoG power was

extracted in the different frequency bandwidths and analyzed during the first 3 minutes of object investigation.

We first needed to ascertain that the NOR task is associated with modulation of cortical oscillations, so that the task can be used to reliably compare oscillations in vehicle and WIN-treated mice. We compared power in the different bandwidths (delta, theta, alpha, beta, gamma) during the first 3 minutes of sample and test sessions for both vehicle (Figure 17A-E) and WIN mice (Figure 17F-J). In vehicle-treated mice, gamma (Figure 17A) and delta power (Figure 17E) were significantly higher during the test session than during the sample session (Figure 17A). Power in the beta, alpha, and theta bandwidths did not change (Figures 17). Interestingly, this modulation of gamma and delta power was not evident in the frontal ECoGs of mice treated with WIN in adolescence (Figures 17F, J), nor did power differ between the sample and test session in any other frequency bandwidth (Figures 17G,H,I). Therefore, while exposure to a familiar object increased the power of gamma and delta oscillations in vehicle-treated mice, repeated WIN treatment during adolescence may prevent this task-dependent modulation of frontal ECoG oscillation power.

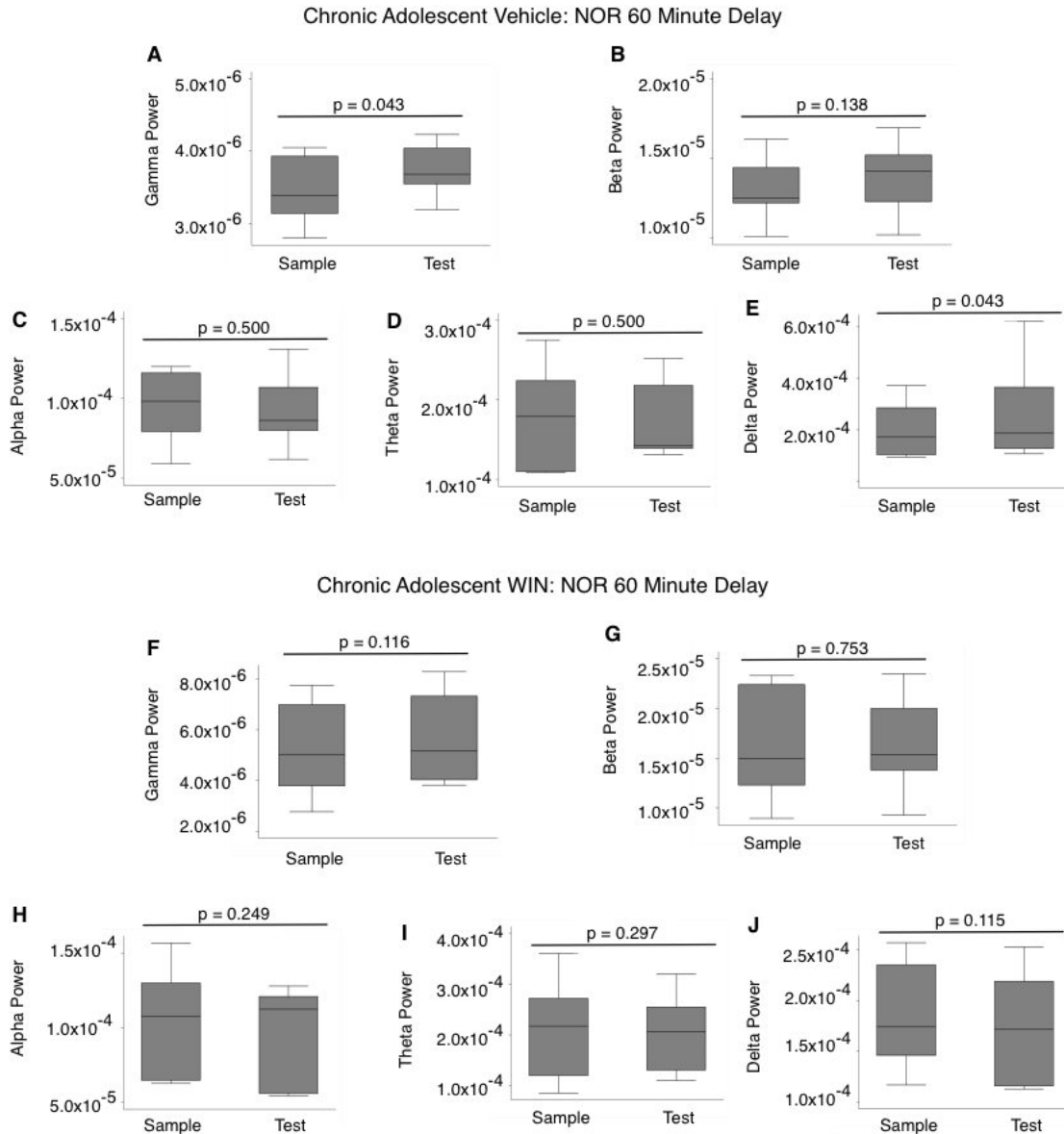


Figure 17: Frontal ECoG gamma and delta oscillation power is significantly higher during the NOR test session than the NOR sample session in adult mice treated with vehicle, but not WIN in adolescence. (A-E) In adolescent vehicle-treated adult mice ($n = 5$), frontal ECoG oscillation power in the (A) gamma (30-80 Hz), (B) beta (13-29 Hz), (C) alpha (8-12 Hz), (D) theta (4-7 Hz), or (E) delta (1-3 Hz) frequency bandwidths during the first 3 minutes of the sample and test sessions of the NOR task after a 60-minute delay. (F-J) In adolescent WIN (1 mg/kg) treated adult mice ($n = 6$), frontal ECoG oscillation power in the (F) gamma, (G) beta, (H) alpha, (I) theta, or (J) delta frequency bandwidths during the first 3 minutes of the sample and test sessions of the NOR task after a 60-minute delay. Wilcoxon matched-pairs signed rank tests were used to compare sample and test session oscillation power in vehicle and WIN-treated mice (significant $p < 0.05$).

Due to the behavioral impairment demonstrated by WIN animals during the NOR test session after a 60-minute delay period, we predicted that oscillation power would be

significantly lower in WIN-treated mice than in vehicle mice during the test session, in parallel with the behavioral results. Surprisingly, WIN animals had significantly *higher* gamma ECoG power than vehicle-treated mice ($p = 0.045$) (Figure 18A) during the first 3 minutes of the NOR test session. Oscillation power in the other bandwidths tested did not significantly differ between vehicle and WIN-treated mice during the NOR test session or during the sample session (data not shown). Gamma power in WIN animals was significantly higher than in vehicle-treated mice during most 30-second epochs of the first 3 minutes of the NOR test session (Figure 18B). Although we wanted to compare animals' behavior with their gamma oscillation power, ECoGs and behavioral data were collected with independent acquisition systems and we were therefore unable to synchronize the data for this analysis.

We tested the intriguing possibility that this unexpected increase in gamma power in WIN-treated test session ECoGs was a physiological feature of WIN animals' impaired working memory performance after a 60-minute delay (Figure 15C). After a 30-minute delay between the sample and test sessions, WIN-treated mice displayed the same novel object preference as vehicle-treated animals (Figure 15A). Interestingly, during this 30-minute delay test session, gamma ECoG power did not significantly differ between vehicle and WIN-treated mice (Figure 18C). This was the case both when we analyzed average gamma power in the entire 3-minute session, as well as during 30-second bins (Figure 18D). This increase in frontal gamma ECoG activity in adolescent WIN-treated mice therefore appeared to be specific to the NOR test session in which they demonstrate a behavioral impairment.

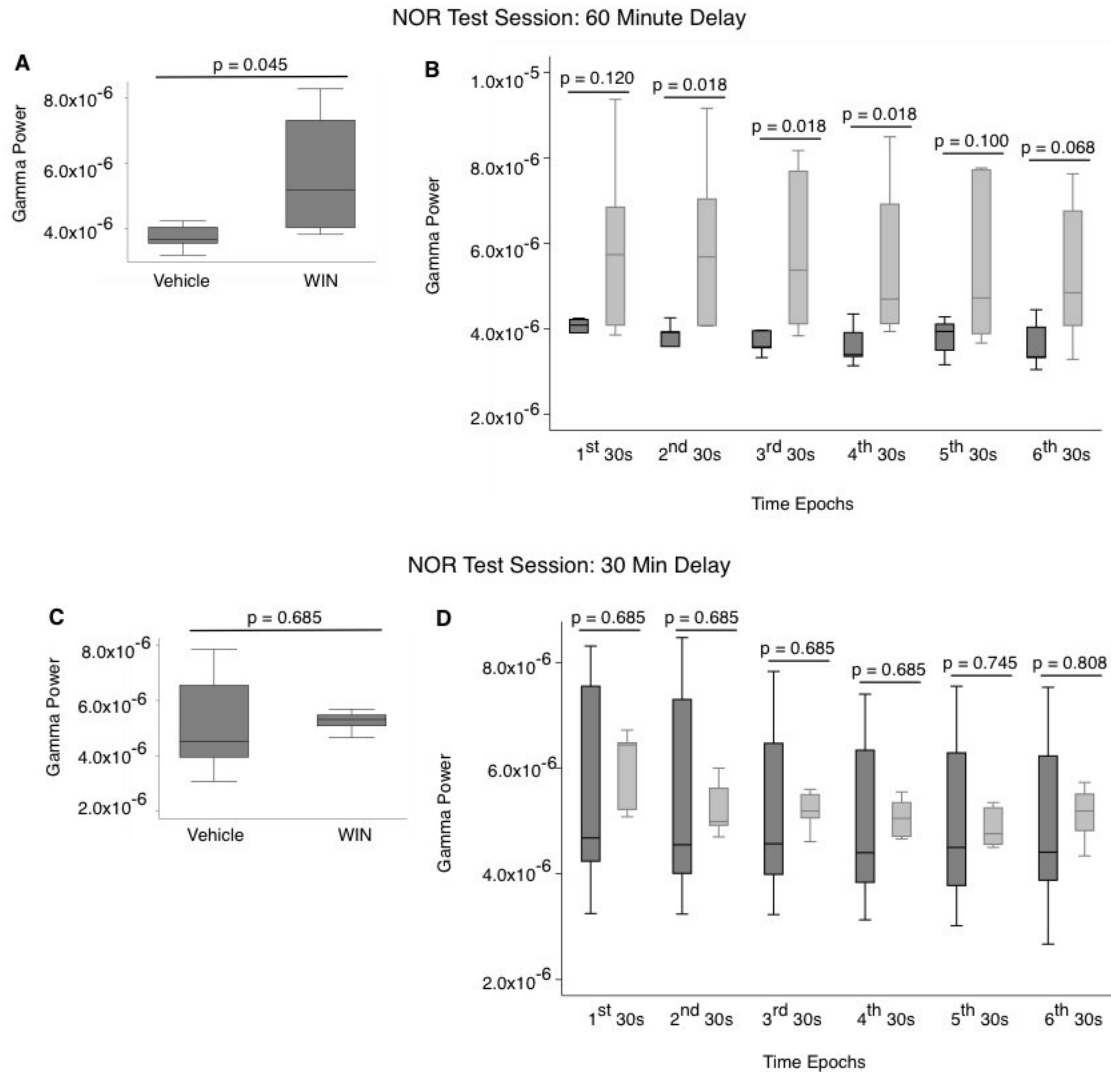


Figure 18: Frontal ECoG gamma power is significantly higher in chronic adolescent WIN than vehicle-treated mice during the NOR test session following a 60, but not a 30-minute delay. (A) Box-and-whisker plot of total frontal ECoG gamma power in vehicle (n = 5) or WIN (n = 6) treated mice during the first 3 minutes of the NOR test session after a 60-minute delay. (B) Box-and-whisker plot of frontal ECoG gamma power in 30-second bins during the first 3 minutes of the same test session as analyzed in (A) for adolescent vehicle (dark gray) and WIN (light gray) treated mice. (C) Box-and-whisker plot of total frontal ECoG gamma power in adolescent vehicle (n = 7) and WIN (n = 5) treated mice during the first 3 minutes of the NOR test session after a 30-minute delay. (D) Box-and-whisker plot of frontal ECoG power in 30-second bins during the first 3 minutes of the same test session as in (C) for adolescent vehicle (dark gray) and WIN (light gray) treated mice. MWU tests were used to determine an effect of adolescent WIN treatment (significant $p < 0.05$).

Unfortunately, technical limitations prevented us from time-locking behavior with ECoG activity to better understand what might account for the surprising finding of

increased gamma oscillation power in WIN treated mice. Furthermore, ECoG recordings lack adequate spatial resolution and may not be able to distinguish whether adolescent cannabinoid exposure alters cortical oscillations directly in mPFC. To more rigorously test the prediction that behaviorally-evoked prefrontal cortical oscillations are altered by chronic adolescent cannabinoid exposure, we recorded LFPs *in vivo* during the novel object recognition task. By synchronizing behavioral and electrophysiological data acquisition, we tested the prediction that LFP activity specifically in mPFC was modulated by the different behavioral epochs of the NOR task.

B. Chronic Adolescent WIN or THC Exposure Produced Inconclusive Effects in Novel Object Recognition Behavior and LFP Activity in mPFC

Male CD-1 mice were chronically treated during adolescence (P35-P55) with either vehicle, WIN (1 mg/kg) or THC (5 mg/kg) and implanted with multi-electrode arrays in mPFC after reaching adulthood (> P100). NOR testing was performed as previously described. LFPs and behavioral videos were acquired using the Plexon Cineplex Capture system, behavior was analyzed using Cineplex Markup, and Neuroexplorer was used to analyze behaviorally-synched LFP activity.

Although we prepared 21 mice for these experiments, 11 of these animals lost their electrode implants before we were able to obtain data from them. We completed NOR testing and LFP recordings and analysis on the remaining 10 mice, 4 of which were exposed to vehicle, 3 that were injected with WIN, and 3 with THC during adolescence. As discussed in the Methods section, these small sample sizes prevented us from conducting statistical analyses on LFP, but not on the behavioral data. Therefore, LFP

oscillation data will be presented in figures and described in text, but no statistical conclusions will be made.

1. NOR Task: Behavior

We were unable to replicate our previous finding of a working memory deficit in the NOR task in chronic adolescent WIN-treated mice, likely due to the unexpectedly small samples size for each treatment condition. We first analyzed time spent investigating the novel and familiar objects and compared the novel object preference between the different treatment conditions (Figure 19A), as previously described in Chapter 3. Vehicle and WIN-treated mice demonstrated equal preferences for the novel object [vehicle (n = 4 mice): novel object preference = $50.0 \pm 8.3\%$; WIN (n = 3 mice): novel object preference = $60.0 \pm 23.5\%$; $p = 0.455$]. Surprisingly, mice treated in adolescence with THC displayed a significantly *higher* preference for the novel object than vehicle-treated mice [vehicle (n = 4 mice): novel object preference = $50.0 \pm 8.3\%$; THC (n = 3 mice): novel object preference = $64.4 \pm 2.5\%$; $p = 0.036$]. Total investigation time of both objects approached, but did not reach significance between vehicle and WIN-treated mice (vehicle: 33.0 ± 8.9 sec; WIN: 15.9 ± 10.0 sec; $p = 0.058$) and THC and vehicle-treated mice spent the same total amount of time investigating objects (vehicle: 33.0 ± 8.9 sec; THC: 21.8 ± 6.4 sec; $p = 0.127$) (Figure 19B). Similar to data reported in Chapter 3, neither vehicle, WIN, nor THC animals displayed a significant side-bias during the NOR test session (vehicle: chi-square test: $p = 0.66$; WIN: chi-square test: $p = 0.17$; THC: chi-square test: $p = 0.28$) (Figure 19C), and again, the object location (left or right) of the novel object was counterbalanced in this behavior session. Overall, it is difficult to draw conclusions regarding the effect of adolescent WIN or THC exposure

on NOR working memory performance, as vehicle-treated animals do not appear to act as a positive behavioral control during this run of the NOR task, as indicated by their devoting equal attention to the novel and familiar objects.

We examined the animals' behavior during the sample session to determine whether vehicle animals failed to investigate the objects during their initial exposure to them, which could underlie the absence of a novel object preference during the test session. This was not the case. WIN and THC exposed mice investigated the objects for equal amounts of time as vehicle-treated animals (vehicle: 43.6 ± 8.8 sec; WIN: 26.3 ± 13.0 sec; $p = 0.086$; THC: 38.6 ± 7.5 sec; $p = 0.461$) (Figure 19D). Furthermore, only THC-treated mice displayed a significant directional bias during the sample session with a preference for the right object (vehicle: chi-square test: $p = 0.24$; WIN: chi-square test: $p = 0.46$; THC: chi-square test: $p = .04$) (Figure 19E). This bias was not present in the THC group during the test session, allowing us to exclude the possibility that THC animals performed better than vehicle-treated mice due to the novel object consistently being located on their preferred side of the behavioral testing arena.

