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Schizophrenia and Endocannabinoid Signaling in the Prefrontal Cortex

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Schizophrenia and Endocannabinoid Signaling in the Prefrontal Cortex

A Dissertation submitted in partial satisfaction of the requirements for the degree of

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in

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by

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December 2014

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Dedication

For my mother, Linda May Lovelace. Your unconditional love and support had always been a comfort to me, knowing that even if I would fail, it wouldn’t matter to you and you would always be proud of whatever person I have and will become.
Developmental factors involved in schizophrenic pathology are known to exist, but complex chemical interactions during critical stages of brain development are unclear. Environmental and genetic factors contribute to the development of cortical brain circuits and disruption during specific critical times in development could have lifelong consequences. N-methyl-D-aspartate receptors (NMDARs) are of critical importance for synaptic formation as well as synaptic plasticity throughout the lifespan. Hypofunctioning NMDARs have been implicated in schizophrenic patients and have been extensively used as the basis of animal models to study schizophrenia in a lab environment. Here we use a developmental model induced by drugs that block NMDARs during early stages of postnatal development in order to model schizophrenia in mice. These mice have shown permanent reductions in endocannabinoid (eCB) signaling in the medial prefrontal cortex (mPFC). These findings emphasize a possible involvement of eCB signaling in the development of schizophrenia. eCBs are unusual in
that they act retrogradely at the synapse and are released on demand under high levels of post synaptic activation. Since the overall effects of eCBs at the synapse are inhibitory, it has been suggested that eCBs may play an important role in maintaining a nominal level of neuronal activation in the cortex. This also led us to investigate a well-known risk factor of schizophrenia, adolescent cannabis abuse. Cannabis contains Δ⁹-tetrahydrocannabinol (THC) which directly activates cannabinoid receptors in the brain. Prolonged exposure to cannabis during adolescence has been speculated to have long-term effects in some people lasting into adulthood. An adolescent cannabis abuse model induced by a specific agonist of the primary cannabinoid receptor (CB₁R) in the brain shows permanent reduction in the eCB system signaling including impaired eCB-dependent synaptic plasticity and CB₁R function. In addition, the cannabis abuse model shows deficiency in a function of another presynaptic receptor involved in control of presynaptic release, metabotropic glutamate receptor 2/3 (mGluR2/3). CB₁Rs and mGluR2/3 receptors both share the same intracellular signaling pathways which suggest that the critical maladaptation underlying the etiology of psychosis may involve signaling mediated by presynaptic metabotropic receptors or downstream processes. These data open a new avenue for understanding and treatments of schizophrenia taking into account important developmental factors.
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Introduction

It has not been until recently in our history that mankind has been able to attribute the bizarre behavioral symptoms of what we now call “schizophrenia” to biological factors. In modern science, we instead find ourselves in a unique position of not only being able to identify schizophrenia as biological pathology, but we also have the tools to begin treating and potentially preventing this mental disorder. In order to develop the best treatment, and even more importantly an effective prevention, it is critical to first understand the causes and roots of this devastating brain ailment. Schizophrenia has been described as a developmental pathology both in terms of its biological (Behrens and Sejnowski, 2009; Bitanihirwe et al, 2009; Cannon et al, 1999; Wang et al, 2011; Wong et al, 2010) and psychological perspectives (Granic, 2005; Granic and Hollenstein, 2003). It is important to keep in mind these developmental aspects of the schizophrenia etiology when conducting research in this field, because developmental factors could be crucial in understanding the best ways to treat or prevent the disease. Since it is becoming clearer that much of schizophrenic pathology involves developmental factors that carry on into adulthood, some have proposed that the best treatment for at-risk patients might be early prevention (Fusar-Poli et al, 2007; Lieberman, 2007). It is well known that schizophrenia symptomology usually expresses during early adulthood, but there are cognitive signs at even earlier stages of development (Reichenberg et al, 2010). Establishing behavioral, anatomical, and physiological consequences in models using a variety of environmental factors at different stages of brain maturation could guide development of future
preventative treatments, and may even contribute to public policy in raising awareness of possible risk of drug abuse during development.

A well established hallmark of schizophrenia is prefrontal cortex (PFC) hypofunction as demonstrated in human subjects using fMRI and behavioral studies (Fusar-Poli et al, 2007; Molina et al, 2005). The mPFC is important for performing memory tasks including working memory, extinction, and discriminative fear learning (Grayson et al, 2007; Nelson et al, 2011; Vieira et al, 2014). Other studies which use computational models based on biological evidence in humans also show abnormal mPFC information processing (Cohen et al, 1996; Kristiansen et al, 2010a). The medial prefrontal cortex (mPFC) is a well known hub of input from various sub cortical regions of the brain which include (but not limited to): the amygdala (Orozco-Cabal et al, 2006), medial dorsal thalamus (Jodo et al, 2010), and the hippocampus (Jay and Witter, 1991; Parent et al, 2009). Each of these brain regions are also associated with behaviors which are disrupted in schizophrenic patients and mouse models (emotion, social interaction, and memory respectively)(Morris et al, 2005). Additionally, the PFC is one of the last areas of the brain to develop while major pruning occurs during adolescence and early adulthood (Gogtay et al, 2004). Coincidently it is also the same time in which psychotic symptoms begin to emerge in most schizophrenics. It seems reasonable to assume that synaptic activity and measures of plasticity in the mPFC may also be disrupted in animal models of schizophrenia and is therefore the primary region of interest in this report.

It is widely believed that the risk of developing schizophrenia relies on a variety of risk factors, and not just a single genetic or environmental insult. Knowing how these
different risk factors interact could be a crucial piece of the puzzle in understanding schizophrenic pathology. Genetic factors are definitely connected to schizophrenia, with twin studies showing ~50% risk of developing schizophrenia having a schizophrenic twin (Gottesman, 1989; Gottesman and Bertelsen, 1989; Mowry and Gratten, 2012). Studies indicate that interplay between genetic and environmental risk factors are likely to be involved in schizophrenia pathology (Beaton and Simon, 2011). Few studies looking at “multiple hits” during critical periods of development have been done in animals to further our understanding of environmental influence during different stages of development (Chen et al, 2011). One of the major factors that may increase risk for schizophrenia is dopamine regulation. For example, genetic variations of catechol-O-methyltransferase (COMT Val158Met allele), an enzyme that helps regulate dopamine transmission, is well-characterized risk factor for schizophrenia (Goldbeg Weinberger 2003). In fact, most current drug treatments for schizophrenia target dopamine signaling (Fragou et al, 2012; Grace, 1991; Melnik et al, 2010). It has also been demonstrated that the relative risk of developing psychosis following use of cannabis is increased in people carrying mutation within COMT (Val158Met allele) gene (Caspi et al, 2005). This effect was only observed in people using cannabis before the age of 18, suggesting an important developmental interaction (Caspi et al, 2005). Additionally, AAT-repeats in the gene that codes for the CB1R has been shown to be a risk factor in certain subtypes of schizophrenia (Ujike and Morita, 2004). Cannabis use during adolescence is a risk factor and has been investigated and verified several times (Manrique-Garcia et al, 2011; Ujike et al, 2004). In humans, cannabis use has been linked to increased DA release in the
striatum (Bossong et al., 2009), which not only links it to possible addiction, but also to psychiatric disorders such as schizophrenia. Another study in which THC was administered to 3 different groups: healthy cannabis users, patients with psychotic disorder, and first degree relatives, showed that only patients and relatives displayed increased signs of DA release, while control subjects did not (Kuepper et al., 2013). Furthermore is seems that DA regulation of PFC function undergoes dramatic changes in adolescence which could also be linked to cannabis abuse during the same time (O'Donnell, 2010). This information combined with a cannabis abuse interaction with COMT polymorphisms seems to support an important interplay between eCB activity and dopamine during adolescence as potential interacting risk factors for developing schizophrenia.

Another well known genetic risk factor is on the DISC1 gene, important for development of healthy synapses and neuron morphology, as well as of N-methyl D-aspartate receptor (NMDAR) expression (Wei et al., 2014; Wexler and Geschwind, 2011; Yates, 2012). One study has showed that knock down of the NR1 subunit causes reduced levels of DISC1 at synapses in addition to immature spine development in the striatum, displaying a close relationship between DISC1 and NMDARs (Ramsey et al., 2011). Mouse models in which DISC1 genes are altered show many schizophrenic like behaviors including hypersensitivity to amphetamine, impaired sensorimotor gating, deficits in working memory, and abnormal sociability. In addition several biological hallmarks also exist, including decreased neurogenesis, reduction in PV+ interneurons, and lower cortical dopamine levels (Brandon and Sawa, 2011). In a recent report a
dominant negative DISC1 model showed a reduction on CB₁R expression levels in the prefrontal cortex (Kaminitz et al., 2014). In vivo knockdown of DISC1 also results in increased NMDAR current in layer 5 pyramidal cells in the prefrontal cortex. Additionally this knock down also seems to trigger an over expression of NR2A containing subunits in NMDARs(Wei et al., 2014). The downstream effects of alteration in DISC1 are extensive; however it is clear that interactions with NMDAR function, CB₁R expression, and its contribution to synaptic development make it an important risk factor in further research.

Differential patterns of NR2B (NMDA receptor subunit) expression have also been observed in post mortem schizophrenic patients (Grimwood et al., 1999; Kristiansen et al., 2010a; Kristiansen et al., 2010b). Two NR1 subunits are obligatory for NMDAR formation along with two NR2 subunits. However, unlike NR1 which has variations through genetic splicing of GRIN1, NR2s are created through 4 genetic isoforms (GRIN2A-D). During very early stages of postnatal development NR2s are almost exclusively NR2B. Overtime NR2B subunits are replaced with NR2A as synapses maturate (Sheng et al., 1994). NMDARs with NR2B subunits remain open for longer showing slower kinetics when compared to NR2A containing receptors(Wang et al., 2011). This difference in kinetics could be important for initial strengthening and establishment of immature cortical and subcortical synapses, while NR2A could be utilized for more nuanced signaling once pathways have been established. In fact, studies have been done by genetically replacing NR2B subunits with NR2A resulting in mice that have a series of behavior deficiencies as well as impaired α-Amino-3-hydroxy-5-
methyl-4-isoxazolepropionic acid receptor (AMPAR) regulation and homeostatic synaptic plasticity (Wang et al., 2011). This study highlights the critical importance of NMDAR containing NR2B during very early postnatal development. This switch has been shown to be dependent upon neural activity and experience (Williams et al., 1993), as well as NMDAR and mGluR5 activation (Matta et al., 2011). Therefore, hypofunctioning NMDARs during very early stages of development could have adverse effects on timing the switch from NR2B to NR2A. Developmental importance of the NR2 subunit switch along with abnormal NR2B expression levels in post mortem schizophrenic brains lends support to the idea that the NMDAR is a central aspect of schizophrenic pathology.

Irregularities in neuregulin 1 (NRG1) is also a well characterized risk factor for schizophrenia with research being done on gene/environment interactions (Karl, 2013). NRG1 is a growth factor which promotes dendritic spine growth and synaptic formation in brain development (Barros et al., 2009). Receptor tyrosine-protein kinase 2 and 4 (ErbB2 and ErbB4) are receptors for NRG1 and their genes are also susceptibility genes for schizophrenia (Benzel et al., 2007). Intact NRG1/ErbB signaling pathways are crucial for dendritic spine maturation through binding of post synaptic density 95 (PSD95) and subsequent recruitment of NMDARs to the maturing spines. If NRG1/ErbB signaling is disrupted, it will then also have an impact on the recruitment of NMDARs to the synapse and have further consequences to synaptic plasticity and brain development (Barros et al., 2009). The list of potential genetic and environmental factors is long, and all the variations and subtypes of schizophrenic diagnosis are probably due to unique
combinations of risk factors, but it seems that many risk factors converge on NMDAR hypofunction in general (Snyder and Gao, 2013).

Administration of NMDAR antagonists has been shown to elicit both positive and negative symptoms in normal subjects that are indistinguishable from schizophrenia (Adler et al., 1999; Morris et al., 2005). Multiple pharmacological animal models for schizophrenia have been established using NMDAR antagonists such as phencyclidine (PCP), ketamine, and MK801 (Bickel and Javitt, 2009; Neill et al., 2010). All of these models are attempts to mimic “hypofunctioning glutamate” systems associated with schizophrenia (Bitanihirwe et al., 2009; Konradi and Heckers, 2003b; Neill et al., 2010). Rats that are given sub-chronic injections of PCP have been shown to develop long-lasting behavioral deficits in working memory and social behavior analogous to psychosis in humans (Mouri et al., 2007; Neill et al., 2010). It is well established that behavioral symptoms such as working memory, object recognition, pre-pulse inhibition, and a variety of anxiety and social tasks have shown deficits in multiple animal models for schizophrenia, which mimic schizophrenic symptoms in humans (Mateos et al., 2010; Nakatani-Pawlak et al., 2009; Pollard et al., 2012; Rubino et al., 2008; Schneider and Koch, 2003, 2005b; Schneider et al., 2008; Sircar, 2003; Sircar and Soliman, 2003; Vigano et al., 2008; Wang et al., 2008; Wegener and Koch, 2009; Wiley et al., 2003; Zamberletti et al., 2010). Other PCP induced animal models have shown reduction in dendrite density (Nakatani-Pawlak et al., 2009), increases in apoptotic markers and neuronal death (Wang et al., 2001; Wang and Johnson, 2005). The most common phenotype using various PCP models is reduction in PV+ cell immunoreactivity (Abdul-
Monim et al, 2006; Abekawa et al, 2007; Cochran et al, 2003; Nakatani-Pawlak et al, 2009; Reynolds et al, 2004; Wang et al, 2008). Not as much has been done investigating the effects of cannabinoid treatments on PV+ expression specifically, but some modulation of GABAergic neuron morphology has been reported (Behan et al, 2012; Lovinger, 2008). Interactions between CB$_1$R activity and PV+ expression may not be directly connected, since it is well established that CB$_1$Rs are not readily expressed on PV+ fast spiking terminals (Harkany et al, 2008; Ohno-Shosaku et al, 2011). The effects of systemic PCP treatment during different stages of brain development are no doubt complex and intertwined, which makes direct and simple claims about mechanistic downstream effects difficult to make. However, there is no reason to believe that natural phenomenon occurring in schizophrenic patients is any less complex.

In addition to NMDARs direct role in synaptic plasticity and development in schizophrenia, it also interacts with the dopamine (DA) system. Probably the longest standing biological hypothesis for the symptoms of schizophrenia is based on dopamine signaling. The so called “classic antipsychotic” medications developed are DA antagonists and have been shown to be quite effective in treating positive symptoms of schizophrenia. One of the most studied DA-NMDAR interaction is D1 receptor mediated potentiation of NMDAR current (Cepeda et al, 2009; Chen et al, 2004). This interaction could be important for reward signaling. Released DA potentiates Ca$^{2+}$ signals through NMDARs, thereby leading to increased long term potentiation of synapses involved in processing of the reward stimulus. Conversely if DA is overactive, an excess of Ca$^{2+}$ can lead to excitotoxicity through overly active NMDARs, a possible link to
neurodegeneration in schizophrenia (Konradi and Heckers, 2003a; Plitman et al, 2014). In addition glutamate signaling can directly control release of DA in different regions of the brain (Sesack and Carr, 2002). Glutamate synapses from the PFC project directly to neurons in the ventral tegmental area (VTA) and can facilitate dopamine release (Takahata and Moghaddam, 1998). Hypofunctioning NMDARs could reduce this drive and thus reduce DA release into the PFC contributing to a hallmark of schizophrenia, hypofunctioning mesocoritcal DA activity resulting in negative symptoms.

Hypofunctioning NMDARs can also contribute to positive symptoms through PFC control of the mesolimbic DA pathway. Glutamatergic projections from the PFC synapse in the VTA onto GABAergic interneurons which then control the release of DA to the nucleus accumbens. Hypofunctioning NMDARs in this circuit will cause disinhibition of DA neurons, and increase levels of DA in the nucleus accumbens (Sesack et al, 2002). Additionally, direct physical interactions have been reported between NMDARs and D1 receptors which affect NMDAR functionality (Lee et al, 2002). These NMDAR-D1 complexes add another level of complexity that needs to be considered. Alterations in either NMDAR composition or function could have profound impact on DA signaling and schizophrenic symptoms.

