The Endocannabinoid, 2-arachidonoylglycerol, Regulates Resilience to Stress-induced Anxiety

By

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Dedicated to my family of strong, thoughtful, adaptable individuals who have, by biology and environment, imbued me with my own resilience.

And to MJL, whose love and wit both support and challenge me in the best ways.

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2-AG 2-arachidonoylglycerol
AA ..... arachidonic acid
AAV ..... adeno-associated virus
ABHD6, ABHD12 ..... alpha/beta hydrolase domain containing 6, 12
ACSF ..... artificial cerebrospinal fluid
AEA ..... arachidonoylethanolamine, anandamide
ANOVA ..... analysis of variance
ANS ..... autonomic nervous system
BL ..... baseline
BLA ..... basolateral amygdala
Ca2+ ..... calcium cation
CB1R, CB2 ..... cannabinoid receptor subtype 1, 2
CNS ..... central nervous system
CRE ..... cre recombinase
CRH ..... corticotropin releasing hormone
CSDS ..... chronic social defeat stress
CUS ..... chronic unpredictable stress
DAGL ..... diacylglycerol lipase
DMSO ..... dimethylsulfoxide
DNA ..... deoxyribonucleic acid
DNMT ..... DNA-methyl transferase
DSE ..... depolarization induced suppression of excitation
DSI ..... depolarization induced suppression of inhibition
DSM5 ..... diagnostic and statistical manual 5
eCB ..... endogenous cannabinoid
ELISA ..... enzyme-linked immunosorbent assay
EPM ..... elevated plus maze
EPSC ..... excitatory postsynaptic current
FAAH ..... fatty acid amide hydrolase
FRT ..... flippase recognition target

FS ..... foot-shock
GFP ..... green fluorescent protein
GPCR ..... g-protein coupled receptor
HPA ..... hypothalamic-pituitary-adrenal
IEI ..... inter-event interval
LC/MS/MS ..... liquid chromatography tandem mass spectrometry
LD ..... light-dark
LH ..... learned helplessness
MAGL ..... monoacylglycerol lipase
MDD ..... major depressive disorder
mGluR ..... metabotropic glutamate receptor
mPFC ..... medial prefrontal cortex
NAc ..... nucleus accumbens
NCT ..... novel cage test
NMDAR ..... n-methyl-d-aspartate receptor
oDSE ..... optical depolarization induced suppression of excitation
OFT ..... open field test
PBS ..... phosphate buffered saline
PCR ..... polymerase chain reaction
PFA ..... paraformaldehyde
PHARC ..... polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract
PIP2 ..... phosphatidylinositol 4,5-bisphosphate
PLCβ ..... phospholipase C β
PTSD ..... posttraumatic stress disorder
qRT-PCR ..... quantitative real-time polymerase chain reaction
RDoC ..... research domain criteria
RNA ..... ribonucleic acid
rNIH ..... repeated novelty-induced hypophagia
SAG ..... steroyl-arachidonoylglycerol
sEPSC ..... spontaneous excitatory postsynaptic current
SPT ..... sucrose preference test

SSRI ..... selective serotonin reuptake inhibitor
TBS ..... tris-buffered saline
THC Δ9-tetrahydrocannabinol
TRPV1 ..... transient potential vanilloid receptor 1
TST ..... tail-suspension test
vHIP ..... ventral hippocampus

CHAPTER I

INTRODUCTION

Social and Economic Burden of Affective Disorders

An international effort to characterize the global impact of specific diseases has demonstrated that mental, neurological, and substance use disorders account for approximately 10% of global disease burden, and nearly 30% of the nonfatal global disease burden (Whiteford et al. 2013). Within the category including mental, neurological and substance use disorders, 42% of disability-adjusted life years are due to mood and anxiety disorders (collectively termed affective disorders) (Patel et al. 2016). Mood disorders are characterized by sad, empty, or irritable mood with cognitive changes that, together, cause difficulty with participation in typical daily activities; these symptoms may persist for many weeks or months but often recur with intervening periods of remission. The symptomology of the most common mood disorder, major depressive disorder (MDD), can also include a combination of sleep dysfunction, fatigue, psychomotor changes, weight changes, reduced interest or pleasure in daily activities, feelings of worthlessness or guilt, attention deficits, and suicidal ideation. Anxiety disorders are characterized by incapacitating fear (autonomic fight or flight responses) or anticipation of fear-inducing stimuli (enhanced vigilance and caution or avoidance). Post-traumatic stress disorder (PTSD) fits this general description and was previously classified as an anxiety disorder, although in the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM5) it has been reclassified under the new category of stressor- and trauma-related disorders. This new category of disorders is overtly linked to stress exposure but also closely related to mood,

anxiety, and compulsive disorders. Indeed, one of the reasons these disorders were split into their own category is the "variable expression of clinical distress following exposure to catastrophic or aversive events". The responses to trauma can vary from primarily fear and anxiety to anger and aggression to loss of interest and pleasure. The shared risk factors and high comorbidity between mood, anxiety, and trauma-related disorders strongly suggest the potential for some shared mechanisms of dysfunction (Spinhoven et al. 2014). In support of this, the current standard of treatment (selective serotonin reuptake inhibitors; SSRIs) for mood, anxiety, trauma-related, and even some eating disorders is the same and exhibits similar efficacy across these disorders (Kaufman et al. 2015). Indeed, while DSM classifications are useful diagnostic tools there has also been increasing interest, particularly within the research community, in focusing more on domains of behavioral dysfunction that may be shared between several diseases than on specific disease states. This is epitomized by the National Institute of Health's (NIH) development of the Research Domain Criteria (RDoC). This type of approach could potentially lead to the development of therapeutics that work across diagnoses to target particular types of symptoms which are likely to be regulated by similar molecular or anatomical processes: negative valence, positive valence, cognitive, social, and arousal domains.

A significant societal economic burden accompanies the negative impact on quality of life for those who experience mood and anxiety disorders. Interestingly, it has been estimated that the return on investment for improving the current global under-treatment of mood and anxiety disorders is approximately 4:1 when taking enhanced labor participation and productivity with adequate treatment into account (Chisholm et al. 2016, Thornicroft et al. 2017). In addition, affective disorder diagnoses are associated with subsequent development of arthritis and other chronic physical conditions suggesting a possibility that early treatment of these mental disorders

could further reduce the global burden of disease by either directly or indirectly reducing the prevalence of other physical disabilities (Aguilar-Gaxiola et al. 2016, Scott et al. 2016). Furthermore, estimates of enhanced labor participation inherently depend upon the efficacy of intervention and would, therefore, be meaningfully amplified by the discovery of more effective treatment options.

Limitations of Treatment for Affective Disorders

Two factors besides access substantially limit treatment of affective disorders: 1) undereducation and stigma about psychiatric diagnoses, and 2) suboptimal properties of pharmacological interventions. Only ~16.5% of people who meet diagnostic criteria for major depression receive adequate treatment each year - partly due to a lack of knowledge or stigma, both of which prevent people from seeking treatment (Thornicroft et al. 2017). This is a serious problem that demands better communication from the scientific community to the public. For those patients who seek treatment for a mood or anxiety disorder, SSRIs have been the first line treatment since the 1980s (Lecrubier 2007, Kaufman et al. 2015). SSRIs exhibit reduced side effect profiles compared to the monoamine modulators that they replaced (tricyclics and monoamine oxidase inhibitors). Monoamine neurotransmitters (serotonin, norepinephrine, dopamine) were first targeted for the treatment of depression because it was hypothesized that the basis of affective disorders was a reduced monoaminergic tone (Schildkraut 1995). While this hypothesis has fallen out of favor, SSRIs remain one of the most effective treatments available for affective dysfunction. However, only 40% of depressed patients achieve remission with the first prescribed antidepressant (Rush et al. 2006). This presents a significant difficulty as the antidepressant and anxiolytic effects of SSRIs often do not emerge before 4-8 weeks of

treatment and symptoms may even worsen during that delay (Salchner and Singewald 2002, Burghardt et al. 2004, Drapier et al. 2007). With a second prescription 55% of depressed patients achieve remission, and by a fourth (representing >16 months of failed treatment) this figure only climbs to 67% (Rush et al. 2006). Currently, there is no good way to predict which antidepressant will be effective, although many studies have attempted to associate treatment responses with neuroimaging, genetic, psychosocial, cognitive, and psychophysiological measures (Kemp et al. 2008). Also problematic, up to 18.4% of patients with pure depression are wrongly prescribed only anxiolytics (Lecrubier 2007).

Selective norepinephrine uptake inhibitors, benzodiazepines, tricyclic antidepressants, monoamine oxidase inhibitors, anticonvulsants, and atypical antipsychotics are the other major classes of pharmacotherapy available for the treatment of anxiety disorders. Selective norepinephrine reuptake inhibitors appear to share both the efficacy and drawbacks (particularly slow onset of action) of SSRIs. Tricyclics and monoamine oxidase inhibitors are prescribed less often for mood and anxiety disorders than SSRIs because of their harsher side effect profiles and benzodiazepines, while more rapidly effective, have a strong potential for abuse (Hoffman and Mathew 2008). Additionally, benzodiazepines do not have antidepressant effects, so they should not be used for anxiety disorders comorbid with depression. A limited number of anticonvulsants and atypical antipsychotics have shown reasonable efficacy in treating anxiety disorders, but also produce sedation and weight gain (Hoffman and Mathew 2008). Exposure therapy, rather than pharmacotherapy, is currently recommended as a first-line treatment for PTSD although SSRIs have shown some efficacy in treating comorbid depressive symptoms (Hoffman and Mathew 2008). Interestingly, clinical studies based on preclinical research demonstrating that glutamate signaling through N-methyl-D-aspartate receptors (NMDARs) mediates extinction, have

indicated that D-cycloserine, a partial NMDAR agonist (at the glycine binding site), can increase the efficacy of exposure therapy by enhancing extinction learning (Kaufman et al. 2015, Singewald et al. 2015).

Using preclinical research to construct a better understanding of how genetic and environmental factors contribute to the development or treatment of affective dysfunction should, as in the case of D-cycloserine, lead to improvements in treatment options and efficacy. Indeed, another example of this is the relatively recent discovery of the rapid antidepressant properties of ketamine, an NMDAR antagonist. Rodent studies showed that chronic, but not acute, treatment with typical antidepressants inhibited glycine binding to the NMDAR (which is required for its activation) and, subsequently, that direct NMDAR antagonism also exhibited antidepressant-like effects (Trullas and Skolnick 1990, Paul et al. 1994). Based on these studies, a small clinical trial confirmed that acute ketamine treatment produces rapid antidepressant effects in depressed patients (Berman et al. 2000). While this represents a huge step forward for the field, ketamine itself has a relatively high potential for abuse and can cause cognitive deficits; further research is being conducted to develop a more useable pharmaceutical with similar efficacy (Morgan et al. 2004). These examples establish the importance and utility of using preclinical research to determine the molecular mediators of environmental factors and treatments that produce or reduce affective dysfunction. With this goal in mind, the project herein is focused on elucidating how the endogenous cannabinoid 2-arachidinoylglycerol (2-AG) regulates the impact of the primary environmental risk factor for the development of affective dysfunction – stress exposure.

The Endogenous Cannabinoid System

The endogenous cannabinoid (eCB) system interacts extensively with the hypothalamic pituitary adrenal (HPA) axis and autonomic nervous system, two major components of the stress response which will be discussed further in subsequent sections of this introduction (Wamsteeker et al. 2010, Gray et al. 2015, McEwen et al. 2015, Morena et al. 2016). The elucidation of the eCB system began with the search for a mechanism underlying the effects of the *Cannabis sativa* plant and its primary psychoactive component, Δ^9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam 1971). Long before the determination of its psychoactive components, the cannabis plant had been used for its mood-altering and other therapeutic properties (Adams and Martin 1996). The two cannabinoid receptors, CB1R and CB2, were discovered and cloned in the early 1990s and the CB1R was shown to be almost ubiquitously expressed in the central nervous system (CNS) with the CB2 subtype preferentially expressed in the periphery (Matsuda et al. 1990, Munro et al. 1993, Glass et al. 1997). Discovery of arachidonoylethanolamine (anandamide; AEA) and 2-AG, the two main eCB ligands rapidly followed (Devane et al. 1992, Sugiura et al. 1995). Several other related lipids have been suggested to bind CB1R, but their presence at biologically relevant levels in the mammalian CNS has been questioned (Huang et al. 2001). The CB1R is a transmembrane G-protein coupled receptor (GPCR) largely expressed at axon terminals that primarily couples to $G\alpha_{i/o}$ and is ~97% homologous between rodents and humans (Chakrabarti et al. 1995). CB1R activation is transduced by several downstream signaling cascades consistent with $G\alpha_{i/o}$ signaling including inhibition of adenylate cyclase and voltage gated calcium (Ca^{2+}) channels that have the overall effect of reducing synaptic vesicle fusion and neurotransmitter release (Pertwee 1997, Kano et al. 2009). While this appears to be the typical mode of signaling, there is also evidence for CB1R binding to $G\alpha_q$ and $G\alpha_s$ to activate

alternative signaling cascades, an effect which may be controlled at least partially by ligand-bias or heterodimerization with other GPCRs (Lauckner et al. 2005, Chen et al. 2010, Hudson et al. 2010, Laprairie et al. 2016). Interestingly, the CB1R ligand binding pockets are located within the transmembrane helices indicating that ligands likely diffuse in laterally from the plasma membrane (Song and Bonner 1996, Makriyannis et al. 2005). CB1Rs are generally localized to the perisynaptic membrane on the presynaptic terminal and are expressed throughout the mammalian brain, including limbic brain regions relevant for regulating affective function (Kawamura et al. 2006, Yoshida et al. 2011, Ramikie et al. 2014).

AEA and 2-AG are both lipid-derived, arachidonic acid-containing, retrograde CNS signaling molecules. AEA is expressed at very low levels and acts as a partial agonist at the CB1R but can also activate transient potential vanilloid 1 (TRPV1) receptors (Chavez et al. 2010). Several biochemical pathways that can synthesize AEA exist, but which of them mediate the production of AEA for CNS signaling remains unclear (Astarita et al. 2008, Simon and Cravatt 2008, Ueda et al. 2013). Fatty acid amide hydrolase (FAAH) is the primary enzyme that terminates AEA signaling by degradation (Cravatt et al. 1996). For further information on AEA-related biosynthetic pathways see Ueda et al. 2013.

2-AG sits at a nexus of bioactive lipid metabolism (Ueda et al. 2013, Grabner et al. 2017). In fact, it was originally thought to be simply an intermediate in a mechanism to convert diacylglycerols into free arachidonic acid (AA) (Prescott and Majerus 1983). While 2-AG is now recognized as a physiologically relevant signaling molecule in its own right, it is still thought to be an important intermediate in the regulation of AA levels. Neurons primarily synthesize 2-AG 'on demand' in response to postsynaptic depolarization and Ca^{2+} influx, activation of $G_{q/11}$ coupled GPCRs, and even more robustly in response to simultaneous Ca^{2+} influx and $G_{q/11}$

activity (Kreitzer and Regehr 2001, Ohno-Shosaku et al. 2001, Kano et al. 2009, Hashimotodani et al. 2013, Ramikie et al. 2014). Several biochemical pathways that can lead to the synthesis of 2-AG have been described, although most evidence indicates that the activity of diacylglycerol lipase α (DAGLα) is required for 2-AG mediated retrograde synaptic inhibition, its primary mode of action in the CNS (Stella et al. 1997, Bisogno et al. 2003, Piomelli 2003, Jung et al. 2007, Tanimura et al. 2010, Oudin et al. 2011, Fonseca et al. 2013, Aaltonen et al. 2014). DAGLα catalyzes the hydrolysis of diacylglycerols (DAG) into monoacylglycerols. It is hypothesized that the primary precursor for 2-AG is the DAG, 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG), which is produced by the phospholipase Cβ (PLCβ)-mediated cleavage of PIP_2, a ubiquitous component of the plasma membrane (Aaltonen et al. 2014). SAG is suggested to be the major precursor for 2-AG synthesis because it is one of the most abundant DAGs in the brain and it is significantly decreased in DAGLα overexpressing rat neuroblastoma derived cells (Jung et al. 2007). Further evidence for this will be presented in Chapter 2. The PLCβ-DAGLα pathway likely controls 'on demand' synthesis of 2-AG as PLCβ requires Ca^{2+} and can be stimulated by the activation of $G_{q/11}$ coupled metabotropic receptors including metabotropic glutamate receptors (mGluR 1 and 5) and muscarinic acetylcholine receptors which have both been shown to enhance eCB-mediated synaptic signaling (Maejima et al. 2001, Kim et al. 2002, Ohno-Shosaku et al. 2002, Ohno-Shosaku et al. 2003, Fukudome et al. 2004, Ramikie et al. 2014). Additionally, it has been demonstrated that PLCβ activation and 2-AG signaling are potentiated to a similar degree by simultaneous depolarization and $G_{q/11}$-coupled receptor activation, suggesting that PLCβ may act as a 'coincidence detector' that amplifies 2-AG production when both signals coincide (Hashimotodani et al. 2005).

Two diacylglycerol lipase isoforms (DAGL α and β) have been cloned, differing primarily by DAGLα's longer C-terminal tail, which likely facilitates interactions with scaffolding proteins (Bisogno et al. 2003, Jung et al. 2007, Oudin et al. 2011). In mature neurons, DAGLα is prominently expressed in dendrites immediately adjacent to CB1R expressing axon terminals while DAGLβ is expressed more broadly in peripheral tissues (Bisogno et al. 2003, Katona et al. 2006, Yoshida et al. 2006, Uchigashima et al. 2007). Mice lacking DAGLα produce up to 80% less 2-AG in the CNS and exhibit impaired CNS 2-AG signaling while mice lacking DAGLβ have less than a 50% reduction in brain 2-AG content and no impairment in 2-AG mediated synaptic signaling (Gao et al. 2010, Tanimura et al. 2010). These data indicate that DAGLα is largely responsible for 2-AG synthesis in the context of retrograde synaptic signaling (Oudin et al. 2011). Altogether, the evidence strongly suggests that the PLCβ-DAGLα pathway plays an important role in the biosynthesis of 2-AG at synapses. However, the effect of PLC and DAGL inhibitors on 2-AG mediated synaptic signaling is not always consistent indicating that regulation of synaptic 2-AG is not likely to be so simple (Safo and Regehr 2005, Edwards et al. 2006, Hashimotodani et al. 2008, Min et al. 2010, Zhang et al. 2011). As such, further investigation of the other proposed biosynthetic pathways, which have been largely ignored in recent years, is necessary (Ueda et al. 1993, Tsutsumi et al. 1994, Nakane et al. 2002).

While there are also several biochemical pathways implicated in 2-AG degradation, approximately 98% of 2-AG is hydrolyzed into arachidonic acid and glycerol by serine hydrolases, with the majority of this activity (~85%) in the CNS attributed to monoacylglycerol lipase (MAGL) (Dinh et al. 2002, Blankman et al. 2007, Hoover et al. 2008, Hermanson et al. 2013). Two additional serine hydrolases, ABHD6 and ABHD12, account for a further 13% of 2-AG hydrolysis in rat brain membrane preparations (Blankman et al. 2007). Interestingly, reduced

activity of each of these serine hydrolases has distinct effects: ABHD6 inhibition reduces epileptic activity and the negative impact of traumatic brain injury (Tchantchou and Zhang 2013, Naydenov et al. 2014); mutations in ABHD12 lead to a neurodegenerative disease called PHARC (Fiskerstrand et al. 2010); and MAGL inhibition has a host of effects including antidepressant and anxiolytic actions which will be discussed further in other sections of this chapter (Fiskerstrand et al. 2010, Kinsey et al. 2011, Nomura et al. 2011, Kinsey et al. 2013, Tchantchou and Zhang 2013, Naydenov et al. 2014, Zhong et al. 2014). Knockdown of MAGL in cultured cells enhances the accumulation of 2-AG following application of ionomycin, which stimulates the production of 2-AG by elevating intracellular Ca^{2+} (Dinh et al. 2004). Additionally, immunodepletion of MAGL from rat brain homogenate results in a significant reduction in 2-AG hydrolysis (Dinh et al. 2004). Whereas DAGLα is expressed postsynaptically, MAGL is co-expressed with CB1R in presynaptic compartments allowing for tight coupling of synthesis, retrograde signaling, and degradation of 2-AG (Gulyas et al. 2004). Genetic deletion and chronic high-dose MAGL inhibition lead to extreme elevations in 2-AG and CB1R desensitization, but chronic low dose MAGL inhibition maintains anxiolytic and antidepressant efficacy in mice without causing CB1R downregulation (Schlosburg et al. 2010, Pan et al. 2011, Kinsey et al. 2013). Importantly, both AEA and 2-AG signaling can interact with the HPA axis stress response suggesting a clear potential for eCB signaling to directly modify the effects of stress on affective function. For a review of these interactions see Hill and Tasker 2012.

Stress Exposure is a Profound Risk Factor for Affective Dysfunction

Both acute and chronic stress elicit a multitude of functional changes in the CNS including modifications of neuronal proliferation, morphology, plasticity, and circuitry. These

changes, in general, elicit behavioral responses (arousal, vigilance, avoidance, etc.) that represent an attempt to adapt to environmental pressure but these responses can become maladaptive. Indeed, physical and psychosocial stress have repeatedly been associated with subsequent affective disorder diagnoses (McLaughlin et al. 2010, Boschloo et al. 2014, Spinhoven et al. 2014, van Loo et al. 2015, Biaggi et al. 2016, Mullins et al. 2016). Additionally, many of the same brain regions that exhibit altered function or volume in affective and trauma-related disorders are responsive to stress in humans, in particular: the amygdala, hippocampus, and medial prefrontal cortex (mPFC) (Buijs and Van Eden 2000, Tillfors et al. 2002, Liberzon et al. 2007, Drevets et al. 2008, Pruessner et al. 2008, Milad et al. 2009, Hartley and Phelps 2010, Kim et al. 2011, Veer et al. 2011, Calhoon and Tye 2015). These regions are components of the limbic system which primarily serves to process and respond to emotionally salient stimuli, including stressors. The overarching hypothesis has been that the amygdala combines sensory input from the brainstem and periphery with contextual information from the hippocampus and information about previous experiences from the mPFC to produce an overall judgement about the salience and valence of stimuli. The amygdala then guides behavioral responses to stimuli via its outputs to the hypothalamus (HPA axis), periaqueductal gray (freezing responses), etc. Both the mPFC and hippocampal inputs have been generally suggested to have an inhibitory effect on the amygdala, although this is likely to be an oversimplification (Kim et al. 2011, McDonald and Mott 2017). This section will focus on establishing a role for the amygdala, mPFC, and hippocampus in stress responses and affective dysfunction in humans while details of the stress response itself will be elaborated in the next section.

Neuroimaging studies have consistently shown hyperactive amygdala responses combined with hypoactive mPFC responses to negative stimuli in affective disorders. In PTSD

patients this is consistent with the hypothesis that many PTSD symptoms stem from inappropriate, or overgeneralized activation of the stress response (Yehuda and LeDoux 2007, Liberzon and Sripada 2008, Ressler 2010). One case study has shown that a patient with PTSD demonstrated dramatic improvement following deep brain stimulation for inhibition of the basolateral amygdala (BLA) (Langevin et al. 2016). Amygdala hyperactivity and prefrontal cortex hypoactivity have also been consistently demonstrated in MDD (Almeida et al. 2009, Delvecchio et al. 2012, Hamilton et al. 2012, Phillips et al. 2015). Additionally, 6-8 weeks of SSRI treatment of patients with MDD reduces amygdala responsivity to negative images and increases resting state cortico-amygdala connectivity (Sheline et al. 2001, Anand et al. 2007, Victor et al. 2010). Postmortem tissue from MDD patients shows decreased immediate early gene expression in the PFC, consistent with hypoactivity, and deep brain stimulation of the anterior cingulate cortex has shown promise in treatment-resistant depression (Covington et al. 2010). Some studies have even reported that direct transcranial magnetic stimulation of the PFC can reduce depressive symptoms, although others have been less successful (Kekic et al. 2016). MDD is also consistently associated with reduced mPFC gray matter and reduced hippocampal volume in some patient cohorts and smaller hippocampal volume may be a risk factor for PTSD (Rauch et al. 2006, Drevets et al. 2008). These changes in volume, activity, and connectivity of the hippocampus, amygdala, and mPFC are at least partly recapitulated in various rodent models that use stress exposure to produce anxiety- and depressive-like phenotypes and will be discussed in more detail in subsequent parts of this introduction.

The amygdala, mPFC, and hippocampus also strongly respond to, or modulate their response, after stressful stimuli in humans. Long recognized as a regulator of fear responses and associative fear learning, the amygdala is activated by stressful stimuli and the ability to respond

to threat-predictive cues is lost in amygdala-lesioned patients (LaBar et al. 1998, Funayama et al. 2001, Whalen et al. 2001, Phan et al. 2002). Some subregions of the mPFC show increased activity, while others show decreased activity, during stress, which is consistent with rodent data indicating that specific subregions activate while others inhibit fear-related behavior (Wang et al. 2005, Kumar et al. 2015). For example, stress-induced activation of the mPFC is inversely correlated with amygdala activation and cortisol release, while stress-induced activation of more lateral PFC is positively associated with cortisol (Kern et al. 2008). Additionally, acute stress reduces reward-related activation of the mPFC (Ossewaarde et al. 2011) and after acute stress exposure, the hippocampus is more activated by fearful faces than neutral faces during a recall task (Li et al. 2014). Interestingly, multiple studies have shown that early life stress is associated with decreased hippocampal volumes, particularly in patients with affective dysfunction (Woon and Hedges 2008, Hanson et al. 2015). Many other brain regions have been shown to respond to stress, but these three have been inextricably linked to affective disorders and will be the primary regions discussed herein.

Stages and Consequences of the Stress Response

In order to determine how stress can function as a risk factor for affective dysfunction, the stress response must first be understood. A stressor is a physical or psychological stimulus that is, or is perceived as, a threat to homeostasis. Exposure to a stressor elicits biological responses that, in general, facilitate a resolution (fight or flight) and then counteract the effects of the stressor and return the biological system to homeostasis. Briefly, sensory information from the periphery is routed through the brainstem, thalamus, and sensory cortices for initial processing and then sent to limbic brain regions, like the amygdala and nucleus accumbens, for

assignment of salience (importance) and valence (good or bad), and the prefrontal cortex and hippocampus for integration with context and previous experience (Likhtik et al. 2005, Hermans et al. 2014, Namburi et al. 2015, Namburi et al. 2016). Based on the integration of information from all of these limbic regions, if a stimulus is determined to be a threat, the HPA axis will be activated (Janak and Tye 2015).