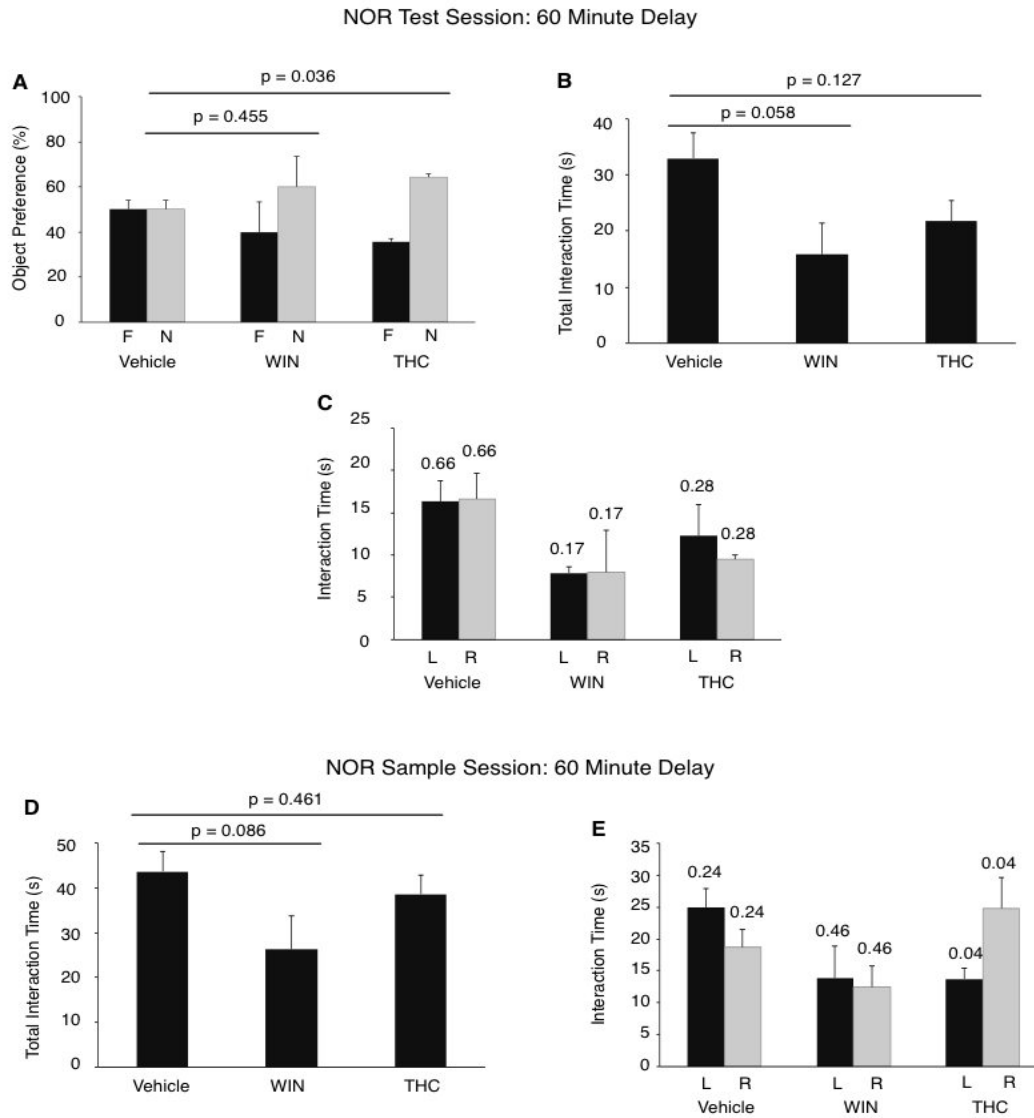


Figure 19: Attempted replication of the NOR deficit by chronic adolescent WIN exposure was unsuccessful in a small sample of adult mice. (A) Mean preference for the familiar (F) or novel object (N) object (% preference = time investigating F or N objects/total time investigating both objects) during the first 3 minutes of the test session after a 60-minute delay by adolescent vehicle (n = 4), WIN (1 mg/kg; n = 3), or THC (5 mg/kg; n = 3) treated adult mice. Novel object preference is compared between vehicle and WIN animals with 2-tailed Student's t-tests (significant $p < 0.05$) (B) Mean total object investigation time during the 3-minute test session after a 60-minute delay by adolescent vehicle, WIN, or THC-treated adult mice are compared between vehicle and WIN or vehicle and THC-treated mice with 2-tailed Student's t-tests. (C) Mean time investigating the object located on the left (L) and right (R) side of the behavioral arena during the test session. *P* values from chi-square tests are presented from comparisons of the actual time animals' spent with L and R objects compared to the expected time (50% of total time spent with each object) if animals' investigated each object equally. (D) Mean total number of object contacts during the test session in (A-C). (E) Mean total object investigation time during the 3-minute sample session before a 60-minute delay in adolescent vehicle, WIN, or THC-treated adult mice. Statistical analyses are performed as in (B). (E) Mean time investigating the L and R objects during the sample session. Statistical analyses are performed as in (C). Throughout, all error bars represent the standard error of the mean (SEM).

Behavior results from the NOR task after a 60-minute delay did not replicate our previous results of a working memory impairment in adolescent, cannabinoid-treated mice, as both vehicle and WIN treated mice had similar novel object preferences, and THC-treated animals displayed a significantly higher preference for the novel object than mice treated in adolescence with vehicle. These unexpected findings are probably due to inadequate sample sizes in each treatment condition and high behavioral variability in WIN-treated mice during the sample session.

2. NOR Task: LFP Activity in mPFC

Despite the absence of a NOR behavioral impairment in adolescent cannabinoid-treated mice, we analyzed mPFC LFP oscillations during both the sample and test sessions to determine whether the NOR task evoked detectable differences in mPFC LFP oscillations during different behavioral epochs, as well as to test the prediction that adolescent-cannabinoid treatment would alter oscillations during this cognitive task. As described in the Methods section, we could not conduct statistical analyses on LFP oscillation data due to the inadequately small sample sizes.

An example vehicle-treated LFP including novel and familiar object investigation epochs is shown in Figure 20A, in which there appears to be a reduction in LFP amplitude during novel object investigation. We computed average peri-event spectrograms centered around the onset of either novel or familiar investigation to determine whether LFP oscillations were modulated by investigation of either type of object, examples of which are shown in Figure 20B,C. Lower frequency (theta and alpha) power markedly increased approximately 0.5 seconds after novel object investigation onset in this vehicle-treated example (Figure 20B). Interestingly, this increase was not

apparent after the onset of familiar object investigation (Figure 20C). A similar increase in low frequency power was present approximately 0.5 seconds after the onset of object investigation during the test session, regardless of novel or familiar object identity (Figure 19D) that was likely driven by increased power after novel object investigation initiation. This object-related increase at 0.5 seconds was not present during object investigation during the NOR sample session (Figure 20E). Power in the lower frequency bandwidths appeared to increase approximately 1 second after the onset of object investigation. However, after expanding the time-scale after object investigation onset, we discovered that this low frequency increase repeated approximately every 0.5 seconds after its first appearance, potentially due to multiple object contacts within this time-window. While we were encouraged by the appearance of event-related oscillatory power in this vehicle-treated animal, subsequent analysis of network activity in other animals did not reveal a consistent pattern, nor did this peri-event oscillation activity correlate with NOR test session performance (data not shown).

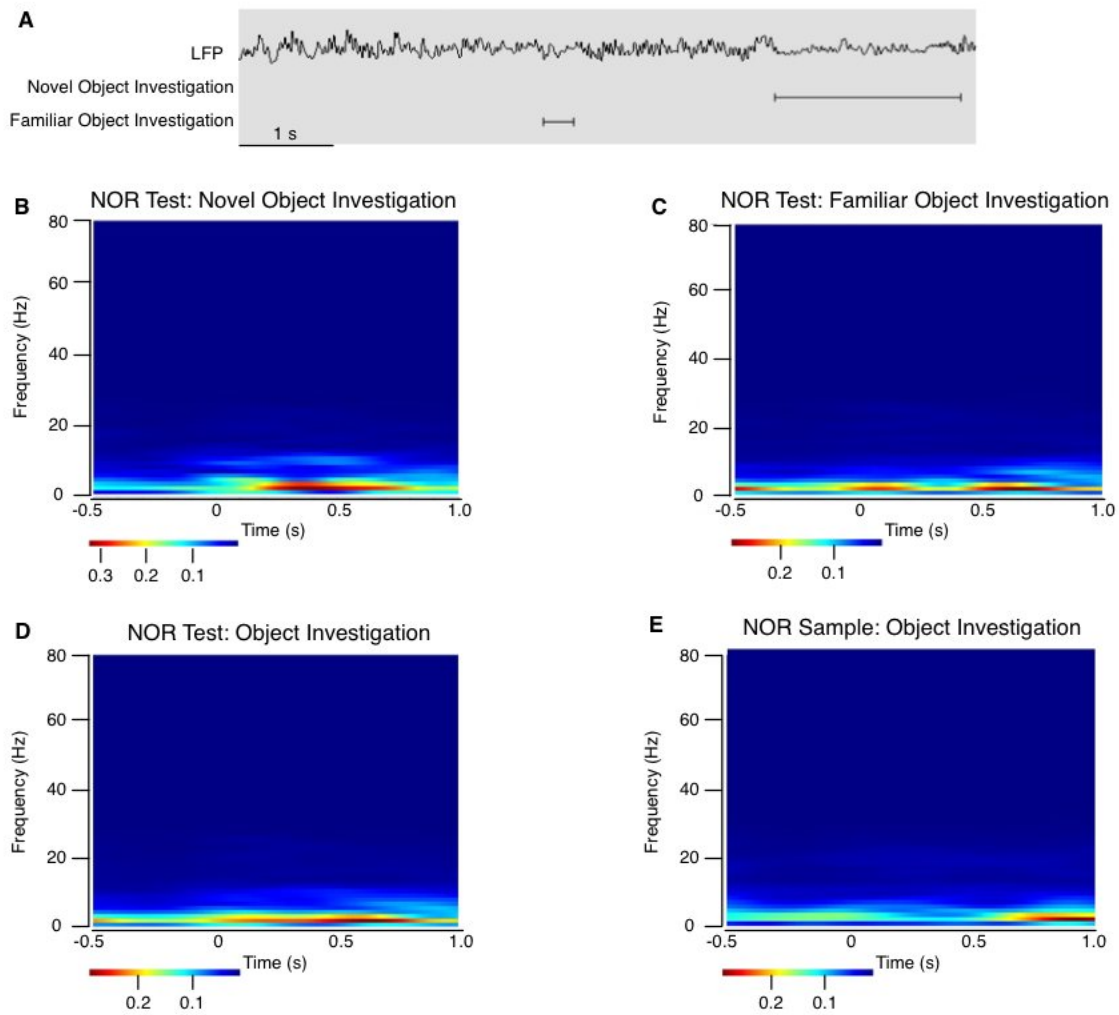


Figure 20: Object investigation in the NOR task does not reliably evoke oscillations in an mPFC LFP recorded in an adolescent vehicle-treated adult mouse. (A) Example mPFC LFP recording in an adolescent vehicle-treated adult mouse during the NOR test session after a 60-minute delay while investigating both novel and familiar objects. (B) Example peri-event spectrogram averaged over 11 novel object contacts during the same recording as in (A). (C) Example peri-event spectrogram averaged over 18 familiar object contacts during the same recording as in (A,B). (D) Example peri-event spectrogram averaged over 29 object contacts, regardless of novel or familiar identity, during the same recording as in (A-C). (E) Example peri-event spectrogram averaged over 35 object contacts during the NOR sample session before a 60-minute delay for the same animal as in (A-D). For all spectrograms, time = 0 is the initiation of object investigation as identified during off-line behavioral analysis.

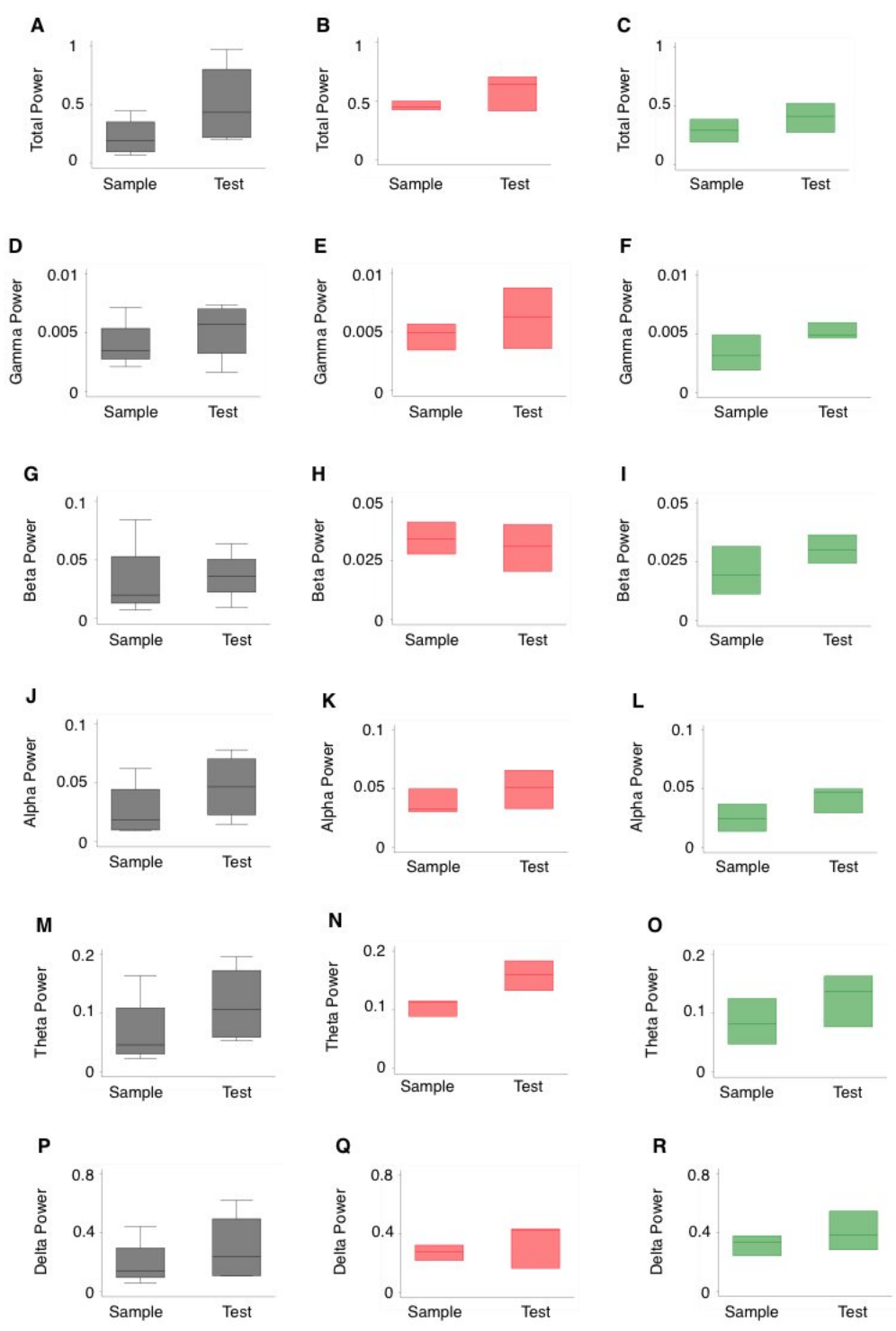
3. LFP Analysis: Entire Behavior Sessions

Nevertheless, we persisted in our analysis of LFP activity during the NOR task to determine whether oscillations were behaviorally-modulated, and to establish a protocol

for LFP analysis in the context of animals' behavior that can be employed in future experiments. As we found in frontal ECoGs (Figure 17), we predicted that, in vehicle-treated animals, gamma and delta power would be significantly higher during the NOR test session than during the sample session. We predicted that similar behavioral modulation of oscillations would not be present in animals exposed to 1 mg/kg WIN or 5 mg/kg THC in adolescence. We extracted power from LFPs during the entire NOR sample and test sessions. Box-and-whisker plots in Figure 21 show total oscillation power in adult animals treated in adolescence with vehicle (dark gray boxes; Figure 21A), WIN (red boxes; Figure 21B), or THC (green boxes; Figure 21C), as well as power in the distinct frequency bandwidths (Figures 21D-R). Although these sample sizes are too small for statistical analysis, it appears that, in adolescent vehicle-treated mice, total oscillation power increases from the sample session to the test session (Figure 21A). Similar increases may be present in WIN (Figure 21B) and THC-treated mice (Figure 21C), although the sample size of $n = 3$ in each of these conditions makes such a conclusion tenuous. Frontal ECoG analysis indicated that gamma and delta power increased significantly in vehicle, but not WIN-treated mice, between the sample and test session in which WIN-treated animals performed significantly poorer (Figure 17), suggesting that recognition of an object's familiarity evokes gamma and delta oscillations. While we do not find a similar behavioral difference in these small samples of adolescent vehicle and WIN-treated animals, adolescent THC-treated mice demonstrated a significantly higher novel object preference than vehicle animals. Interestingly, in this THC-treated sample, mPFC LFP gamma power appears to be higher during the test session than the sample session (Figure 21F), as we previously found in the ECoGs of

vehicle-treated animals (Figure 17A), suggesting that recognizing an object's familiarity evokes gamma oscillations directly in mPFC. The same suggested relationship was not as clearly apparent in delta power recorded in THC-treated animals (Figure 21R), although, again, the small sample size makes these comparisons difficult. Clearly, increasing the sample size in all three treatment conditions is necessary if we are to make definitive conclusions regarding modulation of mPFC LFP oscillations during the different sessions of the NOR task.

Figure 21: LFP oscillation power in mPFC during the entire NOR sample and test sessions in adult mice treated in adolescence with vehicle, WIN, or THC. (A, D, G, J, M,P) Box-and-whisker plots of (A) total oscillation power, (D) gamma power, (G) beta power, (J) alpha power, (M) theta power, and (P) delta power in LFPs recorded in the mPFC in chronic adolescent vehicle-treated adult mice (n = 4) during the NOR test and sample sessions after a 60-minute delay. (B, E, H, K, N, Q) Box-and-whisker plots of (B) total oscillation power, (E) gamma power, (H) beta power, (K) alpha power, (N) theta power, and (Q) delta power in LFPs recorded in the mPFC in chronic adolescent WIN (1 mg/kg) treated adult mice (n = 3) during the NOR test session after a 60-minute delay. (C, F, I, L, O, R) Box-and-whisker plots of (C) total oscillation power, (F) gamma power, (I) beta power, (L) alpha power, (O) theta power, and (R) delta power in LFPs recorded in the mPFC in chronic adolescent THC (5 mg/kg) treated adult mice (n = 3) during the NOR test session after a 60-minute delay. Dark gray = vehicle, red = WIN, and green = THC-treated mice.