The role of endocannabinoids in schizophrenia and psychosis has raised a lot of interest, not only because of the potential new therapeutic pharmacological targets, but because of social and political ramifications regarding cannabis legalization in the United States and the potential harmful effects of adolescent abuse. In parallel with the developmental progression of behavioral symptoms of schizophrenia, new evidence is
emerging relevant to neural networks abnormalities at the synaptic level in humans including the CB$_1$R (Dalton et al., 2011; Wong et al., 2010). Studies in which cannabinoid agonists are administered to adolescent animals have shown many long term behavioral, biochemical, and physiological effects (Abush and Akirav, 2012; Behan et al., 2012; Lisboa et al., 2010; O'Tuathaigh et al., 2010; Raver et al., 2013; Schneider et al., 2003; Schneider et al., 2008; Tan et al., 2011). These models take into account endocannabinoid signaling regulation during critical periods of brain development in adolescence. A major advantage of these models is that they mimic drug abuse patterns observed in society, which makes these studies directly relevant to serious environmental risk factors today.

Endocannabinoids are particularly unusual neurotransmitters that are active throughout the nervous system. The most prominent cannabinoid receptor in the central nervous system is the CB$_1$R which mediates most endocannabinoid synaptic transmission in the brain (Heifets and Castillo, 2009). The CB$_1$R is a metabotropic receptor coupled to the G$_{i/o}$ protein pathway. CB$_1$Rs are located predominately on presynaptic terminals in the central nervous system (Lovinginer, 2008; Ohno-Shosaku et al., 2011), but is also readily expressed in astrocytes (Han et al., 2012), mitochondria (Calabresi et al.), and growth cones (Berghuis et al). The CB$_2$R is less expressed in the central nervous system and tends to express more in the periphery in places like: spleen, thymus, lymph nodes, immune cells, and liver (Buckley, 2008). More recently there have been reports that the G protein-coupled receptor 55 (GPR55), which has not been typically thought of as cannabinoid receptor, responds to cannabinoid ligands (both endogenous and exogenous). Research in endocannabinoids and their receptors are still in its infancy and there are
probably additional receptors that have yet to be identified. However, since CB₁Rs are the most widely expressed cannabinoid receptor in the central nervous system, and more importantly for this report in the mPFC, this will be the focus of this dissertation.

The first functional evidence of endocannabinoids in the brain came from the discovery that depolarization-induced suppression of inhibition (DSI) in the cerebellum and hippocampus was dependent upon CB₁R activation (Kreitzer and Regehr, 2001; Ohno-Shosaku et al, 2001; Wilson and Nicoll, 2001). Briefly, DSI is a reduction of synaptically driven inhibitory current onto postsynaptic cells which is triggered by prolonged post synaptic depolarization (1-10 seconds). This experimental setup requires that some information must travel from the postsynaptic site, which received the prolong depolarization, to the presynaptic terminals, which releases γ-Aminobutyric acid (GABA) and triggers subsequent inhibitory currents in the post synaptic cell. Since almost all synaptic neurotransmission occurs in a pre-to-post fashion, the discovery of a retrograde signal was, and still is, quite novel. Using CB₁R antagonists effectively blocks DSI (Yoshida et al, 2002). This same effect has also been found to exist on excitatory terminals and termed depolarization-induced suppression of excitation (DSE) (Kreitzer et al, 2001). Both DSI and DSE are transient effects that last on the order of seconds. It involves a reduced probability of neurotransmitter release by inhibiting presynaptic voltage-gated calcium channels, activation of potassium channels, and modulation of vesicle fusion machinery via G_{i/o} pathway activation (Lovinger, 2008). In order for a retrograde signal to occur, eCBs need to be released from the post synaptic terminal. The 2 main eCBs in the brain are 2-Arachidonoylglycerol (2-AG) and N-
arachidonylethanolamine (anandimide, AEA). 2-AG is in the highest concentration in the brain, and has shown to be responsible for most synaptic eCB signaling on excitatory terminals in the mPFC over AEA (Lafourcade et al, 2007). 2-AG is synthesized postsynaptically on demand and is not stored in vesicles for release like typical neurotransmission. The signaling pathway usually requires activation of both postsynaptic mGluR1/5 and voltage-gated calcium channels. mGluR1/5 receptors are coupled to Go_q/11, and when activated causes phospholipase C (PLC) to cleave phospholipid phosphatidylinositol (PI) into diacylglycerol (DAG). Subsequently, membrane-bound diacylglycerol lipase (DAGL) synthesizes 2-AG from DAG and since 2-AG is lipid soluble it is able to diffuse through the lipid membrane and act on its presynaptic targets (CB_1Rs). However, diffusion of 2-AG is also thought to be enhanced through transport proteins on post synaptic terminals (Ohno-Shosaku et al, 2011). Inactivation of 2-AG is mainly through hydrolysis by presynaptic monoacylglycerol lipase (MAGL), although other routes include postsynaptic oxidation by cyclooxygenase-2 (COX-2) and hydrolysis by 2-arachidonoylglycerol hydrolase (ABHD6)(Vandevoorde and Lambert, 2007). This unique retrograde signaling system allows for dynamic regulation of neurotransmitter release and another level of synaptic modulation which could be crucial for regulating and maintaining homeostasis in neuronal circuits.

More recently, eCB signaling has been implicated in long term forms of plasticity as well, namely eCB mediated long term depression (eCB-LTD)(Heifets et al, 2009; Heifets et al, 2008; Lafourcade et al, 2007). eCB-LTD occurs at excitatory synapses, while inhibitory long term depression (I-LTD) occurs at inhibitory synapses. The
signaling pathways for eCB-LTD are the same as described for DSE but requires a much longer period of stimulation, on the order of minutes (Lafourcade et al., 2007; Mato et al., 2005). Presynaptic CB₁R and subsequent Gaₐ₁b activation is required. This in turn inhibits adenylyl cyclase (AC) and reduces the production of cyclic adenosine monophosphate (cAMP). With less cAMP, protein kinase A (PKA) is then less active, and is unable to phosphorylate critical target proteins (which currently are mostly unknown). One candidate is likely Rab3 interacting molecule 1 alpha (RIM1α), which is an important modulator at the active sites for fusion of vesicles in presynaptic terminals. PKA activity is also competing with presynaptic phosphotase activity such as calcineurin (CaN) which dephosphorylates the same target proteins in the presence of presynaptic calcium (Heifets et al., 2009; Ohno-Shosaku et al., 2011). If the balance between CaN and PKA activity are imbalanced long enough, prolonged reduction in neurotransmitter release lasting for hours is induced. One interesting difference between eCB-LTD and I-LTD is that I-LTD is inherently a heterosynaptic form of plasticity that requires release of 2-AG through post synaptic glutamate synapses, which is then released and diffuses out towards inhibitory terminals (Heifets et al., 2008). eCB-LTD on the other hand can be either homosynaptic or heterosynaptic. The functional relevance of eCB-LTD is still widely speculative, but homeostatic control of neurotransmitter release seems to be the most direct role. Others have shown association with eCB-LTD to behaviors such as working memory (Han et al., 2012), fear extinction but not acquisition (Marsicano et al., 2002), and drug addiction (Kasanetz et al., 2010; Kasanetz et al., 2013). There is also some speculation that eCB-LTD is important weakening of synapses from sensory deprivation.
and could potentially play a role in Parkinson’s disease as well as synaptic pruning processes (Heifets et al., 2009; Kreitzer and Malenka, 2007). Much more work needs to be done to understand the full scope of eCB-LTD and its function in neural circuits and behavior as well as the interactions with other neurotransmitter systems.

Chapter I:

Impaired fear memory specificity associated with deficient endocannabinoid-dependent long-term plasticity.

Abstract

In addition to its central role in learning and memory, N-methyl D-aspartate receptor (NMDAR)-dependent signaling regulates central glutamatergic synapse maturation and has been implicated in schizophrenia. We have transiently induced NMDAR hypofunction in infant mice during post-natal days 7-11, followed by testing fear memory specificity and presynaptic plasticity in the prefrontal cortex in adult mice. We show that transient NMDAR hypofunction during early brain development, coinciding with the maturation of cortical plasticity results in a loss of an endocannabinoid (eCB)-mediated form of long-term plasticity (eCB-LTD) at adult central glutamatergic synapses, while another form of presynaptic plasticity mediated by the metabotropic glutamate receptor 2/3 (mGluR2/3-LTD) remains intact. Mice with this selective impairment of presynaptic plasticity also showed deficits in fear memory specificity. The observed deficit in cortical presynaptic plasticity may represent a neural maladaptation contributing to network instability and abnormal cognitive functioning.
Introduction

The ability to discriminate between similar, yet different, contexts is critical for episodic memory (Tulving, 2002). Episodic memory is demonstrably abnormal in schizophrenia (Pelletier et al., 2005) and imbalanced memory specificity and generality may occur independent of associative learning in schizophrenia patients (Tamminga, 2013). Although studies on context discrimination have focused on the hippocampal circuit (Leutgeb et al., 2007; McHugh et al., 2007; Sahay et al., 2011), recent studies have demonstrated that the neural circuit for memory specificity and generalization comprises an anatomically widespread circuit including the medial prefrontal cortex (mPFC) (Navawongse and Eichenbaum, 2013; Xu and Sudhof, 2013).

Clinical data and neuroimaging studies indicate that schizophrenia-specific cognitive deficits related to prefrontal circuit pathophysiology are present prior to the onset of psychosis (Reichenberg et al., 2010). In addition, there is converging evidence linking the schizophrenia cognitive endophenotype with NMDAR hypofunction in the corticolimbic system (Coyle et al., 2003; Tamminga, 1998). Exposure to dissociative anesthetics such as phencyclidine (PCP) or ketamine, which are NMDAR antagonists, produces schizophrenia-like symptoms (Javitt and Zukin, 1991) and acute, chronic or perinatal treatment with NMDAR antagonists produce cognitive deficits similar to schizophrenia-like phenotypes in rodents (Morris et al., 2005).

Although the idea of deficient memory specificity and overgenerality in psychosis is appealing, there has been no experimental data to support this hypothesis. Conversely,
the fundamental question regarding how NMDARs function during early postnatal development to sculpt the capacity for presynaptic forms of plasticity has not been elucidated. To address these questions, we examined fear memory specificity and presynaptic forms of cortical plasticity in the mPFC in an adult mouse model with induced transient PCP-mediated NMDAR hypofunction during early brain development coinciding with the maturation of cortical plasticity. PCP-treated mice exhibited impairment in contextual discrimination tasks, indicating deficient fear memory specificity. We also examined multiple forms of presynaptic plasticity at the cortical layer 2/3 to 5 (L2/3→L5)–glutamatergic synapse in the mPFC in PCP-treated mice. Unexpectedly, PCP-treated mice showed a strong deficit in the endocannabinoid (eCB)-dependent long-term depression (eCB-LTD) in the mPFC, while the metabotropic GluR2/3 receptor (mGluR2/3)-dependent plasticity and forms of presynaptic short-term plasticity were spared. Thus, the maturation of presynaptic long-term plasticity at this cortical glutamatergic synapse is sensitive to NMDAR hypofunction during early postnatal development. The observed loss of eCB-LTD may result from a maladaptation in the excitatory circuitry, which is associated with deficient cognitive functioning.

Results

PCP-treated mice exhibit deficit in fear memory specificity

To investigate the developmental role of NMDARs on cognitive function in adulthood, we induced transient PCP-mediated NMDAR hypofunction in perinatal mice on postnatal days P7-11 (Wang et al., 2001), which overlap with a period of extensive
cortical plasticity maturation (Zhang, 2004). We found that PCP-treated mice performed similar to vehicle-treated controls (CTRL) in the contextual version of the fear conditioning task (Figure 1.6A) and showed the same freezing after a 24 h delay (CTRL: 24.91 ± 4.90 %, n = 19; PCP-treated: 25.09 ± 5.18 %, n = 16; t\(_{(33)}\) = 0.979, p = 0.979, r = 0.17). Next, we subjected the PCP-treated mice to a context discrimination task (McHugh et al, 2007) to evaluate the role of NMDARs during early postnatal development in the establishment of neural mechanisms underlying fear memory specificity (Figure 1.1A). PCP-treated and control mice exhibit comparable freezing to both contexts during the first block trial (Figure 1.1D, two-way ANOVA of context and treatment during trial block 1; Context x Treatment: F\(_{(1,66)}\) = 0.06, p = 0.810). In fact, the similar level of freezing was observed in both groups during entire generalization phase (see Figure 1.1B-C). This indicates that context A was similar enough to context B that strong generalization was observed during generalization phase and freezing in Context A and B was comparable in both groups.

The control group began to freeze significantly less to context B compared to context after training on context discrimination task, demonstrating the ability to consistently distinguish between similar yet different contexts (RT-ANOVA of trial blocks 1-5 and context: Context: F\(_{(1,18)}\) = 23.33, p = 0.0001; Trial block, F\(_{(4,72)}\) = 4.74, p = 0.002; Trial block x Context: F\(_{(4,72)}\) = 4.70, p = 0.002). *Post hoc* analysis using Bonferroni correction for multiple comparisons indicates that differences were present during the discrimination phase: trial blocks 4 (p = 0.0008) and 5 (p = 0.0002) only. In contrast to control animals, PCP-treated mice exhibit a deficit in fear memory specificity (Figure 1.1C, RT-ANOVA
of trial blocks 1-5 and context: Context: $F_{(1,15)} = 6.41$, $p = 0.023$; Trial block: $F_{(4,60)} = 0.86$, $p = 0.49$; Trial Block x Context: $F_{(4,60)} = 0.79$, $p = 0.535$).

PCP-treated animals demonstrated strong deficit in memory specificity during discrimination phase when compared to controls (Figure 1.1B-C, RT-ANOVA, Treatment x context x trial block 3-5: $F_{(2,66)} = 3.411$, $p = 0.039$). Control mice acquired the ability to distinguish context A and B during trial block 4, $t_{(18)} = 4.026$, $p = 0.0008$, $r = 0.69$ and also showed strong fear memory specificity during trial block 5, $t_{(18)} = 4.754$, $p = 0.0002$, $r = 0.75$. Contrary to the control, PCP-treated mice were not able to discriminate context A and B on trial block 4 (a two-way ANOVA of treatment and context on trial block 4: Context x Treatment $F_{(1,66)} = 4.64$, $p = 0.035$. PCP-treated: $t_{(15)} = 0.776$, $p = 0.450$, $r = 0.20$). During the last trial block (Figure 1.1C), PCP-treated mice slightly improved their ability to discriminate Context A and B (PCP-treated: $t_{(15)} = 2.429$, $p = 0.028$, $r = 0.53$), which suggests that PCP-treated mice overcame their context discrimination deficit with additional training compared to controls (a two-way ANOVA of treatment and context on trial block 5: Context x Treatment: $F_{(1,66)} = 2.88$, $p = 0.094$). In order to clarify this effect, we have also compared context discrimination ratios between groups during trial blocks. Figure 1.1E shows a marked difference on trial block 4 ($t_{(33)} = 2.121$, $p = 0.042$, $r = 0.74$), but no difference between groups on trial block 5 ($t_{(33)} = 1.670$, $p = 0.10$, $r = 0.28$), indicating that as training continues, PCP-treated animals’ performance become closer to control animals’ performance. In summary these data strongly suggest that transient NMDAR hypofunction during early postnatal development (P7 - 11) resulted in deficient fear memory specificity in the adult.
In addition, PCP-treated mice showed normal levels of uninduced locomotor activity in novel environment (Figure 1.6D-F; Average Velocity: PCP-treated: 4.85 ± 0.15 cm/s, n = 33; CTRL: 5.07 ± 0.20 cm/s, n = 30; \( t_{(61)} = 0.859, p = 0.3939, r = 0.11 \). Total Distance Traveled: PCP-treated, 43.42 ± 1.28 m, n = 33; CTRL, 45.56 ± 1.77 m, n = 30; \( t_{(61)} = 0.998, p=0.3262, r = 0.13 \) and normal anxiety-related responses (PCP-treated: 29.95 ± 0.98 %, n = 33; CTRL: 26.48 ± 1.84 %, n = 30; \( t_{(61)} = 1.709, p = 0.0926, r = 0.21 \) (Figure 1.6F) which is consistent with previous study (Nakatani-Pawlak et al, 2009). Consistent with a previous report (Grayson et al, 2007; Nakatani-Pawlak et al, 2009; Wiley et al, 2003), PCP-treated mice exhibited abnormal behavior in the novel object recognition task (Figure 1.8 A-B) but normal performance in the object location task (Figure 1.8 C-F). One difference between these two behavioral tasks is that novel object recognition memory test involves exposure to novelty (which may generate mild fear) while object location test relies entirely on familiar context and objects.