Early investigations of exposure to a broad range of stressors produced the hypothesis of a "General Adaptation Syndrome" comprised of three stages: alarm (48 hours), resistance (1-3 months), and exhaustion (Selye 1938, Selye 1998). Overall this syndrome is understood as an adaptive process, though its continual activation and entry into the third stage produces negative physiological consequences. During the alarm stage, under the regulation of the limbic system, the autonomic nervous system and the HPA axis are triggered; for a review of the autonomic nervous system (ANS) and HPA axis responses to stress see Ulrich-Lai and Herman 2009. With continued or repeated stress exposure, the resistance stage begins during which there is a prolonged activation of these autonomic and HPA signaling mechanisms which can, for a time, allow normal functioning despite altered physiology (Selye 1998). This process has been termed allostasis, which means "achieving stability through change" (McEwen 2006). Finally, if the stressor persists, the animal enters the exhaustion stage in which the protracted activation of stress-responsive signaling pathways becomes deleterious (Selye 1998). This has also been described in terms of "allostatic overload", indicating that a prolonged stress response requires chronically increased effort of multiple systems which then accumulate more damage than normal and may eventually malfunction (McEwen 2006). For example, in addition to their role in affective dysfunction, chronically elevated glucocorticoids can produce or exacerbate symptoms of cardiovascular disease (Grippo et al. 2002), reduce cognition (McEwen 2001,

Abush and Akirav 2013), and increase inflammatory responses relevant for colitis, cancer, and diabetes (Stetler and Miller 2011, Peters et al. 2012, Vicario et al. 2012, Sanghez et al. 2013).

Providing an additional layer of control, cortico-limbic signaling mechanisms continue to interact with these stress response systems. These cortico-limbic circuits can elicit either habituation or sensitization depending on the predictability and intensity of the threat stimuli (Bhatnagar and Dallman 1998, Kamprath and Wotjak 2004). The limbic system's strong regulatory influence over the ANS and HPA axis suggests that an imbalance in limbic signaling could produce inappropriate activation or maintenance of the stress response contributing to the development of mood, anxiety, and trauma-related disorders. A better understanding of the signaling associated with acute and chronic stress responses in the limbic system may lead to novel approaches for reducing or preventing affective dysfunction precipitated by stress exposure.

Cortico-limbic Regulation of Stress, Fear, and Anxiety

Importantly, several factors have been shown to reduce the negative impact of stress exposure including positive affect and outlook (Pressman and Cohen 2005, Steptoe et al. 2005), physical activity (Vankim and Nelson 2013, Silverman and Deuster 2014), and social support (Cohen and Wills 1985, Seeman et al. 2002, Hackett et al. 2012). It has been hypothesized that these factors represent differences in, or act by modifying, top-down control over the stress response. Many studies have attempted to utilize conscious emotional regulation to modify emotional responses, and some success has been shown in using neurofeedback to increase prefrontal cortex activation and decrease amygdala activation, but the efficacy of these efforts within patient populations remains unclear (Ray et al. 2005, Phelps 2006, Urry et al. 2006, Paret

et al. 2016, Zotev et al. 2016). Most details about cortico-limbic control of stress-induced fear and anxiety responses comes from rodent studies, including evidence for stress-induced dysfunction in the amygdala, mPFC, and hippocampus and, perhaps most importantly, altered synchrony between the three (Likhtik and Gordon 2014, Likhtik et al. 2014). The amygdala and hippocampus are reasonably conserved between rodents and humans in general structure, connectivity, and function (Janak and Tye 2015). There has been some controversy over using rodent mPFC to draw conclusions about human mPFC because it is a much simpler structure and a smaller portion of the brain in rodents (Uylings and van Eden 1990). However, several subregions do seem to be analogous to human prefrontal cortical structures and the rodent PFC has been clearly shown to regulate affective function (Likhtik et al. 2005, Sierra-Mercado et al. 2011, Hubner et al. 2014).

Stress induces changes in the amygdala, mPFC, and hippocampus of rodents which are consistent with the functional responses to stress in humans described above, as well as consistent with structural and functional changes seen in affective disorders. For example, chronic stress has been consistently shown to reduce adult neurogenesis in both the subventricular zone and the dentate gyrus of the hippocampus, which could represent the underlying mechanism for the volumetric changes seen in human hippocampus (Tanti et al. 2013, Morais et al. 2014). The precise implications of this change are not fully understood, but it can be prevented and reversed by antidepressants (Boldrini et al. 2009, Pinnock et al. 2009, Su et al. 2009). Chronic stress also reduces dendritic branching of adult neurons in the hippocampus and the medial prefrontal cortex, but can increase or decrease dendritic growth in the amygdala depending on the predictability of the stressor (Shors and Servatius 1997, Conrad et al. 1999, McEwen and Magarinos 2001, Wellman 2001, Vyas et al. 2002, Drevets et al. 2008, Radley et

al. 2008). Both acute and chronic stress increase excitatory drive to the BLA in rodents (Kavushansky and Richter-Levin 2006, Mitra and Sapolsky 2008, Rosenkranz et al. 2010). Interestingly, many of the effects of chronic stress are phenocopied in germline CB1R knockout mice: anxiety-like behavior, impaired hippocampal neurogenesis, reduced dendritic complexity in the PFC, and increased dendritic length in the BLA (Hill et al. 2011). Acute pharmacological blockade of CB1R also induces anxiety- and depressive-like behaviors in multiple animal models and clinical trials investigating CB1R antagonists as weight-loss therapeutics were discontinued due to increased incidence of anxiety, depression, and suicidality (Christensen et al. 2007, Steiner et al. 2008, Dubreucq et al. 2012, Gamble-George et al. 2013). Direct CB1R agonists are often reported to have antidepressant and anxiolytic effects, but some studies find that CB1R agonists can be anxiogenic, typically at higher doses (Rey et al. 2012).

eCB Signaling in Cortico-limbic Regulation of Stress, Fear, and Anxiety

As mentioned above, the amygdala is one of the key limbic structures whose activity promotes HPA axis activation (Hill et al. 2010). Local administration of a CB1R agonist into the BLA reduces activation of the HPA stress axis while CB1R antagonism increases neuronal activation in the BLA and corticosterone release in response to stress exposure (Steiner et al. 2008, Ganon-Elazar and Akirav 2009, Hill et al. 2010). Some conflicting evidence has been reported, including that increased 2-AG in the BLA impairs fear extinction (Hartley et al. 2016). This may suggest that 2-AG's role in regulating emotional learning is distinct from its role in regulating affective phenotypes. As discussed above, 2-AG production and signaling is typically activity-dependent in that it usually occurs as a result of postsynaptic depolarization, $G_{q/11}$-coupled receptor activation, or both, so its production follows increased neuronal activation.

Both acute and chronic stress exposure have been shown to increase neuronal activity and 2-AG in multiple limbic brain regions including the amygdala (Morena et al. 2016). In addition to elevating amygdala 2-AG, stress enhances Fos immunoreactivity of BLA pyramidal neurons, but not interneurons (Patel et al. 2005, Patel et al. 2005, Patel et al. 2009, Sumislawski et al. 2011). This suggests that increased 2-AG may be acting to buffer a specific increase of glutamatergic signaling in the BLA following stress, but the impact of stress on 2-AG-mediated suppression of glutamatergic signaling in the BLA has not been sufficiently examined.

CB1R activity also regulates signaling relevant to anxiety-like behavior in the mPFC and ventral hippocampus (vHIP). Interestingly, not only does 2-AG within the mPFC enhance emotional learning but CB1R activation in the BLA increases mPFC activity and enhances fear conditioning (Laviolette and Grace 2006, Tan et al. 2011, Lisboa et al. 2015). Several studies have demonstrated a role for AEA signaling in the vHIP regulating anxiety-like behavior (Roohbakhsh et al. 2009, Campos et al. 2010). Evidence for anxiolytic 2-AG signaling in the vHIP is sparser, but it has been shown that 2-AG in the vHIP can reduce both fear and pain responses (Rea et al. 2014). Further highlighting the importance of investigating eCB-mediated suppression of glutamatergic signaling in the context of stress, conditional CB1R deletion from forebrain glutamatergic neurons eliminates the anxiolytic and antidepressant effects of low-dose CB1R agonists (Kamprath et al. 2009). Most of these experiments, while they have clearly demonstrated an important role for 2-AG-CB1R signaling in the BLA, mPFC, and vHIP in regulating affective-like behavior, have utilized broad manipulations of eCB signaling that affect all the glutamatergic inputs to each brain region. Optogenetic studies investigating the microcircuitry of various brain regions, like the amygdala, have made it increasingly clear that

moving forward it will be important to investigate the effects of manipulations on distinct inputs to specific regions (Janak and Tye 2015).

Inter-individual Differences in Risk for Stress-related Affective Diagnoses

Given that not everyone who experiences stressful or traumatic life events develops affective or trauma-related disorders, we and others have become very interested in discovering factors that provide resilience to stress-induced dysfunction. One of the simplest explanations would be genetic polymorphisms in genes that regulate affect. Indeed, one of the first polymorphisms reported to interact with stressful life events was in the promoter region for the serotonin transporter (Caspi et al. 2003). A host of studies have attempted to identify additional genes that increase risk for affective disorder in the context of stressful life events, but taken together there are very few consistent candidate genes. Particularly relevant here, two studies have implicated polymorphisms in the CB1R with MDD and suggested an enhanced risk in combination with stressful life events (Juhasz et al. 2009, Agrawal et al. 2012, Mandelli and Serretti 2013). However, numerous studies investigating the heritability of affective disorders have indicated that the genetic component is likely to involve complex interactions between multiple genes (Drevets et al. 2008). In line with the RDoC initiative described above, it has been hypothesized that this complexity may indicate that specific combinations of genetic differences produce particular constellations of symptoms within this set of heterogeneous disorders, although there is currently little direct evidence for this hypothesis (Power et al. 2017).

More recently, the increased understanding of epigenetics has allowed an expansion on the basic concept of gene by environment interactions. Substantial evidence indicates that stress has profound effects on the epigenome (Mill and Petronis 2007). Given that differential

responses to stress are apparent even in inbred lines of mice, like the commonly used C57Bl/6J strain, epigenetic studies may provide long elusive answers about individual differences in genetically similar populations. Early life stress clearly affects the epigenome in a multitude of ways that may increase risk for affective dysfunction on their own or in combination with additional environmental factors (McGowan et al. 2009, Elliott et al. 2010, Konishi et al. 2010, Zhang and Meaney 2010, Weder et al. 2014, Kundakovic and Champagne 2015). Epigenetic changes in the mPFC and hippocampus are also numerous and long-lasting following stress exposure in adult rodents (Mychasiuk et al. 2016). While the methods for specifically measuring epigenetic changes are not yet commonplace, there is an increasing appreciation for measuring molecular differences in individuals with differential responsivity to the same stimulus. For example, several models relevant to MDD and at least one that models aspects of PTSD have been developed in which rodents are characterized by their behavioral responses to stress as either stress-susceptible (or vulnerable) or stress-resilient (or resistant). The specific definitions of susceptibility differ between paradigms (several of which will be described in the next section), but using this approach and comparisons between stress-naïve, -resilient, and -susceptible individuals allows for a distinction to be made between adaptive and maladaptive stress-induced molecular changes. This approach could lead to the identification of resilience factors that may be druggable targets or biomarkers for affective disorders (Yehuda et al. 2006).

Animal Models of Individual Differences in Stress-induced Affective Dysfunction

Initial models of stress-related psychiatric disease utilized simple control versus stress comparisons in the attempt to elucidate how stress produces affective dysfunction. However, this approach cannot distinguish between stress responses that promote adaptation to and survival of

environmental changes and those that have become maladaptive. Over the last two decades, research that utilizes the innate variability in stress responsivity to identify factors that promote resilience to stress-induced dysfunction has exploded (Krishnan 2014). The comparison of innately stress-resilient and -susceptible individuals is relatively new but the approach provides a significant advance over simple comparison of naïve and stressed groups since some stress-induced changes may be primarily adaptive. Thus, the attempt to reverse or prevent all stress-related changes is misguided. Instead, enhancing responses that are found to promote resilience may provide even more effective protection against stress-induced dysfunction than methods that reverse specific stress-induced changes.

Several rodent models of MDD have been developed that utilize chronic stress exposure to elicit depressive-like phenotypes (they also elicit anxiety-like behavior, but are typically described as models of MDD) including chronic unpredictable stress (CUS), chronic social defeat stress (CSDS), and learned helplessness (LH). All of these models have produced incredible insights about how stress affects the central nervous system and the molecular mediators of various antidepressant interventions, although the comparison of innate individual differences has been most prevalent in CSDS. Importantly, these studies have conclusively demonstrated that resilience is not merely the absence of negative changes induced by stress exposure, but is an active process composed of a plethora of adaptive changes (Krishnan et al. 2007). These models of inter-individual differences in stress responsivity have produced substantial advances in understanding the role that specific signals and circuits play in regulating affective function either positively or negatively in the context of stress.

The CUS paradigm involves several weeks of exposure to an unpredictable rotation of multiple, distinct, stressful stimuli which prevents adaptation to any particular stressor. CUS has

long been used as a model of depression, but its use to compare resilient and susceptible individuals based on the variable development of depressive phenotypes is relatively recent. As such, the model has produced many insights about chronic stress and antidepressant effects but fewer about how stress responses differ in susceptible and resilient individuals. For example, adolescent predictable stress promotes resilience to CUS in adulthood (Suo et al. 2013); ketamine treatment reverses CUS-induced reduction of glutamate transporter expression in the hippocampus (Zhu et al. 2017); and CUS corticosterone-dependently reduces tonic GABA inhibition in the amygdala (Liu et al. 2014). CUS also changes CB1R expression in the vHIP in a sex-dependent manner (Reich et al. 2009). The few studies that have also specifically examined variable responsivity to CUS have shown that increases in prolactin are associated with resilience to CUS (Faron-Gorecka et al. 2014), and trait anxiety and exploratory behavior predict differential behavioral deficits following CUS (Castro et al. 2012). The Strekalova lab has utilized the development of anhedonia to characterize individuals as either susceptible or resilient (~30-50%) to CUS and demonstrated that while several phenotypes are associated with anhedonia, anxiety-like phenotypes are exhibited equally in both groups (Strekalova et al. 2004, Strekalova and Steinbusch 2010). From these studies, it is clear that CUS can be used to examine differential effects of stress on resilient and susceptible individuals, but this approach has not yet been incorporated widely.

The CSDS model has been especially well adapted for examining differential responsivity to stress and manipulating circuits and signals that promote resilience. In CSDS, repeated exposure to an aggressor elicits depressive-like phenotypes specifically in individuals that develop a social-avoidance phenotype (approximately 1/3 of the population) (Krishnan et al. 2007, Golden et al. 2011, Krishnan 2014). Some of the first studies to use this approach

demonstrated important roles for dopamine signaling in the nucleus accumbens. Recent studies have expanded on these findings by showing that while susceptibility is associated with hyperactive ventral tegmental area dopamine neuron activity, further increasing dopamine neuron activity promotes resilience (Berton et al. 2006, Krishnan et al. 2007, Friedman et al. 2014). Other groups have also shown important roles for the amygdala and mPFC in the CSDS model: susceptibility is associated with increased BLA activity (Chou et al. 2014); *in vivo* electrophysiological recordings indicate individual differences in the intrinsic firing rate of mPFC neurons predict stress-resilience and increased mPFC-amygdala theta coupling during stress exposure is associated with resilience (Kumar et al. 2014). One important limitation of this paradigm, given that MDD is more common in females than males, is that it cannot easily be used to study depressive-like phenotypes in females due to the low levels of aggression in female rodents (Krishnan 2014).

The LH paradigm has also been used to compare the differential stress responses of susceptible and resilient individuals with approximately 35% of rats exhibiting a resilient phenotype (Yang et al. 2015). Studies using this paradigm have shown that hippocampal brain-derived neurotrophic factor is more inhibited by histone deacetylase 5 (HDAC) in helpless than resilient individuals but this is reversed by both HDAC inhibition and SSRI treatment (Su et al. 2016); parvalbumin expressing interneuron activation in the mPFC promotes resilience (Perova et al. 2015); antidepressant-like effects of ketamine may be mediated through the infralimbic mPFC (Shirayama and Hashimoto 2016); and activation of the vHIP abolishes depressive phenotypes in helpless individuals (Seo et al. 2016).

All of these models utilize chronic stress exposure, which may reduce the detection of early stress-induced changes that could be harnessed to promote resilience. Additionally,

equivalent levels of anxiety-like behavior are seen in stressed resilient and susceptible groups, so in general these models cannot currently be used to examine molecular changes that are specifically important for the development of stress-induced anxiety-like phenotypes. One recent CSDS study, however, did demonstrate that while anxiety-like phenotypes do not covary with social avoidance, individual differences in anxiety-like responses can be examined using a clustering analysis (Bosch-Bouju et al. 2016). This adaptation of the CSDS paradigm suggests that, with fairly minor modifications, any of these models *could* be utilized to investigate susceptibility to stress-induced anxiety-like phenotypes. The examples herein are a small sample of the brain regions and signaling pathways implicated in either susceptibility or resilience using these models of MDD. Hopefully this recent adaptation of CSDS and the studies described herein will pave the way for further discovery of both distinct and shared mechanisms of susceptibility to stress-induced depressive- and anxiety-like phenotypes. Currently, variability in the specifics of the chronic stress protocols used by different labs and conflicting results makes forming a cohesive picture of stress-induced changes that promote resilience extremely difficult, although they are largely consistent with changes seen in human patients. It is clear, however, that in each of these models both reward- and fear-related circuitry play important roles and that these paradigms have not adequately modeled susceptibility to stress-induced anxiety-like phenotypes. Although there have also been some efforts to model susceptibility to additional domains of PTSD symptomology, there is a general dearth of research investigating susceptibility to the development of anxiety phenotypes (Tsoory et al. 2007).

Evidence for Cannabinoid Regulation of Stress-induced Affective Dysfunction

As mentioned above, one recent effort to address this gap in our understanding of stress-

induced anxiety mechanisms used a clustering analysis to characterize mice as anxious or non-anxious (~50% of individuals) following CSDS. This study showed that impaired eCB signaling in the nucleus accumbens (NAc) was associated with the anxious phenotype (Bosch-Bouju et al. 2016). While few studies have investigated the role of cannabinoid signaling in the context of promoting resilience to stress-induced affective dysfunction it has been shown that: CB1R activation in the mPFC promotes active coping responses to CUS (McLaughlin et al. 2013); CUS reduces 2-AG signaling in the hippocampus while pharmacologically enhancing 2-AG prevents the emergence of CUS-induced depressive phenotypes and increases neurogenesis (Hill et al. 2005, Wang et al. 2014, Zhang et al. 2015); and transcranial magnetic stimulation with antidepressant effects increases CB1R expression in the hippocampus (Wang et al. 2014). Conversely, a gene expression study comparing resilient and susceptible mice showed decreased CB1R transcript in the hippocampus of individuals resilient to learned helplessness (Yang et al. 2015). Despite some conflicting results, overall increases in eCB signaling have been linked to reducing anxiety- and depressive-like phenotypes (Hill and Patel 2013). Low dose inhibition of 2-AG degradation with the MAGL inhibitor JZL-184 is consistently anxiolytic in the marble-burying test, an animal model of obsessive-compulsive-like anxiety behavior, although in most behavioral tests MAGL inhibition is largely ineffective except after stress exposure or in aversive contexts (Kinsey et al. 2011, Sciolino et al. 2011, Sumislawski et al. 2011). For example, in another anxiety test, the elevated plus maze, the proven anxiolytic diazepam increases open arm time (indicative of reduced anxiety) in both dim and bright lighting conditions (Sciolino et al. 2011). JZL-184 treatment, however, only increases open arm time under the more aversive bright lighting condition (Sciolino et al. 2011). This could indicate that acute environmental aversiveness modulates the responsivity of the eCB system although there is

no direct evidence for a mechanism underlying this phenomenon. The same phenomenon is seen when mice are treated with a FAAH inhibitor to increase AEA, suggesting either that this aversiveness-enhanced responsivity is downstream of the eCB ligand, or that a common mechanism is able to regulate the signaling of both eCB ligands (Haller et al. 2009). Enhancing 2-AG signaling during chronic stress exposure reduces the anxiogenic and depressant effects of both chronic homotypic and chronic heterotypic stress (Sumislawski et al. 2011, Zhong et al. 2014). Direct CB1R agonists tend to exhibit biphasic effects with low doses being anxiolytic or antidepressant (mediated by CB1R on glutamatergic terminals) and high doses producing the characteristic cannabinoid 'triad' (catalepsy, hypothermia, and thermal antinociception) and anxiety-like behavior (mediated by CB1R on GABAergic terminals) (Rey et al. 2012, Grim et al. 2016).

Interestingly, peripheral 2-AG is significantly reduced in women with MDD and patients with PTSD as compared to healthy controls suggesting that impairments in 2-AG may be involved in either susceptibility or pathogenesis of these disorders, although it is not known how peripheral eCB levels are related to central eCB levels (Hill et al. 2008). In addition, many patients who are diagnosed with affective disorders report social withdrawal or isolation and a recent study has shown that chronic isolation stress significantly reduces circulating 2-AG in humans (Yi et al. 2016). While these correlations are compelling, prior to the studies described in Chapter 2, there was no causal evidence demonstrating that specific impairment of 2-AG signaling elicits anxiety- or depressive-like phenotypes. Although DAGLα knockout mice have existed for some time, and it had been shown that adult neurogenesis was impaired suggesting a strong possibility that DAGLα KOs would exhibit anxiety and depressive phenotypes similar to CB1R KOs and chronically stressed mice, very little relevant behavioral testing had been

reported (Gao et al. 2010, Hill et al. 2011). This shortage of data led directly to the studies reported in Chapter 2.

As established above, amygdala hyperactivity is a common feature of affective disorders. Studies indicate that acute and chronic stress elevate 2-AG content and signaling within the amygdala. This elevation of 2-AG may represent a partial attempt to buffer stress-related glutamatergic drive into the amygdala and contribute to habituation to repeated homotypic stress. If true, 2-AG signaling in the amygdala could act as a resilience factor. In addition, impaired 2-AG signaling may contribute to the pathogenesis of these stress-related psychiatric disorders. Restoration of 2-AG signaling may constitute an effective treatment for major depression, anxiety disorders, and other stress-related pathologies.

CHAPTER II

GENETIC DISRUPTION OF 2-ARACHIDONOYLGLYCEROL SYNTHESIS REVEALS A KEY ROLE FOR ENDOCANNABINOID SIGNALING IN ANXIETY MODULATION

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ABSTRACT

Endocannabinoid (eCB) signaling has been heavily implicated in the modulation of anxiety, depressive behaviors and emotional learning. However, the role of the most abundant endocannabinoid 2-arachidonoylglycerol (2-AG) in the physiological regulation of affective behaviors is not well understood. Here we show that genetic deletion of the 2-AG synthetic enzyme diacylglycerol lipase α (DAGLα) in mice reduces brain, but not circulating, 2-AG levels. DAGLα deletion also results in anxiety-like and sex-specific anhedonic phenotypes associated with impaired activity-dependent eCB retrograde signaling at amygdala glutamatergic synapses. Importantly, acute pharmacological normalization of 2-AG levels reverses both phenotypes of DAGLα deficient mice. These data suggest 2-AG deficiency could contribute to the pathogenesis of affective disorders and that pharmacological normalization of 2-AG signaling could represent a novel approach for the treatment of mood and anxiety disorders.

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Introduction

Endogenous cannabinoid (eCB) signaling is mediated by cannabinoid receptors (type 1; CB1R, and type 2; CB2), which are activated by several endogenous ligands including anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Piomelli 2003, Kano et al. 2009). Over the past decade eCB signaling has been implicated in the regulation of multiple physiological functions in the nervous system and periphery, including a primary role in the modulation of anxiety and depressive behaviors (Kathuria et al. 2003, Lutz 2009, Ruehle et al. 2012, Hill and Patel 2013). Specifically, pharmacological blockade or genetic deletion of CB1 receptors increases anxiety in multiple animal models and in humans under some conditions (Hill and Gorzalka 2004, Patel and Hillard 2006, Christensen et al. 2007, Moreira et al. 2009, Gamble-George et al. 2013). Consistent with these data, anxiety in various animal models is decreased by low doses of exogenous cannabinoids (Patel and Hillard 2006, Rey et al. 2012) and by pharmacological or genetic augmentation of eCB levels (Kathuria et al. 2003, Patel and Hillard 2006, Moreira et al. 2008, Rossi et al. 2010, Sciolino et al. 2011, Sumislawski et al. 2011, Hermanson et al. 2013). These data strongly suggest a key role for eCB signaling in the physiological regulation of anxiety and depressive behaviors.

2-AG is the most abundant eCB ligand in the brain and is the primary mediator of phasic eCB-mediated retrograde suppression of neurotransmitter release in the nervous system (Gao et al. 2010, Tanimura et al. 2010, Ohno-Shosaku et al. 2012). The synaptic signaling pools of 2-AG are synthesized and degraded primarily by the postsynaptic DAGLα and presynaptic monoacylglycerol lipase (MAGL), respectively, in the adult brain (Dinh et al. 2002). Several recent studies have demonstrated that pharmacological elevation of 2-AG signaling can reduce anxiety-like behaviors in animal models (Busquets-Garcia et al. 2011, Sciolino et al. 2011,

Sumislawski et al. 2011, Zhong et al. 2014), while some clinical studies have demonstrated reduced peripheral 2-AG levels in patients with PTSD and women with major depression compared to control subjects (Hill et al. 2008, Hill et al. 2013). Although these data suggest a close association between 2-AG signaling and affective pathology, a causal relationship between endogenous 2-AG signaling and the physiological expression of anxiety and depressive behaviors has not been demonstrated. Here we show *Dagla* knockout mice exhibit reductions in limbic 2-AG levels associated with anxiety-like behaviors and anhedonia, both of which are normalized by acute pharmacological normalization of 2-AG deficiency. These data provide causal evidence supporting 2-AG signaling deficiency as a key mechanism subserving development of affective pathology and support pharmacological 2-AG augmentation as a viable treatment approach for mood and anxiety disorders.