4. LFP Analysis: Object Investigation vs. No-Object Investigation Behavior Epochs

Selectively attending to stimuli and integrating sensory information evoke cortical oscillations (Fries *et al.*, 2001; Singer and Gray, 1995). We therefore predicted that oscillation power would be higher during behavioral epochs in which animals were investigating, compared to epochs in which they were not investigating objects. This may not be the case. We compared mPFC LFP oscillation power during object investigation (OI) and no object investigation (NI) behavioral epochs during the NOR test session and sample session, in animals treated during adolescence with vehicle, WIN, or THC. Box-and-whisker plots of LFP power during these epochs in the test session are presented in Figure 22, and are presented for the NOR sample session in Figure 23. Interestingly, it appeared that investigation of both objects (familiar and novel combined) did not obviously change oscillation power in vehicle-treated animals during the test session (Figures 22A,D,G,J,M,P) although there may be a trend towards lower gamma (Figure 22D) and beta power (Figure 22G) during epochs of object investigation. Lower beta and alpha power appears to emerge in WIN (Figures 22H,K) and THC-treated animals (Figures 22I,L) during the test session while they were engaged with the objects. This distinction may be more apparent during the NOR sample session, in which oscillation power in most frequencies appears lower during object investigation epochs, compared to periods in which animals are not interacting with the objects (Figure 23), independent of adolescent treatment. Again, no statistical analyses were performed on these data due to small sample sizes, although it is interesting to note that locomotion and grooming behaviors—the predominant behaviors that animals engage in while not investigating objects—may be associated with higher oscillation power than interaction with objects.

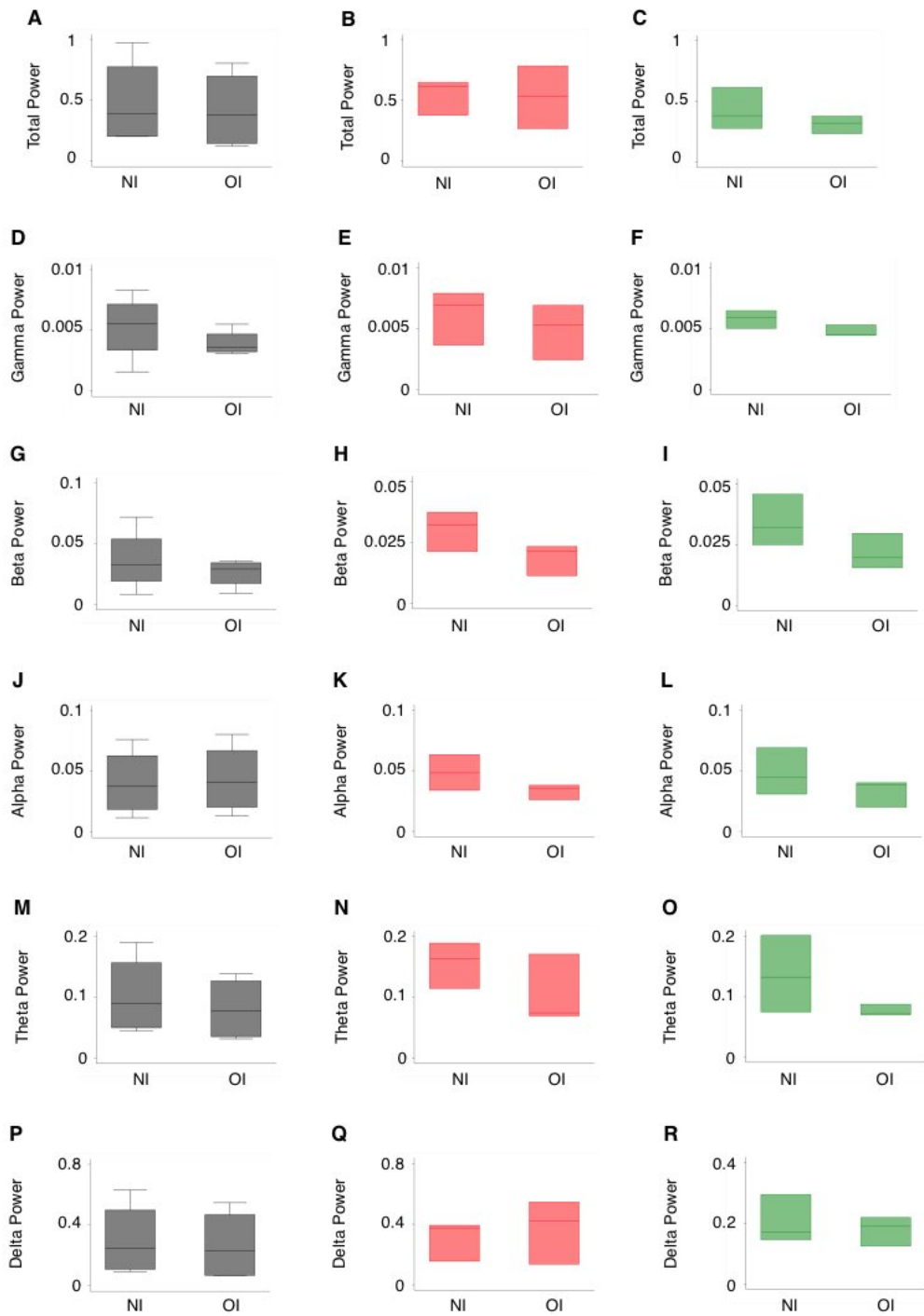


Figure 22: LFP oscillation power in mPFC during the object investigation and non-object investigation behavioral epochs of the NOR test session in adult mice treated in adolescence with vehicle, WIN, or THC. (A, D, G, J, M,P) Box-and-whisker plots of (A) total oscillation power, (D) gamma power, (G) beta power, (J) alpha power, (M) theta power, and (P) delta power in LFPs recorded in the mPFC in chronic adolescent vehicle-treated adult mice ($n = 4$) during object investigation (OI) or non-object investigation (NI) segments in the NOR test session. (B, E, H, K, N, Q) Box-and-whisker plots of

(B) total oscillation power, (E) gamma power, (H) beta power, (K) alpha power, (N) theta power, and (Q) delta power in LFPs recorded in the mPFC in chronic adolescent WIN (1 mg/kg) treated adult mice (n = 3) during object investigation (OI) or non-object investigation (NI) segments in the NOR test session. (C, F, I, L, O, R) Box-and-whisker plots of (C) total oscillation power, (F) gamma power, (I) beta power, (L) alpha power, (O) theta power, and (R) delta power in LFPs recorded in the mPFC in chronic adolescent THC (5 mg/kg) treated adult mice (n = 3) during object investigation (OI) or non-object investigation (NI) segments in the NOR test session. Dark gray = vehicle, red = WIN, and green = THC-treated mice.

Figure 23: LFP oscillation power in mPFC during the object investigation and non-object investigation behavioral epochs of the NOR sample session in adult mice treated in adolescence with vehicle, WIN, or THC. (A, D, G, J, M,P) Box-and-whisker plots of (A) total oscillation power, (D) gamma power, (G) beta power, (J) alpha power, (M) theta power, and (P) delta power in LFPs recorded in the mPFC in chronic adolescent vehicle-treated adult mice (n = 4) during object investigation (OI) or non-object investigation (NI) segments in the NOR sample session. (B, E, H, K, N, Q) Box-and-whisker plots of (B) total oscillation power, (E) gamma power, (H) beta power, (K) alpha power, (N) theta power, and (Q) delta power in LFPs recorded in the mPFC in chronic adolescent WIN (1 mg/kg) treated adult mice (n = 3) during object investigation (OI) or non-object investigation (NI) segments in the NOR sample session. (C, F, I, L, O, R) Box-and-whisker plots of (C) total oscillation power, (F) gamma power, (I) beta power, (L) alpha power, (O) theta power, and (R) delta power in LFPs recorded in the mPFC in chronic adolescent THC (5 mg/kg) treated adult mice (n = 3) during object investigation (OI) or non-object investigation (NI) segments in the NOR sample session. Dark gray = vehicle, red = WIN, and green = THC-treated mice.

5. LFP Analysis: Familiar vs. Novel Object Investigation Behavior Epochs

Gamma power increases in frontal ECoGs of human subjects during recognition of a familiar object in novel object recognition tasks (Gruber *et al.*, 2008). We therefore predicted that oscillation power would be higher during epochs of familiar object investigation than in epochs in which animals investigated novel objects. Preliminary data collected from these small numbers of adolescent cannabinoid treated animals suggest that this may be the case across most frequencies in vehicle and THC, but not WIN-treated mice (Figure 24). Specifically, gamma power appears lower in vehicle treated animals while they investigate novel objects (Figure 24D) relative to power during familiar object investigation. A similar trend may emerge for THC-exposed animals (Figure 24F). However, adolescent WIN-treated animals appear to demonstrate increased gamma power while they investigate the novel object, compared to an object that they have encountered previously (Figure 24E). Furthermore, median novel object investigation power appears higher than familiar object investigation power in all other frequencies as well in animals treated with WIN (Figures 24B,E,H,K,N,Q). Based on these very preliminary comparisons of the distribution of oscillation power during familiar and novel object investigation epochs, it appears that mPFC LFP oscillations are more robust during investigation of a previously encountered object. Future experiments will need to be conducted to determine whether increased oscillation power during these familiar object epochs coincides with behavioral recognition of the familiar object, as would be inferred by a preference for the novel object.

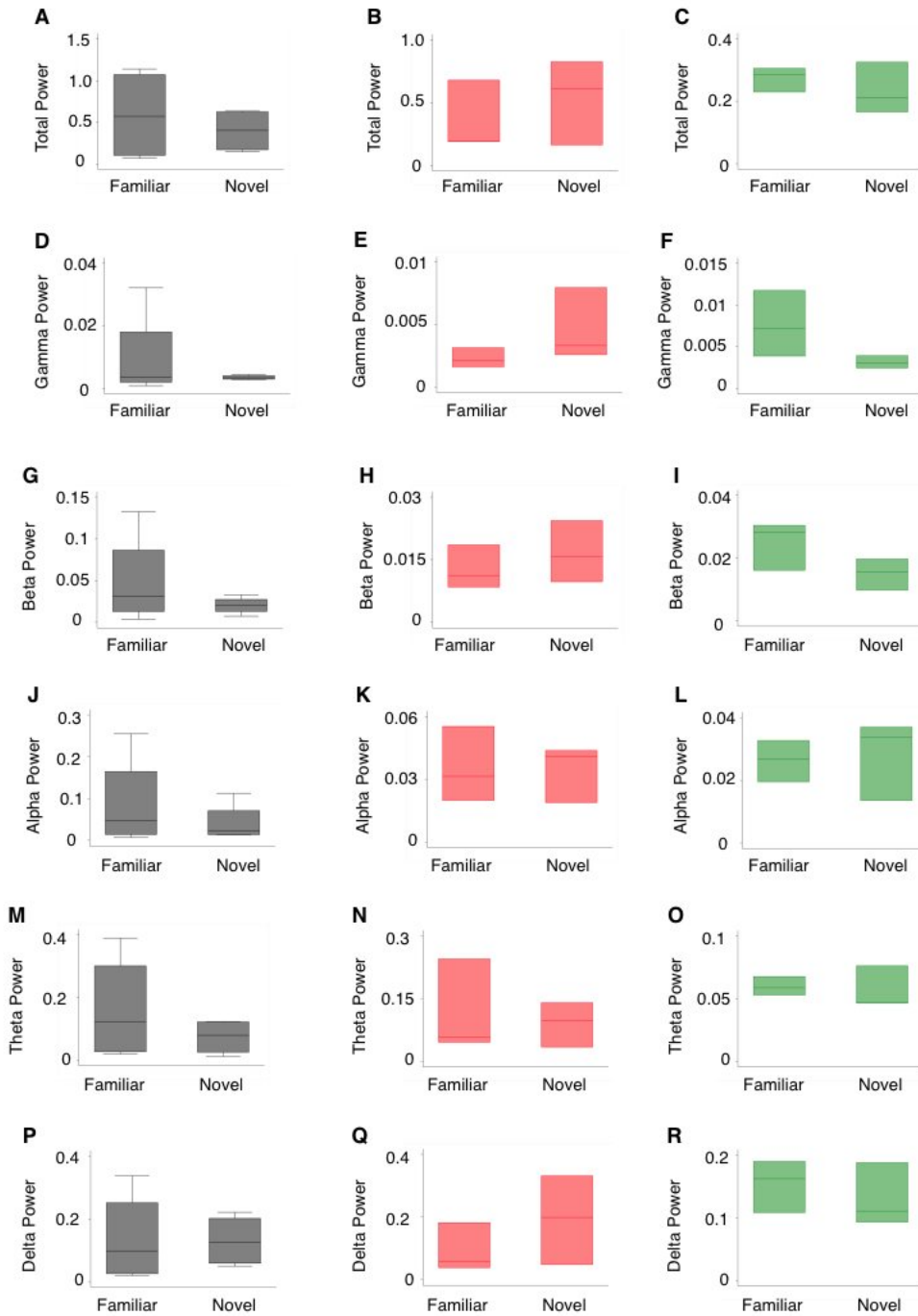


Figure 24: LFP oscillation power in mPFC during the familiar and novel object investigation behavioral epochs of the NOR test session in adult mice treated in adolescence with vehicle, WIN, or THC. (A, D, G, J, M,P) Box-and-whisker plots of (A) total oscillation power, (D) gamma power, (G) beta power, (J) alpha power, (M) theta power, and (P) delta power in LFPs recorded in the mPFC in chronic adolescent vehicle-treated adult mice (n = 4) during familiar or novel object investigation segments in the NOR test session. (B, E, H, K, N, Q) Box-and-whisker plots of (B) total oscillation power, (E) gamma power, (H) beta power, (K) alpha power, (N) theta power, and (Q) delta power in LFPs recorded in the mPFC in chronic adolescent WIN (1 mg/kg) treated adult mice (n = 3) during familiar or novel object

investigation segments in the NOR test session. (C, F, I, L, O, R) Box-and-whisker plots of (C) total oscillation power, (F) gamma power, (I) beta power, (L) alpha power, (O) theta power, and (R) delta power in LFPs recorded in the mPFC in chronic adolescent THC (5 mg/kg) treated adult mice (n = 3) during familiar or novel object investigation segments in the NOR test session. Dark gray = vehicle, red = WIN, and green = THC treated mice.

6. LFP Analysis: Comparison of Chronic Adolescent WIN, THC, or Vehicle-

Administered Animals

We observed that LFP oscillations recorded in mPFC of adult animals treated with WIN or THC in adolescence appeared to be of lower power than those recorded in vehicle-treated animals, regardless of the behavioral epoch analyzed. Again, we could not perform statistical analyses on these comparisons, but the distribution of oscillation power in the different frequencies is presented in Figure 25. Interestingly, total and gamma power may be higher in WIN-treated mice during the entire session than in vehicle-treated animals (Figure 25A,B), which would be in line with our previous findings that adolescent WIN-treated gamma oscillation power was significantly higher than in vehicle-treated frontal ECoGs (Figure 18) during the NOR test session.

Oscillation power in most frequencies appeared to be somewhat modulated in vehicle-treated animals by the different behavioral epochs of the NOR test session, but this was not as readily apparent in data from WIN or THC animals. More specifically, gamma, beta, and alpha power was more variable during familiar object investigation epochs in vehicle-treated mice than in either WIN or THC-exposed animals (Figures 25B,C,D).

Preliminary data suggest that oscillations in mPFC may be higher in WIN-treated animals during the entire NOR test session, but lower during familiar object investigation epochs, although additional animals are required to definitively test the prediction that chronic adolescent cannabinoid exposure attenuates oscillations *in vivo* during cognitive behavioral tasks.

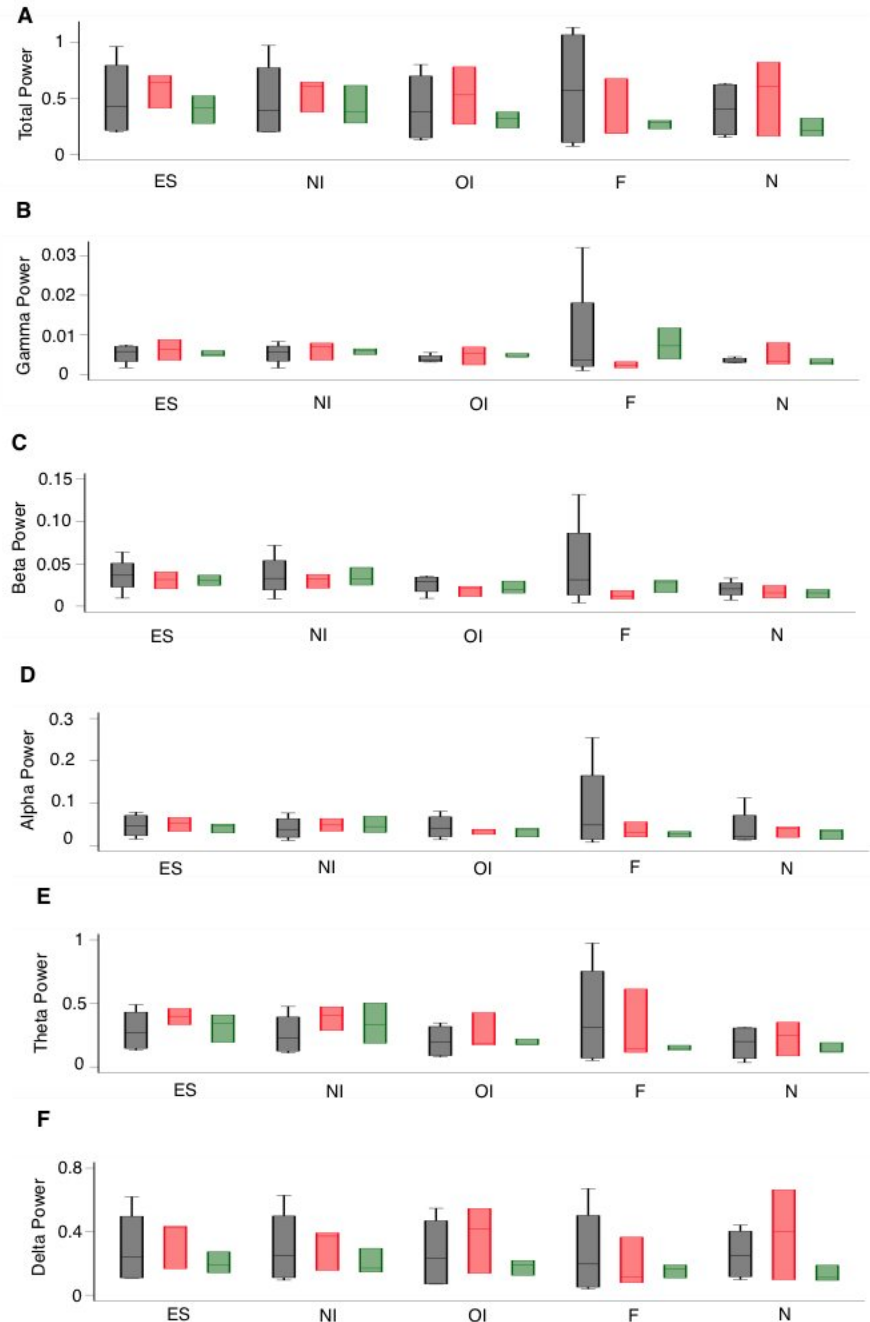


Figure 25: Oscillation power in mPFC LFPs in chronic adolescent vehicle, WIN, or THC treated mice during the different behavioral epochs of the NOR test session. (A) Box-and-whisker plots of (A) total oscillation power, (B) gamma power, (C) beta power, (D) alpha power, (E) theta power, and (F) delta power in vehicle (n = 4)(dark grey), WIN (1 mg/kg; n = 3) (red), and THC (5 mg/kg; n = 3) (green) treated mice during the different behavioral epochs of the test session after a 60-minute delay. ES = entire 3 minute session, NI = all no object investigation epochs, OI = all object investigation epochs, F = all familiar object investigation epochs, N = all novel object investigation epochs.