**mGluR2/3-dependent LTD is unaffected in PCP-treated mice.**

Convergent cortical and subcortical pathways are integrated in cortical layer L2/3 pyramidal neurons, which form abundant contacts with pyramidal neurons in cortical L5, the output of the cortex. To investigate the developmental role of NMDA receptors in the maturation of presynaptic plasticity, we tested synaptic activity at the L2/3→L5 glutamatergic synapse (Hempel et al, 2000; Morris et al, 1999) (Figure 1.2-1.3 and Figure 1.10). One way to capture how a synapse transforms signals is to analyze relation between its input and output (I/O). Analysis of the shape of the I/O curve may provide
valuable information about additive operations (shift along the input axis) or gain (slope change). I/O relations appear to be the same in PCP-treated and control mice (Figure 1.10C). Additional detail analysis of I/O relations was done to determine if differences in the shape of the curves existed using different metrics obtained from Boltzmann sigmoidal analysis. Comparing means of individual Boltzmann fitted parameters revealed no differences between PCP-treated and control mice in maximum asymptote (A2: PCP-treated, 0.68±0.057 mV, n=11; CTRL: 0.78 ± 0.06 mV, n = 9; t test: p > 0.05), in the center (x0: PCP-treated: 79.46 ± 9.98 µA, n = 11; CTRL: 81.63 ± 10.34 µA, n = 9; p > 0.05), or in the time constant (dx: PCP-treated: 22.36 ± 3.67, n = 11; CTRL: 27.63 ± 4.53, n = 9; t test: p>0.05). This suggests that there are no differences in all tested characteristics of the I/O curves. Thus by these measures, PCP-treated mice showed unaltered synaptic transmission in the L2/3→L5 pathway indicating normal synaptic density within the population and that synaptic transmission was equally effective in response to a single stimulus.

mGluR2/3 receptors activation is known to induce LTD (mGluR2/3-LTD) with a presynaptic locus of expression in PFC (Robbe et al, 2002). We tested mGluR2/3-LTD in PCP-treated mice in the L2/3→L5 pathway. Success or failure of LTD expression in individual groups was determined using a paired Wilcoxon signed rank test (Wilcoxon test) by comparing the mean fPSP amplitude during baseline (10 min before stimulation) and 50-60 min after LTD induction. Figure 1.2 shows that the mGluR2/3 agonist LY379268 was effective in inducing robust mGluR2/3-LTD in the control and PCP-treated mice (Wilcoxon test: CTRL: 50.83 ± 6.03 %, Z = -2.366, n=7, p=0.018; PCP-
treated: 45.31 ± 4.41 %, Z = -2.366, n = 7). There was no difference between the control and PCP-treated mice in the level of mGluR2/3-LTD expression (Figure 1.2C, RT-ANOVA: Time x Treatment: F_{(2,24)} = 1.125, p = 0.305), indicating that mechanisms controlling presynaptic mGluR2/3-LTD, including those shared with eCB-LTD (see below), are intact in PCP-treated mice.

**Short-term plasticity in the L2/3->L5 pathway.**

To examine the effects of NMDAR hypofunction during a critical time for maturation of plasticity in the PFC circuit, we tested short-term plasticity in the L2/3->L5 pathway in the mPFC in adult PCP-treated mice. Multiple forms of short-term plasticity can be expressed at the L2/3->L5 excitatory synapse, including the paired-pulse ratio of peak amplitudes (PPR), short-term potentiation (STP) and short-term depression (STD) (Hempel et al, 2000; Morris et al, 1999). PPR, STP and STD were tested at 30% and 70% of the maximum stimulus intensity since the abnormality in eCB-LTD was observed in recordings performed at 70% of the maximum stimulus intensity. There was no difference in PPR, STP and SDT expression between the PCP-treated and control mice at 30% (Figure 1.10D-F) or at 70% of the maximum stimulus intensity (Figure 1.10G-J), suggesting that the deficit at the L2/3->L5 glutamatergic excitatory synapse in PCP-treated mice might be limited to the eCB system-dependent plasticity.
PCP-treated mice show deficient endocannabinoid-dependent cortical plasticity.

eCB-LTD is a widespread form of cortical plasticity that provides activity-dependent inhibitory control of neurotransmitter release. The cannabinoid receptor type 1 (CB₁R) is required for the induction of eCB-LTD (Lovinger, 2008). Similar to mGluR2/3-LTD, eCB-LTD is also a presynaptic form of plasticity that is negatively coupled to the cAMP/PKA signaling pathway via Gi/o (Lovinger, 2008). Thus, we examined eCB-LTD in PCP-treated mice in the L2/3→L5 pathway. We used a moderate stimulation protocol of 10 Hz for 10 min at 70% stimulus intensity to induce eCB-LTD at the L2/3→L5 glutamatergic synapse in the mPFC (Figure 1.3A), which has been shown to produce long-term depressive effects lasting for at least 1 hour (Lafourcade et al., 2007). A comparative analysis of the mean fPSP amplitude during baseline and 50-60 min after LTD induction revealed robust LTD expression in control animals (Figure 1.3A, Wilcoxon test: 54.30 ± 7.34 %, Z = -2.521 n=8, p = 0.012). It is well established that the expression of this form of LTD is coupled with an eCB receptor (CB₁R) (Lafourcade et al, 2007; Lovinger, 2008). Not surprisingly, bath application of CB₁R inhibitors such as AM251 (Figure 1.3A) or SR141716 (Rimonabant or RMNT) (Figure 1.9D), blocked the induction of eCB-LTD in acute brain slices isolated from control animals in the L2/3→L5 pathway (Figure 1.3C, Wilcoxon test: AM251: 94.26 ± 5.25%, Z = -0.734, n = 6, p = 0.463; Rmnt: 92.05 ± 11.41 %, Z = -0.813, n = 5, p = 0.416). Comparative analysis of the last 10 min of recordings (Figure 1.3D) reveals difference between control and AM251- or Rimonabamt-treated slices (AM251: 94.26 ± 5.25%, n = 6; CTRL: 54.30 ±
7.34 %, n = 8; AM251 vs. CTRL: t(12) = -4.134, p = 0.00138, r = 0.77; Rmnt: 92.05 ± 11.41 %, n = 5; Rmnt vs CTRL: t(11) = -2.929, p = 0.0137, r = 0.66).

Unexpectedly, the PCP-treated mice showed a strong deficit in eCB-LTD induction in the L2/3→L5 pathway in the mPFC (Figure 1.3B, Wilcoxon test: 90.29 ± 12.6 %, Z = -0.866, n = 10, p = 0.386). In contrast to the control animals, where tested L2/3→L5 pathway showed a robust and reproducible expression of the eCB-LTD in response to the 10 Hz stimulus, the recordings in the PCP-treated mice showed strong deficit in eCB-LTD expression in response to the 10 Hz stimulation protocol (Figure 1.3E, CTRL: 54.30 ± 7.34 %, n = 8; PCP-treated: 90.29 ± 12.6 %, n = 10; t(16) = 2.211, p = 0.0420, r = 0.48).

Blockade of the monoacylglycerol lipase (MAGL), the primary enzyme responsible for degrading the endocannabinoid 1-arachidonoylglycerol (2-AG) (Makara et al., 2005), with the specific inhibitor JZL184 (Long et al., 2009) reversed eCB-LTD deficit found in the PCP-treated mice. (Figure 1.3B, Wilcoxon test for PCP-treated+JZL184: 49.38 ± 3.78 %, Z = -2.366, n = 7, p = 0.018; PCP-treated vs. PCP-treated+JZL184: 49.38 ± 3.78 %, n = 7; t(15) = 2.509, p = 0.024, r = 0.54). All together, these experiments strongly suggest that the eCB system is disrupted in PCP-treated mice.

**CB1R signaling in mPFC is reduced in PCP-treated mice**

Induction of eCB-LTD can be effectively abolished via the direct inhibition of CB1R function (Figure 1.3C), which is consistent with previous reports (Lovinger, 2008). It is reasonable to assume that the observed deficits in eCB-LTD could result from the development of abnormal CB1R-dependent signaling in PCP-treated mice as a
compensatory mechanism of the mPFC network to the abnormal maturation of a glutamatergic synapse. Thus, we examined CB₁R expression in PCP-treated mice using immunohistochemistry (Figure 1.4A-B). The PCP-treated mice showed a modest decrease in CB₁R in the mPFC (PCP-treated: 0.81 ± 0.037, n = 33; CTRL: 1±0.036, n=23; t(54) = 3.605, p = 0.0007, r = 0.44). Next, we measured the direct responsiveness of CB₁Rs to its well-characterized agonist Win55,212-2 using an acute brain slice preparation in PCP-treated and control animals. Evoked fPSPs in L5 stimulated at the L2/3 inputs in the mPFC were strongly inhibited by a bath perfusion of the 1 µM WIN55,212-2 in control animals (Figure 1.5) while the PCP-treated mice showed a modest reduction of inhibitory responses (Figure 1.5A, RT-ANOVA: Time x Treatment: F(2,27) = 8.27, p = 0.000003) which correlated with a decrease in CB₁R immunoreactivity (Figure 1.4A). The size of PPR responses measured before and during 1 µM Win55,212-2 bath application corresponded to the level of recorded depression of fPSPs (Figure 1.5B). This increase in PPR is consistent with a presynaptic locus of action. The observed decrease in CB₁R function is consistent with the observed loss of eCB-LTD (Figure 1.3B) and provides direct evidence for deficient endocannabinoid signaling in PCP-treated mice. However, we have not found any abnormality in functionality of CB₁R-dependent signaling at the CA3→CA1 synapse in the hippocampus in PCP-treated mice (Figure 1.5E, RT-ANOVA: Time x Treatment: F(2,0, 20,0) = 1.077, p = 0.384). In addition, the total levels of CB₁R in CA1 (including CA3→CA1 synapses) within hippocampus did not differ between PCP-treated and control animals (Figure 1.4 D-E) (PCP-treated: 1.03±0.017, n = 28; CTRL: 1±0.022, n=22; t(48) = 0.911, p = 0.3667, r = 0.013), while in
DG region (including cortical projections from Entorhinal cortex) were slightly increased in PCP-treated mice (Figure 1.4 F-G). These data suggest that the disruption in the eCB signaling observed in PCP mice is not widespread.

**Discussion**

NMDAR hypofunction during development is known to produce structural and functional abnormalities of cortical networks in the adult brain, although the neural mechanisms of emergent pathology and its consequences on cognitive function are unclear. It has been shown that PCP-mediated NMDAR hypofunction during early postnatal development before PND 12 results in cognitive dysfunction, including working memory deficits (Nakatani-Pawlak et al, 2009) and object recognition deficits (Grayson et al, 2007; Nakatani-Pawlak et al, 2009; Wiley et al, 2003) in rodent models of psychosis. Our data strongly suggested that transient PCP-mediated NMDAR hypofunction during early postnatal development (PND 7-11) resulted in imbalanced fear memory specificity and generalization in the adult.

Theoretical and experimental models predict coexistence of two anatomically distinctive neural processes underlying specificity of episodic memory (Leutgeb et al, 2007; Marr, 1971; McHugh et al, 2007; O'Reilly and McClelland, 1994). Pattern separation is a putative neural process underlying memory specificity, in which memories are encoded and retrieved as unique representations, while pattern completion enables recall based on partial cues and memory generalization. It is uncertain if the abnormality in context discrimination found in PCP-mediated NMDAR hypofunction resulted from
overgeneralization or a deficit in pattern separation. The possibility for a disruption of hippocampus-dependent pattern separation/completion in schizophrenia has been previously discussed (Tamminga et al., 2010). However, the neural circuit for memory specificity and generalization also involves PFC and thalamus (Navawongse et al., 2013; Xu et al., 2012; Xu et al., 2013), and there is converging evidence linking the schizophrenia cognitive endophenotype with NMDAR hypofunction-induced abnormalities in the thalamus, prefrontal cortex (PFC) and hippocampus circuit, consistent with the NMDAR hypofunction theory (Coyle et al., 2003; Tamminga, 1998). We suggest that impairment in fear memory specificity observed in adult animals may be associated with an abnormal maturation of plasticity as revealed in PCP-mediated NMDAR hypofunction mice.

PCP-treated mice exhibited a loss of eCB-dependent synaptic plasticity at a central glutamatergic synapse suggesting that abnormality in fear memory specificity and generalization may be associated with deficient eCB-dependent plasticity. Recent studies indicate that schizophrenia pathology may be linked to the eCB system (Caspi et al., 2005; Zammit et al., 2011). Furthermore, mutations in CNRI (a gene encoding CB1R) have been associated with a susceptibility to schizophrenia in human genetic studies (Leroy et al., 2001; Ujike et al., 2002). Studies of mutant mice with the CNRI gene deletion revealed that the schizophrenia-like behavioral responses to an NMDAR inhibitor depended on a functional CB1R (Haller et al., 2005). Thus, our data indicating an abnormality in the eCB system in a schizophrenia model are consistent with initial findings in humans and rodents.
Our initial observation indicated a loss of eCB-LTD (Figure 1.3B), which coincides with a decrease in CB₁R function in PCP-treated mice (Figure 1.5). However, this modest decrease in CB₁R function may be insufficient to explain the loss of eCB-LTD induction, and it is uncertain at this point if additional components of the eCB system are dysregulated in PCP-treated mice. For example, either decreased endocannabinoid synthesis or increased endocannabinoid degradation will attenuate eCB-LTD. This might occur if PCP-treatment leads to enduring changes in expression of the enzymes mediating endocannabinoid synthesis and degradation (see below). eCB-LTD was first discovered in the glutamatergic striatal synapse (Calabresi et al, 1992), although the role of the eCB system including CB₁R in this form of plasticity was not known until later (Gerdeman et al, 2002). At the L2/3→L5 synapse in the mPFC, the retrograde eCB signaling system depends on complex presynaptic and postsynaptic signaling (Lovinger, 2008) through the CB₁R (Lafourcade et al, 2007), metabotropic glutamate receptor (mGluR5, that transduces the level of glutamate onto the postsynaptic compartment) (Calabresi et al, 1992; Lafourcade et al, 2007), diacylglycerol lipase (DAGL α, essential for the endocannabinoid 2-arachidonoylglycerol (2-AG) synthesis) (Lafourcade et al, 2007; Lovinger, 2008), Ca²⁺ influx (Di Marzo et al, 1994), and monoacylglycerol lipase and alpha beta hydrolase domain 6 (MAGL and ABHD6, respectively, enzymes that degrade 2-AG released from the postsynaptic terminal) (Marrs et al, 2010; Piomelli, 2003). The eCB-LTD mechanism may also involve serotonin receptors (5-HT2) (Best and Regehr, 2008), dopamine receptors (D2) (Calabresi et al, 1992) or muscarinic acetylcholine receptors (M1/3) (Kim et al, 2002). In addition, eCB-LTD is a widespread type of long-
term synaptic plasticity found at excitatory and some inhibitory synapses in the CNS (Lovinger, 2008). The extent to which the eCB system is disrupted in PCP-treated mice has yet to be determined, but it is unlikely that this form of synaptic plasticity is diffusely deficient throughout the brain.

The induced NMDAR hypofunction in PCP-treated mice is overlapping at a time when the intrinsic characteristics of the pyramidal neurons in L5 and the properties of L2/3 inputs in the mPFC are developing to form mature plasticity mechanisms at this glutamatergic pathway (Zhang, 2004). Our data indicated that NMDARs are required for the maturation of eCB-dependent presynaptic plasticity at the glutamatergic synapse in addition to the well-studied maturation of postsynaptic plasticity.