Results

Biochemical Validation of DAGL$\alpha^{-/-}$ mice

Homozygous *Dagla* knockout mice (DAGL$\alpha^{-/-}$) mice were generated as described in Experimental Methods and identified by standard PCR approaches, exhibiting the expected shift in size of amplification product associated with targeting cassette insertion upstream of exon 8 of the *Dagla* gene (Fig. 2.1A). Adult male and female DAGL$\alpha^{-/-}$ mice showed a complete lack of brain DAGLα protein expression ($p<0.0001$ for both sexes) as assessed by western blot analysis and reductions in enzymatic activity ($p<0.05$ for both sexes) confirming effectiveness of the gene disruption strategy (Fig. 2.1B-D). Mass spectrometry analysis revealed a significant reduction of forebrain 2-AG levels (male $p<0.05$, female $p<0.0001$; Fig. 2.1E). To provide insight into the specific diacylglycerol (DAG) species that may be DAGLα substrates *in vivo*, we analyzed

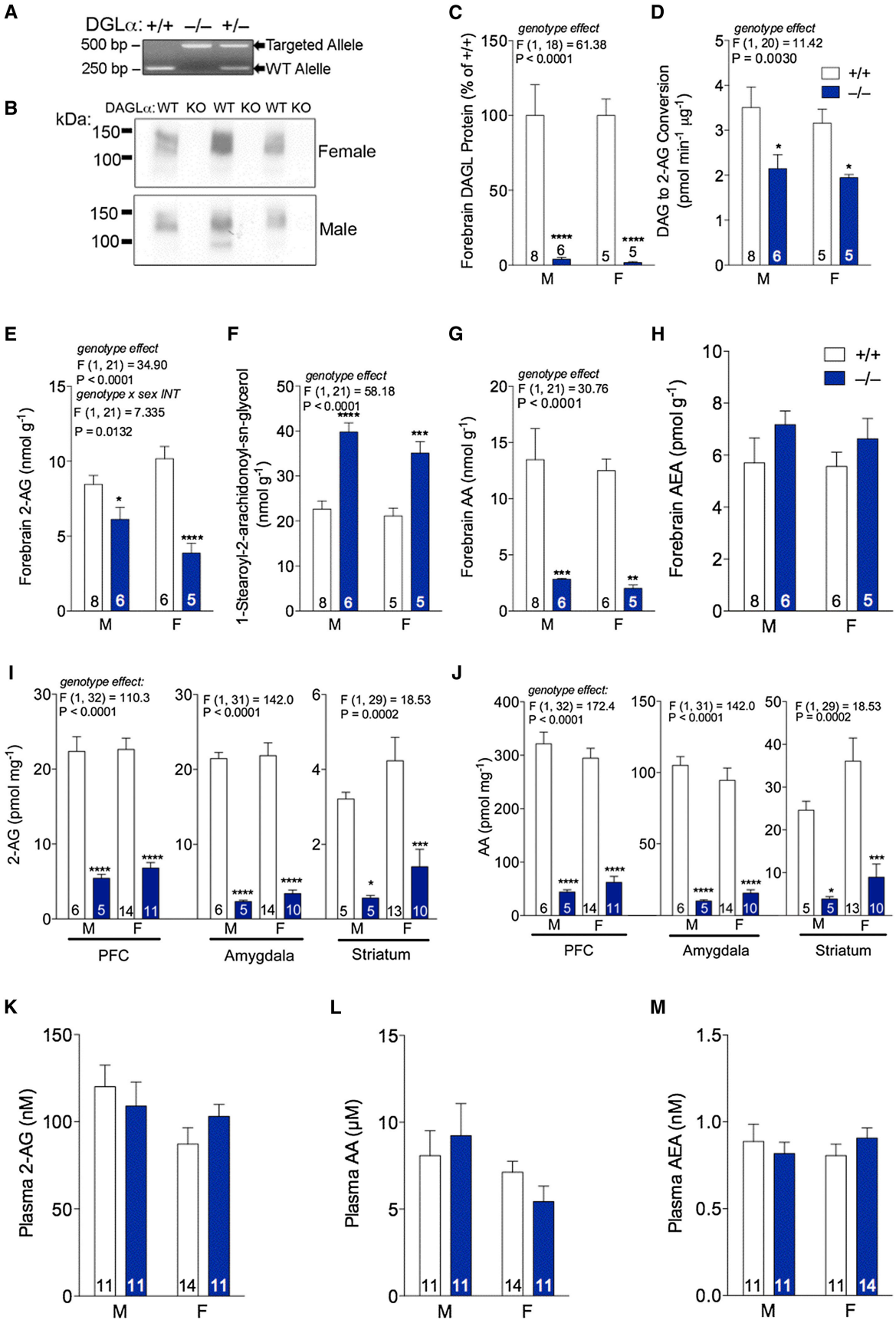
A
DGLα: +/+ –/– +/–
500 bp –
250 bp –
Targeted Allele
WT Alelle
B
kDa:
DAGLα: WT KO WT KO WT KO
150
100
Female
Male
C
genotype effect
F (1, 18) = 61.38
P < 0.0001
Forebrain DAGL Protein (% of +/+)
D
genotype effect
F (1, 20) = 11.42
P = 0.0030
DAG to 2-AG Conversion (pmol min-1 µg-1)
+/+
–/–
E
genotype effect
F (1, 21) = 34.90
P < 0.0001
genotype x sex INT
F (1, 21) = 7.335
P = 0.0132
Forebrain 2-AG (nmol g-1)
F
genotype effect
F (1, 21) = 58.18
P < 0.0001
1-Stearoyl-2-arachidonoyl-sn-glycerol (nmol g-1)
G
genotype effect
F (1, 21) = 30.76
P < 0.0001
Forebrain AA (nmol g-1)
H
Forebrain AEA (pmol g-1)
I
genotype effect:
F (1, 32) = 110.3
P < 0.0001
F (1, 31) = 142.0
P < 0.0001
F (1, 29) = 18.53
P = 0.0002
2-AG (pmol mg-1)
PFC
Amygdala
Striatum
J
genotype effect:
F (1, 32) = 172.4
P < 0.0001
F (1, 31) = 142.0
P < 0.0001
F (1, 29) = 18.53
P = 0.0002
AA (pmol mg-1)
PFC
Amygdala
Striatum
K
Plasma 2-AG (nM)
L
Plasma AA (µM)
M
Plasma AEA (nM)
M
F

Figure 2.1 Characterization and validation of DAGLα$^{-/-}$ mice (A) Conventional PCR gel showing the amplified products from primers that anneal to endogenous DNA sites that flank the targeting cassette, which was inserted upstream of exons 8. The resulting product in DAGLα$^{-/-}$ mice demonstrates the expected ~250 bp shift as a result of the successful insertion of the targeting cassette. (B) Representative western blot gel from male "M" and female "F" and DAGLα$^{-/-}$ forebrain, which is summarized in bar graph (C). (D) DAGL activity in forebrain extracts from male and female DAGLα$^{-/-}$ mice. (E-H) Forebrain levels of 2-AG, 1-steroyl-2-arachidonoyl glycerol (SAG), arachidonic acid (AA), and AEA in male and female DAGLα$^{-/-}$ and WT mice. (I-J) 2-AG and AA levels in the prefrontal cortex (PFC), amygdala, and striatum of male and female DAGLα$^{-/-}$ mice. (K-M) Plasma levels of 2-AG, AA, ad AEA in DAGLα$^{-/-}$ mice. * P < 0.05, ** P<0.01, *** P<0.001, **** P<0.0001 by Sidak's post hoc test. Error bars indicate SEM. See also Figure S1. (A-H BCS; I-J RJB & DJH; K-M RJB & BCS)

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levels of arachidonate-containing DAGs. The most abundant arachidonic acid (AA)-containing DAG detected, 1-stearoyl-2-arachidonoylglycerol (SAG), was increased in both male ($p<0.0001$) and female ($p<0.001$) DAGLα$^{-/-}$ forebrains (Fig. 2.1F), indicating its importance as a physiological substrate for DAGLα in the brain, and confirming previous work in cultured neuroblastoma cells (Jung et al. 2007). Several less abundant AA containing DAG species were also detected, some of which also displayed sex-specific changes in DAGLα$^{-/-}$ mice (Fig. S2.1A-J). In contrast, none of the non-AA-containing DAGs measured were different between genotypes of either sex (data not shown). Consistent with reductions in 2-AG levels, the primary hydrolytic metabolite of 2-AG, AA, was also reduced in DAGLα$^{-/-}$ mice (male $p<0.001$; female $p<0.01$; Fig. 2.1G). There were no changes in levels of the other major eCB, anandamide (Fig. 2.1H). Brain regional analysis revealed reductions in 2-AG and AA in the prefrontal cortex (PFC), amygdala and striatum of both male and female DAGLα$^{-/-}$ mice (Fig. 2.1I-J). Levels of the non-eCB monoacylglycerol, 2-oleoylglycerol (2-OG), were also significantly reduced in the PFC, amygdala and striatum of male and female DAGLα$^{-/-}$ mice (Fig. S2.1K). These data indicate DAGLα$^{-/-}$ mice have the expected alterations in DAGLα protein, enzymatic activity,

and 2-AG metabolism with reduced 2-AG levels, increased 2-AG precursor levels, and reduced levels of the 2-AG degradation product AA. Lastly, given the increasing interest in using peripheral eCB levels as biomarkers for neuropsychiatric diseases, we measured plasma 2-AG, AEA, and AA concentrations and found no changes in DAGL$\alpha^{-/-}$ mice relative to WT littermates (Fig. 2.1K-M). Thus, plasma 2-AG levels are not dependent on DAGLα under basal conditions.

DAGLα deletion increases anxiety-like behaviors

Having established that DAGLα deletion results in reduced central 2-AG levels, we next evaluated the effects of this 2-AG deficiency on anxiety- and depressive-like behaviors. Separate cohorts of male and female DAGL$\alpha^{-/-}$ mice and WT littermates were sequentially tested for a series of anxiety and depressive-like behaviors (Fig. 2.2). In the open-field assay, male ($p<0.01$) and female ($p<0.05$) DAGL$\alpha^{-/-}$ mice showed reduced vertical exploration without changes in total distance traveled indicating decreased exploratory drive without gross motor abnormality (Fig. 2.2A-B). Only female mice showed a reduction in percent center distance traveled ($p<0.05$) suggestive of enhanced anxiety-like behavior (Fig. 2.2A-B). In the light-dark box (L-D Box) test, both male ($p<0.01$) and female ($p<0.01$) DAGL$\alpha^{-/-}$ mice showed reduced exploratory distance traveled, reduced percent light-zone ambulation (males $p<0.001$ and female $p<0.01$), and reduced time spent in the light compartment (males $p<0.05$ and females $p<0.01$; Fig 2.2C-D). We next tested mice using a more ethologically-relevant behavioral assay, the novelty-induced hypophagia (NIH) test, which is highly sensitive to eCB modulation (Gamble-George et al. 2013). In this test, both male and female DAGL$\alpha^{-/-}$ mice showed an increase in feeding latency as demonstrated by a difference in the cumulative feeding latency distribution (males $p<0.0001$ and females $p<0.0001$ by K-S test), increased mean feeding latency (females $p<0.001$), and reduced consumption (males $p<0.05$ and females $p<0.05$; Fig 2.2E-F). To exclude the possibility

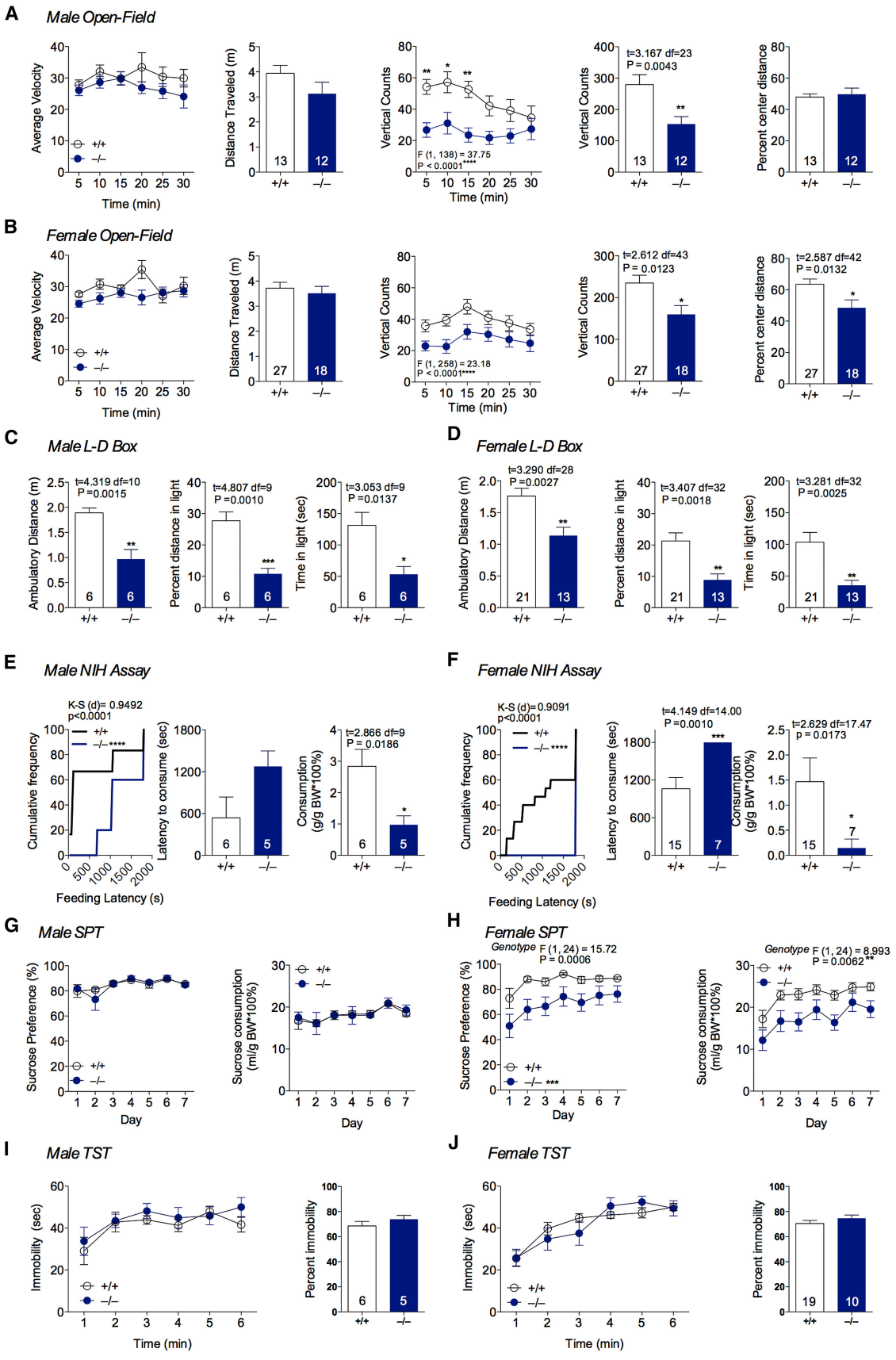
A
Male Open-Field
Average Velocity
Time (min)
+/+
–/–
Distance Traveled (m)
Vertical Counts
F (1, 138) = 37.75
P < 0.0001****
t=3.167 df=23
P = 0.0043
Percent center distance
B
Female Open-Field
F (1, 258) = 23.18
P < 0.0001****
t=2.612 df=43
P = 0.0123
t=2.587 df=42
P = 0.0132
C
Male L-D Box
Ambulatory Distance (m)
t=4.319 df=10
P =0.0015
Percent distance in light
t=4.807 df=9
P =0.0010
Time in light (sec)
t=3.053 df=9
P =0.0137
D
Female L-D Box
t=3.290 df=28
P =0.0027
t=3.407 df=32
P =0.0018
t=3.281 df=32
P =0.0025
E
Male NIH Assay
K-S (d)= 0.9492
p<0.0001
Cumulative frequency
Feeding Latency (s)
Latency to consume (sec)
t=2.866 df=9
P = 0.0186
Consumption (g/g BW*100%)
F
Female NIH Assay
K-S (d)=0.9091
p<0.0001
t=4.149 df=14.00
P =0.0010
t=2.629 df=17.47
p = 0.0173
G
Male SPT
Sucrose Preference (%)
Day
Sucrose consumption (ml/g BW*100%)
H
Female SPT
Genotype F (1, 24) = 15.72
P = 0.0006
Genotype F (1, 24) = 8.993
P = 0.0062**
I
Male TST
Immobility (sec)
Time (min)
Percent immobility
J
Female TST

Figure 2.2 DAGLα deletion increases anxiety-like and depressive behavior (A-B) Behavioral analysis of male (A) and female (B) DAGLα$^{-/-}$ mice in the open-field assay. (C-D) Behavioral analysis of male (C) and female (D) DAGLα$^{-/-}$ mice in the light-dark box test (L/D Box). (E-F) Behavioral analysis of male (E) and female (F) DAGLα$^{-/-}$ mice in the NIH assay. (G-H) Behavioral analysis of male (G) and female (H) DAGLα$^{-/-}$ mice in the sucrose preference test (SPT). (I-J) Behavioral analysis of male (I) and female (J) DAGLα$^{-/-}$ mice in the tail suspension test (TST). * P < 0.05, ** P<0.01, *** P<0.001, **** P<0.0001 by ANOVA, t-test, or K-S test as indicated in the panel. Error bars indicate SEM. See also Figure S2. (A-J RJB)

that impaired motor coordination contributes to the observed behavioral effects, we tested DAGLα$^{-/-}$ mice on the accelerating rotarod, but did not find any differences in motor performance between genotypes (Fig. S2.2A-B)

DAGLα deletion results in sex-specific depressive-like behavior

We next examined more traditional depressive-like behaviors using the sucrose preference test (SPT) and the tail suspension test (TST). No differences in sucrose preference, sucrose consumption, or water consumption (not shown) were observed in male DAGLα$^{-/-}$ mice relative to WT littermates (Fig. 2.2G). In contrast, female DAGLα$^{-/-}$ mice showed reduced sucrose preference ($p<0.001$), reduced sucrose consumption ($p<0.01$), and increased water consumption ($p<0.001$; not shown) over the course of the experiment after normalization to body weight (Fig. 2.2H and see Fig. S2.2C-D for body weight data). Neither male nor female DAGLα$^{-/-}$ mice showed differences in immobility time during the tail suspension test relative to WT littermates (Fig. 2.2I-J).

DAGLα deletion impairs amygdala eCB retrograde synaptic signaling

eCB signaling, and specifically 2-AG signaling, is known to inhibit excitatory glutamatergic transmission in the amygdala (Azad et al. 2003, Domenici et al. 2006, Yoshida et al. 2011) and eCB-mediated reductions in anxiety likely require CB1R-mediated inhibition of glutamate, but not GABA, release (Haring et al. 2012, Rey et al. 2012, Ruehle et al. 2013).

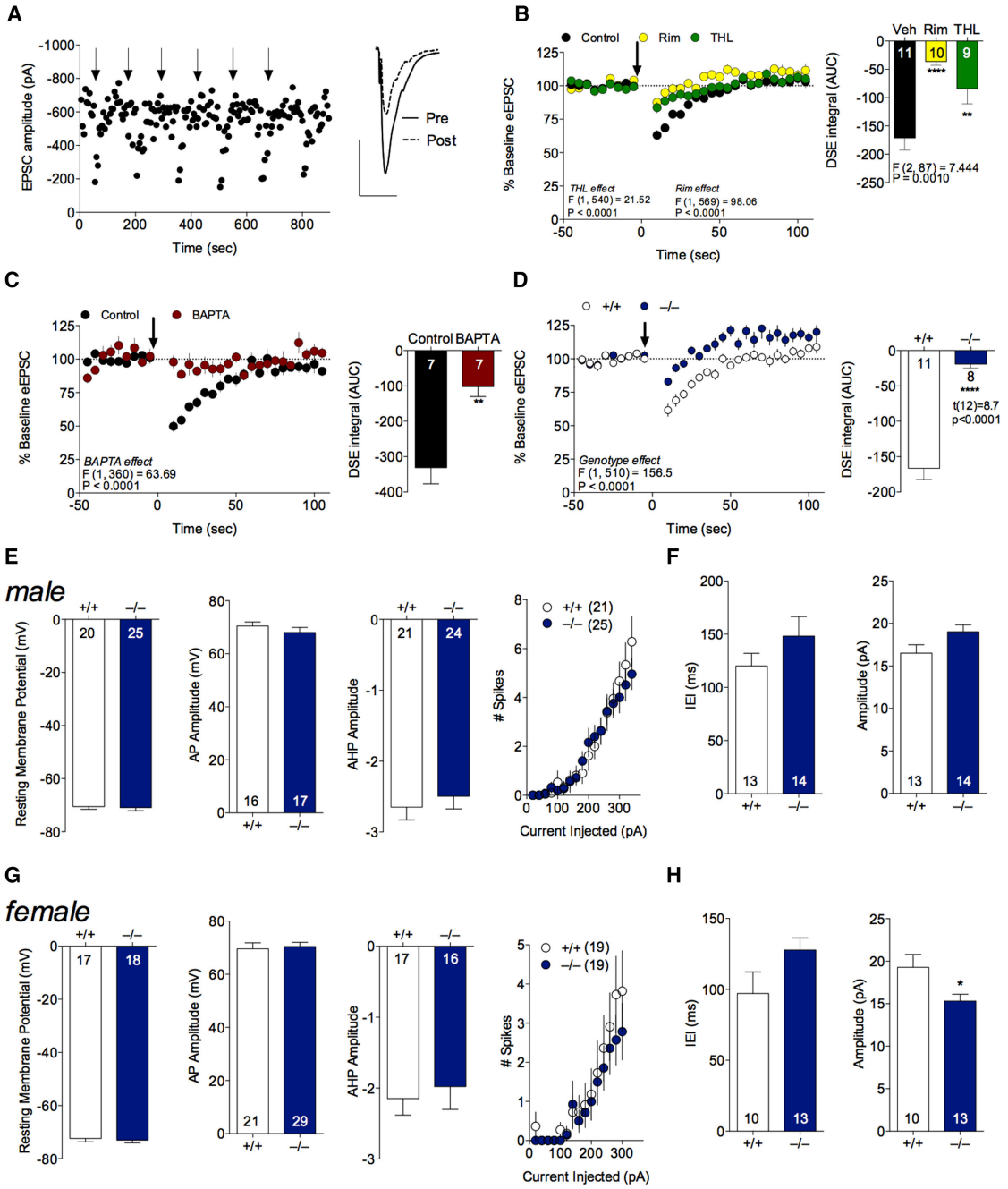

Figure 2.3 DAGLα deletion impairs eCB-modulation of amygdala glutamatergic transmission (A) Representative example of DSE in the BLA with arrows indicating time of 10 second depolarization from -70 mV to +30 mV. (B) Effects of the CB1 receptor antagonist Rimonabant and DAGL inhibitor THL on BLA DSE. (C) Effects of post-synaptic calcium chelation with BAPTA on BLA DSE. (D) Effects of DAGLα deletion on BLA DSE. (E-F) Analysis of BLA neuron (E) membrane properties, excitability, and (F) sEPSC amplitude and inter-event interval (IEI) in male DAGLα$^{-/-}$ mice. (G-H) Analysis of BLA neuron (G) membrane properties, excitability and (H) sEPSC amplitude and IEI in female DAGLα$^{-/-}$ mice. ** P<0.01, **** P<0.0001, by ANOVA, Sidak's post hoc analysis, or t-test as indicated in the panel. Error bars indicate SEM. See also Figure S3. (A-C TSR; D-H TSR, RB, & RJB)

Therefore, we hypothesized that DAGL$\alpha^{-/-}$ mice would have impaired eCB-mediated suppression of basolateral amygdala (BLA) glutamatergic transmission, which could contribute to the observed anxiety-like behavioral phenotype. To test this hypothesis, we used the most well established form of 2-AG-mediated retrograde endocannabinoid signaling expressed widely throughout the CNS, depolarization-induced suppression of excitation (DSE) (Alger 2002, Tanimura et al. 2010). Whole-cell patch-clamp recordings from BLA pyramidal neurons revealed that postsynaptic depolarization caused a transient suppression of excitatory postsynaptic current (EPSC) amplitude (Yoshida et al. 2011) (Fig. 2.3A), which was blocked by pre-incubation with the CB1 receptor antagonist Rimonabant ($p<0.0001$). BLA DSE was attenuated by pharmacological inhibition of DAGL with THL ($p<0.01$; Fig. 2.3B) and by postsynaptic calcium chelation with 40 mM BAPTA ($p<0.01$; Fig. 2.3C). Importantly, DSE was essentially absent in DAGL$\alpha^{-/-}$ mice relative to wild-type controls ($p<0.0001$; Fig. 2.3D). Since 2-AG levels were decreased in the striatum as well, we also confirmed that DSE was absent in this brain region of DAGL$\alpha^{-/-}$ mice (Fig. S2.3). These data support 2-AG as a key mediator of retrograde 2-AG signaling at excitatory synapses (Gao et al. 2010, Tanimura et al. 2010).

Lastly, we evaluated potential compensatory consequences of the prolonged impairment of eCB-mediated inhibition of glutamatergic signaling on measures of excitatory synaptic transmission, membrane properties, and excitability of BLA principle neurons of male and female DAGL$\alpha^{-/-}$ mice. Neither male nor female DAGL$\alpha^{-/-}$ mice exhibited changes in membrane properties or cellular excitability (Fig. 2.3E and G). However, there was a slight reduction of spontaneous EPSC (sEPSC) amplitude specifically in females, while neither sex exhibited significant changes in sEPSC inter-event interval (IEI; Fig. 2.4 F and H). Overall, these measures indicate no major compensatory changes in basal glutamatergic transmission or cell excitability

as a result of long-term DAGLα deletion.

2-AG restoration normalizes the behavioral phenotype of DAGLα$^{-/-}$ mice

Thus far, our data clearly support *necessity* for 2-AG signaling in the physiological regulation of anxiety and depressive behaviors. However, establishing a causal link between 2-AG signaling deficiency and the behavioral phenotypes observed in DAGLα$^{-/-}$ mice also requires experimental support for *sufficiency* of 2-AG signaling in the regulation of physiological anxiety and depressive behaviors. Therefore, we utilized a pharmacological approach to restore 2-AG levels in DAGLα$^{-/-}$ mice and determined the impact on the behavioral phenotype of DAGLα$^{-/-}$ mice. Administration of the MAGL inhibitor JZL-184 (20 mg/kg) robustly increases brain 2-AG levels in WT mice (Long et al. 2009) (Fig. S2.4), suggesting this approach could also be useful for restoring deficient 2-AG levels in DAGLα$^{-/-}$ mice.