Increased oscillation power may also be present in adolescent WIN-treated animals during the NOR sample session, as shown in Figure 26. Although these sample sizes are very small, it is interesting to note that WIN, but not THC oscillation power in most frequencies appears to be higher than vehicle power during the entire session and during the non-object interaction epochs. Of course, it remains to be seen whether any of these comparisons will achieve statistical significance if more data were added to the analysis. In the absence of statistical analyses on LFP oscillations recorded during the NOR task, we can only conclude that the NOR task appears to be evoking oscillations directly in mPFC and that the power of these oscillations may be modulated by the different behavioral epochs of the NOR task.

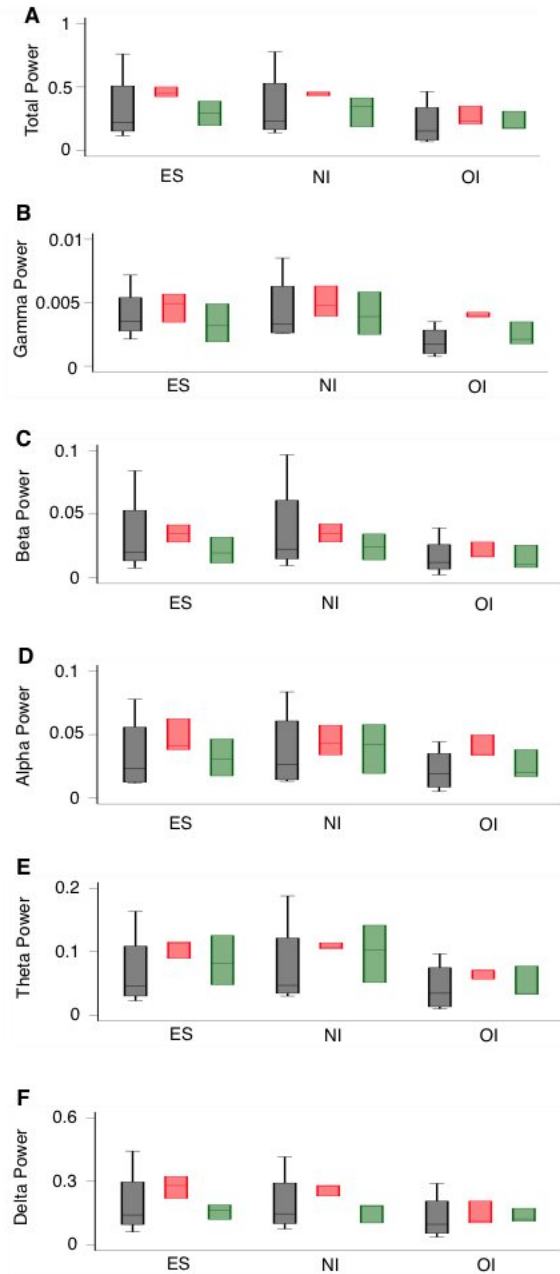


Figure 26: Oscillation power in mPFC LFPs in chronic adolescent vehicle, WIN, or THC treated mice during the different behavioral epochs of the NOR sample session. (A) Box-and-whisker plots of (A) total oscillation power, (B) gamma power, (C) beta power, (D) alpha power, (E) theta power, and (F) delta power in vehicle (n = 4)(dark grey), WIN (1 mg/kg; n = 3) (red), and THC (5 mg/kg; n = 3) (green) treated mice during the different behavioral epochs of the sample session before a 60-minute delay. ES = entire 3-minute session, NI = all no object investigation epochs, OI = all object investigation epochs.

C. Chronic Adolescent WIN Exposure Does Not Alter ECoG Oscillations in vivo

During the Object Recency Task

We recorded frontal ECoGs while mice performed the object recency (OR) task and compared oscillations between animals exposed to WIN (1 mg/kg) or vehicle in adolescence to determine whether adolescent WIN exposure altered oscillatory power during this working memory task, that reportedly engages the mPFC (Mitchell and Laiacona, 1998; Hannesson *et al.*, 2004; Barker *et al.*, 2007; Nelson *et al.*, 2011). After 45 minute delays between behavioral sessions, there was no difference in the recognition of the least recently viewed object (S1 preference) between vehicle and WIN-treated mice, regardless of whether we analyzed the first 3 or 1 minute of behavior (Figure 16). Nevertheless, we analyzed the ECoGs collected during this task to determine whether there existed any detectible differences in oscillations between treatment conditions. We analyzed the first minute of ECoG activity during the behavioral sessions, as this period is reportedly the most sensitive to animals' object preferences (Dix and Aggleton, 1999; Barker *et al.*, 2007). We first analyzed gamma (Figure 27A), beta (Figure 26B), alpha (Figure 27C), theta (Figure 27D), and delta (Figure 27E) oscillations during the OR test session. We found no statistically significant differences between vehicle and WIN-treated mice in any of the frequencies examined, in parallel with identical behavioral performance between vehicle and WIN-exposed animals. We therefore reasoned that the OR task, as it was run, was not an appropriate metric to detect possible effects of chronic adolescent 1 mg/kg WIN exposure on cognitive ability and behaviorally-evoked oscillations, and so we did not further pursue this behavioral method.

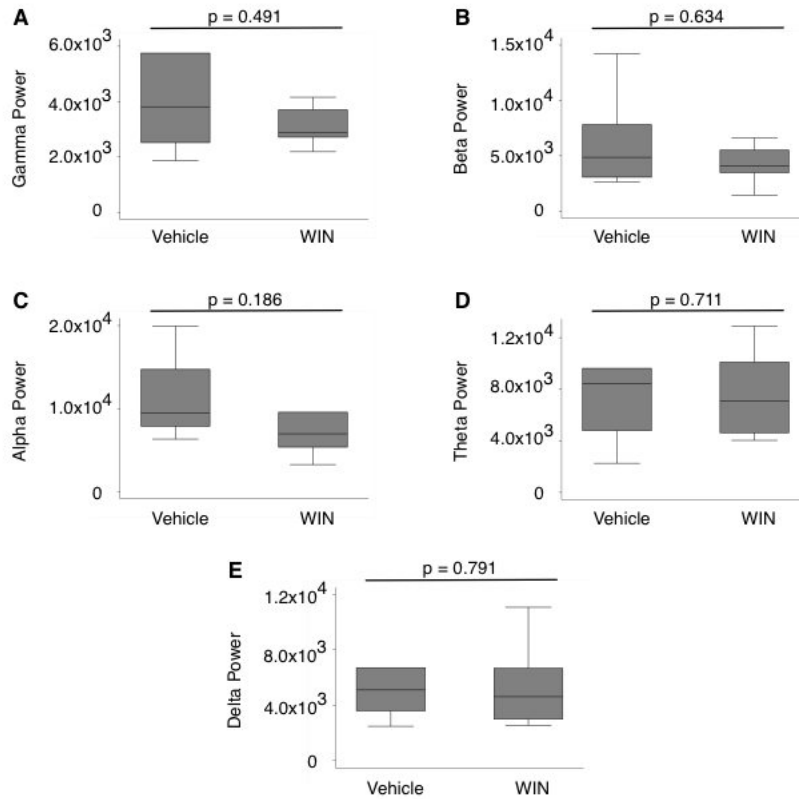


Figure 27: Oscillation power in frontal ECoGs does not differ between adult mice chronically exposed to vehicle or WIN during adolescence during the OR test session. Box-and-whisker plots of frontal ECoG (A) gamma power, (B) beta power, (C) alpha power, (D) theta power, (E) delta power during the first 1 minute of the OR test session in adolescent vehicle (n = 7) or WIN (1 mg/kg; n = 9) treated adult mice. Statistical comparisons between treatments were performed with MWU tests (significant $p < 0.05$).

D. Robust Auditory Stimuli-Evoked Oscillations Were Not Apparent in mPFC ECoGs

Robust oscillations can be evoked *in vivo* by presenting trains of auditory stimuli at various frequencies, and we employed this approach to test for predicted effects of adolescent cannabinoid exposure on *in vivo* oscillations in intact adult animals.

Frequency-dependent auditory steady-state evoked responses (ASSRs) can be elicited in cortical ECoGs by presenting trains of auditory stimuli at different frequencies to which ECoGs will entrain (Franowicz and Barth, 1995; Brenner *et al.*, 2009). These ASSRs have been reported to be abnormal in human chronic marijuana users (Skosnik *et al.*, 2012), and we predicted that they would be similarly impaired in adult mice chronically

exposed to cannabinoids in adolescence.

We presented animals with auditory stimuli at 20 or 40 Hz to try to generate robust beta and gamma oscillations, respectively, that we predicted would be suppressed in animals exposed to cannabinoids in adolescence. Figure 28A shows an event-triggered average, unfiltered ECoG in the auditory cortex (ACx), in response to multiple presentations of 40 Hz auditory stimuli, while Figure 28B shows that same averaged response filtered with a 30 Hz high-pass filter. Note that a clear response to the stimulus was only apparent when low-frequency contributions to the ECoG were removed with filtering. Because we were primarily interested in examining oscillatory activity in the prefrontal cortex, we recorded ECoGs from PFC in response to the same 40 Hz auditory stimuli. However, in PFC ECoG recordings, no clear response to the 40 Hz stimuli was apparent in either average unfiltered (Figure 28C) or 30 Hz high-pass filtered responses (Figure 28D). Because the PFC ECoG did not demonstrate clear stimulus-evoked oscillatory activity, and because of other technical aspects of this task discussed in greater detail later, we declined to pursue this method of evoking cortical oscillations *in vivo*.

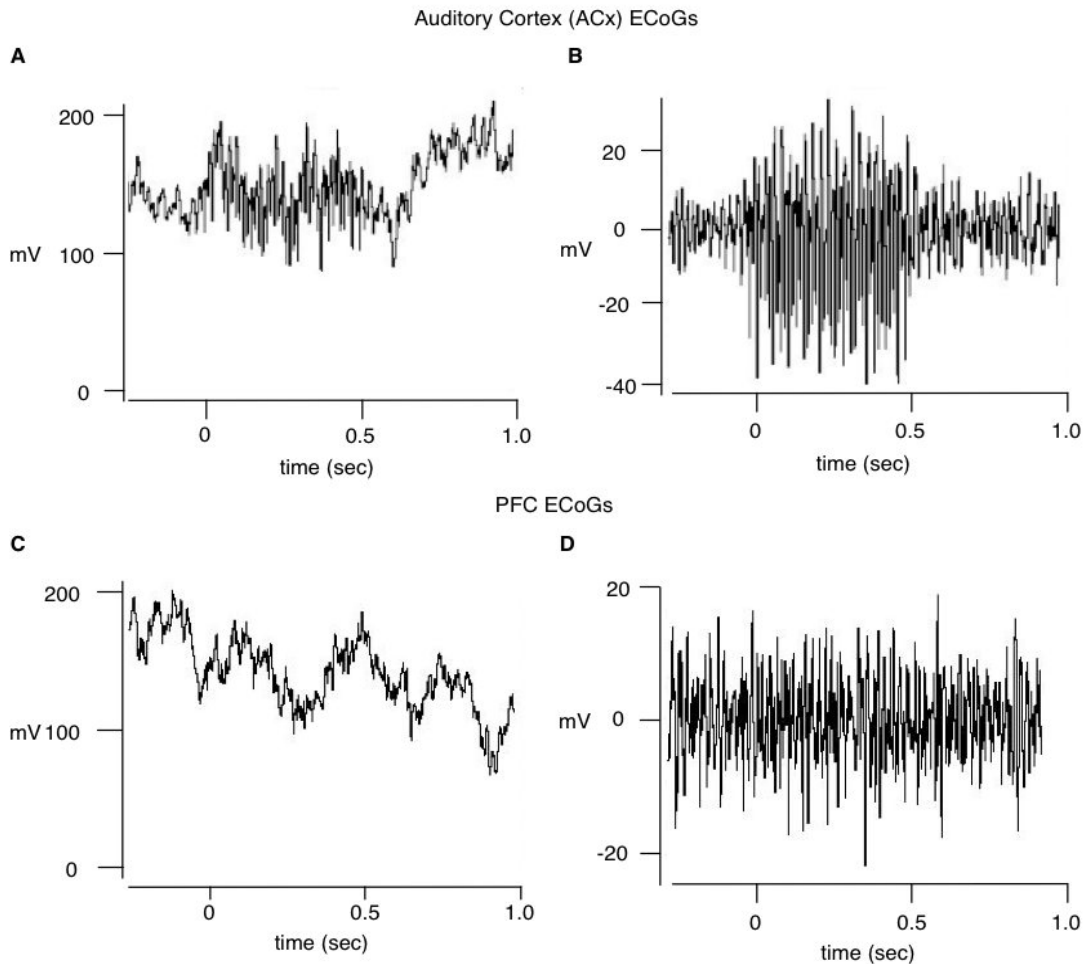


Figure 28: ECoG recordings during 40 Hz auditory stimuli generates 40 Hz oscillations in auditory cortex, but not PFC. (A) Unfiltered ECoG recorded in the auditory cortex (ACx) during presentation of 40 Hz auditory stimuli (time = 0) in a naïve adult mouse. (B) 30 Hz high-pass filtered ECoG recorded as in (A). (C) Unfiltered ECoG recorded in the PFC as in (A). (D) 30 Hz high-pass filtered ECoG recorded as in (A).

E. Chronic Adolescent WIN Exposure Does Not Alter Resting-State ECoGs

Thus far, we have found some evidence that *in vivo* oscillations recorded during cognitive behavioral tasks are altered by adolescent cannabinoid exposure, and that these findings may compliment oscillation abnormalities in patients with schizophrenia.

Resting state oscillatory activity recording during “baseline” conditions has also been reported as abnormal in EEGs/ECoGs recorded from patients with schizophrenia

(Boutros et al., 2008; Rutter et al., 2009; Kikuchi et al., 2011). We therefore tested the

prediction that cannabinoid exposure in adolescence alters resting state ECoG oscillations during “baseline” conditions, i.e. when mice are not engaged in a behavioral task, or are not being presented with discrete sensory stimuli. We recorded cortical ECoGs during two “baseline” behavioral states: when animals were alert and active by being introduced to a novel environment, and when animals were relaxed in their home-cages during overnight ECoG recording sessions, and predicted that oscillatory power would be altered by adolescent WIN (1 mg/kg) treatment.

Mice were placed into a novel environment and allowed to freely explore for 50 minutes, during which frontal ECoGs were recorded and analyzed (Figure 29) for their component frequency content in gamma (Figure 29A), beta (Figure 29B), alpha (Figure 29C), theta (Figure 29D), and delta (Figure 29E) bandwidths. We found no significant difference in oscillation power in any frequency between WIN and vehicle animals, when power was averaged across the entire 50-minute recording session. We also compared oscillations in 10-minute bins during the 50-minute novel environment recording session (Figure 30) and found no significant differences between vehicle (dark gray boxes) and WIN-treated mice (light gray boxes) at any frequency. These data suggest that oscillations during novel environment exploration are not affected by chronic adolescent WIN exposure at the dose that we administered.

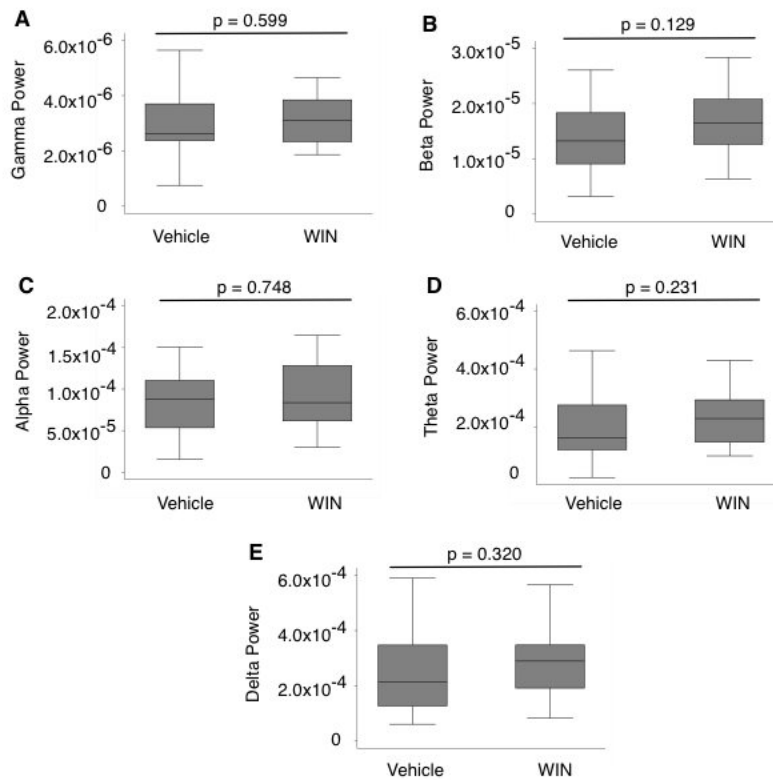


Figure 29: Frontal ECoG oscillation power does not differ between chronic adolescent vehicle and WIN-treated mice during exposure to a novel environment. Box-and-whisker plots of total frontal ECoG (A) gamma power, (B) beta power, (C) alpha power, (D) theta power, and (E) delta power during 50 minutes in a novel environment in chronic adolescent vehicle ($n = 20$) and WIN (1 mg/kg; $n = 18$) treated adult mice. Statistical comparisons between treatments were performed with MWU tests (significant $p < 0.05$).