The retrograde eCB inhibitory system demonstrates a number of properties that make it a viable candidate for a homeostatic mechanism controlling neural stability that can be permanently disrupted via adaptive mechanisms. There are two critical characteristics that make the eCB system an effective component of the neuronal activity-dependent homeostatic mechanism. First, endocannabinoids are produced locally and postsynaptically. Second, the retrograde release of endocannabinoids exhibits temporal and spatial patterns that faithfully follow the patterns of neuronal activity at presynaptic terminals (Lovinger, 2008). However, recent studies have indicated that the eCB system is vulnerable to disruption via maladaptive mechanisms induced through environmental insults. Both epilepsy and chronic exposure to a CB₁R antagonist induce long-lasting changes in the expression of CB₁R (Chen et al, 2003; Hsieh et al, 1999; Rinaldi-Carmona
et al, 1998). Repeated self-administered cocaine resulted in an impairment of two forms of long-term plasticity, such as eCB-LTD and metabotropic glutamate receptor 2/3 (mGlu2/3)-mediated LTD in the mPFC (Kasanetz et al, 2012). The loss of eCB-LTD was also observed in response to a single exposure to $^9$THC (tetrahydrocannabinol; THC) in the nucleus accumbens as a result of functional tolerance and the desensitization of CB$_1$R (Mato et al, 2004). However, prolonged exposure to this drug induces compensatory mechanisms and rescues LTD via mGlu2/3-dependent homeostatic synaptic adaptation (Mato et al, 2005). Excitatory synapses onto indirect-pathway medium spiny neurons selectively express dopamine D2 receptor-dependent eCB-LTD, which is absent in Parkinson’s disease models, and can be rescued through the pharmacological restoration of eCB-LTD expression (Calabresi et al, 1992; Gerdeman et al, 2002; Kreitzer et al, 2007). Thus, long-term synaptic depression, including eCB-LTD, is involved in cortical and subcortical maladaptations, and our current data suggest that the eCB-LTD mechanism might be a target relevant to the developmental etiology underlying neuropsychiatric disorders. However, the specific roles of disrupted eCB-LTD on network stability, synaptic plasticity and cognitive function have yet to be determined.

Methods and Materials

Subjects

C57BL/6 mice were used for all of the experiments following protocols approved by the IACUC at UCR. The animals were housed in plastic cages (2-4 mice/cage) and kept on 12/12 h dark/light cycle with ad libitum access to food and water. All of the behavioral
experiments were performed during the light phase of the cycle. Ten- to seventeen-week-old mice were used for the physiological and behavioral studies. To generate the PCP-treated animals, C57BL/6 mice are subcutaneously injected with a 10 mg/kg/day dose of phencyclidine-HCl (PCP) (Sigma) at the same time of day on postnatal days (P) 7, 9, and 11 as previously described (Wang et al, 2001). All of the behavioral and physiological tests were performed using equal gender distribution (males/females, 50%/50%) during adulthood (P70-P120).

**Behavioral Assays**

Fear Conditioning is performed as described before (Korzus et al, 2004). After being handled, individual mice were exposed to context A. Context A was the unmodified fear conditioning box (Coulburn Instruments Inc.), which was placed inside of a sound attenuated chamber with the house light and house fan on. The chamber was cleaned with Quatracide, 70% ethanol, and distilled water. The individual mice were exposed to Context A for 180 s and received a 0.75 mA, 2 s foot shock (context A – foot shock pairing) and left for another 180 s inside the chamber. For memory retention test, mice are placed back in the training chamber for 180 s. Freezing was scored and analyzed automatically by a Video-based system (Freeze Frame software ActiMetrics Inc.). Video was recorded at 30 frames per s. The Freeze Frame software calculated a difference between consecutive frames by comparing gray scale value for each pixel in frame. Freezing was defined based on experimenter observations and set as sub-threshold activity for longer then 1 s. Freezing was expressed as a % Freezing, which was
calculated as a percent of freezing time per total time spend in testing chamber. Contextual fear conditioning and a 24 h memory test (Figure 1.1A, Days: 1 and 2) were followed by context discrimination assay (Figure 1.1A, Days: 2-12). Context discrimination assay was performed similarly as described before (McHugh et al, 2007). The task is divided into three phases: initial phase, generalization phase and discrimination phase. After being handled, individual mice were exposed to context A. Context A was the unmodified fear conditioning box (Coulburn Instruments Inc.), which was placed inside of a sound attenuated chamber with the house light and house fan on. The chamber was cleaned with Quatricide, 70% ethanol, and distilled water. Context B was the modified fear conditioning chamber, with angular wall inserts, house fan off, and scented with Simple Green. During initial phase (Days 1 and 2), mice were placed in the context A (CS+) for 180 s followed by a single foot shock (arrow). Across 4 consecutive days (generalization phase: Days 3-6; Trial Block 1-2), the individual mice were exposed to Context A for 180 s and received a 0.75 mA, 2 s foot shock, and left for another 60 s inside the chamber. Four hours later, the mice were exposed to the similar Context B for 242 s and receive no footshock. During the generalization phase mice were not able to discriminate between context A and context B. On Days 7-12 (discrimination phase: Trial Block 3-5) mice were placed again in the context A (CS+) for 180 s followed by a single foot shock and left for 60 s after shock, and context B (CS-) for 242 s without any reinforcement. CB57BLJ6 mice acquired ability to discriminate between context A and context B during the discrimination phase after at least 6 days of training. Context A and B were similar but not the same. The protocol included 12 days of training. Thus animals
were exposed to CS+ 12 times before the final test. The order of exposure to different contexts was counter balanced. Additionally, the context cues themselves were counter balanced within each group in order to isolate the effect of the CS+.

**Open Field Activity**

Anxiety-related behavioral responses and locomotor activity were scored and analyzed using an Open Field Activity test performed in novel environment following protocols described before (Korzus et al, 2004). A 17” x 17” x 12” clear Plexiglas arena with a white acrylic floor was used for the open field test. The arena was located in a sound attenuated chamber with lights and a ceiling mounted camera. After sanitizing the arena with Quatricide TB, 70%EtOH, and distilled water, mice were individually placed inside and allowed to explore for 15 min before being returned to their home cage. Videos were analyzed offline using behavioral analysis software TopscanLite (Clever Sys, Inc.) to track anxiety and locomotion measures.

**Object location task**

The mice were habituated in a testing apparatus 3 times for 15 min each for at least 2 days before training. The testing apparatus used in the study was an open field arena (17” x 17” x 12”) with clear Plexiglas walls, a white acrylic floor and visual cues (located on north and east sides), which were visible to the arena. A light and behavior recording camera was installed above the testing arena. The mice performed four training trials for 5 min each trial with an ITI of 3 min. During the first trial, the mice were re-habituated
to the arena without objects. In trials 2-4, two identical objects were placed in the NW and NE corner of the arena, and the mice were allowed to explore the objects for 5 min for each trial. Memory was then assessed during a single, 5 min test trial after a 5 min delay (for short-term memory) or 24-hr delay (for long-term memory). During the test trial, replicas of the training objects were placed in a familiar [NW] corner and a novel [SE] corner. The objects and locations were counterbalanced. Wild type mice showed an exploratory preference for the novel location [SE].

**Novel Object Recognition**

Assays performed accordingly to previously described protocol (Korzus *et al*, 2004). The task is divided into four phases: habituation, familiarization, delay and test. 1) mice were handled 4 times and were placed in the experimental room for two hr before the experiment. 2) During familiarization two identical objects were placed in the home cage with the animals for 5 min. 3) Animals were tested after 2 min. delay. 4) During the test phase both the familiar object (a replica of the original familiar object was used to avoid the use of odor cues) and a novel object were placed in the cage. Object exploration times (for both familiar and novel objects) were recorded for a 2 min test period. We employed computer-assisted scoring using software to measure and analyze performance on the VPC task. Object exploration was scored when the animal’s head was oriented towards the object and vibrissae were moving. The objects varied in color, shape and size and were balanced so that the same objects (replicas) were used for some animals as “familiar” and for others as “novel” in the same session. Also the position of “novel” and “familiar” objects was randomized.
Foot-Shock Sensitivity.

Pain thresholds were assessed similarly as described before (Schrott and Crnic, 1994) in fear conditioning box (Coulburn Instruments Inc.). Mice were received 27 (3 blocks) to 45 (5 blocks) trials of mild foot-shock, Shock duration was 1 s and an intershock interval was 30 s.). Nine foot shocks were delivered to each animal in order of increased intensity. Levels ranged from 0.05 to 1.0 mA. Animal behavioral responses were recorded and given numerical score: no response=0, flinch=1, hop=2, run=3, horizontal jump=4, and vertical jump=5 (maximum response). In addition, we measured a minimum level of the shock (mA) required to evoke vocalization. The same experienced observer who was blind to group designations scored all subjects.

Drugs

Rimonabant (SR141716) and WIN55,212-2 were obtained from NIMH Chemical Synthesis and Drug Supply Program. LY379268, CP55,940, JZL-184, and AM251 was obtained from Tocris, Inc.. All drugs used for in electrophysiological procedures were made from stock solutions and frozen in aliquots at -20C until used on day of the experiment. No stock solutions were used if made more than 1 month prior. Rimanobant (SR141716 ) and WIN55,212-2 were dissolved in DMSO and made into 20mM stock solutions. CP55,940, JZL-184, and AM251 were dissolved in DMSO and stored in 100mM stock solutions. LY379268 was dissolved in water and stored in 10mM stock solutions. The concentration of DMSO was always <0.1% of total concentration and had no effect on synaptic responses.
Electrophysiology

Local field potential recordings were obtained from cortical layer 5 in the PL region of the mPFC stimulated at the layer 2/3 inputs. Acute brain slices containing the mPFC were prepared using the Compresstome VF-300 (Precisionary Instr., Greenville, NC). Ex vivo slice electrophysiology was performed on brain slices containing the PL region of the mPFC bathed in ACSF (124.00 mM NaCl, 4.40 mM KCl, 25.00 mM NaHCO3, 1.2 mM Na2HPO4, 1.3 mM MgSO4-7H2O, 2.5 mM CaCl2-2H2O, and 10 mM glucose) saturated with a 95%O2/5%CO2 gas mixture. Brain slices containing the PL region or dorsal hippocampus were placed in a recording chamber, and continuously perfused [~1.5 ml/min] with carboxygenated ACSF held at 28°C. The slices were viewed under an OlympusTM FV1000 confocal microscope using DIC. In all cases, the local field potential recordings were obtained from cortical layer 5 in the PL region using a 3-5 MΩ resistance glass pipette filled with ACSF guided by visual landmarks and combined with adjusting for the maximal fPSP response within a region ~500-800 µm from the cortical surface. Signals from the head stage were then amplified, filtered, digitized at 10 KHz, and sent to a computer for data storage using the Clampex software and analyzed using Clampfit (Molecular Device Inc). Stimulations were delivered using a bipolar cluster electrode (FHC, Inc.) placed in cortical Layer 2/3 in the PL region of the mPFC, which was visible under DIC as a dark band ~200-300 µm from the surface. The stimulatory electrode was aligned with the recording electrode, which was perpendicular to the cortical surface. Acute mPFC slices from 10- to 17-week-old mice were used for recordings, which were performed according to previously described protocols:
Input/Output (I/O) and paired-pulse facilitation (PPF) (Limback-Stokin et al., 2004), short-term potentiation (STP) (Hempel et al., 2000), endocannabinoid-dependent LTD (eCB-LTD) (Lafourcade et al., 2007), and mGlur2/3 LTD (Huang et al., 2007). The glutamatergic nature of the fEPSP was confirmed from its block with 20 mM DNQX (AMPAR antagonist) DNQX at the end of experiments. Traces from the local field potential recordings were analyzed as previously described (Hempel et al., 2000; Hirsch and Crepel, 1990; Morris et al., 1999). The I/O was recorded by starting with 20 μA stimulation using an isolated stimulator and increasing the stimulation intensity until the amplitude measurements reached a plateau (three consecutive stimulus intensity increases with minimal change). Individual curves were then fitted using Boltzmann’s sigmoidal fit equation: \[ y = \frac{A2 + (A1-A2)}{1 + \exp((x-x0)/dx)} \] and OriginPro software (OriginLab, Inc). An R-square of >0.9 was used as a cut-off criterion for curve fitting. PPF was used to assess the transient synaptic plasticity at increasing inter-stimulus intervals [ISI] ranging from 25 ms. to 300 ms. tested at both 30% and 70% of the stimulation required to reach maximum. The PPF and I/O stimulation was determined by the average of three traces with 15 s intervals between stimulations. STP was induced using a 15-pulse 50 Hz train with at least 20 min of post-stimulus train recording. The test pulses were delivered every 10 s. The STP was measured at both 30% and 70% of stimulation needed to reach maximum. Post-train recordings were compared to baseline recordings as a percentage of the average baseline for amplitude of the postsynaptic potential [PSP]. eCB-LTD was induced according to a previously published protocol (Lafourcade et al., 2007) in which a 10 min 10 Hz stimulation (at 70% stimulus intensity) of layer 2/3 in the PL region of the
mPFC induced long-lasting LTD in layer 5 pyramidal neurons. Long-lasting LTD was then recorded for 1 h, and the test pulses in this paradigm were delivered once per minute. mGluR2/3-LTD was induced at time 0 with 10 minute bath application of 100nM LY379268 (potent mGluR2/3 agonist). Success or failure of LTD expression in individual groups were determined using a paired Wilcoxon signed rank test (Wilcoxon test) by comparing the mean fPSP amplitude during baseline (10 min before stimulation) and 50-60 min after LTD induction. When recording mGluR2/3-LTD WIN55-212-2, and CP55,940 application, test pulses were delivered at a rate of 0.1Hz with at least 10 min of baseline recording before drug application. In the case of WIN55-212-2 and CP55,940 application, a set of 3 paired pulse ratios were delivered at 6 different times during the duration of the experiment: -5 min, 5 min, 15 min, 35 min, 45 min.

**Histology**

Brains isolated from transcardially perfused mice with 4% PFA were additionally fixed by overnight incubation 4% PFA at 4°C and then transferred to Phosphate Buffered Saline solution, pH 7.4 (PBS) with 0.02% NaN2. 100 μm slices including the mPFC (prepared using a Compressstome VF-300 (Precisionary Instr., Greenville, NC)) or 50 μm slices including hippocampus (prepared using Croyostat Leica) were placed in 24-well plates for free floating immunochemistry (IHC). Sections are washed 3 times for 10 min in a Washing Buffer (PBS, 0.3% Triton x-100, 0.02% NaN2) before 1 h incubation in Blocking Buffer (5% normal goat serum in washing buffer) followed by 10 min incubation in the Washing Buffer. Slices were incubated overnight at 4C° with primary antibodies (rabbit anti-CB1R IgG, CB1-L15 from Dr. K. Mackie, 1:1000; monoclonal
anti-NeuroN IgG conjugated with Alexa Fluor 488, 1:1000) in the Blocking Buffer. After 3 washes with the Washing Buffer, slices were incubated with secondary antibodies (Alexa647-goat anti-rabbit IgG; Molecular Probes, 1:1000) in the Blocking Buffer for 2 h at room temperature. Slices were washed again 3 times with the Washing Buffer before mounting for viewing. Immuno-stained tissue was analyzed on a semi-automatic laser scanning confocal microscope Olympus FV1000 controlled by Fluoview software. Fluorescence was measured from mPFC slices using objective 40x/0.80 LUMPlanFL40x objective. Gain and offset of each channel were balanced manually using Fluoview saturation tools for maximal contrast. All settings were tested on multiple slices before data collection and brain slices were imaged using identical microscope settings once established. The background fluorescence for each channel using established image acquisition settings was measured in negative control experiments for each group (secondary without primary) and subtracted from final image calculations. The fluorescence intensity quantification was performed on original images by the use of Olympus Fluoview software without any non-linear image adjustments.

**PFC:** 80 µm z-stacks were taken from the PL region in the mPFC. Regions in the PFC were selected based on the same criteria for selection of recording sites in layer 5 of the PL region. In addition, only the NeuN channel (488) was used to locate appropriate ROI to eliminate experimenter bias of the CB₁R channel. Average intensity of each channel across the entire 640x480 ROI collected at was used as our measure of signal intensity in the PFC. Each image served as its own internal control by expressing CB₁R intensity as a ratio of NeuN intensity. This ratio was then expressed as a percentage of the mean ratio.
(CB$_1$R/NeuN) of all control images. Slices that had non-uniform thickness or signal penetration throughout the z-stack were excluded from analysis.

**Hippocampus**: 50uM z-stacks were taken from the CA1 and DG region in the dorsal hippocampus with 320x600 resolution. CA1 regions were selected by aligning a grid of predetermined ROIs so that the pyramidal cell layer (using the NeuN-488 channel) was centered in the middle of the top row of ROIs (see Figure 1.4E). Data collected for intensity values of NeuN used for normalization was restricted to the cell body layers (ROIs 1 and 2), while the level of CB$_1$R intensity was collected and averaged from all ROIs (1-8, extending into the striatum radiatum) and expressed as a ratio of NeuN from ROIs 1&2. This ratio was then expressed as a percentage of the mean ratio (CB$_1$R/NeuN) of all control images. Identical procedures were used for analysis of DG, except that only ROIs 1-4 were used for analysis of CB$_1$R (since additional ROIs could extend beyond the boundaries of the DG in some slices)(see Figure 1.4G).