In the L-D box, vehicle-treated DAGLα$^{-/-}$ mice again exhibited anxiety-like behavior relative to WT vehicle-treated mice ($p<0.05$ for both measures; Fig. 2.4A-B), while both percent light distance and light time were significantly higher in JZL-184-treated DAGLα$^{-/-}$ mice relative to vehicle-treated DAGLα$^{-/-}$ mice ($p<0.05$ and $p<0.01$ respectively). Ambulatory distance was not affected by any treatment (Fig. 2.4C). In the SPT, baseline sucrose preference was again significantly lower in female DAGLα$^{-/-}$ mice relative to WT mice confirming our previous data ($p<0.001$; Fig. 2.4D). After establishment of a baseline preference, JZL-184 was administered to DAGLα$^{-/-}$ mice while WT mice received vehicle injections. After 1 day of JZL-184 treatment, the sucrose preference of DAGLα$^{-/-}$ mice remained significantly lower than that of vehicle-treated WT mice, but on the second day of drug treatment sucrose preference was not significantly different between vehicle-treated WT mice and DAGLα$^{-/-}$ mice treated with JZL-184 ($p>0.05$; Fig 4D). Importantly, sucrose preference deficits in DAGLα$^{-/-}$ mice relative to WT

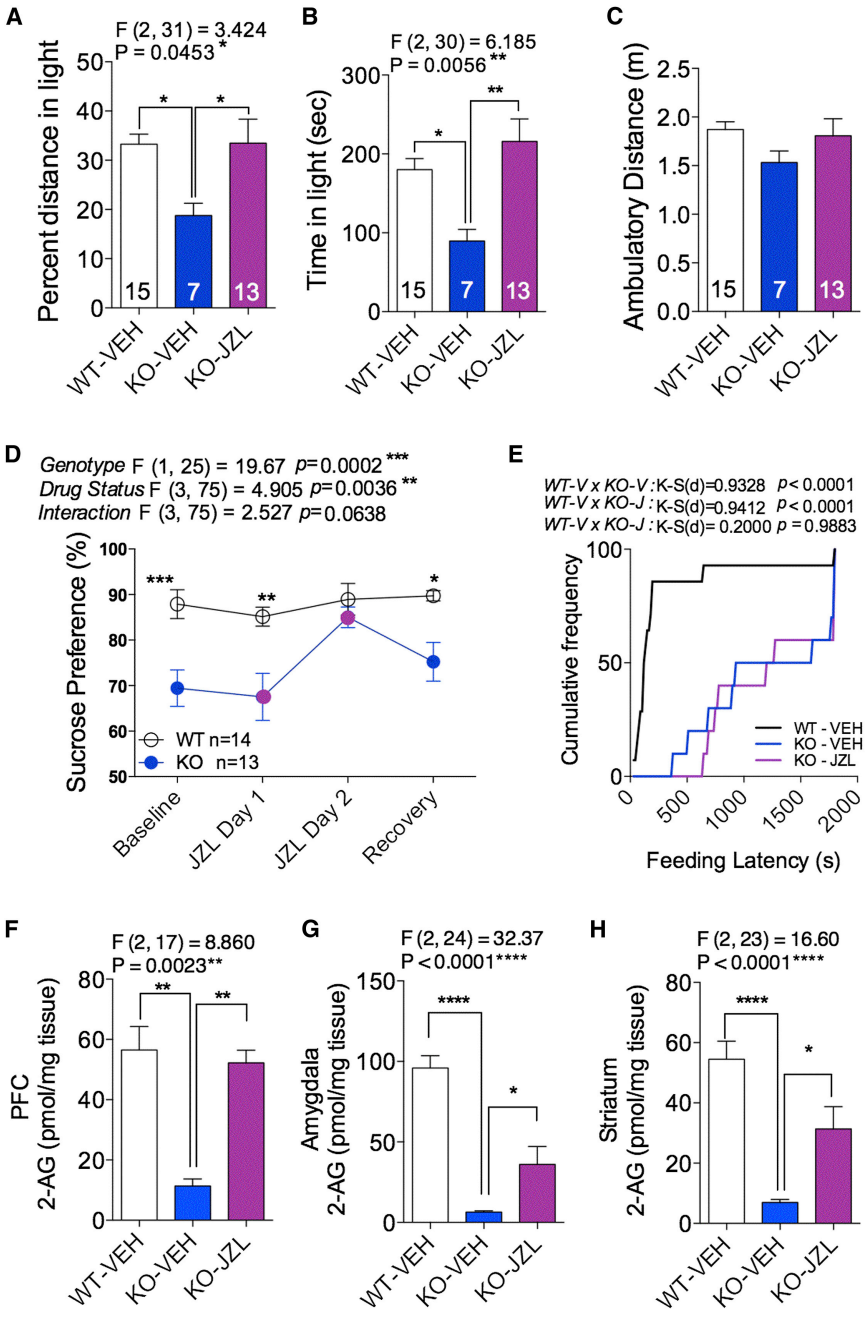

Figure 2.4 JZL-184 reverses anxiety and depressive behaviors in DAGLα$^{-/-}$ mice (A-C) Effects of DAGLα deletion and JZL-184 treatment on percent light distance travelled, light time, and total ambulatory distance in the L-D box. (D) Effects of DAGLα deletion and JZL-184 treatment on sucrose preference in female mice. Note JZL-184 was administered on 2 consecutive days 2h prior to testing, while recovery represents testing under vehicle-treatment conditions 24h after the last drug treatment. (E) Effects of DAGLα deletion and JZL-184 treatment on latency to feed in the NIH assay. (F-H) Effects of DAGLα deletion and JZL-184 treatment on 2-AG levels in the PFC (F), amygdala (G), and striatum (H). * P < 0.05, ** P<0.01, *** P<0.001, **** P<0.0001 by ANOVA followed by Sidak's post hoc analysis or K-S test as indicated in the panel. Error bars indicate SEM. See also Figure S4. (A-H RJB)

reemerged after a 24h drug washout period ($p<0.05$; Fig 2.4D, recovery). Interestingly, although we replicated the robust anxiety-like effect in DAGL$\alpha^{-/-}$ mice in the NIH assay, JZL-184 did not reverse the increased feeding latency exhibited by DAGL$\alpha^{-/-}$ mice (Fig. 2.4E). To confirm this dose of JZL-184 increased 2-AG levels in DAGL$\alpha^{-/-}$ mice we measured 2-AG 2h after 20 mg/kg JZL-184 treatment. Vehicle-treated DAGL$\alpha^{-/-}$ mice showed significant reductions in 2-AG levels in the PFC ($p<0.01$), amygdala ($p<0.0001$) and striatum ($p<0.0001$) (Fig. 2.4F-H). Importantly, treatment with 20mg/kg JZL-184 significantly increased 2-AG levels in the PFC ($p<0.01$), amygdala ($p<0.05$), and striatum ($p<0.05$) relative to vehicle-treated DAGL$\alpha^{-/-}$ mice indicating this dose was sufficient to at least partially restore 2-AG levels in DAGL$\alpha^{-/-}$ mice. These data support sufficiency of 2-AG signaling in regulating anxiety and depressive behaviors.

Discussion

To investigate the physiological role of endogenous 2-AG signaling in the regulation of anxiety and depressive behaviors we utilized a genetic approach to delete a primary 2-AG synthetic enzyme, DAGLα. We find that DAGL$\alpha^{-/-}$ mice have the expected decreases in 2-AG tissue levels and decreases in AA consistent with other DAGL$\alpha^{-/-}$ mouse lines (Gao et al. 2010, Tanimura et al. 2010, Yoshino et al. 2011). Comparison to

previous studies revealed some key differences between our findings and published results. For example, Gao et al. reported ~75% reduction in whole brain 2-AG and AA in DAGL$\alpha^{-/-}$ mice,

while our bulk 2-AG reductions appeared much less dramatic. Reasons for this discrepancy are unclear but could be related to the fact we specifically analyzed forebrain, rather than whole brain. In addition, Gao et al. showed an ~50% reduction in 2-AG levels in DAGL$\beta^{-/-}$ mice (Gao et al. 2010), suggesting this enzyme also contributes to bulk brain 2-AG measurements and that

the residual forebrain 2-AG levels observed in our DAGLα$^{-/-}$ mice may be synthesized by DAGLβ. There may also be significant regional heterogeneity in the effects of DAGLα deletion on 2-AG levels such that forebrain analyses may underestimate highly localized decreases observed in the sub-regional analysis. This suggestion is supported by the *dramatic* region-specific reductions in 2-AG levels observed in DAGLα$^{-/-}$ mice, which confirms robust 2-AG deficiency in key limbic brain regions regulating anxiety and depressive behaviors including the PFC and amygdala. We also show for the first time that the levels of SAG and several other AA-containing DAGs are elevated in the forebrain of DAGLα$^{-/-}$ mice, consistent with previous findings in cultured cells (Jung et al. 2007), revealing these lipids are the precursors of 2-AG formation *in vivo*. Lastly, reductions in brain AEA have also been reported in DAGLα$^{-/-}$ mice (Gao et al. 2010, Tanimura et al. 2010). While we did not observe reductions in forebrain AEA levels in our mice, future studies will be aimed at enhancing detection and quantification of brain regional AEA differences to clarify this discrepancy.

Upon confirmation that DAGLα$^{-/-}$ mice represented a validated limbic 2-AG deficiency model, we undertook a comprehensive behavioral analysis of DAGLα$^{-/-}$ mice to test the hypothesis that endogenous 2-AG signaling is a critical regulator of affective behavior. Our data revealed anxiety-like behaviors in both male and female DAGLα$^{-/-}$ mice, while only female mice exhibit anhedonia as demonstrated by a reduction in sucrose preference. Overall ambulation in the open field and motor coordination on the accelerating rotarod were unaffected by DAGLα deletion, indicating that the observed differences in anxiety-like and depressive behaviors were not likely confounded by motor deficits *per se*. With regard to measures of anxiety, DAGLα$^{-/-}$ mice showed increased anxiety-like behavior in the open-field, L-D-box, and NIH assay, thus supporting a primary role for 2-AG signaling in the regulation of anxiety-like behavior (Patel and

Hillard 2008). In contrast, only female DAGL$\alpha^{-/-}$ mice exhibited anhedonia, while neither male nor female DAGL$\alpha^{-/-}$ mice exhibited despair-like behavior in the TST. The data herein suggest that 2-AG signaling is selectively involved in the regulation of a discrete behavioral dimension of depression, namely anhedonia, while having broader multi-dimensional effects on anxiety-related behavior.

Importantly, we also found that pharmacological elevation of 2-AG levels in DAGL$\alpha^{-/-}$ mice reversed anxiety behavior in the L-D box and anhedonia in the SPT, but was unable to reverse anxiety in the NIH assay. Since the NIH assay is extremely sensitive to CB1R signaling deficiency (Gamble-George et al. 2013), it is possible that more complete 2-AG restoration or longer duration of restoration would be required to see reversal in this test. In support of this, 2 days of JZL-184 treatment was required to produce a reversal of the anhedonic phenotype of DAGL$\alpha^{-/-}$ mice. These data are consistent with recent findings demonstrating that pharmacological elevation of 2-AG reduces stress-induced anxiety (Sciolino et al. 2011, Sumislawski et al. 2011, Zhong et al. 2014). Taken together, our data showing that the increase in anxiety and depressive behavior in DAGL$\alpha^{-/-}$ mice is reversed by normalization of deficient 2-AG levels provides causal evidence to support 2-AG signaling deficiency as the mechanism subserving the behavioral phenotype of DAGL$\alpha^{-/-}$ mice. However, some caveats to this interpretation remain. For example, in addition to 2-AG reductions, we detected profound loss of arachidonic acid throughout the brain, potentially confounding our interpretation of the causal role for 2-AG deficiency in our observed phenotypes. However, inhibition of 2-AG degradation also produces dramatic reductions in AA (Long et al. 2009), but *reduces* anxiety-like and depressive behaviors (Busquets-Garcia et al. 2011, Sciolino et al. 2011, Sumislawski et al. 2011, Zhong et al. 2014); thus, reductions in AA levels cannot explain the anxiety-like behavior

observed in DAGL$\alpha^{-/-}$ mice. It is also possible that reductions in levels of other monoacylglycerols such as 2-OG could contribute to the observed phenotype of DAGL$\alpha^{-/-}$ mice; a hypothesis that will require further testing.

Increased anxiety and depressive behaviors in humans and laboratory animals is highly associated with increased activity in the amygdala (Levine et al. 2001, Etkin and Wager 2007, Roozendaal et al. 2009). Given that one key physiological function of 2-AG signaling is retrograde synaptic suppression, we evaluated the effects of DAGLα deletion on eCB-mediated short-term synaptic suppression at amygdala glutamatergic synapses. BLA DSE was absent in DAGL$\alpha^{-/-}$ mice suggesting an impaired activity-dependent feedback inhibition of BLA glutamatergic transmission in DAGL$\alpha^{-/-}$ mice. These data are consistent with previous studies showing impaired DSE and DSI in hippocampus and cerebellum of DAGL$\alpha^{-/-}$ mice (Gao et al. 2010, Tanimura et al. 2010). Although the behavioral phenotype of DAGL$\alpha^{-/-}$ mice is likely a consequence of perturbed limbic circuit interactions, rather than a consequence of impaired DSE at a single synapse, these data confirm that 2-AG signaling is impaired at one key synapse heavily implicated in the regulation of affective behavior and emotional learning.

These data could also have implications for cannabis use disorders, which are expected to rise given recent shifts in the prohibition against cannabis use. Specifically, the most common reason cited for continued cannabis use in chronic users is reduction in tension and anxiety (Reilly et al. 1998, Hyman and Sinha 2009), and stress "coping" motives are heavily reported by cannabis users (Chabrol et al. 2005, Hyman and Sinha 2009, Fox et al. 2011, Bujarski et al. 2012). These data are consistent with the "tension-reduction hypothesis" of substance use, which posits that negative reinforcement is a primary driver of continued substance use and that inherent negative affect associated with anxiety could drive cannabis use in an attempt to reduce

symptom severity (see (Buckner et al. 2007)). Our data provide compelling support for an "endocannabinoid deficiency" state causing anxiety and depressive effects (Russo 2008), which could drive cannabis use in an attempt to relieve these symptoms. That restoration of 2-AG signaling reverses anxiety and depressive behaviors induced by eCB deficiency supports therapeutic approaches aimed at normalizing endocannabinoid deficiency to treat cannabis use disorders and facilitate abstinence in some individuals (Clapper et al. 2009).

CB1 receptor activity and AEA signaling have been heavily implicated in the regulation of anxiety- and depressive-like behaviors (Kathuria et al. 2003, Gobbi et al. 2005, Viveros et al. 2005, Moreira et al. 2008, Lutz 2009, Bambico et al. 2010, Hill et al. 2010, Hill and Patel 2013). However, the physiological function of the most abundant eCB ligand in the CNS, 2-AG, has until now remained relatively elusive. Our data provide causal evidence for 2-AG signaling in the physiological regulation of anxiety and depressive-like behaviors and suggest the novel hypothesis that 2-AG deficiency could contribute to the pathogenesis of some mood and anxiety disorders. These data also suggest pharmacological approaches aimed at normalizing 2-AG deficiency could represent a viable eCB-based therapeutic strategy for the treatment of mood and anxiety disorders.

Methods

Animals

All studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Vanderbilt University Institutional Animal Care and Use Committee. Adult mice (9-16 weeks) were housed on a 12:12 light-dark

cycle with lights on at 06:00. All experiments were conducted during the light phase. Food and water were available *ad libitum*.

Production of DAGL$\alpha^{-/-}$ mice

DAGL$\alpha^{-/-}$ mice were generated by homologous recombination using the JM8.N4 ES cell line derived from Black C57BL/6N, which were purchased from The European Conditional Mouse Mutagenesis Program (EUCOMM). The targeting construct was inserted into position 10261203 of Chromosome 19 upstream of exon 8. Chimeric mice were produced by injecting JM8.N4 ES cells into albino C57Bl/6 mice. Resulting male chimeric mice were bred with female WT C57Bl/6N mice to produce founder DAGL$\alpha^{+/-}$ mice which were interbred to produce experimental mice. Genotypes for all offspring of DAGL$\alpha^{+/-}$ breeders were determined by PCR of mouse ear punch samples using the following primers: Forward, 5'-TGAGCCAGAGACATTTGCTG-3' and Reverse, 5'-CTGGTGAGGCCAAGTTTGTT-3'.

Membrane Preparation for DAGL Immunoblots and Activity Assay

Mouse forebrain was homogenized by a polytron tissue grinder in lysis buffer containing 20 mM HEPES (pH 7.5), 2 mM DTT, 250 mM sucrose, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mM PMSF, and 1 µM microcystin. Following homogenization, membranes were pelleted by centrifugation at 10,000xg for 25 min at 4ºC. The membrane pellet was resuspended in a dounce homogenizer in buffer containing 20 mM HEPES (pH 7.5), 2 mM DTT, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mM PMSF and 1 µM microcystin.

DAGL Immunoblots

Isolated membrane fractions of mouse forebrain were solubilized by the addition of 2% SDS (final concentration) to the above mentioned resuspension buffer followed by sonication. The samples were then resolved by SDS-PAGE and Ponceau-S stained for normalization to total

protein levels, followed by western blotting with rabbit anti-DGLα, which has been characterized previously(Katona et al. 2006, Keimpema et al. 2010).

DAGL Activity Assay

Activity of DAGL from mouse forebrain was performed as described previously(Shonesy et al. 2013). Briefly, membrane fractions containing 5 μg of total protein were incubated with 1-steroyl-2-arachidonoylglycerol (SAG; Cayman Chemical) for 15 minutes at 37ºC. The reaction was stopped with 100% methanol containing 125 pmol 2-AG-d_8, and 2-AG produced during the reaction was measured using LC/MS methods described below.

LC/MS/MS detection of lipids

LC/MSMS detection of endocannabinoids and arachidonic acid was performed as previously described (Hermanson et al. 2013). Briefly, figure 1 samples were homogenized in acetonitrile and centrifuged; the resulting supernatant was dried under nitrogen and resuspended in 70:30 Methanol:Water. Figure 4 samples were homogenized directly in methanol and centrifuged; water was added to the supernatant for a final ratio of 70:30 Methanol:Water. Sample (20 μl) was injected into a C-18 column (50 × 2 mm, 3 μm; Phenomenex) under the following gradient: 20% A and 80% B from 0 to 0.5 min, increased to 0% A and 100% B from 0.5 to 3.5 min and held for 1 min, and returned to 20% A and 80% B from 4.5 to 6.5. Component A was water and B was methanol, and each component contained 80 μM silver acetate and 0.1% glacial acetic acid (v/v). Analytes were detected via selective reaction monitoring (as $[M+Ag]^+$ complexes except AA, which is ionized as $[(M–H)+2Ag]^+$) in the positive ion mode using the following reactions (the mass in parentheses represents the mass of the deuterated internal standard): AA (*m/z* 519(527) → 409(417)); 2-AG (*m/z* 485(493) → 411(419)); and AEA (*m/z*

454(462) → 432(440)). Quantification was achieved via stable-isotope dilution for AA, 2-AG and AEA.

DAGs were resolved on a 10 cm long x 2.1 mm wide x 3 μM particle size Hypersil GOLD C4 column (Thermo Scientific, San Diego, CA) using a gradient of 1:1 Methanol:Water, 5 mM ammonium acetate, pH = 3.5 with glacial acetic acid (A) and 4:1 Acetonitrile:isopropanol with 0.1% glacial acetic acid (B). DAG species were analyzed by mass spectrometry using multiple reaction monitoring in positive ion mode with parent ions of $[M+NH_4]^+$ to loss of a selected fatty acid tail (i.e., 38:4 DAG 662.5 → 341.5) and quantified using SAG-d_8 as an internal standard (Cayman chemical, Ann Arbor, MI).

Behavioral assays

DAGL$\alpha^{-/-}$ mice were sequentially tested in the open field test, light-dark box, novelty-induced hypophagia assay, sucrose preference test, tail suspension test, and accelerating rotarod. Tests were conducted at least 48 hours apart. Not all mice were used in each behavioral test. For each sex, behavioral data from a minimum of 2 independent cohorts of age-matched WT and DAGL$\alpha^{-/-}$ mice were combined in final data analysis and figures. Cohorts of male and female mice were tested separately. For behavioral reversal experiments, mice were intraperitoneally injected with 20mg/kg of JZL-184 dissolved in dimethylsulfoxide at a volume of 1μl/g bodyweight 2 hours before testing. Mice were sequentially tested in light-dark and novelty-induced hypophagia and females were additionally tested in sucrose preference with a minimum of 1 week between tests. At least 5 days after the final test, mice were again injected with 20 mg/kg JZL-184 and after 2 hours brain samples were harvested for regional analytical lipid measurements.

Novel Open-Field: As described previously (Sumislawski et al. 2011) exploration of a novel open field arena contained within a sound-attenuating chamber was monitored for 30 minutes (27.9×27.9 cm; MED-OFA-510; MED Associates, St. Albans, Vermont). Beam breaks from 16 infrared beams were recorded by Activity Monitor v5.10 (MED Associates) to monitor position and behavior.

Light-dark box: As described previously (Hermanson et al. 2013) exploration of open field chambers containing dark box inserts that split the chamber into light (~25 lux) and dark (<5 lux) halves (Med Associates ENV-511) was recorded by Activity Monitor v5.10 as above. Position and behavior were monitored as described above for 10 minutes.

Novelty Induced Hypophagia: Testing was conducted as described previously (Gamble-George et al. 2013). Individually housed mice were acclimated to testing rooms under red light for at least 30 minutes before habituation and testing. Mice were habituated to a novel, palatable food (liquid vanilla Ensure®, Abbott Laboratories, Abbott Park, IL) in their home cages for 30min/day under red light (<50 lux) for 4 days before testing. After acclimation on the fifth day, mice were transferred to a novel, empty cage in a brightly lit room (~300 lux) and again given access to liquid vanilla Ensure® for 30 minutes. For each mouse, the latency to feed and total consumption was recorded.

Sucrose Preference Testing: Individually housed mice were given *ad libitum* access to water and 1% (w/v) sucrose. Weights of drinking bottles were recorded daily for seven days and liquid weight consumed was normalized to body weight. For behavioral reversal testing, a 4 day baseline preference was established after which JZL-184 was administered for 2 consecutive days, with an additional 1 day washout period. Bottle position was alternated daily to control for side preference. Sucrose preference

was calculated as sucrose consumption divided by total sucrose and water consumption multiplied by 100%.

Tail Suspension Testing: Testing was conducted as previously described with minor modifications (Gamble-George et al. 2013). Each mouse was suspended by the tail from a vertical stainless steel force transducer in an individual cubicle for six minutes during which immobility was recorded using MED Associates Tail Suspension software (Version 3.30).

Accelerating Rotarod: In each trial, the rod was started at 5 RPM, and accelerated to 40 RPM over 5 minutes. Trials were repeated 3 times with 30 min spacing on the same day and the time for each animal to fall (sec) is measured in each trial.

Ex vivo slice electrophysiology

Whole-cell patch clamp electrophysiological experiments were carried out in 4-6 week old female and male DAGL$\alpha^{-/-}$ mice and WT littermates with minor modifications to those described previously (Sumislawski et al. 2011). Mice were anesthetized with isoflurane and transcardially perfused with cold, oxygenated (95% v/v O_2, 5% v/v CO_2) N-methyl-d-glucamine (NMDG)-containing artificial cerebrospinal fluid (ACSF), described below. Following decapitation, the brain was removed and a 3 mm coronal block containing the amygdala or striatum was cut using a cold brain matrix. A Leica VT1000S vibratome (Leica Microsystems, Bannockburn, IL) was used to cut 200-300 mm coronal slices in cold, oxygenated NMDG-ACSF comprised of (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 25 glucose, 5 ascorbate, 20 HEPES, 3 Na-pyruvate, 0.5 $CaCl_2 \cdot 4H_2O$, 10 $MgSO_4 \cdot 7H_2O$. NMDG-ACSF was titrated to pH 7.3-7.4 with HCl. Slices were transferred to a recovery chamber containing the same NMDG-ACSF kept at 34˚C for 10 minutes after which they were held at 24˚C in HEPES-ACSF pH 7.3-7.4 containing (in mM): 92 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20

HEPES, 25 glucose, 5 ascorbate, 3 Na-pyruvate, 2 $CaCl_2 \cdot 4H_2O$, 2 $MgSO_4 \cdot 7H_2O$. Recordings were performed up to 6 hours after slicing in recording ACSF containing (in mM): 113 NaCl, 2.5 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 20 glucose, 1 ascorbate, 3 Na-pyruvate, 2.5 $CaCl_2 \cdot 2H_2O$, 1.2 $MgSO_4 \cdot 7H_2O$. Voltage-clamp and current-clamp recordings were performed on BLA pyramidal neurons or striatum medium spiny neurons identified by morphology. Slices were continuously perfused with oxygenated recording ACSF (30-32˚C) at a flow rate of 2-3 ml/min. Patch electrodes were pulled on a Flaming/Brown microelectrode puller (Sutter Instruments) to a resistance of 2.0-4.0 MΩ. Electrodes were filled with a solution containing (in mM): 130 Potassium-gluconate, 4 NaCl, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, and 10 Na-phosphocreatine (pH 7.25–7.35, adjusted with KOH). For all experiments, access resistance was monitored online and cells that demonstrated a >20% change were excluded from analysis. Additionally, experiments were not initiated until at least 5 minutes post break-in to allow for exchange of the internal pipette solution. Monosynaptic evoked excitatory postsynaptic currents (eEPSCs) were elicited via constant-current stimulation of local fibers via an ACSF-filled glass electrode placed ~100μm from the cell soma. All recordings were carried out at a holding potential of –70mV in the presence of 50μM picrotoxin to block $GABA_A$ receptor currents. eEPSC amplitudes were adjusted to 500-1200 pA. Due to the use of adult animals in electrophysiological experiments, dihydroxyphenylglycine (DHPG) was added to the ACSF at a final concentration of 25μM for depolarization-induced suppression of excitation (DSE) experiments to elicit Ca^{2+} enhanced receptor driven endocannabinoid release. For DSE experiments responses were evoked with a single stimulation delivered every 5 seconds for 50 sec before and 100 sec after DSE induction. To induce DSE, a depolarizing pulse (–70 to +30 mV) was applied to the postsynaptic neuron for 10 seconds. For each DSE trial, eEPSC

amplitudes were normalized to the averaged baseline response and data from two DSE trials per cell were averaged for analyses.

Statistical Analysis

All data were analyzed using GraphPad Prism version 6. Data that included more than 2 groups or 2 factors were analyzed by One-Way or Two-Way ANOVA respectively, as indicated in the figures, with post hoc Sidak's multiple comparisons test. Multiplicity corrected p values are noted in figures. For analysis of two groups, an unpaired two-tailed t-test was used. For analysis of cumulative feeding latencies, K-S test was used. $P<0.05$ was considered significant throughout. Data are presented as mean ± SEM throughout.