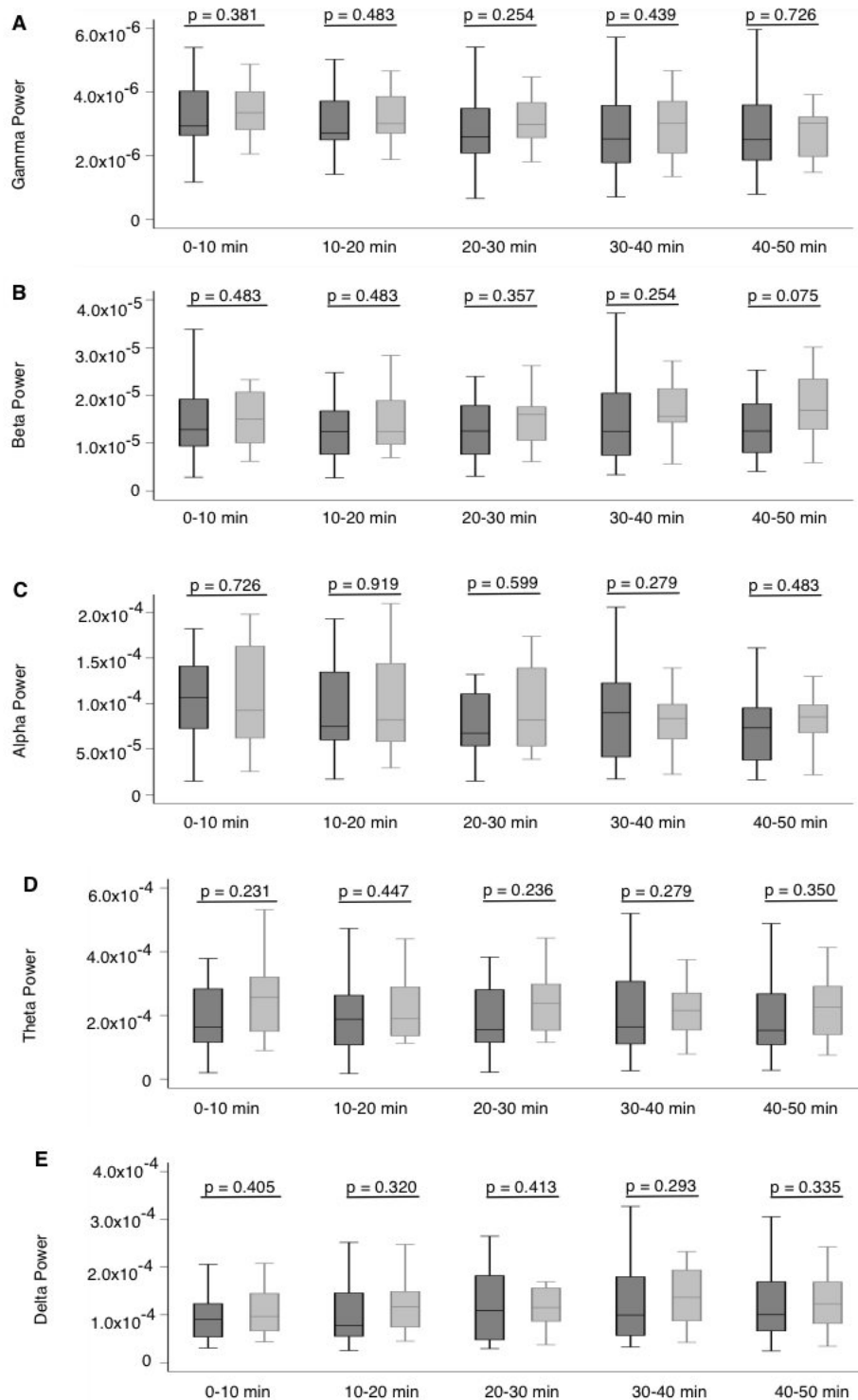


Figure 30: Frontal ECoG oscillation power does not differ during any 10-minute time epoch between chronic adolescent vehicle and WIN-treated mice during exposure to a novel environment. Box-and-whisker plots of total frontal ECoG (A) gamma power, (B) beta power, (C) alpha power, (D) theta power, and (E) delta power during 10-minute time bins in a novel environment in chronic adolescent vehicle (n = 20)(dark gray) and WIN (1 mg/kg; n = 18) (light gray) treated adult mice. Statistical comparisons between treatments were performed with MWU tests (significant $p < 0.05$).

We also recorded ECoG activity in the middle of the animals' dark cycle to determine whether "baseline" ECoGs during relaxed quiet wakefulness/sleep were altered by adolescent WIN exposure. Animals were placed in a darkened room and ECoG activity was recorded for approximately 14 hours overnight. A 3-hour segment of ECoG activity, occurring midway through the dark cycle, was analyzed and time-frequency spectrograms were computed. Figure 31 shows gamma (A), beta (B), alpha (C), theta (D), and delta power (E) during the entire 3-hour segment. WIN animals' oscillatory power does not significantly differ from that of vehicle-treated animals in any frequency. Oscillatory power also did not differ between adolescent WIN or vehicle-exposed animals during the 3 hours overnight when binned into 30 minute segments (Figure 32). These data suggest that resting-state oscillations, recorded during a period when mice are relaxed and undisturbed, are not altered by chronic adolescent WIN exposure at the dose that we tested.

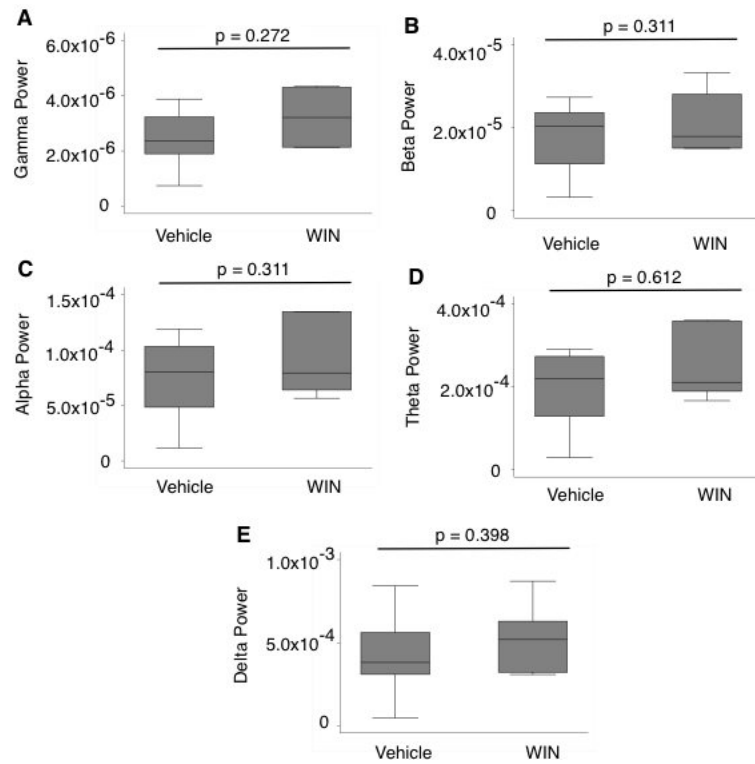


Figure 31: Frontal ECoG oscillation power does not differ between chronic adolescent vehicle and WIN-treated mice during an overnight recording session. Box-and-whisker plots of total frontal ECoG (A) gamma power, (B) beta power, (C) alpha power, (D) theta power, and (E) delta power during 3 hours of ECoG recordings during an overnight recording session in chronic adolescent vehicle ($n = 12$) and WIN (1 mg/kg; $n = 7$) treated adult mice. Statistical comparisons between treatments were performed with MWU tests (significant $p < 0.05$).

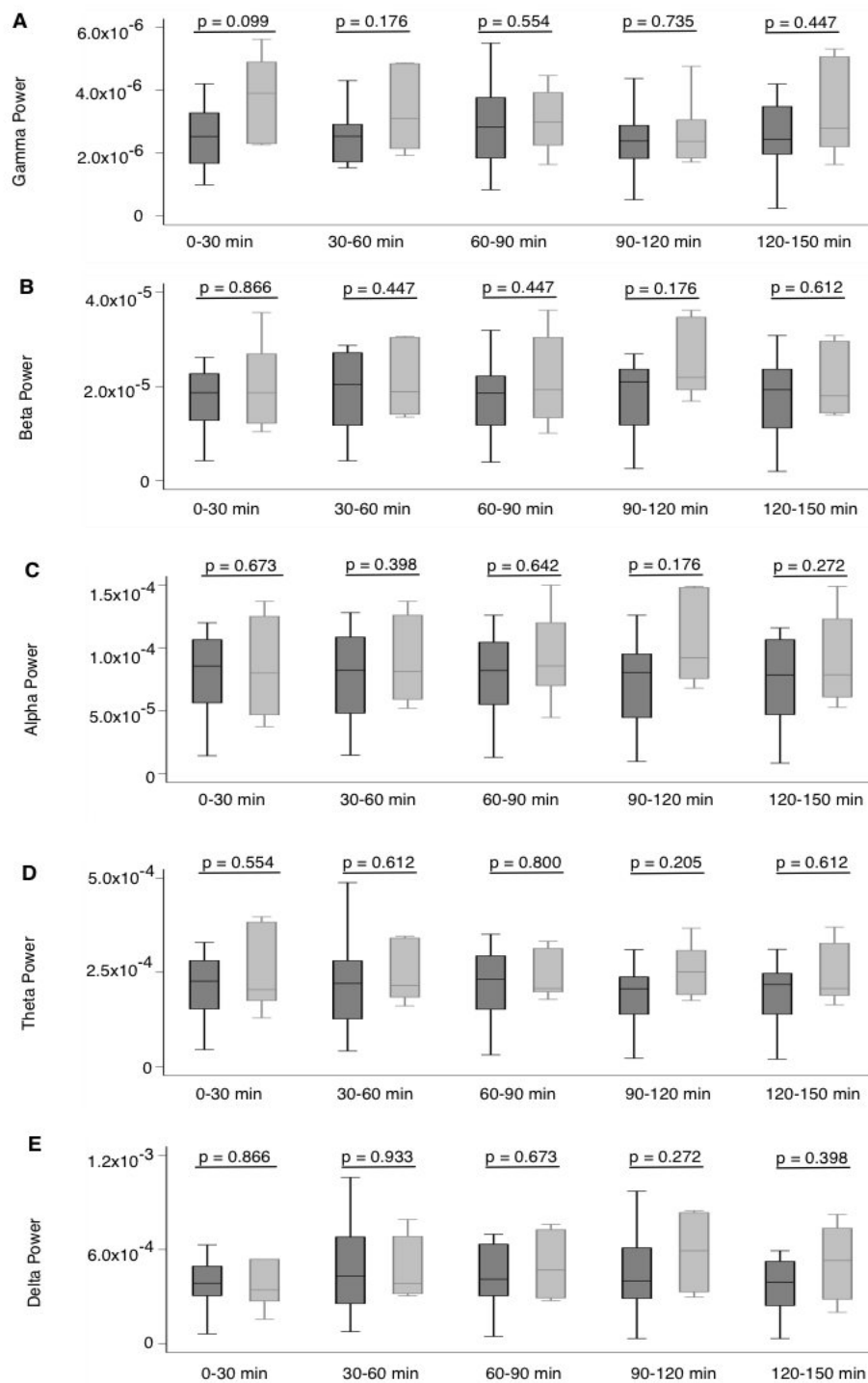


Figure 32: Frontal ECoG oscillation power does not differ during any 30-minute time epoch between chronic adolescent vehicle and WIN-treated mice during an overnight recording session. Box-and-whisker plots of total frontal ECoG (A) gamma power, (B) beta power, (C) alpha power, (D) theta power, and (E) delta power during 30-minute time bins of a 3-hour segment of an overnight recording in chronic adolescent vehicle (n = 12) (dark gray) and WIN (1 mg/kg; n = 7) (light gray) treated adult mice. Statistical comparisons between treatments were performed with MWU tests (significant $p < 0.05$)

IV. Discussion

In Chapter 4 we test the prediction that chronic adolescent cannabinoid exposure permanently alters cortical oscillations recorded *in vivo* from intact adult mice. We test this prediction by analyzing frontal ECoGs and mPFC LFPs recorded while mice perform the novel object recognition (NOR) and object recency (OR) tasks of working memory, both of which purportedly engage the prefrontal cortex (Watson *et al.*, 2012; Mitchell and Laiacona, 1998; Hannesson *et al.*, 2004; Barker *et al.*, 2007; Nelson *et al.*, 2011). We predicted that these tasks would modulate frontal cortical oscillations, and that this modulation would be impaired in adult mice chronically exposed to cannabinoids in adolescence. In support of our prediction, we found that frontal ECoG gamma and delta power increased significantly between the sample and test sessions of the NOR task in animals exposed to vehicle, but not to WIN (1 mg/kg) in adolescence. Surprisingly, we report a significant and unexpected increase in gamma power in frontal ECoGs of adolescent WIN-treated mice during the NOR test session in which they demonstrate a behavioral impairment. However, subsequent attempts to replicate this gamma increase, localize it to mPFC, and determine whether mPFC oscillations parallel behavior in the NOR task have been largely unsuccessful due to inadequate sample sizes. Because of the small n's in these experiments, we could not perform non-parametric rank-based statistical testing on LFP oscillation data recorded during the NOR task, as would be required by the non-normal distribution of these data. Furthermore, in these small samples, we do not replicate our previously reported reduced preference for the novel object in adolescent WIN-treated mice compared to vehicle-treated animals. Again, surprisingly, the 3 adolescent THC-treated animals analyzed in the NOR task

demonstrated a significantly higher preference for the novel object compared to vehicle animals. To ensure that this is not merely an artifact of the small sample size, we need to repeat this experiment with more animals. We find no significant effect of adolescent WIN exposure at the dose tested on ECoG oscillations recorded during the object recency task, or during baseline periods, when mice are not engaged in a cognitive task. These *in vivo* data suggest that the oscillation abnormalities we report in adolescent cannabinoid-exposed animals are subtle and may only become apparent when oscillations are robustly generated with exogenous factors, such as pharmacological tools or during cognitive tests – such as the NOR task – in which these mice demonstrate poor performance.

A. Novel Object Recognition Test: Frontal ECoGs

The NOR test of working memory has been reported to engage the mPFC (Watson *et al.*, 2012) and increase the power of gamma oscillations in frontal cortical regions (Gruber *et al.*, 2008). We therefore predicted that this task would modulate oscillatory power in frontal ECoGs. Indeed, in vehicle-treated mice, gamma and delta power is significantly higher during the NOR test session than during the sample session, suggesting that recognition and memory of a previously encountered object evokes oscillations in gamma and delta frequencies. These findings are in accordance with previous reports that gamma power is associated with familiar object recognition (Martinovic *et al.*, 2008) and that low frequency (including delta) oscillations may be engaged by working memory processes (Wang, 2010). Interestingly, frontal ECoGs recorded in adult mice treated in adolescence with 1 mg/kg WIN do not reveal behavioral modulation during the NOR task; oscillation power in all frequencies is identical between the sample and test sessions. These findings may complement those from the

schizophrenia literature in which task-specific modulation of oscillations is impaired in patients with the disease (Cho *et al.*, 2006; Uhlhaas and Singer, 2010). While EEGs recorded in healthy control subjects reveal increased gamma power as the difficulty of a working memory task increases (Howard, 2003; Haenschel *et al.*, 2009), gamma oscillations in patients with schizophrenia are not modulated by increased cognitive demand (Cho *et al.*, 2006; Basar-Eroglu *et al.*, 2007). A similar phenomenon may account for the lack of behavioral modulation in ECoG gamma and delta power apparent in adult mice exposed to WIN in adolescence during the NOR task.

We report an unexpected increase in frontal ECoG gamma power in adolescent WIN-treated mice, relative to vehicle-treated animals during the NOR test session in which WIN animals demonstrate impaired working memory. This difference appears specific to this test session, as it is not present after a 30-minute delay, when WIN animals perform the NOR test comparably to vehicle-exposed mice. Therefore, contrary to our prediction, increased gamma power in WIN-treated animals may correlate with impaired NOR task performance. We find these results surprising, as acute cannabinoid exposure attenuates the power of gamma oscillations in mPFC during a working memory task (Kucewicz *et al.*, 2011) and we find that pharmacologically evoked gamma oscillations are suppressed both *in vitro* and *in vivo* in adolescent WIN-treated mice. However, we must entertain the possibility that adolescent WIN-treated animals may experience the NOR task during the 60 minute delay session differently than vehicle-treated control mice. This is evident in their significantly reduced preference for the novel object during the test session, increase in total object investigation time, and bias for the object located on the left side of the testing arena during the sample session, as described

in Chapter 3 (Figure 14). It is therefore possible that this unexpected increase in gamma power relative to vehicle animals during the NOR test session is a maladaptive corollary of WIN animals' altered NOR test experience. These findings may also be relevant to reports that patients with schizophrenia have impaired modulation of gamma activity during cognitive tasks (Cho *et al.*, 2006; Uhlhaas and Singer, 2010) that may be partly attributed to the increased baseline gamma power reported in these individuals (Flynn *et al.*, 2008; Kikuchi *et al.*, 2011; Spencer, 2011). Therefore, while vehicle-treated animals demonstrate modulation of gamma power during the NOR task of working memory, adolescent WIN-exposed animals do not display similar gamma modulation, perhaps due to consistently high gamma activity in their frontal ECoG recordings. These surprising data warrant follow-up experiments intended to both replicate this result and to assess oscillatory power in LFPs localized to mPFC.