**Data analysis**

Experimenters were blind to group designations. Data represent Means ± SEM. Statistical analysis was done using Excel (Microsoft Inc.) or SPSS (IBM Inc.). The Student’s t test or repeated measures ANOVA (RT-ANOVA) is used for statistical comparisons. For t – tests, Pearson’ correlation ($r$) was used as an effect size. In cases where repeated measures ANOVA was utilized and assumptions of sphericity were violated (via Mauchly’s Test), analysis was completed using the Greenhouse-Geisser correction. A critical probability of $p<0.05$ was applied. Statistical significance: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. 
Figure 1.1

Context discrimination training protocol:

<table>
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<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<td>B</td>
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<td>B</td>
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<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
</tbody>
</table>

CS+, Context A
CS-, Context B

b

CTRL

% Freezing

***

Trial Block

0% 20% 40% 60%

c

PCP

% Freezing

* 0% 20% 40% 60%

d

Generalization (Trial block 1)

% Freezing

CTRL  PCP

0% 20% 40% 60%

e

Discrimination Index

Discrimination Ratio

0 0.1 0.2 0.3 0.4

CTRL  PCP

1 2 3 4 5
Figure 1.1 Fear memory specificity is deficient in PCP-treated mice.

(a) Experimental design for the context discrimination test. The task is divided into three phases: initial phase, generalization phase and discrimination phase. During initial phase (Days 1 and 2), mice were placed in the context A (CS+) for 180 s followed by a single foot shock (red arrow). On days 3-6 (generalization phase: Trial Block 1-2), mice were placed in the context A (CS+) for 180 s followed by a single foot shock (red arrow) and left for 60 s after shock, and context B (CS-) for 242 s without any reinforcement. On Days 7-12 (discrimination phase: Trial Block 3-5) mice were placed again in the context A (CS+) for 180 s followed by a single foot shock (red arrow) and left for 60 s after shock, and context B (CS-) for 242 s without any reinforcement. Context A and B were similar but not the same. The protocol included 12 days of training. Thus animals were exposed to CS+ 12 times before the final test. (b) During generalization phase CB57BL/J6 control mice (n = 19) were not able to discriminate between context A and context B (RT-ANOVA: Block x Context F(1,18) = 0.356, p = 0.558). However, CB57BL/J6 control mice acquired ability to discriminate between context A and context B during the discrimination phase after at least 6 days of training. (RT-ANOVA: Block x Context F(2,36) = 5.728, p = 0.007). (c) PCP-treated mice (n = 16) exhibit deficit in fear memory specificity during discrimination phase (RT-ANOVA: Block x Context F(2,30) = 0.895, P = 0.419). (d) Freezing in both tested groups had comparable levels to both contexts during the first block. This would indicate that context A was similar enough to context B that generalization was occurring early in training. (e) Analysis of context discrimination ratios (DI) calculated accordingly to formula DI = ((Context A - Context B) / (Context A + Context B)) revealed difference in performance between PCP-treated and control mice during discrimination phase (trial block 4).
Figure 1.2. mGluR2/3-LTD at the L2/3→L5 synapse is unaffected in PCP-treated mice.

mGluR2/3-LTD was induced at time 0 with 10 minute bath application of 100nM LY379268 (potent mGluR2/3 agonist) in the L2/3→L5 pathway. Example traces are shown for each group corresponding to respective time points. The CTRL group (a) showed significant expression of LTD as well as the PCP-treated group (b), suggesting that mGluR2/3 dependent LTD is unaffected in PCP-treated mice. Insets: shown traces indicate representative average from 10 min of baseline (“1”) and the time between 50-60 min of recording (“2”); scale bar: vertical = 0.1 mV horizontal = 5 ms (c) Binned averages of every 10 min across time show similarity in the mGluR2/3-LTD expression levels in the PCP-treated and control animals at the L2/3→L5 synapse. (d) There is no difference in expression of mGLuR2/3-LTD between control and PCP-treated mice after 60 mins (CTRL: 50.82 ± 6.03 %, n=7; PCP-treated: 45.31 ± 4.41 %, n=7; t_{(12)}=0.738, p=0.47, r = 0.21).
Figure 1.3

(a) CTRL and CTRL+AM251

(b) PCP and PCP+JZL184

(c) 10 min Bins

(d) 60-70 min Bin
**Figure 1.3.** eCB-LTD at the L2/3→L5 synapse is deficient in PCP-treated mice.

(a) The CTRL group showed robust expression of eCB-LTD at the L2/3→L5 synapse in the mPFC. eCB-LTD was induced at time 0 (black arrows) with a 10Hz train applied for 10 min. LTD magnitude was estimated from fPSPs registered during the period of 50-60 min (2) after LTD induction as a percentage of baseline fPSP amplitudes. Bath application of 5µM AM251 completely blocked LTD indicating this is an endocannabinoid dependent form of LTD. Insets: Traces shown indicate representative average from 10 min of baseline (“1”) and the time between 50-60 min of recording (“2”) scale bar vertical = 0.1 mV horizontal = 5 ms  
(b) The PCP-treated group showed no induction of LTD at the L2/3→L5 synapse in the mPFC. However, 10Hz10Min stimulation to PCP slices in the presence of JZL184 was able to rescue the deficit (Wilcoxon test: PCP-treated+JZL184: 49.38 ± 3.78 %, Z = -2.366, n = 7, p = 0.018)  
(c) Binned averages of every 10 min show distinct differences between all experimental conditions. (d) Data are plotted from the binned average time point in the rectangle marked during the last 10 min of recording. Control recordings show expression of eCB-LTD, while PCP-treated slices and control slices with bath applied AM251 or RMNT showed no induction of eCB-LTD, JZL184 application to PCP slices brought the level of LTD down to control levels.
Figure 1.4

(a) Normalized CB1R intensity - PL

(b) CB1R - NeuN - Merge

(c) rMPFC - Prelimbic - Layer 5

(d) Normalized CB1R intensity - CA1

(e) CTRL

(f) Normalized CB1R intensity - DG

(g) CTRL
**Figure 1.4.** CB₁R expression is reduced in PCP-treated mice.

(a) Mean intensity of the CB₁R fluorescent signal (normalized to NeuN signal) in layer5 of the PL-mPFC of PCP-treated mice showed a marked decrease when normalized to control group (PCP-treated: 0.81±0.037, n = 33 slices (3 animals); CTRL: 1±0.036, n=23 slices (2 animals); t⁵₄ = 3.61, p = 0.00068, r = 0.44) (b) Z-stacked images at 40x magnification Zoom2x in 100 µm thick slice (scale bar = 25 µm), the first column(left) in green shows CB₁R labeling, second column (middle) in white shows NeuN, and column 3 (right) shows the merge of signals. (c) Representative 10X magnification Z-stacked images taken from dorsal hippocampal sections (scale bar = 200 µm). (d) Mean intensity of the CB₁R fluorescent signal (normalized to NeuN signal taken from pyramidal cell layer) in the CA1 region of the dorsal hippocampus shows no differences between treatment groups (PCP-treated: 1.03±0.017, n = 28 slices (4 animals); CTRL: 1±0.022, n=22 slices (3animals); t⁴₈ = 0.911, p = 0.3667, r = 0.0.13). (e) Representative 40x magnification images taken from the CA1 region used for analysis and quantification. Normalization to NeuN was taken from ROIs 1 and 2 (pyramidal cell layer), and level of CB₁R expression was taken from all ROIs combined. ROIs extended down into the striatum radiatum with an addition 2 rows (ROIs 5,6,7, and 8 not shown) (scale bar = 50 µm). (f) Level of CB₁R in DG was slightly increased (PCP-treated: 1.12±0.027, n = 28 slices (4 animals); CTRL: 1±0.015, n=22 slices (3 animals); t⁵₀ = 3.65, p = 0.0006, r = 0.46). (g) Representative 40x magnification images taken from the DG region used for analysis and quantification. Normalization to NeuN was taken from ROIs 1 and 2 of the granule cell layer, and level of CB₁R expression was taken from all ROIs combined (1-4) (scale bar = 50 µm).
Figure 1.5

(a) mPFC

(b) PPR

(c) mPFC

(d) PPR

(e) CA3-CA1

(f) PPR

Time (Min)

% Baseline (mV)

CTRL
PCP

1μM WIN55,212-2

5μM CP55,940

5μM WIN55,212-2
Figure 1.5. CB$_1$R function is reduced in PCP-treated mice.

(a) The PCP-treated group shows decreased functionality of CB$_1$R at the L2/3→L5 synapse in the mPFC when compared to control mice. After 10 min of baseline recording, continuous application of 1 µM WIN55,212-2 (CB$_1$R agonist) caused a reduction of fPSP amplitude in the control group, which was markedly dampened in the PCP-treated mice. Insets: Traces shown indicate representative average from 10 min of baseline (“1”) and the time between 40-50 min of recording (“2”) scale bar vertical = 0.1 mV horizontal = 5 ms  

(b,d,f) paired pulse ratios were recorded periodically over the course of drug application. Decreases in fPSP (Figure 1.5a) correlate well with PPRs in CTRL and PCP-treated mice over time. This increase in PPR is consistent with a presynaptic locus of action. (c) Continuous application of 5 µM CP55,940 (CB$_1$R agonist) caused a reduction of fPSP amplitude in the control group, which was also markedly dampened in the PCP-treated mice (RT-ANOVA: Time x Treatment: F$_{(2.1, 31.4)}$ = 3.797, p = 0.032). (e) The PCP-treated group shows no difference in functionality of CB$_1$R at the CA3→CA1 synapse when compared to control mice. Continuous application of 5 µM WIN55,212-2 (CB$_1$R agonist) caused a similar reduction of fPSP amplitude in both groups mice (RT-ANOVA: Time x Treatment: F$_{(2.0, 20.0)}$ = 1.077, p = 0.384.)
Figure 1.6. (a) Pavlovian contextual fear conditioning is normal in PCP-treated mice. PCP-treated and control (CTRL) mice show normal acquisition and retention of contextual fear conditioning (a two way ANOVA of Treatment and Baseline/24 h-Test; Treatment: $F_{(1,52)} = 0.0004, p = 0.984$; Baseline/24 h-Test: $F_{(1,52)} = 12.79, p = 0.00076$; Treatment x Baseline/24 h-Test: $F_{(1,52)} = 0.004, p = 0.952$; CTRL: baseline Mean: 8.36±2.29%, 24hr test Mean: 24.91±4.90%; PCP: baseline Mean: 7.98±2.71%, 24hr test Mean: 25.10±5.18%). Contextual fear was tested in Context A 24 h after a single context A-foot shock pairing. CTRL, n = 19; PCP, n = 16. (b) After initial generalization of fear conditioned responses, CTRL mice (n = 19) exhibit robust fear memory specificity during Days 8-11. (c) PCP-treated mice (n = 16) exhibit deficit in fear memory specificity and generalization. (d-e) PCP-treated mice show normal uninduced locomotor activity in novel environment. (f) Anxiety-related responses were unaltered in PCP-treated mice.
Figure 1.7. Pain tolerance is unaffected by perinatal PCP treatment

(a) Pain sensitivity was compared between groups by exposing mice to increasing levels of current. No difference was observed between groups across levels of shock intensity, (RT-ANOVA Intensity X Treatment $F(8,144) = 1.622$, $p = 0.123$). (b) During the pain sensitivity assay, the lowest mA required to evoke mouse vocalization was also recorded, no difference in average mA was observed between Control ($M = 0.120 \pm 0.033$, $N = 10$) and PCP ($M = 0.125 \pm 0.026$, $N = 10$), $t(18) = 0.12$, $p = 0.906$, $r = 0.028$
Figure 1.8

(a) Exploration Time-NOR 5 min delay
- CTRL
- PCP

(b) NOR Test - 5 min delay
- CTRL
- PCP

(c) Exploration Time-NOL 5 min delay
- CTRL
- PCP

(d) NOL Test - 5 min delay
- CTRL
- PCP

(e) Exploration Time-NOL 24 hr delay
- CTRL
- PCP

(f) NOL Test - 24 hr delay
- CTRL
- PCP
Figure 1.8. Memory for novel object recognition and location in PCP-treated mice.

(a) Novel object recognition was compared between Control and PCP treated mice. No difference in total time of exploring during either phase of the assay was observed, (training: \( t_{(43)} = 1.91, p = 0.063, r = 0.280 \). testing: \( t_{(43)} = 0.88, p = 0.383, r = 0.268 \). (b) After a 5 minute delay, during the test phase, control mice (N=21) preferred to explore the novel object as measured by discrimination ratio, while PCP treated mice (N =24) displayed equal preference of novel and familiar objects, indicating a memory deficit \( (t_{(43)} = 6.74, p < 0.000001, r = 0.717) \). (c) Novel location memory was tested and no differences were seen in total exploration time (training: \( t_{(14)} = 0.55, p = 0.593, r = 0.145 \); testing: \( t_{(14)} = 0.12, p = 0.91, r = 0.032 \). (d) Both groups preferred to explore the novel location compared to the familiar \( (t_{(14)} = 0.98, p = 0.340, r = 0.091) \). (e) Using a 24hr delay on the NOL task also revealed no difference between groups exploration (training: \( t_{(18)} = 0.44, p = 0.667, r = 0.103 \); testing: \( t_{(18)} = 0.36, p = 0.726, r = 0.085 \), (f) or discrimination ratio, indicating intact location memory in PCP treated mice \( (t_{(18)} = 0.54, p = 0.599, r = 0.126) \).
Figure 1.9. Functionality of CB₁R in PCP-treated mice.

(a-c) Raw averages of recordings taken every 1 min used for binned graphs in figure 1.5. CB₁R agonism shows differences in mPFC but not dHip CA3->CA1. (d) Raw averages of experiments in which bath application of RMNT was present during 10Hz10Min stimulation in control slices.
Figure 1.10

a) Recording electrode and stimulation electrode

b) Graph showing PSP Amplitude [mV] vs. Stimulus Intensity [μA]

- CTRL
- PCP

Input/Output

PSP Amplitude [mV]

C) Graph showing PPR (50-70%) and STP (50-70%) for CTRL and PCP

- Interstimulus Interval [ms]
- Time (s)
- Pulse#

- % of Baseline (mV)
- % of 1st response

PPR (IPSP2/IPSP1) vs. Interstimulus Interval [ms]

PPR (IPSP2/IPSP1) vs. Time (s)

PPR (IPSP2/IPSP1) vs. Pulse#

CTRL
PCP

STD (50-70%)

CTRL
PCP
Figure 1.10. Short-term plasticity at L2/3→L5 synapse is unaffected in PCP-treated mice.