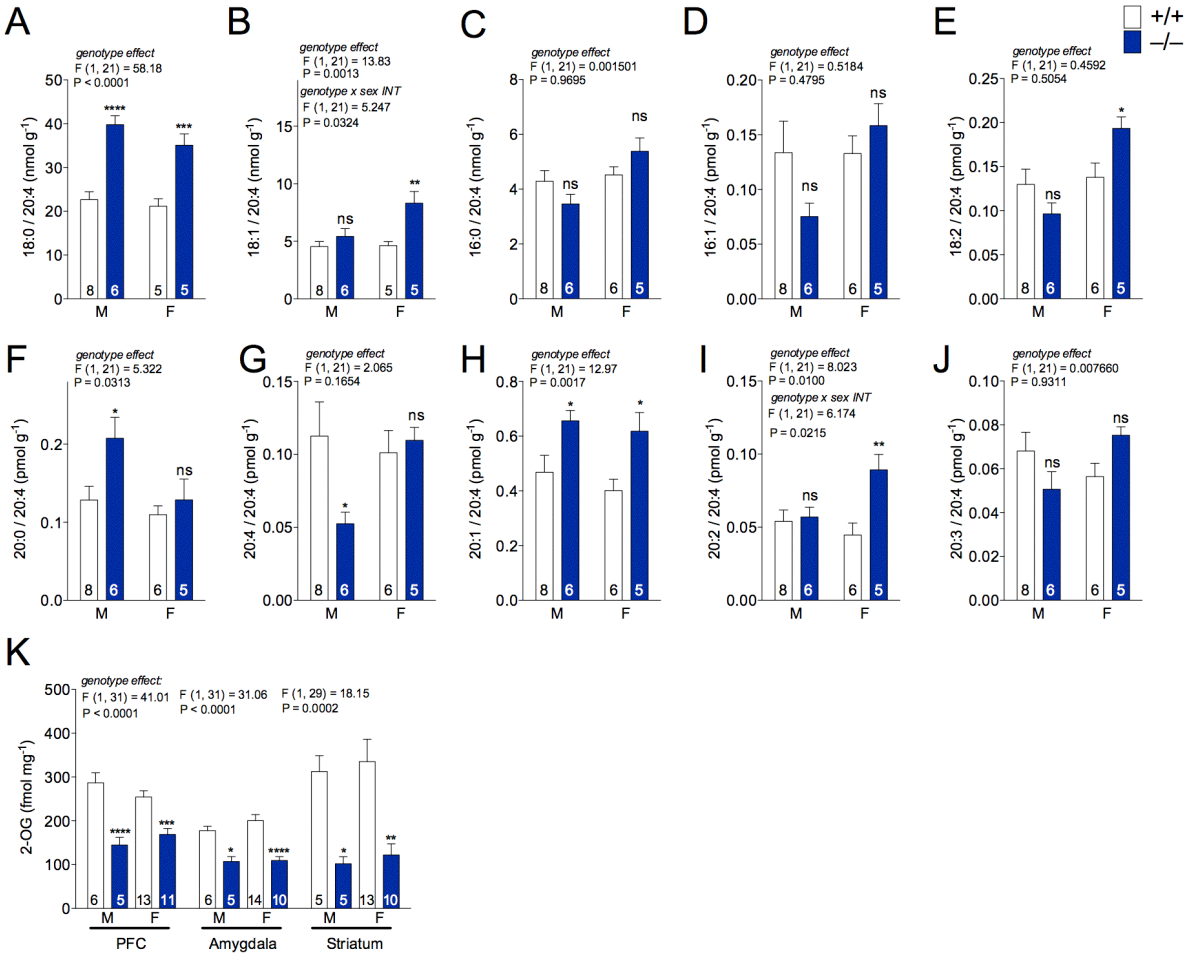

Figure S2.1 Alterations in DAG and 2-OG levels of DAGLα$^{-/-}$ mice, Related to Figure 2.1 (A-J) Forebrain levels of various *sn-2*-AA-containing DAGs in male (M) and female (F) WT and DAGLα$^{-/-}$ mice. Note SAG levels from Fig. 2.1 are shown in (A) for comparison to less abundant DAG species. (K) Levels of 2-OG in prefrontal cortex (PFC), amygdala and striatum of male and female DAGLα$^{-/-}$ mice 2-way ANOVA results indicated in each panel * $P < 0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ by Sidak's post hoc test. Error bars indicate SEM. (A-K BCS)

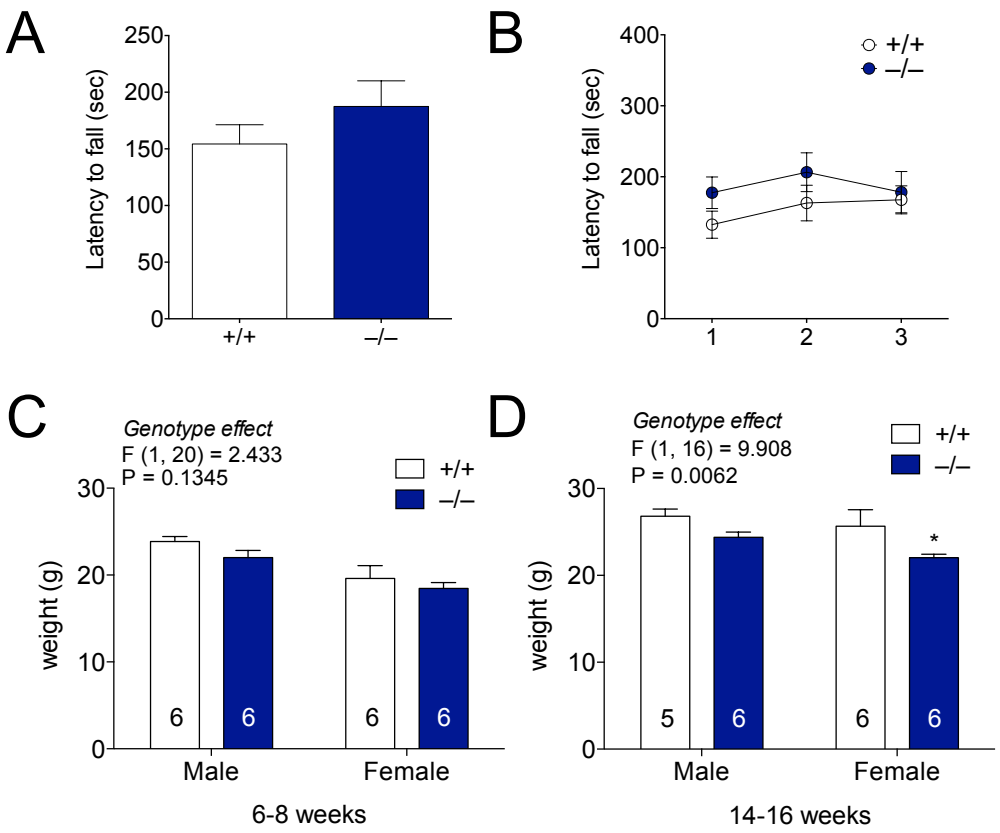

Figure S2.2 Effects of DAGLα deletion on body weight and motor performance evaluated using the accelerating rotarod, Related to Figure 2.2 (A-B) No difference in latency to fall was observed between DAGLα$^{-/-}$ and WT mice during three consecutive training sessions. N=9-10 per group. Significance determined by unpaired two-tailed t-test. (C-D) 14-16 week old Female DAGLα$^{-/-}$ mice showed significantly reduced body weight relative to WT littermates; however, there were no significant differences between males at the same age or either gender at 6-8 weeks. * $P < 0.05$ by Sidak's post hoc test. Error bars indicate SEM. (A-B BCS; C-D RJB)

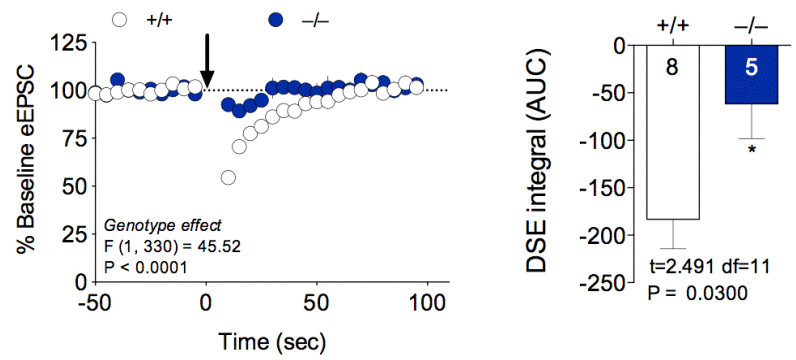

Figure S2.3 Effects of DAGLα deletion on DSE in dorsolateral striatal medium spiny neurons, Related to Figure 2.3 DSE time course and total AUC were significantly reduced in randomly selected medium spiny neurons in the striatum of DAGL$\alpha^{-/-}$ mice relative to WT littermates. * P < 0.05 by two-tailed t-test. Error bars indicate SEM. (BCS)

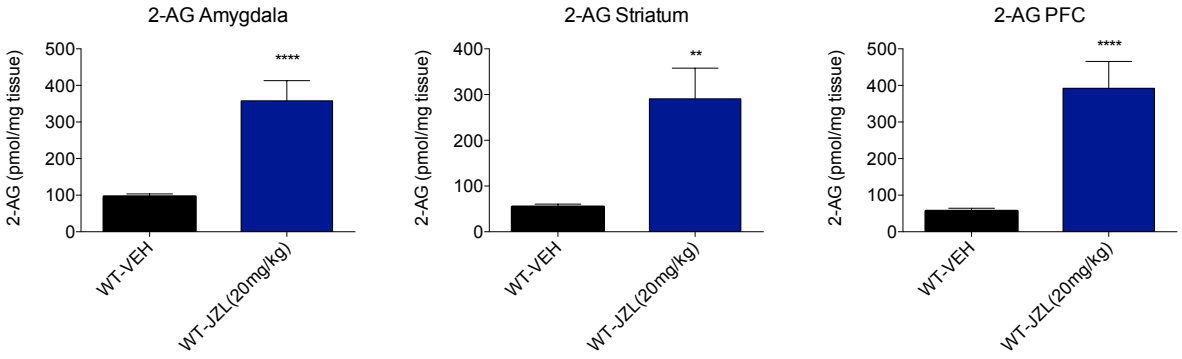

Figure S2.4 Effects of JZL-184 (20 mg/kg) on 2-AG levels in WT mice, Related to Figure 2.4 JZL-184 increases 2-AG levels in the amygdala, striatum, and PFC of WT mice. ** P<0.01, **** P<0.0001 by two-tailed t-test. Error bars indicate SEM. (RJB, BCS)

CHAPTER III

ENDOCANNABINOID SIGNALING MODULATES SUSCEPTIBILITY TO TRAUMATIC STRESS EXPOSURE

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ABSTRACT

Stress is a ubiquitous risk factor for the exacerbation and development of affective disorders including major depression and posttraumatic stress disorder. Understanding the neurobiological mechanisms conferring resilience to the adverse consequences of stress could have broad implications for the treatment and prevention of mood and anxiety disorders. We utilize laboratory mice and their innate inter-individual differences in stress-susceptibility to demonstrate a critical role for the endogenous cannabinoid 2-arachidonoylglycerol (2-AG) in stress-resilience. Specifically, systemic 2-AG augmentation is associated with a stress-resilient phenotype and enhances resilience in previously susceptible mice, while systemic 2-AG depletion or CB1 receptor blockade increases susceptibility in previously resilient mice. Moreover, stress-resilience is associated with increased phasic 2-AG-mediated synaptic suppression at ventral hippocampal-amygdala glutamatergic synapses and amygdala-specific 2-AG depletion impairs successful adaptation to repeated stress. These data indicate amygdala 2-AG signaling mechanisms promote resilience to adverse effects of acute traumatic stress and facilitate adaptation to repeated stress exposure.

Adapted from: Bluett, R. J. et al. Endocannabinoid signalling modulates susceptibility to traumatic stress exposure. Nat. Commun. ***8****, 14782 doi: 10.1038/ncomms14782 (2017).*

Introduction

Stress is a major risk factor for neuropsychiatric disease, particularly major depression and anxiety disorders, and is etiologically causal in posttraumatic stress disorder (PTSD) (Faravelli 1985, Kendler et al. 1999, Goeders 2003, McEwen 2004, Kessler et al. 2005, McEwen 2012, Sharma et al. 2015). Stress-resilience is associated with reduced risk of psychopathology and is an active process of adaptation, not merely the absence of maladaptive changes induced by stress exposure (Krishnan et al. 2007, Norrholm and Ressler 2009, Russo et al. 2012, McEwen et al. 2015, Pfau and Russo 2015, Swartz et al. 2015). Understanding the biological mechanisms promoting stress-resilience could lead to novel treatments for stress-related psychiatric disorders. Here we elucidate a role for endogenous cannabinoid (eCB) 2-arachidonoylglycerol (2-AG) in promoting resilience to acute traumatic stress and successful adaptation to repeated homotypic stress exposure.

The eCB system is composed of the presynaptic cannabinoid CB1 receptor (CB1R), its endogenous ligands including anandamide (arachidonoylethanolamine; AEA) and 2-AG, and enzymes mediating eCB turnover (Mechoulam and Parker 2013, Kano 2014). Neuronal 2-AG is synthesized postsynaptically primarily by diacylglycerol lipase α (DAGLα) (Yoshino et al. 2011, Shonesy et al. 2014), while AEA can be generated via multiple enzymatic cascades (Blankman and Cravatt 2013). After release from the postsynaptic compartment, eCBs travel retrogradely to the presynaptic terminal where they bind CB1Rs, which when activated reduce vesicular neurotransmitter release from the synaptic terminal (Castillo et al. 2012, Kano 2014). 2-AG is primarily degraded presynaptically by monoacylglycerol lipase (MAGL), while AEA is degraded postsynaptically by fatty acid amide hydrolase (FAAH) (Blankman and Cravatt 2013,

Kano 2014), and pharmacological inhibition of MAGL or FAAH can increase 2-AG or AEA-mediated eCB signaling, respectively.

eCBs have been implicated in modulating anxiety, fear learning, and stress responsivity (Lutz 2009, Ruehle et al. 2012, Morena et al. 2016). Pharmacological augmentation of AEA signaling reduces unconditioned anxiety and reduces stress-induced increases in anxiety-like behavior, corticosterone release, and dendritic remodeling (Morena et al. 2016). AEA augmentation also facilitates extinction learning in mice (Morena et al. 2016). Furthermore, stress exposure can decrease brain AEA levels, which are inversely correlated with severity of stress-induced anxiety-like behaviors (Bluett et al. 2014). Although compelling evidence suggests that AEA signaling buffers against stress-related affective pathology (Hill and Patel 2013, Morena et al. 2016), the role of 2-AG signaling in stress-modulation is only now becoming appreciated. For example, pharmacological augmentation of 2-AG signaling can reduce unconditioned anxiety and prevent emergence of stress-induced anxiety-like behaviors (Busquets-Garcia et al. 2011, Sciolino et al. 2011, Sumislawski et al. 2011, Zhong et al. 2014, Lim et al. 2016), while genetic 2-AG deficiency results in increased anxiety-like behaviors (Shonesy et al. 2014, Patel et al. 2015). Moreover, chronic homotypic stressors increase 2-AG levels within the amygdala and prefrontal cortex (Patel et al. 2005, Patel and Hillard 2008). Despite these findings, whether 2-AG signaling within these regions regulates resilience to traumatic stress exposure has not been investigated. To directly address this critical question, herein we develop and validate a model for rapid evaluation of inter-individual differences in stress-resilience. We then utilize pharmacological and circuit-specific electrophysiological approaches combined with a novel conditional genetic model to demonstrate a key role for 2-AG signaling in promoting stress-resilience and successful adaptation to repeated stress exposure.

Results

Augmenting 2-AG reduces stress-induced anxiety-like behavior

To begin to elucidate the role of 2-AG signaling in modulating stress-resilience, we first determined the effects of systemic pharmacological 2-AG augmentation on stress-induced anxiety-like behaviors using the novelty-induced hypophagia (NIH) test, which is highly sensitive to acute traumatic stress and eCB manipulation (Gamble-George et al. 2013, Bluett et al. 2014). Acute administration of the MAGL inhibitor JZL-184 (8 mg/kg) increased 2-AG and decreased its metabolite, arachidonic acid (AA), without significantly affecting AEA in several limbic brain regions (**Fig. 3.1a-c**). JZL-184 significantly reduced anxiety-like behavior two hours after administration, measured as a reduction in latency to consume palatable food in the NIH test 24 hours after one or five days of foot-shock stress, but not in unstressed mice (**Fig. 3.1d**). JZL-184 also increased consumption following one day of stress (**Fig. 3.1e**). The CB1R inverse agonist Rimonabantblocked the effects of JZL-184 after five days of stress (**Fig. 3.1d-e**, diagonal stripes). Visual inspection of the cumulative distribution curves of feeding latency for vehicle vs. JZL-184 revealed larger separation at higher latencies (**Fig. 3.1f-h**), suggesting JZL-184 preferentially reduced the number of mice exhibiting high feeding latencies after stress. Rimonabant alone significantly increased latency and reduced consumption after one or five days of stress (**Fig. 3.1i-k**). Taken together, these data suggest bidirectional modulation of stress-induced anxiety states by enhancing versus inhibiting 2-AG-CB1R signaling.

Given the well-known effects of eCB signaling on food intake(Silvestri and Di Marzo 2013), we confirmed the anxiolytic effects of JZL-184 after stress exposure in another validated assay independent of appetitive motivation. Specifically, JZL-184 significantly increased light-time, light-distance, and % light-distance, and decreased latency to enter the light-zone, without

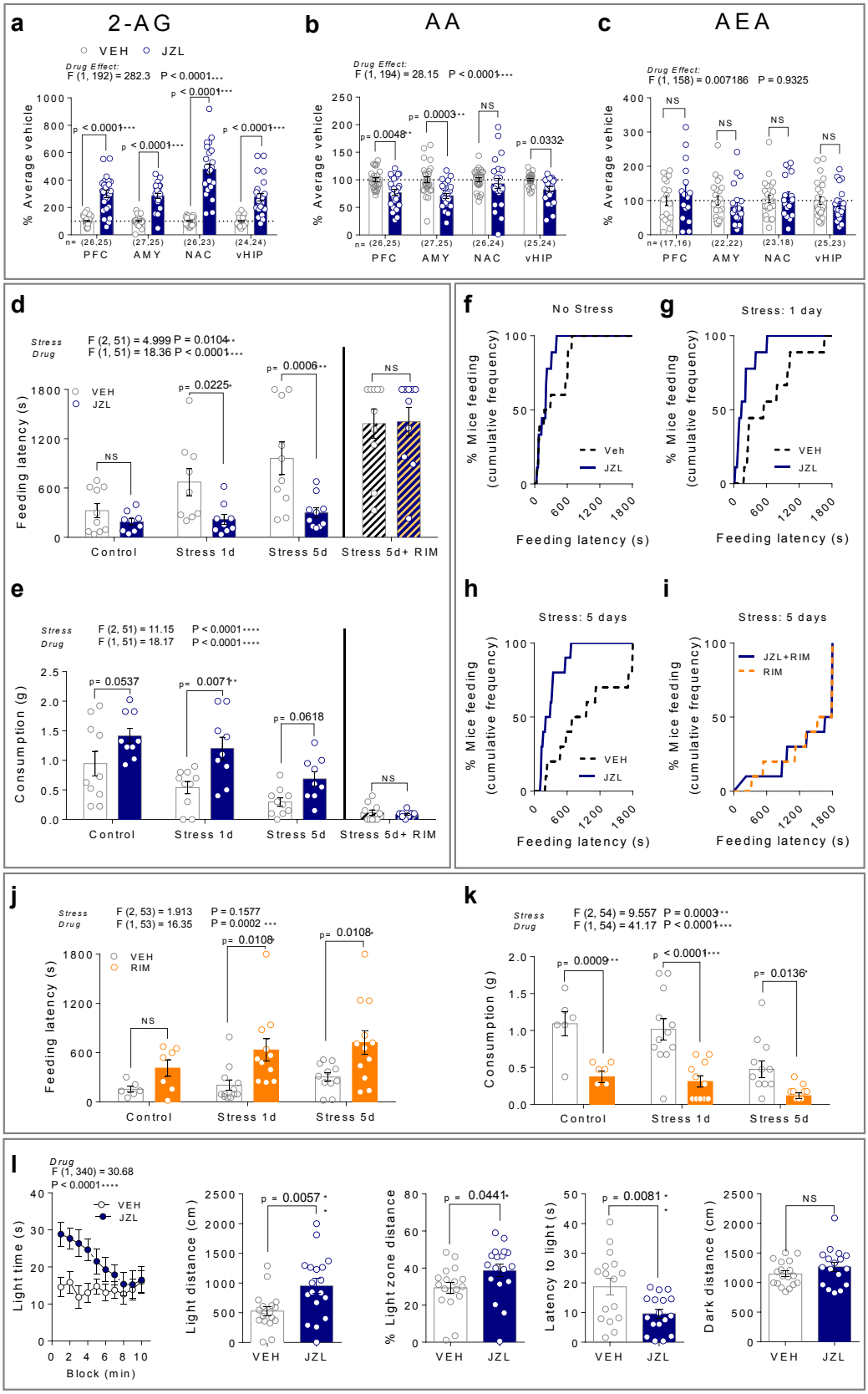
a
2-AG
VEH
JZL
Drug Effect:
F (1, 192) = 282.3 P < 0.0001
% Average vehicle
p < 0.0001
p < 0.0001
p < 0.0001
p < 0.0001
n= (26,25) (27,25) (26,23) (24,24)
PFC AMY NAC vHIP
b
AA
Drug Effect:
F (1, 194) = 28.15 P < 0.0001
% Average vehicle
p= 0.0048
p= 0.0003
NS
p= 0.0332
n= (26,25) (27,25) (26,24) (25,24)
PFC AMY NAC vHIP
c
AEA
Drug Effect:
F (1, 158) = 0.007186 P = 0.9325
% Average vehicle
NS
NS
NS
NS
n= (17,16) (22,22) (23,18) (25,23)
PFC AMY NAC vHIP
d
Stress
Drug
F (2, 51) = 4.999 P = 0.0104
F (1, 51) = 18.36 P < 0.0001
Feeding latency (s)
VEH
JZL
NS
p= 0.0225
p= 0.0006
NS
Control
Stress 1d
Stress 5d
Stress 5d+ RIM
e
Stress
Drug
F (2, 51) = 11.15 P < 0.0001
F (1, 51) = 18.17 P < 0.0001
Consumption (g)
p= 0.0537
p= 0.0071
p= 0.0618
NS
Control
Stress 1d
Stress 5d
Stress 5d+ RIM
f
No Stress
% Mice feeding (cumulative frequency)
Veh
JZL
Feeding latency (s)
g
Stress: 1 day
% Mice feeding (cumulative frequency)
VEH
JZL
Feeding latency (s)
h
Stress: 5 days
% Mice feeding (cumulative frequency)
VEH
JZL
Feeding latency (s)
i
Stress: 5 days
% Mice feeding (cumulative frequency)
JZL+RIM
RIM
Feeding latency (s)
j
Stress
Drug
F (2, 53) = 1.913 P = 0.1577
F (1, 53) = 16.35 P = 0.0002
Feeding latency (s)
VEH
RIM
NS
p= 0.0108
p= 0.0108
Control
Stress 1d
Stress 5d
k
Stress
Drug
F (2, 54) = 9.557 P = 0.0003
F (1, 54) = 41.17 P < 0.0001
Consumption (g)
p= 0.0009
p < 0.0001
p= 0.0136
Control
Stress 1d
Stress 5d
l
Drug
F (1, 340) = 30.68
P < 0.0001
Light time (s)
VEH
JZL
Block (min)
Light distance (cm)
p = 0.0057
VEH
JZL
% Light zone distance
p = 0.0441
VEH
JZL
Latency to light (s)
p = 0.0081
VEH
JZL
Dark distance (cm)
NS
VEH
JZL

Figure 3.1 Modulation of stress-induced anxiety-like behavior by 2-AG signaling. (**a-c**) Effects of JZL-184 (8 mg/kg; blue) on 2-arachidonoylglycerol (2-AG), arachidonic acid (AA), and anandamide (AEA) in the prefrontal cortex (PFC), amygdala (AMY), nucleus accumbens (NAc), and ventral hippocampus (vHIP). Data combined from two independent experiments. (**d-e**) Effects of JZL-184 treatment on feeding latency (top) and consumption (bottom) in the novelty-induced hypophagia test (NIH) without stress, after 1 or 5 days of foot-shock stress, and after 5 days of stress in combination with the CB1R inverse agonist Rimonabant (RIM; 1 mg/kg). (**f-i**) Cumulative feeding latency distributions of vehicle and JZL-184-treated mice without stress, after 1 or 5 days of foot-shock stress, and after 5 days of stress in combination with Rimonabant. (**j-k**) Effects of Rimonabant (orange) on feeding latency and consumption in NIH without stress, and after 1 and 5 days of foot-shock stress. (**l**) Effects of JZL-184 treatment in the light-dark box test after 1 day of foot-shock stress. F and P values for two-way ANOVA shown above individual panels (a-e, j-l). P values shown for pairwise comparisons derived from Holm-Sidak multiple comparisons test after ANOVA or unpaired two-tailed t-test (l). Data are presented as mean ± SEM. (a-k RJB & AH; l DJM)

significantly altering dark-distance in the light-dark test 24h after acute stress (**Fig. 3.1l**). Furthermore, JZL-184 treatment did not affect food consumption 24h after acute stress under less aversive (low light) NIH testing conditions, or increase weight gain over 10 days of treatment (**Fig. S3.1**). JZL-184 also did not significantly impact locomotor behavior in an open-field test at baseline or after five days of stress (**Fig. S3.1**). These data indicate that the ability of JZL-184 to reduce stress-induced anxiety-like behavior in the NIH test is not related to enhanced appetitive or consummatory drive *per se*, or a result of altered locomotor activity.

2-AG augmentation promotes a stress-resilient phenotype

Our data thus far indicate that 2-AG-CB1R signaling modulates stress-induced anxiety-like behavior; however, whether 2-AG signaling affects susceptibility to the development of stress-induced anxiety-like behavior is still unclear. To explicitly test this hypothesis, we developed a repeated NIH testing paradigm, which allowed for the detection of inter-individual differences in stress susceptibility **(Fig. 3.2a)**. Six days after baseline novel-cage testing, mice

were foot-shock stressed and, 24 hours later, evaluated in a 2nd novel-cage test. The change in each individual's latency between baseline and post-stress testing is shown in the population distribution in **Figure 3.2b**. Examination of the population distribution of stress-induced changes in latency revealed a bimodal distribution, with data significantly better fit to two independent Gaussian distributions (resilient n=77, susceptible n=43, $F_{(3,144)}$=112.3, p<0.0001 Extra Sum-of-squares F test, **Fig. 3.2c**). Taking into consideration the observed anti-node between the two distributions in Figure 2b, our aim to generate a meaningful susceptible group (i.e. not affected by low responders), and previous studies indicating that a difference in feeding latency on the order of 1-3 minutes represents a biologically relevant difference in anxiety-like behavior (Dulawa and Hen 2005), we empirically divided the population into susceptible and resilient groups. Susceptible mice were defined as having a stress-induced change in latency ≥120 seconds, while those with a change in latency <120 seconds were categorized as resilient. The means of the major and minor distributions after this categorization were -21 and 168 seconds, respectively. **Figures 3.2c-d** illustrate this cutoff, splitting individuals into stress resilient and susceptible populations. Retrospective examination of baseline NIH latencies of the two groups revealed that their distributions overlapped considerably (resilient mean 258 seconds, susceptible mean 203 seconds), indicating that baseline anxiety-like behavior does not predict stress susceptibility (**Fig. 3.2e-f**). Interestingly, specifically for the resilient subpopulation, there was a significant correlation between baseline latency and post-stress *reduction* in latency, such that the mice exhibiting the highest baseline latencies showed the largest decrease after stress, confirming the resilient nature of this group (**Fig. 3.2g**). There was no correlation between baseline latency and stress-induced *increase* in latency for susceptible mice (n=43, r^2=0.0097, p=0.529, linear regression).