B. Novel Object Recognition Test: mPFC LFPs and Behavior

Unfortunately, recordings of LFPs in mPFC during NOR behavioral testing were mostly inconclusive. More than half of the animals implanted with electrode arrays lost their implants before they were able to generate any data, leaving very small sample sizes in each adolescent treatment condition. In the small number of animals tested in the NOR task, WIN and vehicle-treated animals display equal preferences for the novel object, suggesting no behavioral impairment in these WIN-treated mice. Unexpectedly, mice exposed to THC during adolescence form a significantly greater novel object preference than do vehicle-exposed animals, indicating that they performed the task better than vehicle controls. We infer that all animals are equally interested in the task and had comparable locomotion as we found no significant differences between vehicle and

cannabinoid-treated animals in total object interaction time during both the test and sample sessions. Only THC-treated animals demonstrate a significant side bias during the sample session, which does not persist in the test session and therefore should not contribute to these animals' NOR test performance. It is unlikely that this bias indicates possible anxiety-like behavior, as discussed in the context of WIN-treated animals' side biases during the sample session data presented in Chapter 3, as all parts of the NOR testing arena were equally and dimly illuminated and object investigation time, a proxy for locomotion, does not differ in the THC population. In the absence of an appropriate positive control in the vehicle-treated sample, findings in THC or WIN-treated animals from this run of the NOR task are difficult to interpret. Despite the lack of a clear behavioral impairment in these cannabinoid-treated animals, we analyzed LFP oscillations with the goal of establishing a protocol for LFP and behavioral recording and analysis to be employed in subsequent experiments.

The sample sizes of mPFC LFP data were too small to be analyzed with non-parametric statistical tests, and we therefore can only infer potential relationships between oscillation power during the different behavioral epochs of the NOR test. In mPFC LFPs, oscillation power may be behaviorally modulated by the NOR task, as power in most bandwidths appears to be higher during the NOR test session than during the sample session. However, this trend appears in all adolescent treatment conditions, and we cannot infer a behavioral link between NOR task performance and oscillation power as we have no evidence of a behavioral impairment in these small samples of adolescent cannabinoid-treated mice. Interestingly, contrary to our predictions, oscillation power during object investigation epochs—in both the sample and the test session—

appears to be lower than when animals are not exploring the objects. It is therefore possible that locomotion and grooming — the behaviors that dominate non-object investigation epochs — generate sufficient oscillation power to override gamma oscillations that we predict will coincide with attention to objects. Indeed, beta oscillations are associated with motor planning, and theta and alpha activity predominate during spatial navigation and environmental exploration (Wang, 2010). Gamma activity does appear to be higher during familiar object investigation epochs during the test session than during investigation of a novel object in vehicle and THC-treated mice, reminiscent of increases in gamma power during object recognition tasks in human patients (Gruber *et al.*, 2008); however, this relationship between gamma and familiar object investigation is not as clear in adolescent WIN-treated animals. These intriguing preliminary data warrant follow-up experiments to: a) confirm whether familiar object recognition evokes gamma oscillations; b) test whether this oscillatory activity correlates with successful recognition of the familiar object, as indicated by a novel object preference; and c) determine whether behavioral modulation of gamma oscillations is abnormal in animals exposed to WIN during adolescence. In the absence of larger sample sizes for vehicle, WIN, and THC-treated animals, we are unfortunately unable to fully test our prediction that the NOR task directly modulates oscillations in the mPFC and that this modulation is attenuated by chronic adolescent cannabinoid exposure. Further experiments must be performed to rigorously test this prediction.

C. Object Recency Task: Frontal ECoGs

We chose to pursue the NOR task because adolescent cannabinoid exposure has consistently been shown to impair working memory in adulthood as assessed by this

behavioral test (Schneider & Koch, 2003; O'Shea *et al.*, 2004; Quinn *et al.*, 2008). However, there are other behavioral tests of working memory and other cognitive abilities that may more robustly engage the PFC and are impaired in patients with schizophrenia and young marijuana users, such as tests of attentional set-shifting (Pope *et al.*, 2001; Prentice *et al.*, 2008; Bissonette and Powell, 2012). Subsequent experiments could test the hypothesis that adolescent cannabinoid exposure impairs performance in these PFC-mediated behavioral tasks similarly to patients with schizophrenia, whether these tests generate robust oscillations in the mPFC, and whether these oscillations are abnormal in cannabinoid treated mice.

While object recognition engages the mPFC and is impaired by adolescent cannabinoid exposure, other cortical areas, such as the perirhinal cortex, are also required to discriminate object identity and may be more robustly engaged by NOR tasks (Barker *et al.*, 2007; Nelson *et al.*, 2011). Multiple lesion studies indicate that the ability to determine the relative recency of objects in the OR task requires mPFC (Hannesson *et al.*, 2004; Barker *et al.*, 2007; Nelson *et al.*, 2011), and while there are no previous reports of this behavior being impaired by cannabinoids, we predicted that adolescent WIN exposure would impair OR performance. Our data do not support this prediction, as mice treated with 1 mg/kg WIN in adolescence correctly perform the OR task after 45 minute delay periods. During the OR test session, the power of frontal ECoG oscillations does not differ between vehicle and WIN-treated mice in any frequency examined, which may be expected by their comparable behavioral performance. This test should be repeated using longer (60 minute) delays between the sample sessions to test for impaired performance in WIN-treated mice when the demands of the task are increased. Results

from the NOR task indicate that WIN animals exhibit a behavioral deficit only when the task is made more challenging with a longer delay between object presentations. A similar approach should be adopted for the OR task. Additional experiments could analyze LFP activity in mPFC using similar analysis methods as were employed for NOR LFP recordings to test for changes in oscillations during the different behavioral epochs of the OR task, as it may be better suited to identify abnormalities in mPFC network activity than the NOR task.

D. Auditory Evoked ECoG Oscillations: Lack of Evidence for Active Generation in PFC and Analysis Concerns

Auditory steady-state responses (ASSRs) consist of robust oscillatory activity that entrains to the frequency of the auditory stimuli that are delivered (20 Hz or 40 Hz, for example) (Franowicz and Barth, 1995; Brenner *et al.*, 2009). These responses, and other similarly sensory evoked oscillations, are suppressed in human chronic marijuana users (Skosnik *et al.*, 2012) and in patients with schizophrenia (Kwon *et al.*, 1999; Brenner *et al.*, 2003). We therefore identified ASSRs as a useful ECoG oscillatory signature with which to test our hypothesis.

While we are able to detect a response in multi-trial averaged ACx ECoGs time-locked to the presentation of 40 Hz click trains, this response is not apparent following the presentation of individual stimuli, and requires averaging nearly 100 trials to become evident. Detailed examination of the methods used by other experimenters who use this technique reveal that extensive filtering is also required to clearly identify ASSRs, typically with the boundaries of the filters set uncomfortably close to the target frequency (Griskova *et al.*, 2007; Brenner *et al.*, 2009; Vohs *et al.*, 2010). For example, (Brenner *et*

al., 2009) filter EEG responses between 39-41 Hz to isolate the 40 Hz target frequency, and personal communication with Elyse Sullivan from Dr. Patricio O'Donnell/Dr. Elliott Hong's labs suggests filtering between 37.5 and 42.5 Hz to see a clear 40 Hz ASSR. While these filtering parameters can isolate a clear stimulus-evoked response, the extensive amount of post-processing required to identify these ASSRs makes us question the validity of using this method to evoke oscillations for our experimental purposes.

More germane to our experimental goals is the absence of stimulus-locked oscillatory activity in ECoGs recorded from the PFC. Although many studies present evidence of click-train ASSRs in frontal EEGs/ECoGs (Reyes *et al.*, 2004; Skosnik *et al.*, 2012), it is unclear whether the presence of the signal in frontal cortical areas is due to a contribution or generation of these oscillations by prefrontal cortical areas (Reyes *et al.*, 2004; Reyes *et al.*, 2005), or is merely a consequence of passive signal propagation from the ACx.

E. Resting State Oscillations During Baseline Conditions

We chose to analyze resting-state oscillations in animals exposed to vehicle or 1 mg/kg WIN in adolescence during recording sessions when they are not engaged in cognitive behavioral tasks to test the prediction that WIN exposure alters the generation baseline oscillatory activity. Oscillation power in all bandwidths tested does not differ between animals exposed to vehicle or 1 mg/kg WIN treated animals during either of the "baseline" periods that we tested: when animals are alert and exploring a novel environment or are relaxed and undisturbed during their dark cycle. Although previous reports suggest that these resting state oscillations are abnormal in patients with schizophrenia (Boutros *et al.*, 2008; Rutter *et al.*, 2009), we do not find similar effects in

adolescent cannabinoid-treated mice. This could be due to the poor spatial resolution of the ECoG recordings that we analyzed. However, a more probable explanation for the absence of an effect of adolescent WIN on resting state oscillations may be that the effects of WIN on *in vivo* oscillations may be subtle when synchronous activity is not being robustly generated by pharmacological methods, or internally generated during a challenging working memory task. Furthermore, we must remember that administration of synthetic cannabinoids, or THC, to animals does not recreate human marijuana intoxication, nor do the animals tested in these studies display the genetic risk factors found in some human populations that probably underlie their susceptibility to the chronic effects of marijuana. Therefore, an exact recreation of a schizophrenia-like phenotype in animals chronically exposed to cannabinoids during adolescence is highly unlikely.

General Discussion

I. Summary of Hypothesis and Predictions

In this Dissertation, we test the hypothesis that marijuana use during adolescence persistently alters cortical oscillations and related cognitive behavior in adulthood. We have tested this hypothesis in several predictions presented in the four experimental chapters of this Dissertation.

A. *Chaper 1: Specific Aim I:* Cannabinoid exposure during adolescence suppresses pharmacologically-evoked cortical oscillations in adult mice.

- **Prediction 1:** Chronic adolescent exposure to a CB1R agonist persistently suppresses pharmacologically-evoked cortical oscillations *in vitro*.
 - Data are consistent with this prediction.
- **Prediction 2:** Effects of chronic adolescent CB1R agonist exposure are more pronounced in rostral cortical areas that are less developmentally mature at the time of drug exposure than in caudal, more mature cortical regions.
 - Data are consistent with this prediction.
- **Prediction 3:** Chronic exposure to a CB1R agonist in adulthood does not produce persistent effects on oscillations.
 - Data are consistent with this prediction.
- **Prediction 4:** Chronic exposure to the primary psychoactive ingredient in marijuana, THC, persistently suppresses pharmacologically-evoked oscillations *in vitro*.
 - Data are consistent with this prediction.
- **Prediction 5:** Chronic adolescent exposure a CB1R agonist persistently

suppresses pharmacologically-evoked cortical oscillations *in vivo* in intact, behaving adult mice.

- Data are consistent with this prediction.

B. Chapter 2: Specific Aim II: Cannabinoid exposure during adolescence suppresses pharmacologically-evoked cortical oscillations via CB1Rs.

- **Prediction 1:** Repeated CB1R antagonist administration in adolescence prevents suppression of pharmacologically-evoked oscillations by chronic adolescent cannabinoid exposure.
 - Data are partly consistent with this prediction. Sub-chronic early adolescent WIN exposure suppresses oscillations in mPFC through CB1Rs. Chronic adolescent THC exposure suppresses oscillations in SCx through CB1Rs. The predicted CB1R mechanism of chronic adolescent WIN-induced oscillation suppression could not be fully tested, as chronic exposure to CB1R antagonists alone persistently suppressed oscillations in mPFC and SCx.
- **Prediction 2:** Repeated CB2R antagonist administration in adolescence does not alter suppression of pharmacologically-evoked oscillations by chronic adolescent cannabinoid exposure.
 - Data are inconsistent with this prediction. Chronic adolescent exposure to a CB2R antagonist alone persistently suppresses oscillations in mPFC and SCx, and therefore we could not test whether CB2R antagonist exposure prevented oscillation suppression by WIN.
- **Prediction 3:** Repeated adolescent administration of a CB1R inactive

compound does not persistently suppress pharmacologically-evoked oscillations.

- Data are partly consistent with this prediction. A putative CB1R inactive compound has no persistent effect in SCx, but persistently attenuates oscillations in mPFC.
- **Prediction 4:** Repeated adolescent cannabinoid administration does not alter pharmacologically-evoked oscillations in CB1R knockout mice.
 - Preliminary data are inconclusive. Suppression of oscillations by chronic adolescent CB1R agonist administration was not recreated in CB1R WT mice, and therefore results in CB1R KO mice are currently inconclusive

C. Chapter 3: Specific Aim III: Cannabinoid exposure during adolescence persistently alters cognitive behavior in adult mice.

- **Prediction 1:** Adolescent exposure to a CB1R agonist impairs working memory performance in the Novel Object Recognition Task.
 - Data are partly consistent with this prediction. NOR performance is impaired after a long, but not a short delay period, in adult animals chronically exposed to a CB1R agonist during adolescence.
- **Prediction 2:** Adolescent exposure to a CB1R agonist impairs working memory performance in the Object Recency Task.
 - Data are inconsistent with this prediction.
- **Prediction 3:** Adolescent exposure to a CB1R agonist persistently alters sleep behavior in adult mice.

- Data are inconsistent with this prediction.

D. Chapter 4: Specific Aim IV: Cannabinoid exposure during adolescence alters behaviorally-evoked oscillations *in vivo* in adult mice.

- **Prediction 1:** The Novel Object Recognition (NOR) task modulates behaviorally-evoked frontal ECoG oscillations.
 - Data are partly consistent with this prediction. Gamma and delta oscillation power is slightly, but significantly higher during the NOR test session than the NOR sample session in adult animals exposed to vehicle during adolescence.
- **Prediction 2:** Adolescent exposure to a CB1R agonist impairs modulation of behaviorally-evoked ECoG oscillations in the NOR task.
 - Data are partly consistent with this prediction. The significant elevation in gamma and delta oscillation power during the NOR test session that is apparent in vehicle-exposed animals is not seen in animals exposed to WIN during adolescence. However, gamma oscillation power is significantly higher in WIN-treated animals during the NOR test session during which they demonstrate a behavioral impairment.
- **Prediction 3:** The Novel Object Recognition (NOR) task modulates behaviorally-evoked LFP oscillations in mPFC.
 - Preliminary data are inconclusive due to limited sample sizes. In animals treated with vehicle during adolescence, the NOR test session appears to elevate oscillation power relative to the sample session, and familiar object investigation epochs appear to evoke higher oscillation

power than novel object investigation periods.

- **Prediction 4:** Adolescent exposure to CB1R agonists suppresses modulation of behaviorally-evoked LFP oscillations in mPFC during the NOR task.
 - Preliminary data are inconclusive due to limited sample sizes and an absence of a behavioral impairment in NOR behavior in the limited number of adolescent cannabinoid-treated animals. The emerging patterns of NOR task modulation of behaviorally-evoked oscillations seen in vehicle-treated animals appear to be absent following adolescent WIN exposure, although small sample sizes prevent statistical comparisons
- **Prediction 5:** Adolescent exposure to a CB1R agonist suppresses modulation of behaviorally-evoked ECoG oscillations in the Object Recency task.
 - Data are inconsistent with this prediction, possibly due to the absence of a behavioral impairment in animals exposed to WIN during adolescence during the OR task.
- **Prediction 6:** Auditory stimuli evoke robust sensory stimulus-generated ECoG oscillations in the prefrontal cortex.
 - Data are inconsistent with this prediction.
- **Prediction 7:** Adolescent exposure to a CB1R agonist suppresses spontaneously-generated resting-state ECoG oscillations.
 - Data are inconsistent with this prediction.

II. Overall Summary

Experiments conducted throughout the course of my thesis work reveal that cannabinoid exposure during adolescence induces persistent abnormalities in coordinated, oscillatory activity in the adult neocortex. These include a pronounced and lasting suppression of pharmacologically-evoked oscillation power both *in vitro* in isolated cortical slices, and *in vivo* in the awake, behaving adult mouse. We have confirmed that the adolescent cortex is acutely sensitive to the lasting effects of adolescent cannabinoid exposure, as adult exposure to cannabinoids does not induce the same oscillation suppression. The rostral, medial prefrontal cortex (mPFC) demonstrates higher sensitivity to cannabinoid exposure compared to the caudal, primary somatosensory cortex (SCx). Furthermore, a short period of exposure to the potent, synthetic CB1R/CB2R agonist, WIN, during the early adolescent period, compared to later in adolescence, suppresses oscillations selectively in mPFC that can be antagonized with co-administration of a CB1R antagonist, that alone produces no effects. Chronic exposure to THC, the primary psychoactive ingredient in marijuana, suppresses oscillations in the caudal SCx through a similar CB1R-dependent mechanism. We find that chronic adolescent exposure to WIN impairs working memory performance, as assessed by the novel object recognition task, and alters induced gamma oscillations during this cognitive task. These results may be applicable to the cognitive impairment and increased risk for developing severe psychiatric disorders, such as schizophrenia, in those who frequently used marijuana in adolescence. These findings are largely consistent with my hypothesis and are relevant to the current and ongoing public debate over the medical utility of marijuana, as well as regarding legalization of the drug for recreational use.