(a) Diagram showing configuration of both recording and stimulation electrode within the prelimbic region of the mPFC used in electrophysiological experiments testing synaptic activity at the L2/3→L5 synapse. (b) Application of CNQX shows the glutamatergic component of the fPSP. The red line is an average fPSP of 5 traces with only ACSF in the bath. After 5 min of bath application of 5μM CNQX another average of 5 traces was recorded (black line). Note that the fiber volley is spared while the fPSP is abolished under the CNQX condition. (c) I/O curves were determined by starting at 20 μA and increasing stimulation intensity until amplitude measurements reached a plateau. No differences were detected using independent samples t-tests at any intensity measured (CTRL: n = 9; PCP-treated: n = 12, all p-values > 0.05). Shown is also a curve for each group fitted using Boltzman’s sigmoidal equations. A comparison of the means of individual Boltzmann fitted parameters revealed no differences between the PCP-treated and control mice in the maximum asymptote, in the center, or in the time constant (PCP-treated n = 11; CTRL n = 9; t tests: all p>0.05). These thorough analyses of I/O relations at the L2/3→L5 synapse show that there are no differences between control and PCP-treated mice in all of the tested characteristics of the I/O curves. (d-f) Short-term measures of plasticity were also measured at 30% stimulus intensity. (d) Paired pulse ratios (PPR) were measured at the L2/3→L5 synapse at 5 different inter stimulus intervals (25, 50, 100, 200, and 300 ms) at 30% stimulus intensity. No differences in mean PPR were observed between CTRL (n = 10) or PCP-treated (n = 9) at 25 ms, 50 ms, 100 ms, 200 ms, or 300 ms; t tests: all p-values > 0.05. (e) Short term potentiation (STP) was induced with a brief train of 15 pulses delivered at a rate of 50 Hz at time 0 indicated by a black arrow. Test pulses were delivered every 10s at 30% of maximum stimulation as determined by individual fitted I/O curves. RM-ANOVA between PCP-treated (n = 8) and CTRL (n = 9) was conducted on 6 binned time points between 0 and 600 s (100 s bins). No Time X Treatment interaction was found (F(2,26) = 0.96, p > 0.05). (f) Short term depression (STD) was measured during the train used to simulate STP. Each response in the train was compared to the fPSP amplitude of baseline and plotted as a percentage. Depression across the train was observed in all groups with facilitation being favored in the early portion of the train. RM-ANOVA using 5 binned data points (3 pulse bins) revealed no significant Pulse# X Treatment interaction F(2,22) = 0.36 , p > 0.05. (g-j) Short-term measures of plasticity were also measured at 70% stimulus intensity. (g) No differences in PPR at the 70% stimulus intensity were observed between CTRL (n=9) or PCP-treated (n=9) using independent samples t-tests at 25 ms, 50 ms, 100 ms, 200 ms, or 300 ms, all p-values > 0.05. (i) RM-ANOVA on STP at the 70% stimulus intensity between PCP-treated (n=8) and CTRL (n=8) was conducted on 6 binned time points between 0 and 600 s (100 s bins). No Time x Treatment interaction was observed at this stimulus intensity, F(2,30) = 1.44, p > 0.05. (j) STD at the 70% stimulus intensity was measured. Depression across the train was observed in all groups with facilitation being favored in the early portion of the train, but to a lesser extent than at the 30% stimulus intensity.
Chapter II:

Animal model of adolescent cannabis abuse exhibits permanent deficit in presynaptic long-term plasticity

Abstract

Cannabis continues to be the most accessible and popular illicit recreational drug. Whereas current data link adolescence cannabis abuse to increased risk for dependence on other drugs, depression, anxiety disorders and psychosis, the mechanism(s) underlying these adverse effects remains controversial. Here we show in a mouse model of adolescent cannabis abuse deficient endocannabinoid (eCB)-mediated signaling and presynaptic forms of long-term depression at adult central glutamatergic synapses in the prefrontal cortex. Increasing endocannabinoid levels by blockade of monoacylglycerol lipase, the primary enzyme responsible for degrading the endocannabinoid 2-arachidonoylglycerol (2-AG), with the specific inhibitor JZL184 ameliorates these deficits. The observed deficit in cortical eCB-dependent signaling may represent a neural maladaptation underlying network instability and abnormal cognitive functioning. Our study suggests that adolescence cannabis abuse may permanently impair brain functions, including the brain’s intrinsic ability to appropriately adapt to external influences.

Introduction

Cannabis is the most prevalent illicit recreational drug. A total of 2.6 - 5 % of the world’s population (119 - 224 million people) consume cannabis, whereas 0.3 – 0.4 % of
the population consume cocaine (UNODC, 2012). On exposure to cannabis, individuals experience a variety of psychoactive effects including a general alteration of conscious perception, euphoria, impaired social interactions, disrupted memory and learning, and occasionally anxiety and paranoia. Cannabis abuse is considered to be a significant environmental risk for neuropsychiatric disorders (Arseneault et al, 2004). It is presently accepted that many mental illnesses, including psychosis, are the result of abnormal and synergistic interactions between multiple genes and environmental factors. For example, genetic variation in the gene COMT is itself a well-characterized risk factor for schizophrenia (Goldberg et al, 2003). It has been demonstrated that the relative risk of developing psychosis following the use of cannabis is increased in people carrying a common polymorphism within the COMT (Val158Met allele) gene, but this effect was observed only in people who used cannabis during adolescence (Caspi et al, 2005). While current data link adolescent cannabis abuse to increased risk for dependence on other drugs, depression, anxiety disorders and psychosis (Arseneault et al, 2004), the mechanism(s) underlying these adverse effects remains controversial.

In the brain, cannabis exerts its psychological effects through direct binding of its active ingredient, Δ⁹-tetrahydrocannabinol (THC) (Mechoulam and Gaoni, 1965) to the G-protein-coupled, type 1-cannabinoid receptor (CB₁R) (Howlett et al, 2002; Matsuda et al, 1990) expressed on presynaptic terminals (Freund et al, 2003; Gerdeman and Lovinger, 2001; Huang et al, 2001; Katona et al, 1999; Katona et al, 2006). Endogenous ligands of CB₁R, which are referred as to endocannabinoids (eCBs), act as retrograde
signals inhibiting neurotransmitter release (Choi and Lovinger, 1997a, b). 2-arachidonoyl glycerol (2-AG) is most abundant endogenous ligand of CB₁R in the brain (Stella et al., 1997). In fact, the eCB system represents a major activity-dependent regulatory system in the central nervous system and has been implicated in multiple brain functions, including synaptic plasticity and the homeostatic regulation of network activity patterns (Freund et al., 2003; Gerdeman and Lovinger, 2003; Piomelli, 2003; Raver et al., 2013; Sales-Carbonell et al., 2013). CB₁R-mediated decreased probability of neurotransmitter release underlies transient, depolarization-induced synaptic inhibition (Wilson et al., 2001), long-term depression (Choi et al., 1997b) and the postnatal development of corticostrial synapses (Choi et al., 1997b).

During adolescence, the prefrontal cortex (PFC), one of the latest regions of the brain to mature, undergoes significant developmental modification of its circuits that can be translated to cognitive, emotional and behavioral progression (Gogtay et al., 2004). Although the behavioral effects of administration of the CB₁R agonist WIN55,212-2 during adolescence have been studied (Raver et al., 2013), the possible permanent effects of CB₁R hyperactivity during adolescence on the mPFC network physiology are unclear. To address this issue, we tested multiple forms of presynaptic plasticity at glutamatergic synapses in cortical layers 2/3 to 5 (L2/3->L5) in the mPFC of adult mice treated sub-chronically during adolescence with WIN55,212-2 and investigated the effects of this treatment on presynaptic plasticity. We found that two types of long-term depression (LTD), LTD mediated by metabotropic glutamate receptors 2/3 (mGluR2/3) and LTD
mediated by CB₁Rs, were deficient in adulthood in mice treated with WIN55,212-2. While LTD was disrupted, short-term forms of plasticity remained intact, indicating specificity for disruption selectively of long-term plasticity. Over-activation of the CB₁R during adolescence could therefore lead to permanent developmental changes in the expression of presynaptic LTD in the mPFC at excitatory synapses. These observations could be linked to a maladaptation of the mPFC network during a critical period of cortical development and may underlie the alteration of gamma oscillations in adults after adolescent CB₁R stimulation, as found in earlier studies (Raver et al., 2013; Sales-Carbonell et al., 2013; Skosnik et al., 2012).

**Results**

**Adolescent WIN55 exposure triggers a deficit in eCB-dependent LTD in the mPFC**

To test the hypothesis that elevated levels of cannabinoids during adolescence trigger lasting abnormalities in neural network functioning, we exposed mice to repeated doses of the CB₁R agonist WIN55,212-2 (WIN55-treated mice) or vehicle (control, CTRL) during adolescence as described in methods (Figure 2.1A) and tested synaptic activity at the L2/3→L5 glutamatergic synapses (Hempel et al., 2000; Morris et al., 1999) (Figure 2.1B). These cortical glutamatergic synapses (Figure 2.1C) represent one of the major pathways in the mPFC because convergent cortical and subcortical pathways are integrated in cortical layer L2/3 pyramidal neurons, which form abundant contacts with pyramidal neurons in cortical layer L5, from which the output of the cortex originates.
One way to capture how a synapse transforms signals is to analyze the relationship between its input and its output (I/O). Analysis of the shape of the I/O curve may provide valuable information about additive operations (shift along the input axis) or gain (slope change). I/O relations appear to be similar in WIN55-treated and control mice (Figure 2.1D). Additional detailed analysis of the I/O relationship was performed to determine whether differences in the shape of the curves existed using different metrics obtained from Boltzmann sigmoidal analysis. Comparison of the means of individual Boltzmann-fitted parameters revealed no differences between WIN55-treated and control mice in maximum asymptote (A2: CTRL: 0.88 ± 0.10 mV, n = 8; WIN55-treated, 0.91 ± 0.17 mV, n = 8; \( t_{14} = 0.152, p = 0.788, r = 0.04 \), in the center (x0: CTRL: 57.49 ± 7.01 µA, n = 8; WIN55-treated: 74.16 ± 10.82 µA, n = 8; \( t_{14} = 1.293, p = 0.217, r = 0.33 \), or in the time constant (dx: CTRL: 23.41 ± 2.53, n = 8; WIN55-treated: 24.81 ± 3.60, n = 8; \( t_{14} = 0.318, p = 0.755, r = 0.08 \)). These observations suggest that there are no differences in any of the tested characteristics of the I/O curves. Thus, by these measures, WIN55-treated mice showed unaltered synaptic transmission in the L2/3→L5 pathway, indicating that there was normal synaptic density within the population and that synaptic transmission was equally effective in response to a single stimulus following WIN55 treatment.

eCB-LTD is a widespread form of cortical plasticity that provides activity-dependent inhibitory control of neurotransmitter release. The CB1R is required for the induction of eCB-LTD. eCB-LTD is also a presynaptic form of plasticity that is negatively coupled to the cAMP/PKA signaling pathway via \( G_{i/o} \) (Lovinger, 2008). Thus, we examined eCB-
LTD in the L2/3→L5 pathway in WIN55-treated mice. We used a moderate stimulation protocol of 10 Hz for 10 min at 70% stimulus intensity to induce eCB-LTD at L2/3→L5 glutamatergic synapses in the mPFC (Figure 2.1E); this protocol has been shown to produce long-term depressive effects that persist for at least 1 hour (Lafourcade et al., 2007). The success or failure of LTD expression in individual groups was determined using a paired Wilcoxon signed rank test (Wilcoxon test) by comparing the mean fPSP amplitude at baseline (10 min before stimulation) and 50-60 min after LTD induction. Comparative analysis of the mean fPSP amplitude at baseline and 50-60 min after LTD induction revealed robust LTD expression in control animals (Figure 2.1E, Wilcoxon test: 55.82% ± 4.08%, Z = -2.201 n = 6, p = 0.028). The WIN55-treated mice showed a marked deficit in eCB-LTD induction in the L2/3→L5 pathway in the mPFC (Figure 2.1E, Wilcoxon test: 90.54% ± 7.63%, Z = -1.125, n = 9, p = 0.260). Thus, this adolescent cannabis abuse model shows a strong deficit in eCB-LTD but not in baseline synaptic transmission.

**Short-term plasticity is unaffected in WIN55-treated mice**

To examine the effects of WIN55 administration during a critical time for maturation of plasticity in the PFC circuit, we tested short-term plasticity in the L2/3→L5 pathway in the mPFC from adult WIN55-treated mice. Multiple forms of short-term plasticity can be expressed at the L2/3→L5 excitatory synapse, including the paired-pulse ratio of peak amplitudes (PPR), short-term potentiation (STP) and short-term depression (STD) (Hempel et al, 2000; Morris et al, 1999).
PPR were measured at the L2/3→L5 synapse at 5 different interstimulus intervals (25, 50, 100, 200, and 300 ms) using 30% stimulus intensity (Figure 2.2A). No differences in mean PPR were observed between CTRL (n = 10) and WIN55-treated (n = 10) animals at 25 ms (CTRL = 180.53% ± 18.77%; WIN55-treated = 159.67% ± 14.86%; \( t_{(18)} = 0.871, p = 0.395, r = 0.20 \)), 50 ms (CTRL = 190.55% ± 20.45%; WIN55-treated = 179.68% ± 18.99%; \( t_{(18)} = 0.390, p = 0.702, r = 0.09 \)), 100 ms (CTRL = 175.00% ± 12.39%; WIN55-treated = 177.98% ± 15.42%; \( t_{(18)} = 0.157, p = 0.877, r = 0.16 \)), 200 ms (CTRL = 135.64% ± 8.29%; WIN55-treated = 150.76% ± 6.92%; \( t_{(18)} = 1.400, p = 0.179, r = 0.31 \)), or 300 ms (CTRL = 119.82% ± 9.04%; WIN55-treated = 128.46% ± 3.97%; \( t_{(18)} = 0.875, p = 0.393, r = 0.20 \)) (Figure 2.2A).

Short-term potentiation (STP) was induced by a brief train of 15 pulses delivered at a rate of 50 Hz at time 0 (Figure 2.2B). Test pulses were delivered at a rate of 0.1Hz using 30% of maximum stimulation as determined by individual fitted I/O curves. RM-ANOVA between WIN55-treated (n = 10) and CTRL (n = 7) animals was conducted on 6 binned time points between 0 and 600 s (100 s bins). No time x treatment interaction was found (\( F_{(2.0,30.4)} = 0.771, p = 0.473 \)) (Figure 2.2B).

Short-term depression (STD) was measured during the train used to simulate STP. Each response in the train was compared to the fPSP amplitude at baseline and plotted as a percentage of that value. Depression across the train was observed in all groups, with facilitation being favored in the early portion of the train (Figure 2.2C). RM-ANOVA using 5 binned data points (3 pulse bins) revealed no significant pulse# x treatment interaction \( F_{(2.1,37.8)} = 0.1.25, p = 0.296 \).
We also measured forms of short-term plasticity at 70% stimulus intensity under the same conditions used to test LTD. Thus, PPRs were measured at 70% stimulus intensity using the same ISIs as those shown in Figure 2.2A (Figure 2.2D). No differences were observed between CTRL (n=10) and WIN55-treated (n=9) animals based on independent samples t-tests at 25 ms (CTRL = 130.56% ± 10.83%; WIN55-treated = 124.83% ± 7.75%; t\(_{(17)}\) = 0.422, p = 0.679, r = 0.10), 50 ms (CTRL = 130.28% ± 7.70%; WIN55-treated = 131.58% ± 8.90%; t\(_{(17)}\) = 0.111, p = 0.913, r = 0.03), 100 ms (CTRL = 127.47% ± 9.35%; WIN55-treated = 136.21% ± 4.39%; t\(_{(17)}\) = 0.815, p = 0.426, r = 0.19), 200 ms (CTRL = 116.09% ± 5.85%; WIN55-treated = 125.97% ± 3.19%; t\(_{(17)}\) = 1.436, p = 0.169, r = 0.33), or 300 ms (CTRL = 116.89% ± 7.38%; WIN55 = 118.32% ± 5.11%; t\(_{(17)}\) = 0.156, p = 0.878, r = 0.04).

RM-ANOVA on STP at the 70% stimulus intensity between WIN55-treated (n=10) and CTRL (n=10) was also conducted on 6 binned time points between 0 and 600 s (100 s bins). No time x treatment interaction was observed at 70% stimulus intensity (F\(_{(2.4,43.2)}\) = 0.457, p = 0.671) (Figure 2.2E).

STD at 70% stimulus intensity was also measured (Figure 2.2F). Depression across the train was observed in all groups, with facilitation being favored in the early portion of the train, but to a lesser extent than at 30% stimulus intensity. RM-ANOVA using 5 binned data points (3 pulse bins) revealed no significant pulse# x treatment interaction F\(_{(2.2,38.9)}\) = 0.548, p = 0.596 (Figure 2.2F). Taken together, these results suggest that basic communication and short-term plasticity at L2/3→L5 glutamatergic excitatory synapses in WIN55-treated mice are unaffected by WIN55 treatment during adolescence.
Deficient eCB-LTD at L2/3→L5 synapses is ameliorated by treatment with an inhibitor that degrades endocannabinoid 2-AG

It is well established that the expression of LTD induced by application of a 10-Hz train to the mPFC for 10 min by is coupled to an eCB receptor (CB₁R) (Lafourcade et al., 2007; Lovelace et al., 2014; Lovinger, 2008). Not surprisingly, bath application of CB₁R inhibitors such as AM251 (Figure 2.3A) blocked the induction of eCB-LTD in the L2/3→L5 pathway in acute brain slices isolated from control animals (Figure 2.3C, Wilcoxon test: AM251: 101.18% ± 6.48%, Z = 0, n = 4, p = 1.00). Comparative analysis of the last 10 min of recordings (Figure 2.3D) revealed differences between control and AM251-treated slices (CTRL: 55.82% ± 4.08%, n = 6; AM251: 101.18% ± 6.48%, n = 4; t₈ = -6.274, p = 0.0002, r = 0.91).