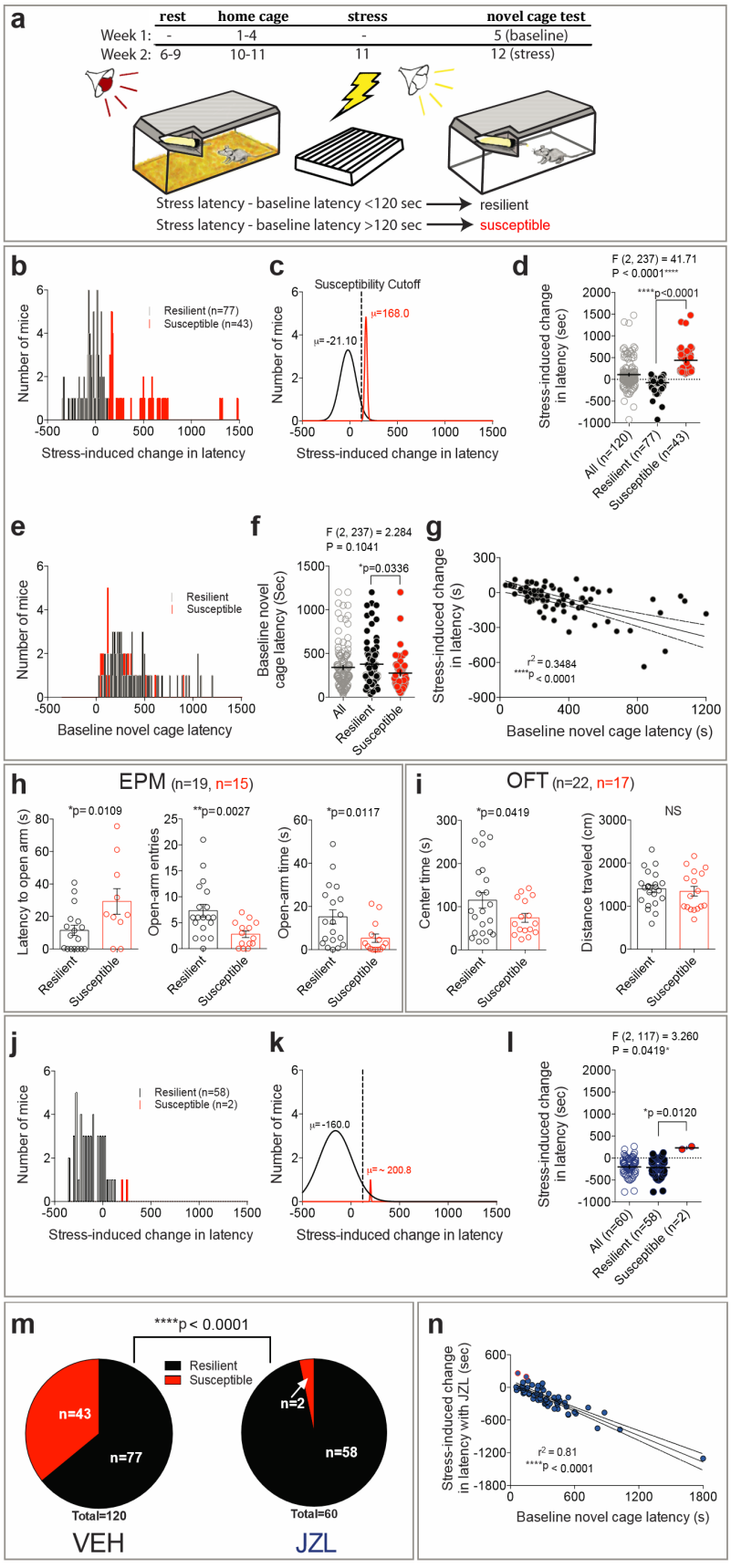
a
rest
home cage
stress
novel cage test
Week 1: - 1-4 - 5 (baseline)
Week 2: 6-9 10-11 11 12 (stress)
Stress latency - baseline latency <120 sec → resilient
Stress latency - baseline latency >120 sec → susceptible
b
Number of mice
Resilient (n=77)
Susceptible (n=43)
Stress-induced change in latency
c
Susceptibility Cutoff
μ=168.0
μ= -21.10
Number of mice
Stress-induced change in latency
d
F (2, 237) = 41.71
P < 0.0001****
****p<0.0001
Stress-induced change in latency (sec)
All (n=120)
Resilient (n=77)
Susceptible (n=43)
e
Number of mice
Resilient
Susceptible
Baseline novel cage latency
f
F (2, 237) = 2.284
P = 0.1041
*p=0.0336
Baseline novel cage latency (Sec)
All
Resilient
Susceptible
g
Stress-induced change in latency (s)
r2 = 0.3484
****p < 0.0001
Baseline novel cage latency (s)
h
EPM (n=19, n=15)
*p= 0.0109
Latency to open arm (s)
**p=0.0027
Open-arm entries
*p=0.0117
Open-arm time (s)
Resilient
Susceptible
i
OFT (n=22, n=17)
*p=0.0419
Center time (s)
NS
Distance traveled (cm)
Resilient
Susceptible
j
Number of mice
Resilient (n=58)
Susceptible (n=2)
Stress-induced change in latency
k
μ=-160.0
μ= ~ 200.8
Number of mice
Stress-induced change in latency
l
F (2, 117) = 3.260
P = 0.0419*
*p =0.0120
Stress-induced change in latency (sec)
All (n=60)
Resilient (n=58)
Susceptible (n=2)
m
****p < 0.0001
Resilient
Susceptible
n=43
n=77
Total=120
VEH
n=2
n=58
Total=60
JZL
n
Stress-induced change in latency with JZL (sec)
r2 = 0.81
****p < 0.0001
Baseline novel cage latency (s)

Figure 3.2 Elevating 2-AG shifts the distribution of stress-susceptibility toward resilience. (**a**) Schematic of behavioral paradigm. (**b**) Histogram of stress-induced change in latency (stress latency minus baseline latency) to consume in the NIH novel-cage test. (**c**) Gaussian curves fitting the resilient (black) and susceptible (red) subpopulations. Dashed line indicates 120-second post-stress latency increase susceptibility cutoff. (**d**) Stress-induced change in latency in the whole population and split into susceptible and resilient subgroups. (**e**) Histogram of pre-stress novel cage latencies categorized by resilience. (**f**) Individuals' pre-stress novel-cage latencies. (**g**) Correlation of resilient subpopulation's baseline and post-stress changes in latency. (**h**) Elevated plus maze (EPM) and (**i**) open-field test (OFT) measured 24h after foot-shock stress, one week after susceptibility characterization. (**j**) Histogram of 24h post-stress changes in latency with JZL-184 treatment 2h prior to testing. (**k**) Gaussian distributions for resilient and susceptible subpopulations with JZL-184 treatment. (**l**) Stress-induced change in latency in the whole population and split into susceptible and resilient subgroups with JZL-184 treatment. (**m**) Proportion of susceptible and resilient mice after either vehicle (VEH) or JZL-184 treatment. (**n**) Correlation between pre-stress latencies and stress-induced changes in latency with JZL-184. Data in a-g was aggregated from 3 cohorts of 40 mice that were used for subsequent experiments (see Online Methods for details). F and P values for one-way ANOVA shown above individual panels (d,f,l). P values shown for pairwise comparisons derived from Sidak multiple comparisons test after ANOVA (d,f,l) or unpaired one-tailed t-test (h-i) shown in each panel. R^2 and P value for linear regression reported in panels (g) and (n). P value from chi-squared test reported with susceptibility ratios (m). Data are presented as mean ± SEM. (a-i RJB; j-n AH & RJB)

To further validate phenotypic separation based on stress-induced NIH latency changes, one cohort of mice was categorized as resilient or susceptible and tested 7 days later in the elevated plus maze (EPM) and open-field test (OFT) 24h after a 2nd stress exposure. As expected, susceptible mice exhibited higher stress-induced anxiety-like behavior than resilient mice in the EPM and OFT (**Fig. 3.2h-i**). In a separate cohort, EPM and OFT were conducted prior to stress, and mice classified *post hoc* as susceptible or resilient using the procedure described above. Using this approach, we show conclusively that baseline (pre-stress) anxiety-like behavior does not significantly differ between groups prior to stress exposure (**Fig. S3.2**). Additionally, in order to be certain that our results were not due to differences in sensory processing of the foot-shock itself we measured foot-shock sensitivity thresholds and behavioral responses to foot-shock stress exposure and found no differences between resilient and susceptible groups (**Fig. S3.2**). Fear learning and recall also did not differ between groups, as indicated by percent freezing

across two foot-shock sessions with week one tone 6 (T6) freezing indicative of within-session learning, week two baseline (BL) freezing indicative of between-session context recall, and week two tone 1 (T1) freezing indicative of context + tone recall (**Fig. S3.2**). In order to determine if susceptibility was primarily due to differential HPA axis responsivity, we also measured stress-induced corticosterone immediately following the week two foot-shock stress session and found no group differences or correlation between stress-induced corticosterone and either NIH susceptibility or foot-shock responsivity (**Fig. S3.2**). These data support the segregation of two populations of mice without differences in basal anxiety-like behavior or acute shock-responsivity, but with differential sensitized anxiety-like behavioral sequelae of stress exposure.

We next wanted to determine if systemic 2-AG augmentation could promote stress-resilience in this model. To examine this experimentally, baseline feeding latencies were obtained, followed seven days later by 24h-post-stress novel-cage testing with JZL-184 treatment. JZL-184 treatment prior to the stress-test shifted the distribution of stress-induced changes in latency toward resilience, nearly eliminating the susceptible subpopulation (**Fig. 3.2j-m**). Analysis of the overall distribution of stress-induced changes in latency between JZL-184 and vehicle treatment revealed a dramatic increase in the resilient proportion at the expense of the susceptible population (**Fig. 3.2b-c vs. 3.2j-k,** and see ratios in **3.2m**). Furthermore, JZL-184 treatment strengthened the correlation between baseline latency and post-stress *reduction* in latency observed in naturally resilient mice (naturally resilient r^2=0.34; total population after JZL-184 treatment r^2=0.81; **Fig. 3.2g vs. Fig. 3.2n**). These data indicate that JZL-184 promotes the expression of a stress-resilient phenotype.

2-AG augmentation converts susceptibility into resilience

Although our data demonstrate that increasing 2-AG can prevent the emergence of stress-susceptibility at a population level, these results could be due to either a profound reduction in latencies specifically of mice that if untreated would have been susceptible to stress, or by an unbiased anxiolytic effect on the entire population. In order to distinguish between these possibilities, we determined the effect of systemic 2-AG augmentation in pre-identified stress-susceptible and resilient populations. To this end, we first established that stress-susceptibility was a relatively stable trait across at least two stress exposures. Extending our data above, we show that two stress exposures one week apart both significantly increase latency relative to baseline specifically in mice categorized as susceptible based on the first stress novel cage test (**Fig. 3.3a-c**). NIH novel-cage test consumption did not significantly change after or between stress exposures across the whole population or in either subpopulation (**Fig. 3.3d-f**). **Figure 3.3g** shows an explicit comparison between the latency change from baseline after the 1st and 2nd stress exposures; importantly, in the stress-susceptible subpopulation there was no significant habituation between the first and second stress-test exposures. While the difference trended downward from the 1st to the 2nd stress-test in both subpopulations, suggesting the possibility of a slight habituation, the decrease was not significant and the latency delta from baseline remained significantly different between the subpopulations after both stress exposures. These data also argue against statistical anomalies such as regression to the mean confounding subsequent drug effect studies (see below). To further exclude the possible confound of between-test habituation, we plotted 1st stress-test latency against the change in latency between the 1st and 2nd stress-tests for the susceptible mice and found that there was no correlation between degree of habituation and latency after the 1st stress exposure (**Fig. 3.3h**). In other words, the degree of stress-induced

anxiety-like behavior did not predict the magnitude of habituation to the second stress-test exposure. Lastly, we analyzed the duration of behavioral dysregulation induced by acute foot-shock stress exposure. In stress-susceptible mice, feeding latency in the NIH assay decreases from 1 day to 3 days, and from 1 day to 14 days after stress exposure; but remains significantly higher at 3 days and 14 days after stress compared to baseline (**Fig. S3.3**). Latencies for the stress-resilient subpopulation do not differ at any time point after stress (**Fig. S3.3**).

These experiments indicate that stress susceptibility is a relatively stable trait across at least two stress-NIH tests, which allows us to explicitly determine the effects of 2-AG augmentation on *a priori* defined stress-susceptible and resilient populations. Using this repeated

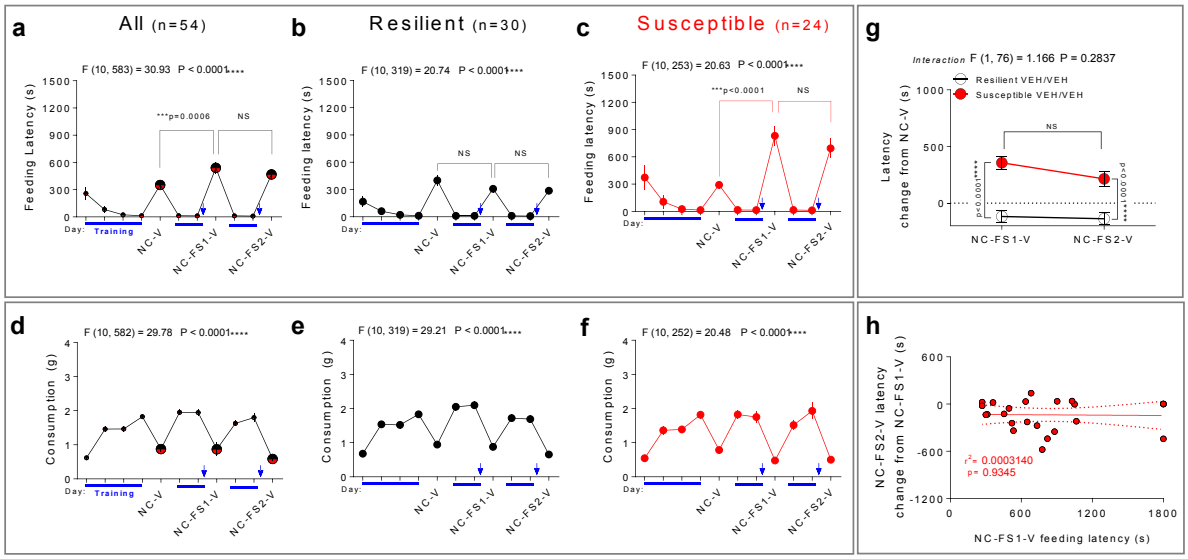

Figure 3.3 Stress susceptibility is a stable trait. (**a-c**) Home cage training (blue lines) and novel cage (NC-V) latencies and (**d-f**) consumption across one baseline and two novel-cage foot-shock stress tests (NC-FS1-V and NC-FS2-V) with vehicle treatment. (**g**) Direct comparison of the latency change from baseline between the two post-stress NIH novel cage tests. (**h**) Correlation between the first stress-test latency and the change in latency between the 2nd and 1st stress novel-cage tests for susceptible individuals. Blue arrows indicate foot-shock stress exposure. F and P values for one-way (a-f) or two-way (g) ANOVA shown above individual panels. P values shown for pairwise comparisons from Holm-Sidak multiple comparisons test after ANOVA. R^2 and P value for linear regression shown in panel (h). Data are presented as mean ± SEM. (a-h AH & RJB)

stress NIH paradigm, we found that JZL-184 was able to reverse established stress-susceptibility. JZL-184 reduced latency and increased consumption after stress across the whole population and Rimonabant blocked these effects (**Fig. 3.4a-b**). In order to examine subpopulation-specific drug effects, mice were split into stress-resilient (**Fig. 3.4 c-d**) and susceptible (**Fig. 3.4 e-f**) subpopulations using the 120 second cutoff criterion established in Figure 2. The paired baseline and post-stress individual latency and consumption data in Figures 4d and 4f illustrate the disparate effect of stress on latency and consumption between resilient and susceptible mice, despite widespread overlap in baseline latencies. JZL-184 reduced stress-test latency in the susceptible subpopulation without affecting latency in the resilient subpopulation (**Fig. 3.4c, e**). This stress-resilience promoting effect of JZL-184 in susceptible mice was completely blocked by co-treatment with Rimonabant. Interestingly, specifically in the resilient subpopulation, Rimonabant treatment increased latency significantly beyond stress-test latency (see **Fig. 3.4e**, NC-FS-V vs. NC-FS-JZL-RIM). These data suggest the possibility that resilient mice have elevated cannabinoid signaling either at baseline or specifically in response to stress, which may be mediating their stress-resilience. Explicit comparison of susceptible and resilient latencies between the 1st stress test with vehicle treatment and the 2nd stress test with JZL-184 demonstrated that JZL-184 reversed susceptibility to stress after it had manifested (**Fig. 3.4g**). Furthermore, the severity of stress-induced anxiety-like behavior was significantly correlated with the effectiveness of JZL-184 treatment (**Fig. 3.4h**), such that larger stress-induced latency increases corresponded with larger JZL-184-induced latency reductions in the subsequent test. As depicted in **Figure 3.4i**, these data indicate that increasing 2-AG-CB1R signaling converted a portion of the stress-susceptible subpopulation into stress-resilient mice. Although we have shown that ability of JZL-184 to reduce feeding latency after acute stress is not driven by

increased appetite (see **Fig. S3.1**), we wanted to confirm this using our repeated NIH testing paradigm. Specifically, when stress-resilient and susceptible mice are tested in the home cage rather than novel cage after repeated stress, JZL-184 does not affect latency or consumption (**Fig. 3.4j**). If increased appetitive drive was responsible for the changes in feeding latency after repeated stress, increases in food intake should be observed under home-cage conditions. Moreover, if increased appetitive drive was responsible for the changes in feeding latency there should be an inverse correlation between novel-cage feeding latency and food consumption; however, we found no correlation between novel-cage latency and consumption further arguing against changes in appetite driving changes in latency measurements with vehicle or JZL-184 treatment (**Fig. 3.4k**). We were not able to detect an anxiolytic-like effect of JZL-184 in the EPM in the context of the repeated testing paradigm, however this may be due to the reduced sensitivity of this assay to 2-AG augmentation. Indeed, a recent study found that MAGL inhibition was not effective in reducing anxiety-like behavior in the EPM in high anxiety rats (Morena et al. 2016) (**Fig. S3.4**).

Female mice displayed similar stress-susceptibility and JZL-184 efficacy (**Fig. S3.5**). However, in this case baseline NIH consumption significantly differed between susceptible and resilient mice, and at a population level moderately predicted stress-induced changes in latency ($n=51$, $r^2=0.12$, $p=0.017$, linear regression; **Fig. S3.5**). These data suggest possible sex differences in acute stress responsivity, which will require further investigation to validate.

2-AG depletion converts resilience into susceptibility

We next tested whether acute systemic pharmacological 2-AG depletion with the DAGLα inhibitor DO34 (Ogasawara et al. 2016), which selectively reduces brain 2-AG and AA but not AEA levels (**Fig. 3.5a-c**), would increase susceptibility to stress-induced anxiety-like

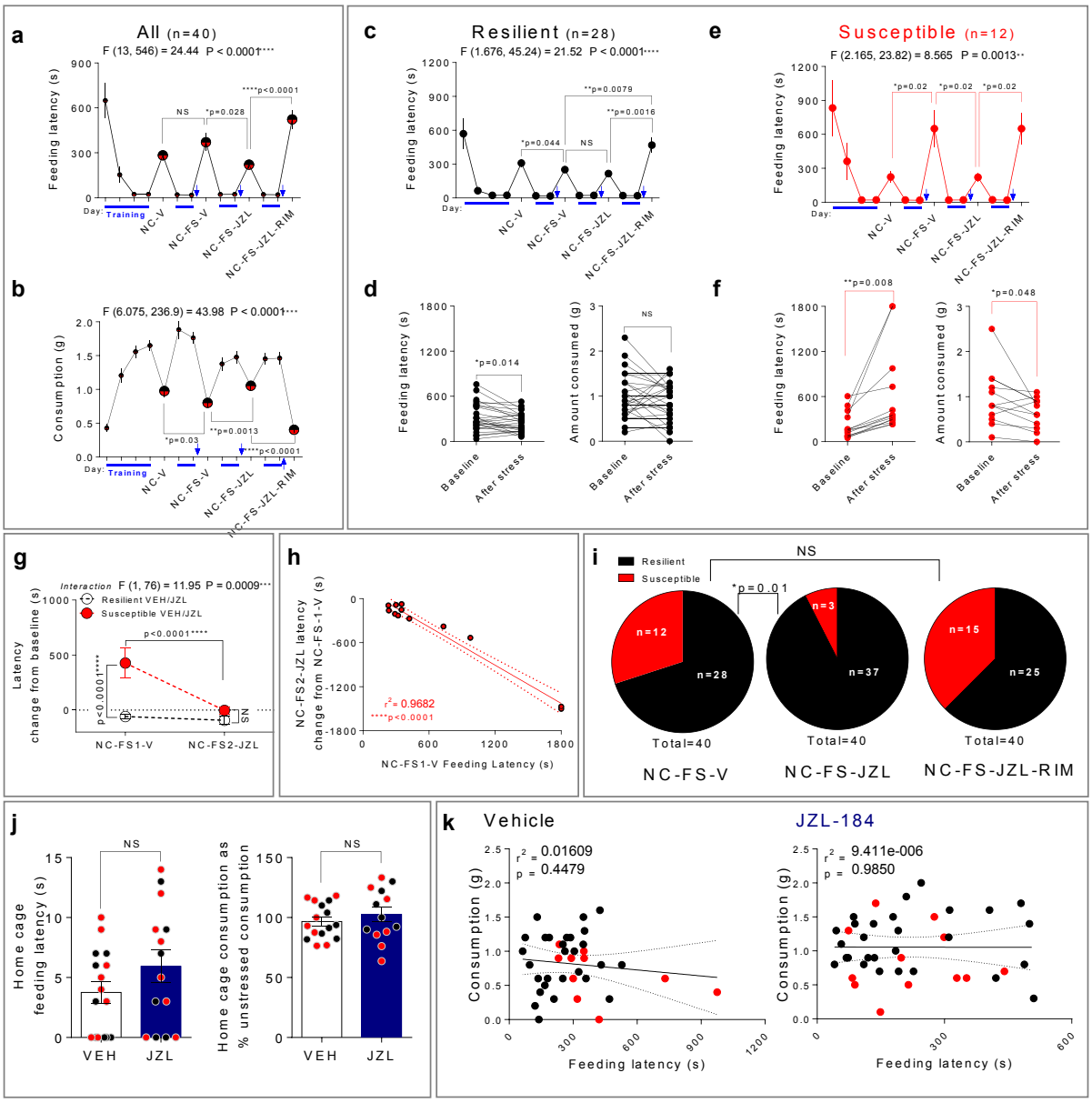

Figure 3.4 2-AG augmentation promotes resilience to acute stress-induced anxiety-like behavior. (**a**) Home cage training (blue lines) and novel cage (NC) latencies and (**b**) consumption before (NC-V) and after (NC-FS-V) foot-shock stress with vehicle (V), JZL-184, and JZL-184+Rimonabant (RIM) treatment. (**c**) Resilient subgroup latencies separated from panel (a). (**d**) Resilient individuals' baseline and post-stress latencies and consumption. (**e**) Susceptible subgroup latencies separated from panel (a). (**f**) Susceptible individuals' baseline and post-stress latencies and consumption. (**g**) Direct comparison of changes in latency from baseline between the 2nd stress test with JZL-184 (NC-FS2-JZL) and the first stress test with vehicle (NC-FS1-V). (**h**) Correlation between stress-test latency and change in latency between JZL-184 and vehicle treatment for susceptible individuals. (**i**) Stress-susceptibility ratios for the same cohort of mice across three weeks after vehicle, JZL-184, or JZL-184+Rimonabant treatment.