On the other hand, multiple attempts to test the CB1R dependence of chronic effects of WIN revealed that chronic *antagonism* of the CB1R or CB2R produced a similar suppression of oscillation power as seen after chronic exposure to CB1R/CB2R agonists. These results prevented us from fully testing the prediction that adolescent CB1R antagonist exposure would prevent the chronic oscillation-suppressing effects of adolescent CB1R agonist administration, and did not support the prediction that CB2R antagonist exposure would neither produce lasting effects on its own, nor attenuate the effects of chronic WIN. While these findings were unexpected, they may be of particularly timely public health relevance. Cannabidiol—which is more heavily concentrated than THC in the *Cannabis indica* strain of marijuana—has recently emerged as an effective strategy in treating severe epilepsy disorders in children that are otherwise resistant to treatment (Porter and Jacobson, 2013), and acts as a potent antagonist of both CB1Rs and CB2Rs in cell systems, although the specificity of this action has not yet been established (Pertwee, 2008). Although cannabidiol-enriched cannabis, or cannabidiol itself, may have promising therapeutic qualities for epilepsy disorders in juveniles, no research, to date, has addressed the safety or tolerability of the cannabinoid in this population, nor the long-term consequences of chronic CB1R/CB2R antagonism. Data that we present from animals chronically exposed to CB1R or CB2R antagonists before adulthood may be relevant to this later concern. Therefore, the findings presented in this Dissertation, in addition to being relevant to the illicit use of marijuana by adolescents and young adults, may be pertinent to emerging medical applications of alternative strains of cannabis or cannabinoids in treating neurological disorders in children.

III. Developmental Trajectory of CB1R During Adolescence and Relevance to mPFC vs. SCx Sensitivity to CB1R Ligands

As predicted by our hypothesis, our studies consistently show that the adolescent mPFC is more sensitive to the persistent cortical oscillation suppressing effects of cannabinoid exposure, compared to the SCx. While the entire cerebral cortex undergoes substantial maturation during adolescence, development continues for longer, and is more pronounced in rostral prefrontal cortical areas than in caudal, primary somatosensory regions (Gogtay *et al.*, 2004; Yurgelun-Todd, 2007). In addition to the considerable maturation of cortical gray and white matter, and of neurotransmitter systems throughout the transition from childhood to adulthood, development of the cortical eCB system is also ongoing at this time. As described in the General Introduction, the eCB system consists of the eCBs themselves, their synthetic and degradative enzymes, and the receptors at which they exert their effects. All three components of this system undergo substantial changes in the cerebral cortex during the adolescent period (Ellgren *et al.*, 2008; Heng *et al.*, 2011; Long *et al.*, 2012; Lee *et al.*, 2013).

Cortical CB1R mRNA expression declines during adolescence (Ellgren *et al.*, 2008), and this reduction is more pronounced in rostral prefrontal cortical areas than in the caudal primary somatosensory cortex (Deshmukh *et al.*, 2007; Heng *et al.*, 2011). While the report by Heng *et al.* reveals that reductions in CB1R expression are more pronounced in mPFC regions than in the primary somatosensory cortex, it was not clear from the analysis presented in these studies whether expression between these two regions differs significantly at any of the postnatal ages examined. Data addressing this question have been generously shared with us by Dr. K.Y. Tseng (Rosalind-Franklin

University, Chicago, IL), and are reprinted here, with permission (personal communication with K.Y. Tseng, 3/3/14) (Figure 33). CB1R mRNA was quantified in slices of mPFC and SCx in male Sprague-Dawley rats at three postnatal ages: juvenile (P25), adolescent (P40) and adult (P70). CB1R mRNA expression in the prelimbic area (PL) of the mPFC and the SCx was compared using a two-factor ANOVA, followed by Newman-Keuls post-hoc tests that reveal a significant main effect of postnatal age ($p < 0.001$) and a main effect of cortical region on CB1R mRNA expression ($p < 0.001$), as well as a significant interaction between age and region ($p = 0.002$) (Figure 33). These analyses indicate that while CB1R mRNA expression decreases significantly throughout adolescence in PL and mildly in SCx during adolescence, expression remains significantly higher in PL than in SCx at all time-points tested (Figure 33).

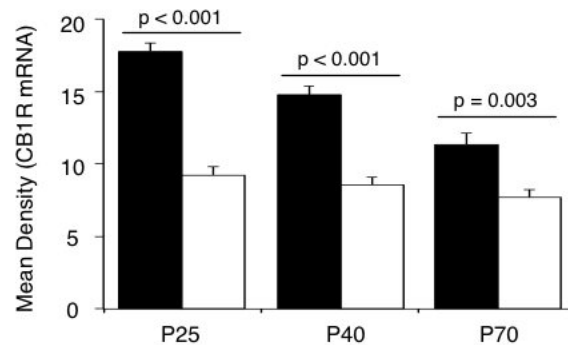


Figure 33: CB1R mRNA expression from juvenile to adult ages in mPFC and SCx. Mean density of CB1R mRNA in the prelimbic region (PL) (black bars) and SCx (white bars) in rats ($n = 6$) at juvenile (P25), adolescent (P40), and adult ages (P70). A two-factor ANOVA was performed to determine main effects of cortical region and age, and p values presented in the figure are the result of Newman-Keuls post-hoc tests comparing PL and SCx at all ages. Summaries of these results are reported in (Heng *et al.*, 2011). Data and statistical analyses were generously provided by Dr. K. Y. Tseng (Rosalind-Franklin University, Chicago, IL) and are reprinted here with permission (personal communication, 3/3/14).

This gradient of CB1R expression parallels the differential sensitivity that we report to chronic and sub-chronic CB1R ligand exposure in mPFC compared to SCx. We find a milder persistent suppression of pharmacologically-evoked oscillations in SCx than

in mPFC in response to chronic adolescent 0.25 mg/kg and 1 mg/kg WIN, and 5 mg/kg THC (Chapter 1). Additionally, while 6-day exposure to 2 mg/kg WIN in early adolescence suppresses oscillation power in mPFC of adult mice, we find no effect of this treatment in SCx (Chapter 2). Finally, unlike in mPFC, we find no suppression of oscillatory activity in response to low doses (0.3 and 0.5 mg/kg) of AM251 or 1 mg/kg AM4113 in SCx (Chapter 2), which could be explained by lower CB1R expression in SCx than in mPFC, as revealed by these data. We have evidence that sub-chronic early adolescent WIN exposure and chronic adolescent THC exposure suppress oscillations in mPFC and SCx, respectively, through CB1Rs, thus supporting a link between the differential sensitivity of mPFC and SCx to WIN and THC that we report, and the gradient of CB1R expression revealed in Dr. Tseng's findings.

IV. Adolescent Cannabinoid Exposure May Lead to a Persistent State of Cortical Disinhibition: Convergence of Hypotheses in the Schizophrenia Field

Our results also complement a recent report from Dr. Tseng's laboratory that shows that a short period of WIN exposure during early (P35-P40) or mid (P40-P45), but not late (P50-P55) adolescence or adulthood (P75-P80) persistently impairs the modulation of LFP amplitude in mPFC (Cass *et al.*, 2014). In these experiments, performed *in vivo* in intact, anesthetized rats, electrical stimulation of the ventral hippocampus delivered at 10 Hz produces a sustained facilitation of LFP amplitude in mPFC. This facilitation is unchanged in adult animals that had received WIN at any age during adolescence. However, stimulation at 20 or 40 Hz elicits a transient (20 Hz) or sustained (40 Hz) suppression of LFP amplitude that is a hallmark of normal mPFC maturation (Thomas *et al.*, 2013). Repeated exposure to WIN during early or mid

adolescence impairs this suppression of ventral-hippocampal evoked LFP amplitude in adult mPFC, thus mimicking an immature state of prefrontal network activity. This impaired modulation of LFP amplitude is mediated by activation of CB1Rs, as co-administration of AM251 returns LFP levels to those seen in vehicle-treated rats, while AM251 alone produces no lasting effects on its own. These results complement our findings of a CB1R-dependent attenuation of oscillatory activity in mPFC after early adolescent (P35-P40) WIN administration, and may allow us to speculate on the underlying mechanisms that might account for the persistent suppression of oscillation power that we have discovered.

Cass *et al.* (2014) find that the pattern of impaired LFP attenuation in mPFC that emerges in animals exposed to WIN during the critical early and mid-adolescent periods may be due to disinhibition of prefrontal network activity. Suppression of LFP amplitude in WIN-exposed rats is restored to control levels by infusion of an allosteric modulator of the GABA-A receptor into the mPFC. Furthermore, while the frequency of spontaneous IPSCs in mPFC pyramidal neurons increases during the transition from juvenile to adult ages, spontaneous IPSC frequency in animals exposed to WIN in early or mid adolescence is indistinguishable from that in animals ages P30-P35 (Cass *et al.*, 2014). These findings suggest that WIN exposure in adolescence transforms the mPFC network to a disinhibited state, reminiscent of that seen in juvenile animals.

A. A Disinhibited Frontal Cortex in Schizophrenia

Prior to the publication of these data by the Tseng lab, we had entertained the possibility that the phenotype we have discovered in animals exposed to cannabinoids in adolescence may arise as a result of a disinhibited cortical network. This idea is in

agreement with a long-standing hypothesis in the schizophrenia field, that proposes that the brains of individuals with schizophrenia are persistently disinhibited, particularly in the frontal cortical areas, which contributes to the aberrant sensory and cognitive function in these patients. It has been proposed that disinhibition impairs the coordinated oscillatory activity of cortical networks that may be crucial for cognitive processes (Lewis *et al.*, 2005) (Gonzalez-Burgos and Lewis, 2008; Uhlhaas and Singer, 2010), and therefore may underlie the cognitive dysfunction that is a core feature of schizophrenia. Data in support of this hypothesis include, but are not limited to, post-mortem studies on the PFC of patients with schizophrenia, revealing decreased expression of the GABA-A receptor (Hoftman *et al.*, 2013), as well as of the GABA synthesizing enzyme GAD-1 (formerly GAD-67), particularly in the interneuron population that expresses the calcium binding protein parvalbumin (PV+ cells) (Akbarian *et al.*, 1995; Volk *et al.*, 2000; Lewis *et al.*, 2012). Additional support for this hypothesis comes from animal studies in which oscillation abnormalities and cognitive impairments characteristic of the disease are recapitulated when GABAergic inhibition is attenuated (Cunningham *et al.*, 2006; O'Donnell *et al.*, 2012; Phillips *et al.*, 2012; Hines *et al.*, 2013).

B. *Glutamatergic Hypofunction on Selected Interneuron Populations in Schizophrenia*

Cortical disinhibition can also result from attenuated excitatory neurotransmission, particularly that mediated through NMDA and AMPA glutamate receptors. NMDA-R hypofunction has been proposed as a mechanism that may account for the pathological cognitive functioning and abnormal network synchrony described in patients with schizophrenia (Olney and Farber, 1995; Gilmour *et al.*, 2012), and has received strong support from both clinical and preclinical studies. Exposure to NMDA receptor

antagonists, such as ketamine, reproduces many of the features of schizophrenia, including psychosis and thought disorder (Krystal *et al.*, 1994; Adler *et al.*, 1999; Lahti *et al.*, 2001), and exacerbates these symptoms in patients with the disease (Malhotra *et al.*, 1997; Lahti *et al.*, 2001; Hong *et al.*, 2010). NMDA-R antagonism induces robust gamma oscillations in humans and animals (Pinault, 2008; Hakami *et al.*, 2009; Lazarewicz *et al.*, 2010), as we have demonstrated in our experiments, and increases the overall excitability of the cortex and the firing rate in cortical pyramidal neurons (Vollenweider *et al.*, 1997; Jackson *et al.*, 2004; Homayoun and Moghaddam, 2007). This increased excitability may be accounted for by the preferential antagonism of NMDA-Rs expressed on parvalbumin containing interneurons, as administration of ketamine decreases the activity of these cells prior to increasing the firing frequency of cortical pyramidal neurons (Homayoun and Moghaddam, 2007). These data, and others (Kocsis, 2011; Carlen *et al.*, 2012; Gonzalez-Burgos and Lewis, 2012), suggest that coordinated recruitment of parvalbumin interneurons, which may be the result of NMDA hypofunction in these cells, may underlie the disinhibited, hyper-excitable cortex that arises in response to ketamine administration, and is a characteristic of schizophrenia. Furthermore, glutamatergic hypofunction primarily within the parvalbumin cells that so critically regulate the output of excitatory pyramidal networks (Markram *et al.*, 2004; Lewis *et al.*, 2012) bridge two prevailing hypothesis in the schizophrenia literature: NMDA-R hypofunction and the disinhibition of cortical networks (Gonzalez-Burgos and Lewis, 2012). A mechanistic link describing the seemingly paradoxical observations that ketamine attenuates parvalbumin-cell recruitment but yet evokes gamma oscillations that are generated the output of parvalbumin-positive interneurons (Cardin *et al.*, 2009), has not yet been described.

However, ketamine-evoked disinhibition and hyper-synchrony of gamma activity may be relevant to repeated reports of increased gamma oscillation power in patients with schizophrenia during resting-state, or baseline conditions (Spencer, 2011), as ketamine mimics many of the psychotic symptoms of the disease (Krystal *et al.*, 1994; Adler *et al.*, 1999; Lahti *et al.*, 2001).

C: Schizophrenia-Related Abnormalities and Hypofunction of Glutamate

Transmission on Parvalbumin-Positive Interneurons

We noted some interesting similarities between the network dysfunction and cognitive impairments that we report in mice exposed to cannabinoids during adolescence, and behavioral abnormalities and oscillatory activity reported in studies in which the glutamatergic recruitment of parvalbumin interneurons is impaired through genetic manipulation of NMDA (Belforte *et al.*, 2010; Carlen *et al.*, 2012) or AMPA receptors (Fuchs *et al.*, 2007) specifically on these interneurons. For example, Fuchs *et al.* (2007) ablated GluR1 or GluR4 subunits of the AMPA receptor specifically in parvalbumin neurons and show that gamma oscillations recorded *in vitro* in CA3 hippocampus in response to kainic acid application are attenuated. These data are similar to our findings of impaired KA+CCh-evoked activity in mPFC and SCx of adult mice treated in adolescence with cannabinoids. Belforte *et al.* (2010) genetically knocked down the crucial NR1 subunit of the NMDA-R in approximately 50% of corticolimbic interneurons, 75% of which were parvalbumin neurons, at either early postnatal ages, or in adulthood. They observed that mice lacking functional NMDA-Rs in this interneuron population from early postnatal life, but not after adolescence, displayed schizophrenia-like behavioral abnormalities, including impaired working memory, anhedonia, sensory-

gating impairments, and impaired social interaction. Many of these behavioral abnormalities could be reversed with antipsychotic administration (Belforte *et al.*, 2010), which points to a probable link between parvalbumin neuron NMDA-R hypofunction and some symptoms of schizophrenia.

Carlen *et al.* (2012) used a more targeted genetic approach to genetically ablate the NR1 subunit specifically from parvalbumin interneurons, and assessed behavior and oscillatory activity *in vivo* in these adult mice. In these mice lacking the NR1 subunit in parvalbumin interneurons, working memory impairments emerge only when the demands of a working memory task are made more difficult, reminiscent of data that we present in adolescent-WIN treated mice. These mice demonstrate *increased* power of induced gamma oscillations during baseline conditions when all animals are under mild isoflurane anesthesia, and thus in the same behavioral state. Interestingly, these same animals display *decreased* evoked gamma activity in response to either optogenetic stimulation of parvalbumin interneurons, which this group has previously shown can reliably generate robust gamma oscillations in cortical networks (Cardin *et al.*, 2009), or following administration of the NMDA-R antagonist MK-801, which evokes gamma activity similarly to ketamine (Kocsis, 2011). These seemingly paradoxical findings—increased, spontaneously-generated gamma oscillations and reduced, exogenously-driven gamma oscillations—are strikingly similar to the findings that we report in adolescent cannabinoid-treated adult mice, as well as to those reported in the schizophrenia literature (Spencer, 2011) and may be fundamentally related. Elevated baseline gamma power in patients with schizophrenia and in animals treated with cannabinoids during adolescence may impair behavioral task or stimulus-dependent modulation of oscillatory activity, and

result in reduced power of gamma oscillations when synchronous activity is evoked using exogenous methods.

Computational modeling performed by Carlen *et al.* supports their, and perhaps our, experimental data by revealing that NMDA-R dysfunction in the parvalbumin interneuron population promotes the emergence of higher spontaneous gamma oscillation power, such as we report during the NOR task in adolescent WIN-treated mice, and decreased responses to exogenous stimuli that evoke robust gamma band activity, such as during KA+CCh or ketamine administration. Again, similar to our findings, these parvalbumin/NR1- mice also did not demonstrate significantly altered gamma oscillation power compared to control animals during investigation of an open field. Although the behavioral states in which Carlen *et al.* and we have discovered increased gamma oscillation activity differ (mild isoflurane anesthesia versus the NOR test session), a common link between these states may be drawn if we reason that the NOR behavioral task engages all animals in a common and somewhat controlled behavioral repertoire, akin to the controlled behavioral state created by anesthesia. This parallel is tenuous, though, and more experiments are clearly called for to determine whether chronic, or sub-chronic, cannabinoid exposure during adolescence persistently impairs inhibitory output specifically from parvalbumin interneurons, as would be predicted by the model proposed by Carlen *et al.* Nevertheless, similarities in impaired evoked gamma activity and enhanced induced gamma oscillation power *in vivo* in mice with impaired recruitment of parvalbumin interneurons, or in mice exposed to cannabinoids during adolescence, may coalesce around a common phenomenon of network disinhibition, as demonstrated by Cass *et al.* (2014) in the mPFC of animals exposed to WIN in adolescence.

V: CB1Rs and Pharmacologically-Evoked Oscillations

How might persistent exposure to cannabinoid receptor ligands during adolescence lead to a persistent state of cortical disinhibition? Previous reports reveal that CB1R activation suppresses pharmacologically-evoked gamma and theta oscillations (see Chapter 2 Discussion), and since we have shown that sub-chronic WIN exposure and chronic THC exposure persistently attenuate oscillation power via CB1Rs in mPFC and SCx, respectively, we will focus on the CB1R as we explore potential mechanisms of oscillation suppression by adolescent cannabinoids. A role in oscillations of CB2Rs, or non-cannabinoid receptors that may be unexpectedly targeted by the cannabinoid receptor ligands administered in our experiments has not yet been reported.