Treatment of adolescent mice with WIN55 induced a long-lasting deficit in eCB-LTD expression when compared with control animals (Figure 2.1E, CTRL: 55.82% ± 4.08%, n = 6; WIN55-treated: 90.54% ± 7.63%, n = 9; t₁₃ = 3.469, p = 0.004, r = 0.69). However, blockade of monoacylglycerol lipase (MAGL), the primary enzyme responsible for degrading the endocannabinoid 2-arachidonoylglycerol (2-AG) (Makara et al., 2005), with the specific inhibitor JZL184 (Long et al., 2009) ameliorated the eCB-LTD deficit in the WIN55-treated mice (Figure 2.3B, Wilcoxon test for WIN55-treated+JZL184: 67.34% ± 6.35%, Z = -2.201, n = 6, p = 0.028). The level of expression of eCB-LTD also differed significantly from that observed in brain slices from WIN55-treated animals without bath application of JZL: WIN55-treated+JZL184 vs. WIN55-
treated: 91.76% ± 8.54%, n = 9; \( t_{(13)} = 2.16, p = 0.050, r = 0.51 \). Together, these results strongly suggest that the eCB system is disrupted in WIN55-treated mice and can be rescued by increasing 2-AG levels.

**Adolescent WIN55 exposure reduces CB\(_1\)R functionality in the mPFC**

Induction of eCB-LTD can be effectively abolished by the direct inhibition of CB\(_1\)R function (Figure 2.3A,C, and D), a finding that is consistent with previous reports. It is feasible to assume that the observed deficits in eCB-LTD result from the development of abnormal CB\(_1\)R-dependent signaling in WIN55-treated animals as a compensatory mechanism within the mPFC network. We measured the direct responsiveness of CB\(_1\)R to its well-characterized agonist WIN55,212-2 using an acute brain slice preparation in WIN55-treated and control animals. Evoked fPSPs in L5 stimulated at the L2/3 inputs in the mPFC were strongly inhibited by bath perfusion of control slices with 1 µM WIN55,212-2 (Figure 2.4A,B), whereas slices from WIN55-treated mice showed a modest reduction in response (Figure 2.4B, RM-ANOVA: time x treatment: \( F_{(2.0,20.1)} = 4.80, p = 0.02 \)).

**Adolescent WIN55 exposure attenuates mGluR2/3-dependent LTD**

Activation of mGluR2/3 receptors is known to induce LTD (mGluR2/3-LTD) in the PFC with a presynaptic locus of expression (Robbe *et al.*, 2002). We examined mGluR2/3-LTD in WIN55-treated mice for the L2/3→L5 pathway. Figure 2.5 shows that the mGluR2/3 agonist LY379268 was effective in inducing robust mGluR2/3-LTD in both control and WIN55-treated mice (Figure 2.5A-B; Wilcoxon test: CTRL: 59.85% ± 3.58%, \( Z = -2.201, n = 6, p = 0.028 \); WIN55-treated: 76.62% ± 2.06%, \( Z = -2.521, n = 8 \),
p = 0.012). However, the level of expression of mGluR2/3-LTD was greater than control compared to WIN55-treated mice (Figure 2.5C, RM-ANOVA: time x treatment: F(6,72) = 3.715, p = 0.003), indicating that mechanisms controlling presynaptic mGluR2/3-LTD, including those shared with eCB-LTD, may be altered in WIN55-treated mice. Additional comparison of the last 10 minutes of recording showed a significant difference in the final level of mGluR2/3 depression in WIN55-treated mice and controls (Figure 2.5D; CTRL: 59.61% ± 3.27%, n = 6; WIN55-treated: 76.62% ± 2.06%, n = 8; t(12) = 4.625, p = 0.001, r = 0.80). These results show that WIN55-treated mice still express mGluR2/3 LTD but that the magnitude of the LTD is suppressed in WIN55-treated mice compared to that of controls. Thus, both forms of presynaptic plasticity, mGluR2/3-LTD and eCB-LTD, are impaired in this mouse model of adolescent cannabis abuse.

**Adolescent WIN55 exposure triggers cognitive deficiency**

To test the hypothesis that adolescent WIN55 exposure leads to a permanent deficit in performance on cognitive tasks, we tested WIN55-treated mice in a novel object recognition test that is sensitive to decreased working memory. Consistent with a previous report (Raver et al, 2013), WIN55-treated mice exhibited abnormal behavior in the novel object recognition task (Figure 2.6). The total time spent with both objects was the same in WIN55-treated and control mice during training (CTRL: 64.31 s ± 5.55 s, n = 21; WIN55-treated: 68.53 s ± 4.28 s, n = 7; t(26) = 0.421, p = 0.677, r = 0.08) and testing (CTRL: 29.32 s ± 2.44 s, n = 21; WIN55-treated: 27.51 s ± 1.64 s, n = 7; t(26) = 0.414, p = 0.682, r = 0.08). The two groups of mice showed no difference in discrimination between objects during training (CTRL: -0.050 ± 0.031, n = 21; WIN55-treated: -0.019 ±
0.025, n = 7; t(20) = 0.544, p = 0.591, r = 0.08). However, whereas control mice displayed a strong bias towards the novel object during the test, WIN55-treated mice were not able to learn to discriminate between the familiar and the novel object (CTRL: 0.363 ± 0.054, n = 21; WIN55-treated: 0.027 ± 0.081, n = 7; t(26) = 3.228, p = 0.003, r = 0.53).

Discussion

During adolescence, extensive maturation of prefrontal circuits coincides with profound psychological changes. Human studies have shown that cannabis abuse during adolescence can increase the risk of psychiatric disorders later in adulthood (Arseneault et al., 2004; Fernandez-Espejo et al., 2009; Luzi et al., 2008; Manrique-Garcia et al., 2011; Rubino et al., 2011; Ujike et al., 2004). The importance of eCB signaling in the psychopathology of this phenomenon is emphasized by the discovery that the presence of AAT repeats in the human CNR1 gene encoding CB₁R has been shown to be a risk factor for certain subtypes of schizophrenia (Ujike et al., 2004), and changes in CB₁R expression have been observed in post-mortem schizophrenic brains (Dalton et al., 2011). Converging evidence from animal studies supports the idea that eCB signaling is particularly susceptible to insults during adolescence. In rodents, strong CB₁R activation during adolescence usually produces permanent biochemical and behavioral changes, while adult brains are resistant to the same treatment (Quinn et al., 2008; Raver et al., 2013; Schneider et al., 2005a; Schneider et al., 2003). In addition, neuroimaging studies in humans indicate that some mild cognitive deficits related to PFC pathophysiology are present before the onset of psychosis (Reichenberg et al., 2010), suggesting that acquired
neural network vulnerability may be a critical step in the etiology of psychopathology. Our current studies demonstrate that adolescent CB₁R hyperactivity in rodent brain triggers a sustained deficiency in presynaptic plasticity that is linked to the activity-dependent regulation of neural circuits. Such a deficit in synaptic activity could potentially lead to abnormalities in neural network homeostasis and, subsequently, to increased vulnerability of the cortical circuits to dysfunction.

One possible explanation for the observed loss of eCB-LTD in WIN55-treated mice is a decreased level of CB₁R expression. Changes in CB₁R expression in the brain have been observed in adolescent mice treated with cannabinoids (Dalton and Zavitsanou, 2010). CB₁R expression decreases in the presence of high levels of cannabinoids, and this may contribute to the development of tolerance to the drug (Bedi et al., 2010; D'Souza et al., 2008; Gonzalez et al., 2005; Martini et al., 2010). Alternatively, attenuation of neurotransmission by CB₁R receptor activation can be the result of desensitization, which involves uncoupling of CB₁R from G-protein. Prolonged agonist activation desensitizes the responses of many G-protein-coupled receptors (GPCRs) (Gainetdinov et al., 2004). In general, GPCR desensitization occurs at a subsequent step after phosphorylation of the receptor by a G-protein coupled receptor kinase (GRK) and interaction of the phosphorylated receptor with an arrestin (Nguyen et al., 2012). Desensitization of CB₁R involves GRK phosphorylation of the receptor at two serine residues, S426 and S430 (Daigle et al., 2008a; Daigle et al., 2008b), a molecular event that contributes to cannabinoid tolerance (Morgan et al., 2014). Thus, the lack of eCB-LTD expression in our WIN55-treated mice could be a result of desensitization of CB₁Rs in response to
higher levels of CB₁R activation during adolescence. Unexpectedly, we also found a
deficit in another form of presynaptic long-term depression at the L2/3→L5 synapse,
mGluR2/3-LTD. Both CB₁R and mGluR2/3 depend on the activity of G-protein-coupled
receptors (GPCRs), and they share the same intracellular Gᵢ/ₒ pathways and have been
shown to actually occlude one another in some cases and to compensate for one another
in other cases (Mato et al, 2008; Mato et al, 2005). While it cannot be ruled out that the
LTD deficits we observed developed independently of each other, the observed deficits in
two presynaptic forms of LTD could be a result of abnormal functioning of shared Gᵢ/ₒ
pathways. It is also possible that the observed deficits in WIN55-treated mice involve
common downstream presynaptic signaling molecules that regulate presynaptic release,
including adenyyl cyclase, protein kinase A, and possibly RIM1α (Heifets et al, 2009;
Ohno-Shosaku et al, 2011). Further studies evaluating components of presynaptic
plasticity at the L2/3→L5 synapse are required to events triggering a loss of both forms
of presynaptic LTD in WIN55-treated mice.

Growing evidence indicates that chronic adolescent, but not adult, exposure to
cannabinoids produces permanent cognitive impairments in animal models (Quinn et al,
2008; Raver et al, 2013; Schneider et al, 2005a; Schneider et al, 2003), (O'Shea et al,
2004) and in humans (Meier et al, 2012; Solowij et al, 2002). In addition, chronic
exposure to the CB₁R agonist WIN55,212-2 during adolescence, but not in adulthood,
attenuates the power of cortical oscillations in the gamma frequency range in the mPFC
in adult mice, a phenomenon that has been associated with cognitive decline (Raver et al,
2013). This finding is consistent with the results of studies involving marijuana users who
initiated use as adolescents (Skosnik et al, 2012). These latter studies demonstrated that chronic cannabis exposure alters the ability to generate neural oscillations in the gamma range (Skosnik et al, 2012). Alterations in gamma oscillation have long been thought to be associated with schizophrenic etiology (Chen et al, 2014; Uhlhaas and Singer, 2013). It is noteworthy that developmental NMDAR hypofunction animal models for psychosis have also shown dysregulation in gamma oscillations in the cortex (Anderson et al, 2014; Hunt and Kasicki, 2013) and loss of eCB-LTD, while mGluR2/3-LTD was spared (Lovelace et al, 2014).

In summary, an animal model of adolescent cannabis abuse shows a loss of activity-dependent presynaptic forms of plasticity. This type of retrograde endocannabinoid regulation appears to be a major regulator of neural network homeostasis and of the brain’s natural ability to adapt to a changing environment. Deficiency in a type of neural control that appears to function as an activity-dependent “brake” is likely to translate to maladaptations that underlie increased vulnerability to psychopathology.

**Methods**

**Subjects**

C57BL/6 mice were used for all of the experiments following protocols approved by the IACUC at UCR. The animals were housed in plastic cages (2-4 mice/cage) and kept on 12/12 h dark/light cycle with ad libitum access to food and water. Ten- to twenty-four-week-old mice were used for the physiological studies. To generate the WIN-55,212-2 treated animals, C57BL/6 mice are intraperitoneally injected twice a day with increasing doses of (+)WIN55, 212-2 (mesylate), once in the morning and once in the afternoon at
the same times of day on postnatal days (PND) 35-45 as in previous studies (Rubino et al, 2008). All of the physiological tests were performed using equal gender distribution (males/females, 50%/50%) during adulthood (P70-P170).

**Drugs - physiology**

WIN55,212-2 was obtained from NIMH Chemical Synthesis and Drug Supply Program. Picrotoxin, 6,7-dinitroquinoxaline-2,3-dione (DNQX), LY379268, JZL-184, and AM251 were obtained from Tocris, Inc.. All drugs used in electrophysiological procedures were made from stock solutions and frozen in aliquots at -20°C until used on day of the experiment. For bath application in physiological studies, WIN55,212-2, JZL-184, AM251, and picrotoxin were dissolved in DMSO and made into 100mM stock solutions. LY379268 was dissolved in water and stored in 10mM stock solutions. The concentration of DMSO was always <0.1% of total concentration and had no effect on synaptic responses. All experiments were done in the presence of 50µM picrotoxin in order to block ionotropic GABA mediated transmission unless stated otherwise.

**Drugs – animal injection**

The WIN55,212-2 injection solution was prepared using a mixture of EtOH / Cremophor / saline in a 1:1:18 ratio (Acheson et al, 2011; López-Gallardo et al, 2012). Stock solutions of WIN55, 212-2 were stored in the same volume of ethanol in varying dilutions for use on different days to achieve the appropriate dose. On the day of injection, WIN55,212-2 stock solution was mixed with cremophor and saline, vortexed, and brought to room temperature before injection. Mice received I.P. injections twice a day at the same time of day on days PND 35-45 as previously described; low doses were
at 0.5 mg/kg (PND 35-36), medium doses at 1 mg/kg (PND 37-41), and high doses at 2 mg/kg (PND 42-45) (Figure 2.1A).

**Electrophysiology**

Local field postsynaptic potential (fPSP) recordings were obtained from cortical layer 5 in the PL region of the mPFC after stimulation at layer 2/3 inputs. Acute brain slices containing the mPFC were prepared using the Compressstome VF-300 (Precisionary Instruments, Greenville, NC). Ex vivo slice electrophysiology was performed on brain slices containing the PL region of the mPFC bathed in ACSF (124.00 mM NaCl, 4.40 mM KCl, 25.00 mM NaHCO3, 1.2 mM Na2HPO4, 1.3 mM MgSO4-7H2O, 2.5 mM CaCl2-2H2O, and 10 mM glucose) saturated with a 95%O2/5%CO2 gas mixture. Brain slices containing the PL region were placed in a recording chamber and continuously perfused [~1.5 ml/min] with carboxygenated ACSF held at 28°C. The slices were viewed under an Olympus TM FV1000 confocal microscope using DIC. In all cases, local field potential recordings were obtained from cortical layer 5 in the PL region using a 1.5-5 MΩ resistance glass pipette filled with ACSF and guided by visual landmarks combined with adjustment for the maximal fPSP response within a region ~500-800 µm from the cortical surface. Signals from the head stage were then amplified, filtered, digitized at 10 KHz, and sent to a computer for data storage using Clampex software and analyzed using Clampfit (Molecular Devices Inc.). Stimulations were delivered using a bipolar cluster electrode (FHC, Inc.) placed in cortical layers 2/3 in the PL region of the mPFC, which was visible under DIC as a dark band ~200-300 µm from the surface. The stimulating
electrode was aligned with the recording electrode, which was perpendicular to the cortical surface.

Acute mPFC slices from 10- to 24-week-old mice were used for recordings, which were performed according to previously described protocols: input/output (I/O) and paired-pulse ratio (PPR) (Limback-Stokin et al, 2004), short-term potentiation (STP) (Hempel et al, 2000), endocannabinoid-dependent LTD (eCB-LTD) (Lafourcade et al, 2007), and mGlur2/3 LTD (Kasanetz et al, 2013). The glutamatergic nature of the fEPSP was confirmed by blocking it with 20 mM DNQX (AMPA receptor antagonist) (no picrotoxin present). Traces from the local field potential recordings were analyzed as previously described (Hempel et al, 2000; Hirsch et al, 1990; Morris et al, 1999). The I/O was recorded starting at 20 μA stimulation using an isolated stimulator, and the stimulation intensity was then increased until the amplitude measurements reached a plateau (three consecutive stimulus intensity increases with minimal change). The individual curves were fitted using Boltzmann’s sigmoidal fit equation: \( y = \frac{[A2 + (A1-A2)]}{[1 + \exp((x-x0)/dx)]} \) and OriginPro software (OriginLab, Inc.). An R-square value of >0.9 was used as a cut-off criterion for curve fitting. PPR was used to assess the transient synaptic plasticity at increasing interstimulus intervals (ISI) ranging from 25 ms to 300 ms tested at both 30% and 70% of the stimulation required to reach maximum (no picrotoxin present). The PPR and I/O stimulation were determined by the average of three traces with 15 s intervals between stimulations.

STP was induced using a 15-pulse 50 Hz train with at least 20 min of post-stimulus train recording (no picrotoxin present). The test pulses were delivered every 10 s. The
STP was measured at 30% and 70% of the stimulation intensity needed to reach maximum. Post-train recordings were compared to baseline recordings as a percentage of the average baseline for amplitude of fPSP. eCB-LTD was induced according to a previously published protocol (Lafourcade et al., 2007) in which a 10-min, 10-Hz stimulation (at 70% stimulus intensity) of layer 2/3 in the PL region of the mPFC induced long-lasting LTD in layer 5 pyramidal neurons. Long-lasting LTD was then recorded for 1 hr, with test pulses delivered at a rate of 0.1Hz. mGluR2/3-LTD was induced at time 0 with a 10-minute bath application of 100 nM LY379268 (a potent mGluR2/3 agonist). The success or failure of LTD expression in individual groups was determined using a paired Wilcoxon signed rank test (Wilcoxon test) by comparing the mean fPSP amplitude during baseline (10 min before stimulation) and 50-60 min after LTD induction. When recording during mGluR2/3-LTD and WIN55,212-2 application, test pulses were delivered at a rate of 0.1 Hz with at least 20 min of baseline recording before drug application.