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Figure 3.4 continued. (**j**) Home cage testing latency (left) and consumption as % of previous day's home cage/no stress consumption (right) 24h after stress exposure with resilient (black circles) and susceptible (red circles) individuals treated with vehicle (VEH) or JZL-184 (blue) one week after susceptibility categorization. (**k**) Whole population correlations between post-stress novel cage test feeding latency and consumption with vehicle and JZL-184 treatment. Blue arrows indicate foot-shock stress exposure. F and P values for one-way (a-c, e) or two-way (g) ANOVA shown above individual panels. P values for pairwise comparisons derived from Holm-Sidak multiple comparisons test after ANOVA, unpaired two-tailed t-test (j), or paired two-tailed t-test (d,f) shown in panels. R^2 and P value for linear regression shown in panels h and k. P values from chi-squared tests reported in panel (i). Data are presented as mean ± SEM. (a-k AH & RJB)

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behavior. As expected, at a population level DO34 administration increases feeding latency and decreases consumption after stress relative to vehicle treatment (**Fig. 3.5d-e**). Subgroup analysis revealed DO34 strongly increases feeding latency in previously resilient mice without significantly affecting latency in susceptible mice (**Fig. 3.5f-h**), and increases the proportion of stress-susceptible mice relative to vehicle treatment (**Fig. 3.5i**). Given the large reduction in food consumption observed after DO34 treatment, we wanted to determine if the anxiogenic-like effect (increased latency) was dependent on changes in appetitive drive. We found that feeding latency and consumption in novel-cage testing after stress were not correlated in either vehicle-treated or DO34-treated resilient groups, again indicating that these two measures are independent (**Fig. 3.5j**). All individuals that did not drink during the entire testing period were excluded from this analysis, although the linear regression remains insignificant even when they are included. We also tested DO34 in a secondary assay, the EPM, to confirm its anxiogenic effect. Using the same experimental design, with the exception that mice were tested in the EPM rather than in the NIH assay in week 3, 24h after the 2nd stress exposure, we found resilient mice treated with DO34 showed higher levels of anxiety-like behavior in the EPM relative to vehicle-treated resilient mice (**Fig. 3.5k**). Taken together, these data indicate that acute depletion of

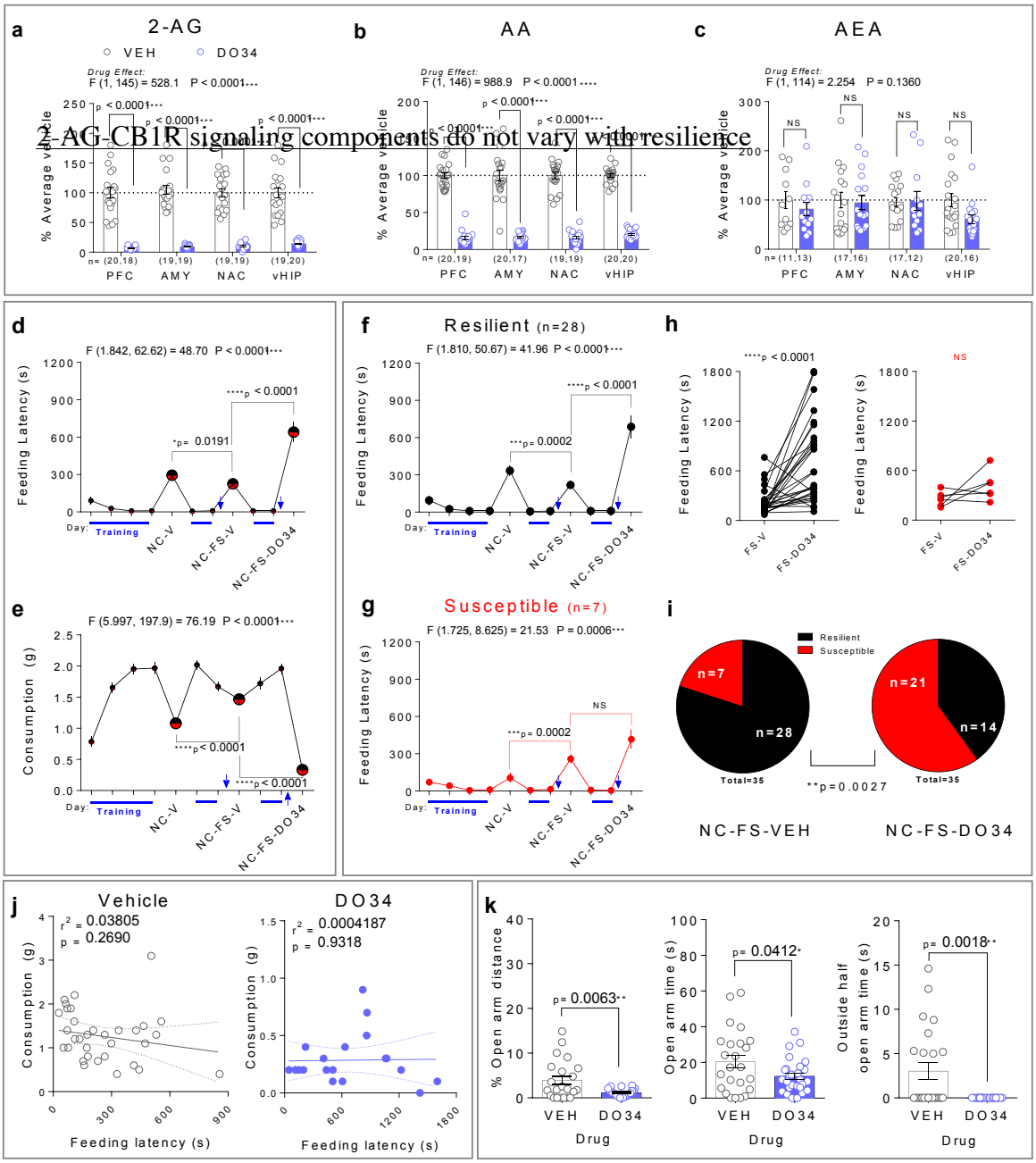

Figure 3.5 2-AG depletion increases susceptibility to acute stress-induced anxiety-like behavior. (**a-c**) Effects of DO34 (50 mg/kg; purple) on 2-arachidonoylglycerol (2-AG), arachidonic acid (AA), and anandamide (AEA) in the prefrontal cortex (PFC), amygdala (AMY), nucleus accumbens (NAc), and ventral hippocampus (vHIP). (**d**) Home cage training (blue lines) and novel cage (NC) latencies and (**e**) consumption before (NC-V) and after (NC-FS-V) foot-shock stress with vehicle (V) or DO34 treatment. (**f**) Resilient and (**g**) susceptible subgroup latencies separated from panel (d). (**h**) Effects of DO34 on feeding latency relative to vehicle treatment in resilient (black) and susceptible (red) mice.

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Figure 3.5 continued. (**i**) Stress-susceptibility ratios for the same cohort of mice across two weeks after vehicle or DO34 treatment. (**j**) Resilient population correlations between novel cage test feeding latency and consumption with vehicle and DO34 treatment. (**k**) Elevated plus maze 24h after stress exposure with resilient individuals treated with vehicle (VEH) or JZL-184 (blue) one week after susceptibility categorization. Blue arrows indicate foot-shock stress exposure. F and P values for one-way (d-g) or two-way (a-c) ANOVA shown above individual panels. P values for pairwise comparisons derived from Holm-Sidak multiple comparisons test after ANOVA, unpaired two-tailed t-test (k), or paired two-tailed t-test (h) shown in panels. R^2 and P value for linear regression shown in panel j. P value from chi-squared test reported in panel (f). Data are presented as mean ± SEM. (a-k ADG, JB, RJB)

2-AG signaling increases susceptibility to the adverse behavioral effects of traumatic stress exposure.

2-AG-CB1R signaling components do not vary with resilience

In an initial effort to elucidate the eCB-related biochemical mechanisms contributing to stress susceptibility, we analyzed levels of CB1R, DAGLα, and MAGL in multiple limbic brain regions. We found no significant differences in levels of CB1R, DAGLα, or MAGL (**Fig. S3.6**) protein in the amygdala (AMY), prefrontal cortex (PFC), nucleus accumbens (NAC), or ventral hippocampus (vHIP) of resilient and susceptible mice. We next used mass spectrometry to directly measure the bulk tissue levels of 2-AG in these brain regions. We found no significant differences in the levels of 2-AG in the amygdala and furthermore show that amygdala 2-AG does not correlate with stress-induced change in latency across the whole population, or specifically in the resilient, or susceptible subpopulations (**Fig. S3.7**). We further found no group differences in 2-AG levels, or population or subpopulation correlations with susceptibility in the prefrontal cortex, nucleus accumbens, or ventral hippocampus (**Fig. S3.7**). Although bulk 2-AG levels and total protein levels of 2-AG metabolic enzymes were not different between stress susceptible and resilient mice, several post-translational mechanisms of regulation have been discovered that may affect the efficiency of 2-AG signaling, including DAGLα phosphorylation

(Shonesy et al. 2013) or localization (Jung et al. 2012), and MAGL sulfenylation (Dotsey et al. 2015). Future studies will be required to test mechanisms by which 2-AG signaling could be differentially regulated in stress resilient versus susceptible mice.

Tetrahydrocannabinol (THC) promotes stress resilience

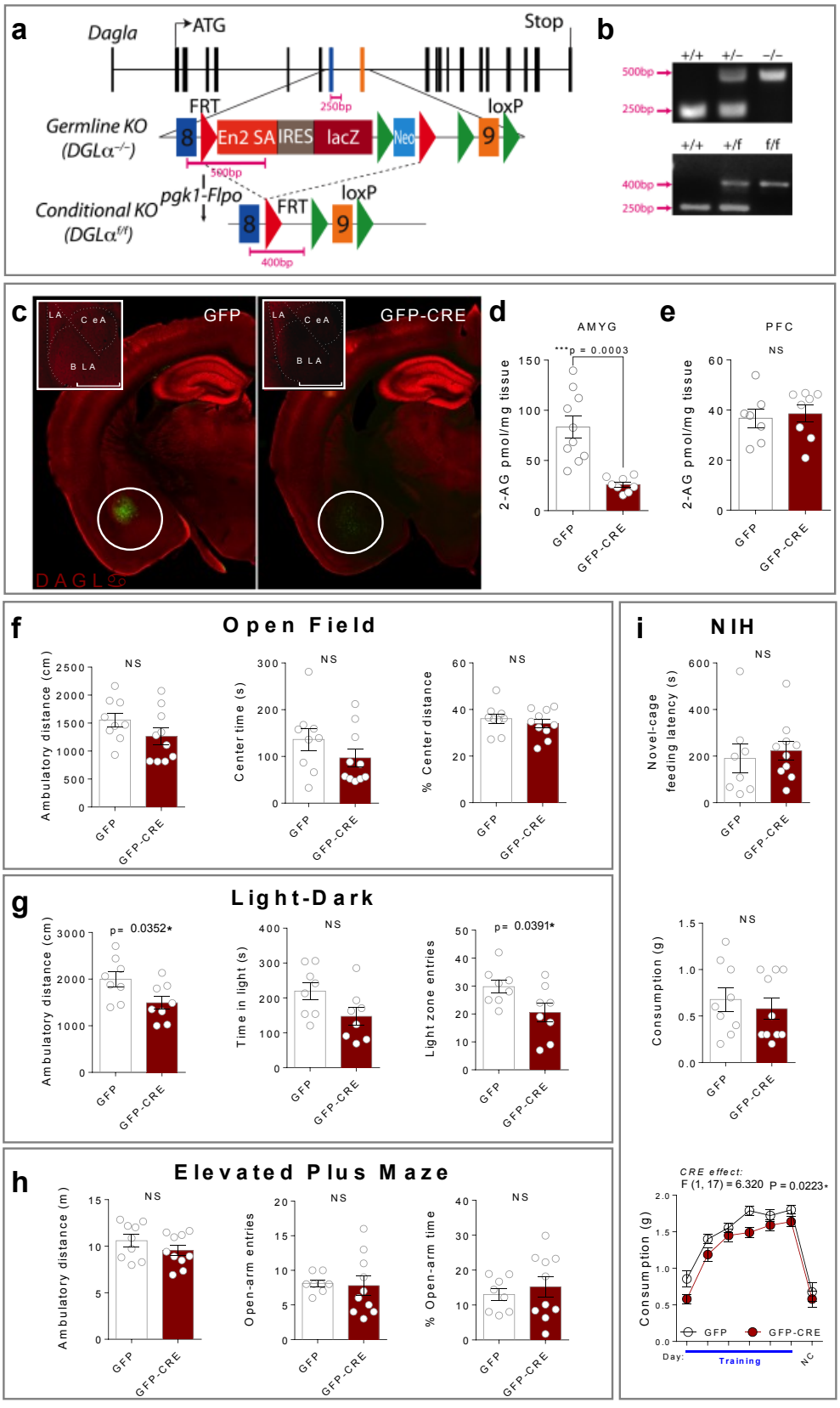
Given the high prevalence of cannabis use in patients with anxiety disorders and PTSD, and the high rate of symptom-coping motives cited by cannabis users with these disorders (Bonn-Miller et al. 2014, Kevorkian et al. 2015), we next sought to determine if this resilience-promoting effect of 2-AG was generalizable to a direct CB1R agonist. Consistent with clinical reports, we found that a low dose of the cannabinoid agonist, delta-9-tetrahydrocannabinol (THC, 0.25mg/kg), also specifically reduced stress-induced anxiety-like behavior in stress-susceptible mice without affecting consumption in either group (**Fig. S3.8**). These data are consistent with several reports showing direct activation of CB1 receptors can attenuate adverse consequences of traumatic stress in laboratory models (Ganon-Elazar and Akirav 2012, Ganon-Elazar and Akirav 2013), and provide additional preclinical support for the tension-reduction hypothesis to explain high rates of cannabis use in patients with mood and anxiety disorders.

Amygdala 2-AG is necessary for adaptation to repeated stress

In order to dissect region-specific necessity of 2-AG signaling in the regulation of stress susceptibility and adaptation to repeated stress exposure, we developed a floxed mouse line for conditional Cre-dependent deletion of the primary central 2-AG synthetic enzyme DAGLα (DAGL$\alpha^{f/f}$) (**Fig. 3.6a-b** and see Methods). We utilized stereotaxic injection of adeno-associated virus serotype 5 encoding a green fluorescent Cre recombinase fusion protein (AAV-GFP-CRE) into the basolateral amygdala (BLA), prefrontal cortex (PFC), and nucleus accumbens (NAc) of

DAGLα$^{f/f}$ mice to achieve substantial reductions in DAGLα immunoreactivity compared to AAV-GFP control injection (**Fig. 3.6c**). As expected, BLA injections of AAV-GFP-CRE also produced a significant reduction of amygdala 2-AG levels (**Fig. 3.6d**), but had no effect in a non-injection region, the PFC (**Fig. 3.6e**). BLA AAV-GFP-CRE injection did not affect locomotor or basal anxiety-like behavior in the OFT (**Fig. 3.6f**). AAV-GFP-CRE injected mice did exhibit a slight basal anxiety-like phenotype in the light-dark test (**Fig. 3.6g**), but not in EPM or NIH (**Fig. 3.6h-i**). BLA-specific DAGLα deletion therefore produced only a slight basal anxiety-like phenotype, in stark contrast to germline deletion (Shonesy et al. 2014, Patel et al. 2015). DAGLα deletion within the PFC or NAc did not affect basal anxiety-like behaviors (**Fig. S3.9**).

We next used the repeated NIH paradigm to test the hypothesis that BLA-specific deletion of DAGLα increases stress-susceptibility. We again found that BLA AAV-GFP-CRE mice exhibited baseline NIH latencies comparable to BLA AAV-GFP injected mice, and additionally that group latencies did not diverge after single stress exposure (**Fig. 3.7a**). However, as others have shown, 2-AG progressively increases in response to repeated homotypic stress exposure (Patel et al. 2005, Patel and Hillard 2008). This progressive increase in 2-AG after repeated homotypic stress has been suggested to represent part of the endogenous stress adaptation response (Patel and Hillard 2008). Based on these data, and our previous experiments showing JZL-184 decreases anxiety-like behaviors after repeated foot-shock stress exposure (see **Fig. 3.1**), we hypothesized that BLA 2-AG signaling may become increasingly important in mediating adaptation across repeated stress exposures. Consistent with this hypothesis, 24h after a 5th foot-shock stress exposure, BLA AAV-GFP-CRE mice exhibited significantly higher NIH latencies than BLA AAV-GFP mice (**Fig. 3.7a**; NC-5FS). Applying the 120 second cutoff criterion to the 5-day stress latencies, we split the AAV-GFP and AAV-GFP-CRE mice into


a
Dagla
ATG
Stop
FRT
250bp
loxP
Germline KO
(DGLα-/-)
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IRES
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560bp
pgk1-Flpo
Conditional KO
(DGLαf/f)
400bp
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+/+
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Consumption (g)
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Figure 3.6 Conditional DAGLα knockout mice and BLA-specific DAGLα deletion. (**a**) Diagram of targeting construct and strategy for the generation of DAGLα$^{f/f}$ mouse. Mice harboring *dagla* gene-trap cassette(Shonesy et al. 2014) were crossed to *pgk-Flpo* mice to generate conditional knockouts with loxP sites flanking exon 9. (**b**) PCR products for genotyping of germline (DAGLα$^{-/-}$) and conditional (DAGLα$^{f/f}$) knockouts. Primer binding sites shown in panel (a). (**c**) Representative coronal brain slices from DAGLα$^{f/f}$ mouse after BLA-AAV-GFP (left) and BLA-AAV-GFP-CRE (right) injection, and 20X magnification of BLA-DAGLα immunoreactivity of BLA-GFP control and BLA-GFP-CRE injected mice (square insets). White circles represent typical brain punch dissections for mass spectrometry. Inset scale bars are 500 μm. (**d**) Amygdala 2-AG levels after AAV-GFP and AAV-GFP-CRE BLA-injection from punch biopsies as indicated by white circles in panel (c). (**e**) PFC 2-AG levels after BLA-AAV-GFP and BLA-AAV-GFP-CRE injection. (**f**) Effect of AAV-GFP vs. AAV-GFP-CRE BLA-injection on behavior in open-field, (**g**) light-dark box, and (**h**) elevated plus-maze. (**i**) Effect of AAV-GFP vs. AAV-GFP-CRE BLA-injection on baseline novelty-induced hypophagia (NIH) testing. P values shown for unpaired one-tailed t-test above each panel (d-i). F and P values for two-way ANOVA shown in panel (i). Data are presented as mean ± SEM. (a-b BCS; c NDH; d-i RJB)

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resilient and susceptible subpopulations (**Fig. 3.7b-c**). A significantly larger proportion of AAV-GFP-CRE mice exhibited susceptibility after 5 days of stress (**Fig. 3.7d**) and, furthermore, feeding latency was significantly higher in susceptible BLA AAV-GFP-CRE mice than susceptible BLA AAV-GFP mice, suggesting an increased severity of the stress-induced anxiety-like phenotype (**Fig. 3.7e-f**). These data indicate that BLA 2-AG signaling is necessary for the physiological adaptation to repeated homotypic stress, and that other regions or the coordinated actions of 2-AG signaling within multiple brain regions promote resilience in response to acute stress exposure. In contrast, mice with PFC or NAc DAGLα deletion did not exhibit differential susceptibility to acute stress- induced anxiety-like behavior or adaptation to repeated stress, relative to GFP-injected controls (**Fig. S3.10**).

Resilience is associated with enhanced BLA 2-AG signaling

In order to elucidate synaptic and circuit-level mechanisms by which 2-AG signaling promotes stress-resilience, we determined the synaptic efficacy of 2-AG signaling in resilient

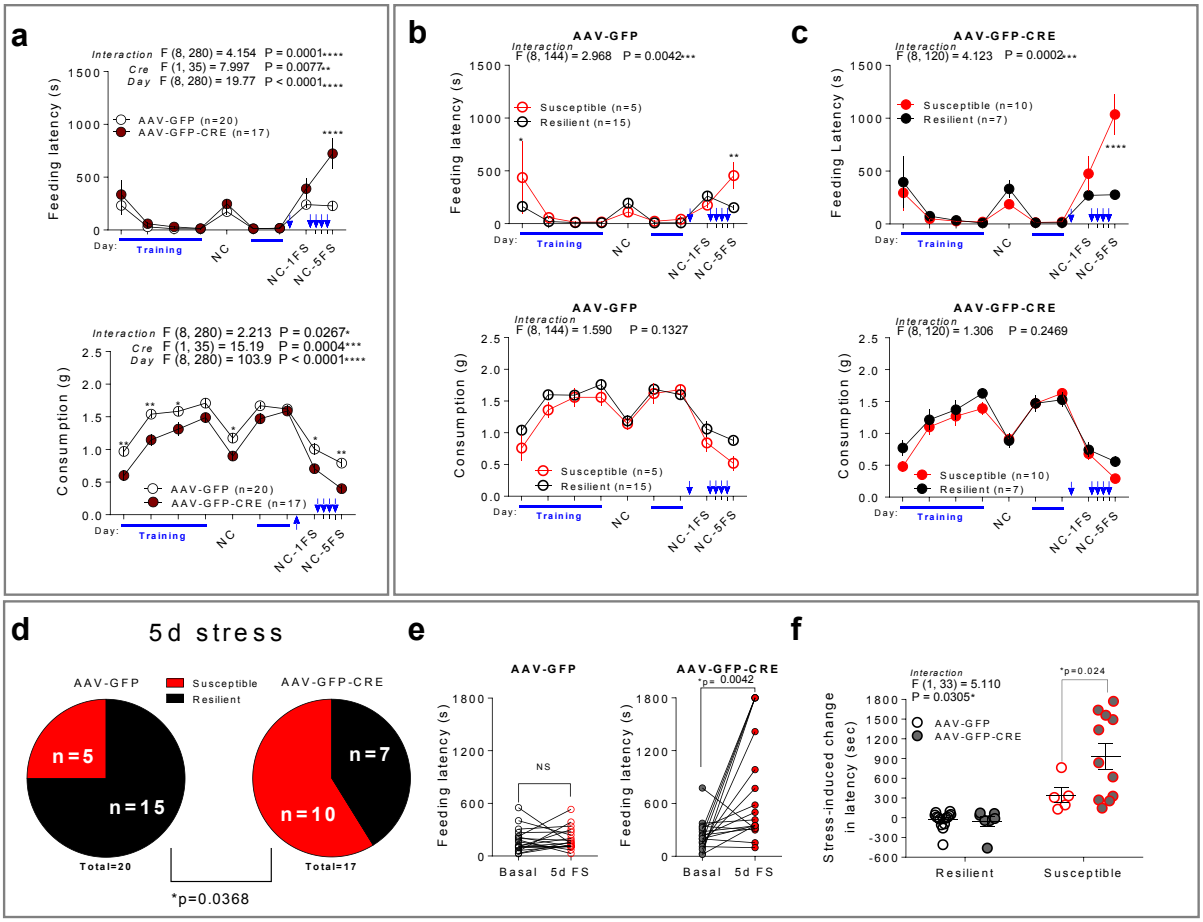

Figure 3.7 BLA-2-AG signaling is required for resilience to repeated traumatic stress. (**a**) Effect of AAV-GFP vs. AAV-GFP-CRE BLA-injection on home cage NIH training (blue lines) and novel cage latency (top) and consumption (bottom) with no stress (NC), 24 hours after 1 day of stress (NC-1FS), and 24 hours after a 5th day of stress (NC-5FS). (**b**) AAV-GFP latency (top) and consumption (bottom) from (a) split into resilient (black) and susceptible (red) groups. (**c**) AAV-GFP-CRE latency (top) and consumption (bottom) from (a) split into resilient (black) and susceptible (red) groups. (**d**) 5-day stress susceptibility ratios for BLA AAV-GFP and AAV-GFP-CRE injected groups. (**e**) Paired individual baseline and post-stress latencies in AAV-GFP and AAV-GFP-CRE BLA-injected groups. (**f**) Direct comparison of stress-induced changes in latency in resilient and susceptible AAV-GFP vs. AAV-GFP-CRE BLA-injected mice. Data combined from 2 independent cohorts. Blue arrows in panels a-c indicate stress exposure, which occurred once per day for 5 consecutive days. F and P values for two-way ANOVA shown above individual panels (a-c, f). P values for pairwise comparisons derived from Holm-Sidak multiple comparisons test after ANOVA, paired two-tailed t-test (e), and chi-squared test reported (d) in each panel. Data are presented as mean ± SEM. (RJB)

and susceptible subpopulations using electrophysiological approaches. We utilized *ex vivo* whole-cell patch-clamp electrophysiology to examine the effect of JZL-184 incubation on the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) onto pyramidal cells in the BLA of control and stress-exposed mice. As expected, in unstressed mice JZL-184 significantly reduced sEPSC frequency (increased inter-event interval; IEI) without affecting sEPSC amplitude (**Fig. 3.8a**). Interestingly, one day after stress, JZL-184 significantly reduced both sEPSC frequency and amplitude (**Fig. 3.8b**). Direct comparison of all four conditions revealed that stress increased sEPSC frequency, and that JZL-184 was more effective at reducing sEPSC frequency after stress (**Fig. 3.8c**), suggesting 2-AG signaling limits stress-related excitatory drive to BLA neurons.

We next examined potential differences in tonic 2-AG signaling at BLA glutamatergic synapses in resilient versus susceptible mice after stress. JZL-184 significantly reduced sEPSC frequency 24h after stress exposure in both populations while Rimonabant increased sEPSC frequency in the resilient subpopulation only; no differences in sEPSC frequency were observed between vehicle-treated susceptible and resilient mice (**Fig. 3.8d**). Neither drug significantly affected sEPSC amplitudes (**Fig. 3.8d**, bottom). Direct comparison of sEPSC frequencies and amplitudes with maximal (JZL-184 incubated) and abolished (Rimonabant incubated) 2-AG signaling revealed that stress-resilience was associated with a greater range of 2-AG signaling capacity at BLA glutamatergic synapses (**Fig. 3.8e**, representative traces in **3.8f**). These data suggest stress-resilient mice utilize eCB signaling to regulate BLA glutamatergic transmission in a wider dynamic range than stress-susceptible mice, and that this broader utilization of eCB signaling could represent a synaptic substrate promoting stress-resilience.

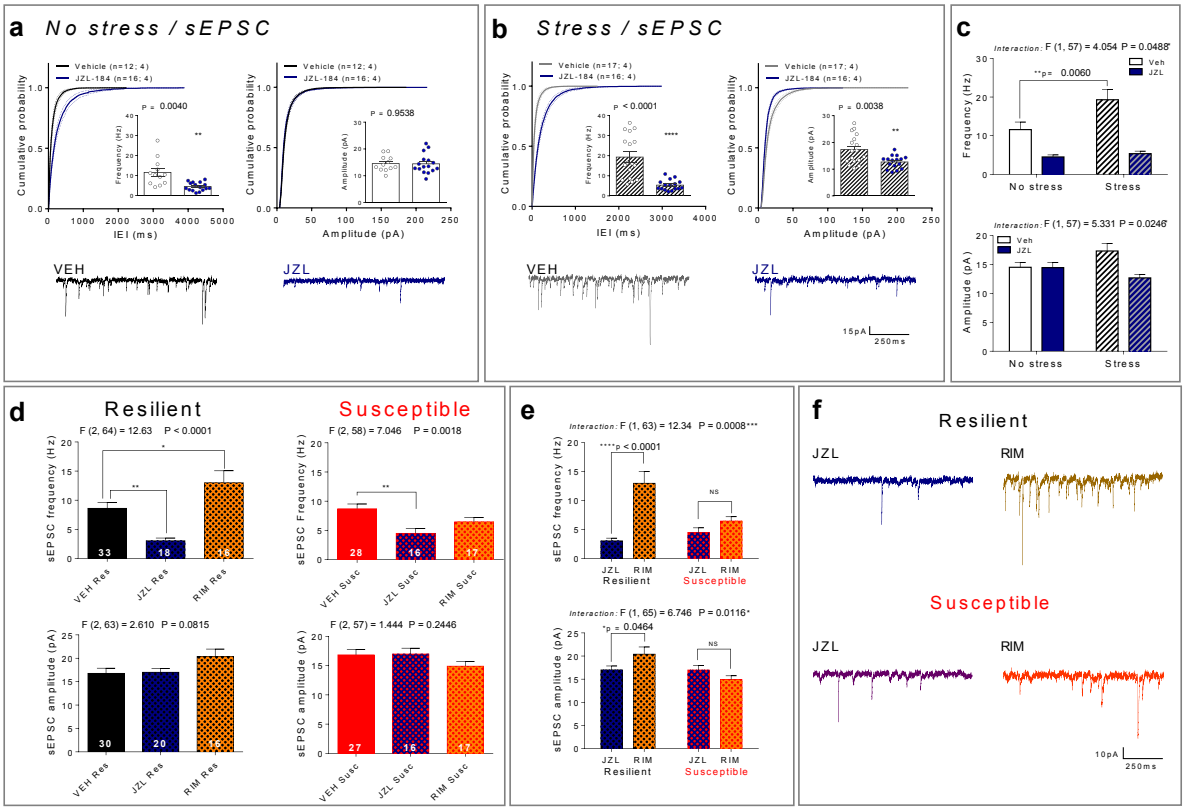

Figure 3.8 Stress-induced increases in sEPSC frequency in the BLA are eliminated by JZL-184 incubation. (**a**) Effect of JZL-184 incubation on the inter-event interval (IEI; left), frequency (left inset) and amplitude (right) of spontaneous excitatory postsynaptic currents (sEPSCs) onto BLA pyramidal cells in control non-stressed mice, and (**b**) 24 h after foot-shock stress exposure. (**c**) Direct comparison of stress effect and JZL-184 effect from (a) and (b). (**d**) Direct comparison of resilient (left; black) and susceptible (right; red) BLA sEPSC frequency (top) and amplitude (bottom) with vehicle (VEH) and either JZL-184 (blue hash) or Rimonabant (RIM; orange hash) incubation. (**e**) Direct comparison of the dynamic range of eCB signaling (defined as the difference in sEPSC frequency (top) and amplitude (bottom) between JZL-184 and Rimonabant conditions shown in panel (d) with representative traces in panel (**f**). Number of cells is shown for each group. Number of (cells, animals) are reported in panels (a-b). Number of cells reported in panel (d). F and P values for one-way (d) or two-way (c, e) ANOVA shown above individual panels. P value for pairwise comparisons derived from Holm-Sidak multiple comparison test after ANOVA (c-e) or unpaired t-test (a-b) shown in individual panels. Data are presented as mean ± SEM. (a-f AH & RB)

2-AG modulation of vHIP-BLA inputs is enhanced in resilience

Our electrophysiological data thus far indicate enhanced 2-AG signaling at BLA glutamatergic synapses in stress-resilient mice; however, the BLA receives a variety of glutamatergic afferents. Indeed, optogenetic projection-targeting approaches combined with *ex vivo* electrophysiology revealed glutamatergic inputs from the prefrontal cortex, ventral hippocampus, and lateral entorhinal cortex (**Fig. S3.11**). Of these afferents, the ventral hippocampal input showed the largest sensitivity to phasic 2-AG-mediated retrograde inhibition in the form of optogenetic depolarization-induced suppression of excitation (oDSE, **Fig. S3.11**). We further verified that the vHIP-BLA oDSE was CB1R-dependent by blocking it with Rimonabant (**Fig. S3.11**). Given that the NIH test relies on perceived novelty of environmental context, and the ventral hippocampal-BLA circuit is considered to be anxiogenic (Herry et al. 2008, Fanselow and Dong 2010), we next focused on elucidating the role of 2-AG signaling at these synapses in stress-resilient and susceptible mice.