A. Gamma Oscillations and Glutamatergic-Localized CB1Rs: A Role for Parvalbumin-Positive Interneurons

As discussed in Chapters 1 and 2, KA + CCh have been used separately, or in combination to reliably evoke robust gamma and beta oscillations *in vitro* in different regions of the neocortex (Oke *et al.*, 2010), including SCx (Buhl *et al.*, 1998), and mPFC (van Aerde *et al.*, 2008; van Aerde *et al.*, 2009), the entorhinal cortex (Cunningham *et al.*, 2003), and the hippocampus (Fisahn *et al.*, 1998). KA+CCh-induced gamma and beta oscillations reportedly require muscarinic cholinergic receptors (mAChRs), AMPA/kainate receptors, and GABA-A receptors, but are insensitive to metabotropic glutamate receptor (mGluR) or NMDA-R blockade (Buhl *et al.*, 1998; Fisahn *et al.*, 1998; Mann *et al.*, 2005). These oscillations appear to be driven by a recurrent feedback loop consisting of excitatory pyramidal neurons and inhibitory interneurons, in which the discharge of pyramidal cells is governed by the perisomatic inhibition provided by

interneurons that are driven by excitatory input (Mann *et al.*, 2005; Gulyas *et al.*, 2010; Holderith *et al.*, 2011). This circuit-based model of gamma generation is called the “pyramidal interneuron network gamma,” or PING model. PING is contrasted with the ING model, or “interneuron network gamma” model, which posits that gamma generation is driven solely by reciprocal inhibitory interactions between perisomatic targeting interneurons that can rhythmically synchronize the output of pyramidal cells, but whose activity is not driven by pyramidal neurons (Whittington *et al.*, 2000; Rotaru *et al.*, 2011; Gonzalez-Burgos and Lewis, 2012). Experimental evidence currently appears to favor the PING, versus the ING model (Gonzalez-Burgos and Lewis, 2012), although it is probable that these two models are not mutually exclusive and may both describe the generation of gamma oscillations under different conditions, or in different brain regions.

Interneurons that primarily target the perisomatic region of pyramidal neurons—and are thus positioned to potentially regulate excitatory output from these neurons—are typically classified as basket cells, and may express parvalbumin or the neuropeptide cholecystokinin (CCK), among other markers (Markram *et al.*, 2004). In the hippocampus and neocortex, CCK+ interneurons express CB1Rs, while parvalbumin cells are thought to lack these receptors (Freund *et al.*, 2003; Freund and Katona, 2007). The presence of CB1Rs on these CCK+ cells presents the possibility that activation of CB1Rs on interneurons may account for the suppression of pharmacologically-evoked gamma oscillations seen following both acute (Hajos *et al.*, 2000; Holderith *et al.*, 2011), and repeated cannabinoid exposure (Raver *et al.*, 2013) (Chapters 1 and 2). However, CB1R-/parvalbumin+, and not CB1+/CCK+ cells are considered the more likely candidate interneurons responsible for gamma oscillation generation, particularly for

gamma activity evoked with pharmacological methods. This may be due in part to the faster kinetics of IPSCs from fast-spiking parvalbumin basket cells, compared to regular-spiking CCK+ interneurons (Freund and Katona, 2007), and to the observation that the output of CCK+ interneurons is largely silenced by CCh exposure in excess of 5 μ M, as is used to drive CCh-evoked gamma oscillatory activity (Gulyas *et al.*, 2010). GABA release from CCK+ neurons is suppressed due to CCh-evoked release of eCBs (Kim *et al.*, 2002). Therefore, rather than targeting the CB1R+/CCK+ population to suppress gamma oscillation power, CB1R agonists appear to attenuate gamma activity in the CA3 region of the hippocampus by activating CB1Rs expressed on the terminals of excitatory pyramidal cells (Holderith *et al.*, 2011). CB1R activation on pyramidal neuron terminals inhibits glutamatergic transmission and reduces the amplitude of excitatory post-synaptic currents recorded in parvalbumin interneurons, subsequently attenuating the frequency and firing precision of parvalbumin fast-spiking basket cells. These collective actions induce smaller and less synchronous field potentials, that in turn cause reductions in the power of network oscillations (Holderith *et al.*, 2011). These experimental results support the PING model of gamma oscillation generation, as attenuation of pyramidal neuron output by CB1R agonist administration results in reduced recruitment of parvalbumin interneurons, causing a dampening of oscillatory power within the interconnected neural network. Together with other studies showing that evoked gamma power is attenuated in conditions in which parvalbumin interneuron recruitment is impaired through selective ablation of glutamatergic transmission in these cells (Fuchs *et al.*, 2007; Carlen *et al.*, 2012), our findings may be partly explained by altered CB1R-mediated suppression of

glutamate release onto these parvalbumin interneurons, as shown by (Holderith *et al.*, 2011).

B. Theta Oscillations and Inhibitory-Localized CB1Rs: A Role for Cholecystinin-Positive Interneurons

However, cannabinoid-induced gamma suppression attributed exclusively to CB1Rs localized on pyramidal neurons, but not on the CCK+ interneurons that predominately express CB1Rs (Katona *et al.*, 1999; Marsicano and Lutz, 1999), presents some paradoxes. For example, if CCK+ interneurons were silenced due to CCh-evoked eCB release and subsequent CB1R-mediated suppression of GABA release (Gulyas *et al.*, 2010), then it is curious that acute administration of a CB1R antagonist on its own has no effect on gamma oscillation power, and that these oscillations are not altered in CB1R knockout mice (Holderith *et al.*, 2011). Holderith *et al.* interpret these findings as indicating a lack of tonic CB1R activation in their CA3 hippocampus slice preparations. This interpretation is at odds with mention by Holderith *et al.* in their Discussion that CCK+ interneuronal output is silenced by CCh via eCB action at CB1Rs (Gulyas *et al.*, 2010), as well as with previous reports from the hippocampus and neocortex (Kim *et al.*, 2002; Ohno-Shosaku *et al.*, 2003; Fortin *et al.*, 2004) that clearly demonstrate CCh-evoked eCB release, and CB1R-mediated suppression of inhibitory activity (depolarization-induced suppression of inhibition; DSI). Indeed, we have discovered that chronic exposure to the CB1R antagonists AM251 and AM4113 in adolescence persistently attenuates gamma oscillation power evoked with pharmacological methods, further implying that tonic eCB signaling is present during either the period of cannabinoid exposure (adolescence), or when oscillations are pharmacologically evoked

(adulthood), and its role in the generation of gamma oscillations. As previous studies have been conducted in the hippocampus, we cannot exclude the presence of regional differences in tonic eCB activity between hippocampal and neocortical networks.

Additional evidence suggesting a role of interneuron-localized CB1Rs in oscillatory activity comes from experiments *in vitro* in the CA1 region of the hippocampus that demonstrate cannabinoid modulation of theta oscillations evoked by CCh or ACh released with optogenetic methods (Reich *et al.*, 2005; Nagode *et al.*, 2011; Nagode *et al.*, 2014). Unlike gamma activity evoked by CCh (Fisahn *et al.*, 1998; Mann *et al.*, 2005), theta oscillations evoked by mAChR stimulation do not require AMPA-Rs, as they persist when excitatory transmission is blocked. Optogenetic release of ACh generates rhythmic IPSCs and LFPs in the theta frequency that are primarily mediated by mAChRs, and that can be suppressed by eCB activity at CB1Rs (Nagode *et al.*, 2011). Because excitatory neurotransmission is blocked in these experiments, these data reveal that ACh activates the class of perisomatic interneurons that are not thought to be crucial for gamma oscillation generation: the CB1+/CCK+ basket cells. Interestingly, optogenetic or pharmacological silencing of the CB1+/CCK+ interneuron population, but not CB1-/parvalbumin cells, suppresses inhibitory theta oscillations (Nagode *et al.*, 2014). These theta oscillations are also suppressed by application of a μ opioid receptor (MOR) agonist, which is surprising, as MORs have classically been thought to be localized exclusively to CB1-/parvalbumin+ interneurons, and be absent from CB1+/CCK+ interneurons (Freund and Buzsáki, 1996; Gulyas *et al.*, 2010). These results reveal the potential presence of an interneuron population capable of potentially regulating theta network activity that is sensitive to both CB1R and MOR agonists. Interestingly, the CB1R antagonist AM251

also acts as an antagonist of MORs (Seely *et al.*, 2012), and therefore presents the possibility that direct modulation of MOR activity on CB1-/parvalbumin+ interneurons, or on another class of MOR+ interneurons, may contribute to the oscillation suppressing effects that we have discovered after chronic AM251 administration.

C: Dual Roles for Glutamatergic and Inhibitory-Localized CB1Rs in Cannabinoid-Induced Suppression of Cortical Oscillations

Although many studies of cannabinoid modulation of oscillations (Holderith *et al.*, 2011; Nagode *et al.*, 2011; Nagode *et al.*, 2014) have been conducted in the hippocampus, our findings of theta and gamma oscillation suppression after adolescent exposure to cannabinoids suggest that altered activity of CB1Rs at excitatory pyramidal neurons and/or CCK+ interneurons may contribute to the persistent suppression that we have discovered in the neocortex. Support for a dual role of CB1Rs expressed on excitatory and inhibitory neurons comes from a recent report in which CB1Rs were genetically ablated from glutamatergic cortical neurons or from all principal forebrain neurons (Glu-CB1Rs), or from all GABAergic interneurons (GABA-CB1Rs)(Sales-Carbonell *et al.*, 2013). Revealingly, CB1R agonist-induced attenuation of ECoG oscillation power faster than 12 Hz was reduced, but still apparent when Glu-CB1Rs were knocked out; Cannabinoid-suppression of ECoG oscillations was enhanced when GABA-CB1Rs were absent. These findings reveal that CB1Rs expressed on both excitatory pyramidal neurons and putative CCK+ inhibitory interneurons may mediate the CB1R-dependent suppression of cortical oscillation power that we have discovered following adolescent cannabinoid exposure.

VI: Long-Term Changes in the eCB System by Chronic Cannabinoid Exposure

A. Decreased CB1R Expression

Repeated cannabinoid administration can alter the expression of multiple different receptors and mediators that affect coordinated network activity, including CB1Rs, eCBs, GABA and glutamate receptors, as well as GABA and glutamate release. Repeated exposure (up to 21 days) of THC or WIN down-regulates and desensitizes the CB1R in multiple brain regions, including the prefrontal cortex (Breivogel *et al.*, 1999; Sim-Selley and Martin, 2002), when assessed immediately after the final drug exposure. Interestingly, CB1R downregulation due to 2-week WIN or THC exposure appears to recover after at least 2 weeks of drug abstinence (Sim-Selley *et al.*, 2006). Similar CB1R desensitization is seen in the prefrontal cortex after adolescent exposure to THC (Burston *et al.*, 2010), although because receptor levels were assessed immediately after administration in that study, it is unclear whether similar recovery of neocortical CB1R expression occurs following *adolescent* THC exposure. Evidence from other brain regions, including the amygdala, hippocampus, nucleus accumbens, and ventral tegmental area, argue against such recovery, as CB1R expression and G-protein coupling in these areas is still suppressed in adulthood when escalating doses of THC are administered for 10 days to adolescent animals (Rubino *et al.*, 2008). Persistently suppressed CB1R expression, or altered receptor function in mPFC and/or SCx, may be similarly induced by chronic, or sub-chronic, cannabinoid exposure, and may underlie the oscillation abnormalities that we report.

B. Altered eCB System Function

Repeated cannabinoid exposure, especially during adolescence, alters other

components of the eCB system, in addition to CB1Rs. For example, WIN administration during early adolescence (P30-P35), but not adulthood (P63-P70), persistently increases eCB uptake and degradation, as mice exposed at these ages demonstrate increased expression of MGL and FAAH, the primary degradative enzymes for 2AG and anandamide, respectively, at postnatal day 120 (Gleason *et al.*, 2012). eCB release may serve to differentiate subgroups of pyramidal neurons that contribute to the excitability of neural networks, based on activity-dependent eCB release and subsequent suppression of inhibition (DSI) from inhibitory synapses that are CB1+. Pyramidal neurons that are less active, and therefore do not release eCBs, are free from CB1R-mediated inhibition, and can subsequently be recruited into the network rhythm by fast-spiking parvalbumin interneurons (Klausberger *et al.*, 2005; Morgan *et al.*, 2008). In Chapter 2, we discussed the likelihood of persistent antagonism of eCBs by chronic administration of CB1R or CB2R antagonists. Therefore, as eCB activity is involved in the generation of synchronous network oscillations, altered eCB release, activity, or breakdown, in addition to changes in CB1R expression or signaling, could contribute to impaired generation of oscillations in adult animals exposed to cannabinoid receptor agonists or antagonists in adolescence.

C. Altered Excitatory and Inhibitory Transmission

Furthermore, chronic or sub-chronic cannabinoid exposure alters components of both the excitatory and inhibitory neurotransmitter systems, revealing functional crosstalk between the eCB, glutamatergic and GABAergic systems. Hippocampal GABA-A receptor density is decreased 24 hours after repeated CB1R agonist administration (Verdurand *et al.*, 2010), and the functional activity of both GABA-A and GABA-B

receptors is reduced in mice that genetically lack the CB1R (Urigen *et al.*, 2011). Adolescent THC treatment persistently decreases NMDA-R binding in the hippocampus (Rubino *et al.*, 20092), and attenuates markers of neuroplasticity and functional excitatory synapses in the prefrontal cortex of adult rats (Rubino *et al.*, 20092). Similarly, immediately following 1 week of THC exposure, hippocampal expression of NMDA and AMPA receptor subunits is significantly decreased in parallel with impaired expression of long-term potentiation (LTP) (Fan *et al.*, 2010). These effects are mediated by CB1Rs, as they are reversed with co-administration of the CB1R antagonist SR141716, and are absent in CB1R KO mice (Fan *et al.*, 2010). Finally, stimulus evoked GABA release is higher, and glutamate release is lower, in adult animals exposed to a CB1R agonist for 11 days during adolescence (Higuera-Matas *et al.*, 2012), that induces in a persistent reduction of the hippocampal excitatory/inhibitory balance. Taken together, these findings support the presence of a link between repeated cannabinoid exposure during adolescence and persistent alterations in glutamatergic and GABAergic functioning that could profoundly alter network activity and neocortical oscillations in adulthood.

VII: *eCB System Complexity and Selectivity of Cannabinoid Receptor Ligands:*
Considerations for Non-CB1R Mediated Effects on Cortical Oscillations

We can speculate on a mechanism accounting for chronic adolescent cannabinoid-induced oscillation suppression that involves altered CB1R expression and/or eCB activity, and potential downstream alterations in the glutamate and/or GABA neurotransmitter systems. However, we would be remiss if we did not briefly discuss the staggering complexity of the eCB system, even as it relates to the CB1R, and the non-specific action of many of the compounds used to study this important neuromodulatory

system. Although CB1R activation is typically associated with the direct inhibition of GABA and, to a lesser degree, glutamate release, CB1Rs regulate the release of other neurotransmitters, including dopamine, serotonin, norepinephrine, acetylcholine, and glycine (Kano *et al.*, 2009), thus revealing even broader neuromodulatory functions of CB1R activation or inhibition. Additionally, while CB1Rs function as homodimers, they may also form functional heterodimers with other GPCRs (Harkany *et al.*, 2007), including MORs (Hojo *et al.*, 2008), dopamine D2 receptors (Kearn *et al.*, 2005), and alpha-2 adrenergic receptors (Pertwee *et al.*, 2005). Because of this heterodimerization, activation of CB1Rs can activate G-protein alpha subunits other than the canonical i/o subtype, including the G-alpha s subunit (Kearn *et al.*, 2005), and thereby stimulate entirely different intracellular signaling cascades than would be expected with CB1R signaling alone. Furthermore, different G-protein subunits (the beta/gamma complex versus the alpha subunit) can act independently of each other and mediate diverse downstream effects of CB1R activation (Chen *et al.*, 2013). CB1Rs have been found in unexpected non-neuronal locations, such as on mitochondrial membranes (Benard *et al.*, 2012), where they regulate neuronal energy metabolism, and on astroglia (Han *et al.*, 2012), where they may play a crucial role in the modulation of synaptic plasticity and working memory. These nuances of CB1R expression and signaling illustrate the incredible diversity of physiological effects that are induced by CB1R activation or inhibition, and present the possibility that the CB1R-mediated suppression of oscillations that we report may be due to unexpected and previously unexplored mechanisms. Finally, although the cannabinoid receptor ligands used in our experiments all have documented potency at their advertised CB1R or CB2R targets, many of these compounds lack

specificity and have reported activity at a variety of non-cannabinoid receptor targets. These include, but are not limited to, TRPV-1 like receptors, receptors for other neurotransmitters, including serotonin, and neuromodulators, including the opioids, as well as unclassified putative non-CB1 or CB2 cannabinoid receptors, and direct modification of ion channel activity (Pertwee, 2006; Pertwee, 2008) (Howlett *et al.*, 2002). The unexpected activities of presumed selective cannabinoid ligands make independent testing of these compounds' specificity necessary in future experiments, if phenotypes are to be correctly attributed specifically to CB1R or CB2R action.

VIII: Overall Conclusion

Experiments conducted during my thesis have tested the hypothesis that marijuana use in adolescence persistently alters the coordinated activity of cortical neural networks in adulthood. We have discovered support for this hypothesis, as we find a persistent attenuation of pharmacologically-evoked cortical oscillations both *in vitro* and *in vivo*, as well as impaired cognitive abilities, and abnormalities in the oscillatory activity generated during cognitive tasks in animals exposed to cannabinoids before adulthood. This work has revealed a novel link between early use of cannabis and persistent impairment in network activity that is considered crucial for normal cognitive and perceptual functioning, and that is grossly abnormal in psychiatric diseases in which these functions are impaired. These findings are highly applicable to public health, and are particularly timely given the ongoing national debate surrounding the use of medical marijuana, and the rapidly advancing effort to legalize recreational consumption of cannabis. While our studies, and those of others, indicate that regular marijuana use in adulthood may not produce lasting and deleterious cognitive or psychiatric consequences,

accumulating evidence suggests that every effort should be made to limit marijuana use during the vulnerable adolescent period.

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