**Novel Object Recognition**

Novel Object Recognition was performed accordingly to a previously described protocol (Korzus et al., 2004). The task is divided into four phases: habituation, familiarization, delay and test. 1) Mice were handled 4 times and were placed in the experimental room for two hr before the experiment. 2) During familiarization two identical objects were placed in the home cage with the animals for 5 min. 3) Animals were tested after a 5 min. delay. 4) During the test phase both the familiar object (a replica of the original familiar object was used to avoid the use of odor cues) and a novel
object were placed in the cage. Object exploration times (for both familiar and novel objects) were recorded for a 2 min test period. We employed computer-assisted scoring using software to measure and analyze performance on the VPC task. Object exploration was scored when the animal’s head was oriented towards the object and vibrissae were moving. The objects varied in color, shape and size and were balanced so that the same objects (replicas) were used for some animals as “familiar” and for others as “novel” in the same session. Also the position of “novel” and “familiar” objects within the cage was randomized.

Data analysis

Experimenters were blind to group designations. Data represent means ± SEM. Statistical analysis was performed using Excel (Microsoft, Inc.) or SPSS (IBM, Inc.). Student’s t test, Wilcoxon signed ranked test or repeated measures ANOVA (RM-ANOVA) was used for statistical comparisons. For t-tests, Pearson’ correlation ($r$) was used as an effect size. In cases where RM-ANOVA was utilized and assumptions of sphericity were violated (via Mauchly’s Test), analysis was completed using the Greenhouse-Geisser correction. A critical probability of $p \leq 0.05$ was applied. Asterisks indicate statistical significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 
Figure 2.1

A. I.P. injections 2x a day

<table>
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Experiments in adulthood

FND 70+

B. Recording electrode and Stimulation electrode

mPFC L6 L5 L2/3

C. Graph showing input/output relationship

Intensity [μA] vs. fPSP [mV]

CTRL WIN55

D. Graph showing eCB-LTD

% Baseline [mV] vs. Time [Min]

CTRL WIN55
Figure 2.1 Adolescent cannabis abuse model shows a deficit in eCB-LTD but not in basic synaptic transmission.

(a) Injection protocol adapted from previous study (Rubino et al, 2008). Mice were injected twice a day during adolescence (PND35-45) with increasing doses of WIN55 and then allowed to mature to adulthood before experimental procedures. (b) Diagram showing the configuration of the recording and stimulation electrodes within the prelimbic region of the mPFC used in electrophysiological experiments to test synaptic activity at the L2/3→L5 synapse. (c) Application of DNQX shows the glutamatergic component of the fPSP. The red line is an average fPSP of 5 traces with only ACSF in the bath. After 5 min of bath application of 20 mM DNQX, another average of 5 traces was recorded (black line). Note that the fiber volley is spared while the fPSP is abolished under the DNQX condition (no picrotoxin present). (d) I/O curves were determined starting at 20 μA stimulus intensity and increasing stimulation intensity until the amplitude measurements reached a plateau. No differences were detected using independent samples t-tests at any intensity measured (CTRL: n = 8; WIN55-treated: n = 8; all p-values > 0.05). A fitted curve for each group obtained using Boltzmann’s sigmoidal equations is also shown. Further detailed analysis of the I/O relationship was performed using different metrics obtained from the Boltzmann sigmoidal analysis to determine whether differences in the shape of the curves were present. A comparison of the means of individual Boltzmann fitted parameters revealed no differences between the WIN55-treated and control mice in the maximum asymptote (A2) p = 0.788, in the center (x0) p = 0.217, or in the time constant (dx) p = 0.755. These thorough analyses of I/O relations at the L2/3→L5 synapse show that there are no differences between control and WIN55 mice in any of the tested characteristics of the I/O curves. (e) eCB-LTD was induced at time 0 (black arrow) with a 10-Hz train applied for 10 min using the previously described protocol (Lafourcade et al, 2007). LTD magnitude was estimated from fPSPs registered during the period 50-60 min after LTD induction as a percentage of baseline fPSP amplitudes. Compared with controls, WIN55-treated mice showed a strong deficit in eCB-LTD expression in response to the 10-Hz stimulation protocol (CTRL: 55.82% ± 4.08%, n = 6; WIN55-treated: 90.54% ± 7.63%, n = 9; t(13) = 3.469, p = 0.004, r = 0.69).
Figure 2.2

A. PPR (SI = 30%)

B. STP (SI = 30%)

C. STD (SI = 30%)

D. PPR (SI = 70%)

E. STP (SI = 70%)

F. STD (SI = 70%)

Interstimulus Interval (ISI)
**Figure 2.2** Short-term plasticity at the L2/3→L5 synapse is unaffected in WIN55-treated mice.

(a-c) Short-term measures of plasticity were measured at 30% stimulus intensity. (a) Paired-pulse ratios (PPR) were measured at the L2/3→L5 synapse at 5 different interstimulus intervals (25, 50, 100, 200, and 300 ms). No differences in mean PPR were observed between CTRL (n = 10) or WIN55-treated (n = 10) animals at any interval tested; all p-values > 0.05. (b) Short-term potentiation (STP) was induced with a brief train of 15 pulses delivered at 50 Hz at time 0 (indicated by a black arrow). RM-ANOVA between WIN55-treated (n = 10) and CTRL (n = 7) samples showed no time x treatment interaction (F(2.0,30.4) = 0.771, p = 0.574). (c) Short-term depression (STD) was measured during the train used to simulate STP. Each response in the train was compared to the fPSP amplitude at baseline and plotted as a percentage. Depression across the train was observed in all groups, with facilitation being favored in the early portion of the train. RM-ANOVA revealed no significant pulse# x treatment interaction (F(2.1,37.8) = 0.125, p = 0.296). (d-f) Short-term measures of plasticity were also measured at 70% stimulus intensity. (d) PPRs were measured at 5 different inter-stimulus intervals (25, 50, 100, 200, and 300 ms). No differences in PPR at 70% stimulus intensity were observed between CTRL (n=10) and WIN55-treated (n=9) animals using independent samples t-tests; all p-values > 0.05. (e) RM-ANOVA on STP at 70% stimulus intensity between WIN55-treated (n=10) and CTRL (n=10) samples showed no time x treatment interaction (F(2.4,43.2) = 0.457, p = 0.671). (f) At 70% stimulus intensity, depression across the train was observed in both groups, with facilitation being favored in the early portion of the train but to a lesser extent than at 30% stimulus intensity. RM-ANOVA revealed no significant pulse# x treatment interaction F(2.2,38.9) = 0.548, p = 0.596.
Figure 2.3

(A) CTRL
- • CTRL-AM251
- ○ CTRL-ACSF

(B) WIN55
- ▲ WIN55-ACSF
- △ WIN55-JZL

(C) CTRL-AM251
- • CTRL-ACSF
- △ WIN55-JZL
- ▲ WIN55-ACSF

(D) Bar graph showing baseline levels for CTRL, WIN55, WIN55+JZL184, and CTRL+AM251 with statistical significance indicated by asterisks: *** (p < 0.001), ** (p < 0.01), * (p < 0.05).
Figure 2.3 Deficient eCB-LTD at the L2/3→L5 synapse is ameliorated by treatment with an inhibitor that degrades endocannabinoid 2-AG.

(a) The CTRL group showed robust expression of eCB-LTD at the L2/3→L5 synapse in the mPFC. eCB-LTD was induced at time 0 (black arrows) by application of a 10-Hz train for 10 min. LTD magnitude was estimated from fPSPs registered during the period 50-60 min after LTD induction and expressed as a percentage of baseline fPSP amplitudes. Bath application of 5 µM AM251 completely blocked LTD, indicating that this is an endocannabinoid-dependent form of LTD. (b) The WIN55-treated group showed no induction of LTD at the L2/3→L5 synapse in the mPFC. However, 10-min stimulation of WIN55 slices at 10 Hz in the presence of JZL184 rescued the deficit (Wilcoxon test: WIN55-treated+JZL184: 67.34% ± 6.35%, Z = -2.201, n = 6, p = 0.028). (c) The binned averages of every 10 min time segment show distinct differences under all experimental conditions. (d) Data are plotted from the binned average time point in the rectangle marked during the last 10 min of recording. Control recordings show expression of eCB-LTD, while WIN55-treated slices and control slices with bath-applied AM251 showed no induction of eCB-LTD. However, JZL184 application to WIN55 slices reduced the level of LTD to the control level.
Figure 2.4

Figure 2.4 CB₁R function is reduced in WIN55-treated mice.

(a) The WIN55-treated group shows decreased functionality of CB₁R at the L2/3→L5 synapse in the mPFC compared to control mice. After 20 min of baseline recording, continuous application of 1 μM WIN55,212-2, a CB₁R agonist, caused a reduction of fPSP amplitude in the control group; this reduction was markedly dampened in the WIN55-treated mice. (b) Analysis of binned averages showed a markedly dampened effect in the WIN55-treated mice (RM-ANOVA: time x treatment: F(2.0, 20.1) = 4.800, p = 0.020).
Figure 2.5

A  CTRL

B  WIN55

C

D

% Baseline (mV)

% Baseline (mV)

% Baseline (mV)

% Baseline (mV)

Time (Min)

Time (Min)

Time (Min)

Time (Min)
**Figure 2.5** mGluR2/3-LTD at the L2/3→L5 synapse is suppressed in WIN55-treated mice.

mGluR2/3-LTD in the L2/3→L5 pathway was induced at time 0 by a 10-minute bath application of 100 nM LY379268, a potent mGluR2/3 agonist. The CTRL group (a) showed significant expression of LTD, whereas LTD occurred to a lesser extent in the WIN55-treated group (b), suggesting that mGluR2/3-dependent LTD is altered in WIN55-treated mice. (c) Binned averages of activity taken every 10 min across time show significant divergence in mGluR2/3-LTD expression levels in WIN55-treated and control animals at the L2/3→L5 synapse; time x treatment: $F_{(6,72)} = 3.715$, $p = 0.003$. (d) There is a significant difference in expression of mGLuR2/3-LTD in control and WIN55-treated mice at 50-60 min (CTRL: 59.61% ± 3.27%, $n = 6$; WIN55-treated: 76.62% ± 2.06%, $n = 8$; $t_{(12)} = 4.625$, $p = 0.001$, $r = 0.80$). These results suggest that treatment of mice with WIN55 during adolescence causes a disruption in the mGluR2/3 signaling pathway.
Figure 2.6. WIN55 treatment during adolescence impairs performance on a novel object recognition task.

Mice were tested on a novel object recognition task with a short (2-minute) delay between the training and the testing phases. (a) Exploration time during training (CTRL: 64.31s ± 5.55s, n = 21; WIN55-treated: 68.53s ± 4.28s, n = 7; t(26) = 0.421, p = 0.677, r = 0.08) and testing (CTRL: 29.32s ± 2.44s, n = 21; WIN55-treated: 27.51s ± 1.64s, n = 7; t(26) = 0.414, p = 0.682, r = 0.08) did not differ in the two groups. (b) While the two groups showed no difference in discrimination between objects during training (CTRL: -0.050 ± 0.031, n = 21; WIN55-treated: -0.019 ± 0.025, n = 7; t(26) = 0.544, p = 0.591, r = 0.08), WIN55-treated mice failed to recognize the familiar object during the test phase (CTRL: 0.363 ± 0.054, n = 21; WIN55-treated: 0.027 ± 0.081, n = 7; t(26) = 3.228, p = 0.003, r = 0.53).
**Conclusion**

It seems clear that cannabinoid signaling contributes to schizophrenic pathology in some way, but much work needs to be done to understand the abnormality at a functional and physiological level. Research has been lacking to a great extent in this area, mostly focusing on gene expression and behavior. Here we show for the first time that eCB dependent plasticity (eCB-LTD), CB₁R expression, and CB₁R function in the mPFC are reduced in a NMDAR hypofunction animal model during early postnatal brain development. We were also able to rescue this loss of eCB-LTD by bath applying an inhibitor of MAGL, the main enzyme responsible for hydrolyzing 2-AG, the active endocannabinoid shown to mediate eCB-LTD in the mPFC (Lafourcade *et al.*, 2007).

While eCB signaling appeared to be disrupted, we observed several other forms of plasticity that remained intact. Short-term forms of plasticity were unchanged including paired pulse ratios, short term potentiation, and short term depression. Interestingly when mGluR2/3-LTD was tested, we found no effect between PCP-treated and control animals.

Both the CB₁ and mGlu2/3 receptors are coupled to the same G_{i/o} intracellular pathway, so the fact that only CB₁R activity was diminished supports the idea that CB₁R functionality was specifically disrupted and not downstream processes shared by both receptors along G_{i/o}. The unique interaction between NMDAR hypofunctioning and CB₁R down regulation is still not well understood, but it is some of the first steps made in the field to understand this interaction on an electrophysiological level. In addition to electrophysiological findings, we also discovered that mice treated with PCP also showed deficits on a memory specificity task, which the PFC is known to be involved (Vieira *et
While a direct causal link between the observed eCB deficit and deficit in discriminative fear learning cannot be made in the current studies, abnormalities in fear discrimination and generalization however could still be used as another behavioral phenotype in schizophrenic models.

Next we investigated a cannabis abuse model in which a CB$_1$R agonist (WIN55,212-2) was administered during an adolescent period of development. We found that eCB-LTD and functionality of CB$_1$R was also decreased and able to be rescued with MAGL inhibition, similar to that of PCP treated animals. It is tempting to speculate that PCP treatment during early postnatal stages triggers elevated 2-AG levels which persist during adolescence and cause the same result as WIN55,212-2 in our model. This interpretation is consistent with other studies in which PCP treatment does in fact cause elevations of 2-AG specifically in the PFC and also reduced G protein activation by CB$_1$R agonists (Vigano et al, 2008). However, using our WIN55,212-2 model, we found that mGluR2/3-LTD was also disrupted, in addition to deficient eCB-LTD signaling. So, during adolescence, it is possible that over activation of CB$_1$Rs causes a general down regulation of G$_i/o$ signaling pathways that are not restricted to the CB$_1$Rs. Another study has shown that a functional switch to mGluR2/3s can actually rescue deficits of CB$_1$Rs caused by chronic THC exposure (Mato et al, 2005). In this study they used a 13Hz10min stimulation protocol similar to the one described in our studies. This stimulation activates both CB$_1$Rs through retrograde transmission mechanisms, but also activates presynaptic mGluR2/3 autoreceptors directly through glutamate release. Since both of these pathways inhibit presynaptic transmitter release they can both act as
homeostatic regulators of activity. However these experiments were done on adult mice only and may not be relevant to adolescent models. Adenosine 1 receptors (A1Rs) express presynaptically and are also coupled to $G_{i/o}$. Tonic activation of A1Rs have been shown to actually inhibit CB$_1$R function, probably through competition of overlapping sets of Ga subunits (Hoffman et al, 2010). This elucidates how complex these interactions can become, and how perturbations of neurotransmitter systems at different stages of brain development can have very specific affects on various signaling pathways.

Schizophrenia, as mentioned, probably has a myriad of factors, and the risk of developing schizophrenia (and its severity) probably depends on how many of those risk factors are present. Studies show abnormal levels of biomarkers of synaptic plasticity in schizophrenic brain such as increased levels of NR2B (NMDA receptor subunit) (Grimwood et al, 1999; Kristiansen et al, 2010a; Kristiansen et al, 2010b) and CB$_1$R (Dalton et al, 2011; Wong et al, 2010). Under normal circumstances these specific proteins undergo changes in levels of expression dependent upon the maturation state of the brain. What is striking is that these natural changes in levels of expression seem to correspond with several factors relevant to schizophrenia: 1) age relevant gene expression, 2) changes in expression mainly located in regions of the brain known to undergo major change during adolescence/early adulthood (prefrontal cortex) 3) the same regions are also known to be involved in cognitive tasks/symptoms. This type of interpretation may help us to better understand the underlying anatomical and physiological abnormalities that are likely occurring at a network level (Insel, 2010).
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