In order to determine whether stress-resilience was associated with relatively enhanced 2-AG-mediated modulation of ventral hippocampal-BLA circuits, AAV-ChR2 was injected into the ventral hippocampus, followed by behavioral separation of mice into stress-susceptible and resilient populations, and *ex vivo* electrophysiological recordings (**Fig. 3.9a**, schematic). Input-output curves indicate that ventral hippocampal-BLA connectivity is stronger in stress-susceptible, relative to resilient, mice while paired pulse ratios do not differ between groups (**Fig. 3.9b**). Importantly, oDSE at hippocampal-BLA synapses is significantly reduced in stress-susceptible, relative to resilient, mice (**Fig. 3.9c**). Moreover, JZL-184 restores maximal oDSE in stress-susceptible mice to levels seen in stress-resilient mice (**Fig. 3.9d**). In a subset of cells, we show that 1 μM JZL-184 wash-on specifically enhances oDSE in the susceptible group as

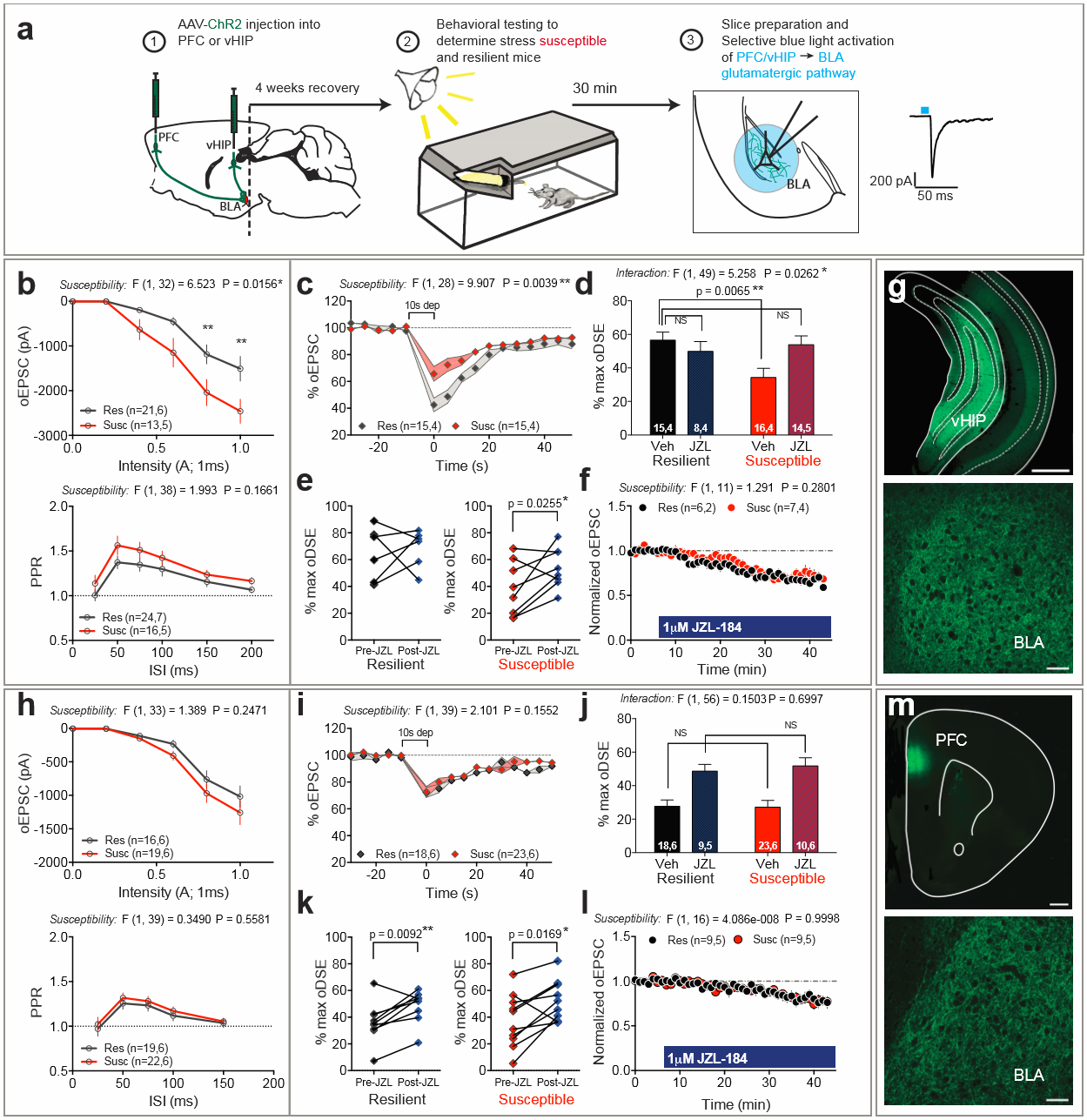

Figure 3.9 Stress-resilience is associated with greater 2-AG modulation of vHIP-BLA glutamatergic synapses. (**a**) Schematic of the experimental procedure for optogenetic recordings. (**b-g**) Optogenetic recordings at vHIP-BLA synapses. (**h-m**) Optogenetic recordings at PFC-BLA synapses. (**b,h**) Optically evoked input-output curves and paired pulse ratio. (**c,i**) Depolarization-induced suppression of excitation (oDSE) in susceptible and resilient population. (**d,j**) Direct comparison of the effect of JZL-184 on the magnitude of oDSE in resilient versus susceptible groups. (**e,k**) Paired comparison of % maximal oDSE in the same cell pre- and post-JZL-184 incubation for resilient and susceptible groups. (**f,l**) 1 mM JZL-184-induced depression of optically evoked EPSCs.

Legend continued on next page.

Figure 3.9 continued. (**g,m**) Representative images of the vHIP and PFC injection sites, and the corresponding BLA recording sites. Number of (cells, animals) are presented within each panel. F and P values for two-way ANOVA shown above individual panels (b-d, f, h-j, l). P values shown for pairwise comparisons derived from Holm-Sidak multiple comparisons test after ANOVA or paired two-tailed t-test (e, k). Data are presented as mean ± SEM. Scale bars are 500 μm for vHIP and PFC images, 50 μm for BLA. (a-m AH & RB)

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demonstrated by paired pre versus post JZL-184 maximal oDSE measurements (**Fig. 3.9e**). However, JZL-184 wash-on does not differentially affect tonic 2-AG mediated oEPSC depression in stress-resilient versus susceptible groups (**Fig. 3.9f**). Injection site and fiber innervation of the BLA is shown in **Fig. 3.9g**. These data reveal enhanced phasic, but not tonic, 2-AG signaling in stress-resilient, relative to susceptible, mice. These studies parallel our behavioral findings and indicate stress-resilient mice have both weaker connectivity of the anxiogenic vHIP-BLA pathway as well as elevated phasic 2-AG-mediated suppression of vHIP-BLA glutamatergic transmission. This elevated phasic 2-AG signaling likely contributes to their stress-resilient phenotype and the anxiogenic effects induced by CB1R blockade in these mice as CB1 receptor function at vHIP-BLA synapses as measured by synaptic depression induced by the direct CB1R agonist CP55940 does not differ between resilient and susceptible individuals (**Fig. S3.12**). In contrast, stress-susceptible mice have relatively reduced phasic 2-AG signaling at vHIP-BLA synapses, which could contribute to their behavioral stress-susceptibility; both of which can be normalized by JZL-184 treatment.

In contrast to the vHIP-BLA pathway, we do not find any changes in synaptic connectivity between stress-susceptible and resilient mice at PFC-BLA synapses (**Fig. 3.9h**). Moreover, oDSE and tonic 2-AG-mediated suppression of glutamate release at PFC-BLA synapses are not different between stress-susceptible and resilient mice (**Fig. 3.9i-l**), and JZL-184 enhances oDSE comparably in both populations (**Fig. 3.9j**). Injection site and fiber

innervation of the BLA are shown in **Fig. 3.9m**. These data support a degree of specificity for eCB effects within the vHIP-BLA circuit in the regulation of stress resilience.

Discussion

Given the prominent role of stress in the development and exacerbation of affective disorders including major depression and PTSD (Kendler et al. 1999, McEwen 2004, McEwen 2012, Sharma et al. 2015), understanding the biological mechanisms contributing to inter-individual differences in stress-susceptibility could lead to the development of susceptibility biomarkers, novel treatments, and preventative approaches (Russo et al. 2012, McEwen et al. 2015). Here we provide converging pharmacological, physiological, and genetic evidence supporting increased 2-AG-CB1R signaling as an endogenous stress-resilience factor that buffers against adverse consequences of stress. These data further support pharmacological 2-AG augmentation as a viable approach for the treatment of stress-related neuropsychiatric disorders.

Utilizing individual differences in stress responsivity to elucidate biological mechanisms subserving stress-resilience/susceptibility has been of increasing interest in recent years. For example, using chronic social defeat stress (CSDS), Krishnan et al. showed that stress-resilience is an active process of adaptation associated with a multitude of changes in gene expression and neural signaling rather than merely an absence of maladaptive changes induced by stress (Krishnan et al. 2007). While social interaction measures after CSDS distinguish subpopulations expressing anhedonia and other depressive-like phenotypes, equivalent levels of anxiety-like behavior are observed in susceptible and resilient groups (Krishnan et al. 2007). The learned helplessness model of depression has also produced important insights about the mechanisms underlying differential susceptibility to the development of depressive-like phenotypes at both

synaptic and circuit levels (Li et al. 2011, Wang et al. 2014, Kim et al. 2016). Here we developed a novel model combining traditional conditioned fear-training (foot-shock+cue/context exposure) and the well-established NIH test of anxiety (Dulawa and Hen 2005) in order to examine individual differences in the generalized anxiety-like response to stress. Overall ~1/3 of mice show a susceptible phenotype, defined as a ≥120 second increase in feeding latency 24h after foot-shock stress relative to baseline latency. It may be important to note that testing was performed on young adult mice and it is possible that stress responsivity could differ in either younger or older populations. The stress-susceptible subpopulation defined using this approach also exhibited increased anxiety-like behavior in the EPM and OFT, further validating our paradigm, without showing changes in depressive-like measures (not shown). Importantly, while the key phenotype in our model has almost completely decayed two weeks following stress exposure it consistently renews following an additional stress exposure which provides a unique opportunity to examine drug effects and manipulate susceptibility in pre-defined populations. Lastly, consistent with the CSDS model (Krishnan et al. 2007), susceptibility in our assay appears to be a latent trait, as neither baseline NIH latencies nor anxiety-like behavior in the EPM or OFT differed between groups prior to stress exposure.

Given the emerging role of 2-AG signaling in the regulation of anxiety and stress-responses (Patel et al. 2015), we utilized this novel behavioral paradigm to test the hypothesis that 2-AG promotes resilience to stress. We show that acute systemic 2-AG augmentation robustly increases the proportion of mice exhibiting resilience to adverse consequences of acute stress, and promotes resilience in previously susceptible mice. In contrast, acute systemic pharmacological 2-AG depletion and CB1R blockade render previously stress-resilient mice susceptible to the development of anxiety-like behavior after acute stress exposure. Taken

together, these data provide causal evidence that 2-AG-CB1R signaling promotes resilience to the adverse effects of acute traumatic stress exposure.

Another important aspect of stress responsivity is habituation or adaptation to repeated homotypic stress exposure, which we have previously suggested may involve 2-AG signaling. Consistent with our previous hypotheses, we found that BLA-specific DAGLα deletion significantly impairs adaptation to repeated stress. Although BLA-specific DAGLα deletion minimally impacts baseline anxiety-like behaviors, it increases the proportion of mice showing anxiety-like behavior after repeated stress exposure and the severity of the anxiety-like phenotype. The preferential effect of BLA-deletion of DAGLα after repeated stress is somewhat surprising in light of the strong converging pharmacological data indicating 2-AG-CB1R modulation of resilience to acute traumatic stress exposure. However, consistent with previous work in other stress models (Rademacher et al. 2008, Hill et al. 2009, Patel et al. 2009), it is likely that endogenous 2-AG plays a more important role in stress modulation after multiple homotypic stress exposures, thus region-specific loss-of-function manipulations may show larger effects after repeated stress. Alternatively, other brain regions besides those tested may be more critical for the modulation of acute stress-induced anxiety or compensatory changes could occur in the weeks after DAGLα deletion that counteract potential increases in acute stress-susceptibility. Despite this issue, these data overall are consistent with previous clinical and preclinical data suggesting 2-AG deficiency could contribute to the development of stress-related psychiatric disorders (Hill et al. 2009, Hill et al. 2013, Shonesy et al. 2014, Jenniches et al. 2015).

A primary function of 2-AG signaling is the retrograde synaptic suppression of afferent neurotransmitter release within limbic nodes including the amygdala and PFC (Azad et al. 2003,

Lafourcade et al. 2007, Castillo et al. 2012, Kano 2014). While dysfunction of multiple limbic regions has been implicated in stress-related psychiatric disorders, hyperactivity of the amygdala, in particular, has been highly associated with affective disorders (Etkin and Wager 2007, Martin et al. 2009, Price and Drevets 2010, Kim et al. 2011), and stress increases BLA neuronal activity in rodents (Mozhui et al. 2010, Masneuf et al. 2014). Interestingly, the anxiolytic effect of low-dose cannabinoid agonist treatment is mediated through CB1Rs on forebrain glutamatergic, but not GABAergic, terminals (Rey et al. 2012, Ruehle et al. 2013), and deletion of CB1Rs from forebrain glutamatergic terminals produces increased fear behaviors (Jacob et al. 2009). Together these data suggest that pharmacological 2-AG augmentation may exert its anxiolytic and resilience-promoting effects by reducing BLA glutamatergic transmission. Indeed, we show that resilient mice have a larger difference in BLA sEPSC frequency between maximal 2-AG-CB1R signaling (JZL-184 incubated) and abolished 2-AG-CB1R signaling (Rimonabant incubated), compared to susceptible mice. These data suggest resilient mice utilize 2-AG signaling to regulate BLA afferent glutamatergic transmission within a broader dynamic range. We suggest this broader dynamic range of 2-AG-signaling could represent part of the adaptive response to traumatic stress that characterizes stress-resilience.

Importantly, we also find that stress resilience is associated with enhanced phasic, but not tonic, 2-AG-mediated suppression of glutamatergic transmission at ventral hippocampal-BLA synapses, and that relatively impaired 2-AG signaling at these synapses in stress-susceptible mice can be normalized by JZL-184 treatment. These data suggest 2-AG signaling could serve to reduce vHIP-BLA circuit activity to promote resilience and successful adaptation to stress. These data are consistent with the known role of the hippocampus in relaying contextual information to limbic output structures to generate appropriate behavioral responses to changes in

environmental context, and with the anxiogenic function of hippocampal-BLA circuits (Felix-Ortiz et al. 2013).

Our data clearly show that systemic 2-AG augmentation promotes resilience to stress, and that stress-susceptible mice have relatively impaired synaptic 2-AG signaling at vHIP-BLA glutamatergic synapses which can be normalized by JZL-184 application *ex vivo*. However, these data do not conclusively localize the resilience-promoting effects of systemic 2-AG augmentation to reductions in activity of the vHIP-BLA circuit per se. Future studies utilizing BLA-specific 2-AG augmentation combined with vHIP-BLA pathway-specific CB1R deletion will be required to conclusively test this hypothesis. The involvement of other neural circuits known to regulate stress-responsivity and anxiety-related behaviors has also not been investigated in the current work.

In summary, here we utilize individual differences in stress-responsivity to demonstrate a causal role for 2-AG-mediated eCB signaling in promoting stress-resilience. Our data demonstrate that on both group (*susceptible vs. resilient*) and individual (*correlational analyses*) levels, the severity of stress-induced generalized anxiety-like behavior predicts the beneficial response to pharmacological 2-AG augmentation. We also find that stress-resilience is associated with enhanced 2-AG-CB1R mediated synaptic suppression of vHIP-BLA glutamatergic transmission, and that BLA 2-AG signaling is required for successful adaptation to repeated traumatic stress. Altogether, these data suggest that pharmacological augmentation of 2-AG signaling could represent a novel approach for the treatment of stress-related neuropsychiatric disorders (Hill et al. 2009, Hill and Patel 2013, Morena et al. 2016), and that 2-AG deficiency states could represent a stress-susceptibility endophenotype predisposing to the development of affective pathology.

Methods

Animals and drugs

All studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Vanderbilt University Institutional Animal Care and Use Committee. Mice were housed on a 12:12 light-dark cycle with lights on at 06:00. All experiments were conducted during the light phase. Food and water were available *ad libitum*. Outbred ICR mice (Harlan, Indianapolis, Indiana, USA) were used for all drug studies, mice were ordered at 5 weeks old and testing began within 2 weeks. JZL-184 (8 mg/kg; AbCam, Cambridge, Massachusetts, USA), Rimonabant (1 mg/kg; APIChem, Hangzhou, Zhejiang, China), and THC (0.25mg/kg; Sigma-Aldrich, St. Louis, Missouri, USA) were prepared in dimethylsulfoxide (DMSO; Sigma Aldrich, Milwaukee, WI, USA) and injected at a volume of 1 µl/g bodyweight. DO34 (50mg/kg) was synthesized as previously described (Ogasawara et al. 2016), prepared in a 1:1:18 mixture of ethanol, kolliphor, and saline, and injected at a volume of 10 µl/g bodyweight. All drugs were administered 2 hours prior to initiation of behavioral testing.

To generate conditional DAGLα knockout mice, germline knockout mice expressing a gene-trap cassette flanked by flippase recognition target (*FRT*) sites were crossed with *pgk1-FLPo* mice (Tg(Pgk1-FLPo)10Sykr; Jackson Laboratories, Stock Number 011065). Following FLPo-mediated *FRT*-site recombination, the resulting conditional knockout allele consisted of *loxP*-sites flanking exon 9 of *dagla* (see Figure 3.6). Offspring of *pgk1-FLPo* and DAGLα$^{-/-}$ crosses were genotyped to identify founders harboring alleles that had undergone FLP-recombination (DAGLα$^{fl/+}$), and DAGLα$^{fl/+}$ founders were then bred to homozygosity (DAGLα$^{fl/fl}$). DAGLα$^{fl/fl}$ mice were maintained by homozygote x homozygote breeding. Genotypes were determined by PCR of mouse ear punch samples using the following primers

(5'-3'): TGAGCCAGAGACATTTGCTG, CTGGTGAGGCCAAGTTTGTT and GGGACAGAAAACCACTTGGA. DAGL$\alpha^{-/-}$ and DAGL$\alpha^{fl/fl}$ mice were bred in house with behavioral testing performed with cohorts of mixed males and females that underwent stereotaxic surgery between 6 and 13 weeks of age and began behavioral testing at least 3 weeks later.

Stereotaxic surgery

Mice were anesthetized with isoflurane. DAGL$\alpha^{fl/fl}$ mice (male and female 7-10 weeks old) underwent bilateral stereotaxic (Neurostar Drill and Injection Robot, Tubingen, Germany) injection of AAV5.CMV.HI.eGFP-Cre.WPRE.SV40 (AAV-GFP-CRE; titer 2 x 10^{13} TU/ml) or AAV5.CMV.PI.eGFP.WPRE.bGH (AAV-GFP; titer 7 x 10^{13} TU/ml) control virus (Penn Vector Core, Philadelphia, Pennsylvania, USA). Viruses were infused into the BLA (250nl, AP: -1.20, ML: ±3.35, DV: 4.95), PFC (150nl, AP: 1.85, ML: ±0.5, DV: 2.18), and NAc (450nl, AP: 1.65, ML: ±0.92, DV: 4.80) of DAGL$\alpha^{fl/fl}$ mice at a rate of 100nl/min. The syringe (10 µl Nanofil, WPI, Sarasota, Florida, USA) was first lowered (0.28 mm/sec) to 0.3 µm deeper than the injection site, after 5 seconds it was raised to the injection site where it paused for 10 seconds before injecting. After the virus was infused, the syringe remained in place for 300 seconds before retracting. Surgery was counterbalanced over time and in each cage so that both conditions were represented in each cage of littermates. Behavioral testing began at least 3 weeks after viral injection.

For electrophysiological studies 3.5-5 week old ICR mice were bilaterally injected with a (2:1) mixture of AAV5.CaMKIIa.hChR2(H134R)-eYFP.WPRE.hGH (AAV-ChR2; titer 1.6 x 10^{13} GC/ml) virus. The constructs were infused into the ventral hippocampus (450nl, AP: -2.90, ML: ±3.25, DV: 4.16), prelimbic prefrontal cortex (110nl, AP: 2.10, ML: ±0.22, DV: 2.10), or

lateral entorhinal cortex (350nl, AP: 0.52 relative to lambda, ML: ±4.56, DV: 4.12). *Ex vivo* electrophysiological recordings were performed at least 3 weeks after viral injection. All viral constructs used in this study were purchased from Penn Vector Core (Philadelphia, Pennsylvania, USA).

Behavior

Foot-shock stress was performed as previously described (Bluett et al. 2014). Foot-shock stress consisted of a 7.5-minute session. After a 60 second baseline, six 0.7 mA foot-shocks were delivered 1 min apart using a MED Associates fear-conditioning chamber (St. Albans, Vermont, USA). Each shock coincided with the last 2 seconds of a 30 second auditory tone. After an additional 60 seconds, mice were returned to their home cages. All post-stress behavioral testing was performed ~24 hours after completion of the final stress exposure. Behavioral responsivity to foot-shock was scored by visual assessment of behavior from videos recorded during these foot-shock stress sessions. Scores were assigned based on the predominant behavioral response over the 2 second shock as follows: 0-no response, 1-flinching/walking, 2-running, 3-jumping. Scores from two independent, blinded observers were averaged.

Foot-shock threshold: analysis was performed at least one week after susceptibility categorization using the repeated NIH procedure described below and occurred in the same chambers as foot-shock stress. A series of 1-second foot-shocks was delivered with an interstimulus interval of 29 seconds with each shock increasing intensity as follows (in mA): 0.075, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7. The intensity at which each mouse first flinched, ran, jumped, and vocalized was recorded and the session was terminated immediately after the first vocalization.

Repeated novelty induced hypophagia (rNIH): testing was based on previously described

methods with some modifications (Gamble-George et al. 2013, Bluett et al. 2014, Shonesy et al. 2014). Individually housed mice were acclimated to testing rooms under red light for at least 30 minutes before home-cage training and novel-cage testing. Mice were habituated to a novel, palatable food (liquid vanilla Ensure®, Abbott Laboratories, Abbott Park, IL) in their home cages for 30 min/day under red light (<50 lux) for at least 4 days before novel-cage testing. After 30+ minute acclimation in their home cages under red light illumination, mice were transferred to a novel, empty cage in a brightly lit room (~300 lux; low light test in Fig. S3.1 <50 lux) and again given access to liquid vanilla Ensure® for 30 minutes. For each mouse, the latency to drink and total weight consumed were recorded. The next week, mice underwent the same home-cage procedure for two consecutive days. After the 2nd home-cage training, mice were exposed to foot-shock stress (described above). Approximately 24 hours later mice underwent novel-cage testing (novel-cage tests were performed at least 7 days apart). In drug trials, mice were injected with vehicle, JZL-184, Rimonabant, or DO34 2 hours before testing. In some cohorts, the same procedure was repeated 1-2 more times. Animals that did not drink in any novel-cage test were excluded from analyses.

The NIH data in Figure 3.2a-f was aggregated from 3 cohorts of 40 mice that completed the baseline and stress testing as a precursor to further experiments, namely those presented in (1) Figure 3.2g-h, (2) Figure 3.4, and (3) Fig. S3.8.

For novel open field testing (OFT): exploration of a novel open field arena contained within a sound-attenuating chamber was monitored for 10 minutes (27.9×27.9 cm; MED-OFA-510; MED Associates, St. Albans, Vermont). Beam breaks from 16 infrared beams were recorded by Activity Monitor v5.10 (MED Associates) to monitor position and behavior.

Light-dark box testing (LD): was performed as previously described (Sumislawski et al.

2011, Shonesy et al. 2014). Exploration of open field chambers containing dark box inserts that split the chamber into light (~300 lux) and dark (<5 lux) halves (ENV-511; MED Associates, St. Albans, Vermont) was recorded by Activity Monitor v5.10 as above. Position and behavior were monitored as described above for 10 minutes.

The elevated plus maze (EPM): consisted of two open arms (30 x 10 cm) and two closed arms (30 x 10 x 20 cm) that met at a center junction (5 x 5 cm). The apparatus was elevated 50 cm from the floor. Light levels in the open arms were approximately 300 lux, while the closed arms were <100 lux. Mice were placed in the center of the maze, facing a closed arm, and allowed to explore for 5 minutes. ANY-maze (Stoelting, Wood Dale, Illinois, USA) video-tracking software was used to monitor and analyze behavior during the test.

LC/MS/MS detection of lipids

Mice underwent cervical dislocation immediately followed by decapitation. The brain was quickly removed, placed in a brain matrix, and covered with ice cold NMDG-ACSF (details in electrophysiology section below). 1-2mm thick coronal sections containing the target brain regions were frozen on a metal block in dry ice. Dissections were performed on the frozen tissue for the production of amygdala-, NAc-, PFC-, and vHIP-enriched samples using a 1 or 2 mm diameter metal micropunch. Samples were stored at -80°C until extraction.

LC/MS/MS detection of endocannabinoids and arachidonic acid was performed as previously described with minor modifications (Shonesy et al. 2014). Briefly, all samples were homogenized directly in methanol, incubated at -20°C overnight, and centrifuged at 10 x g for 15 minutes at 4°C; water was added to the supernatant for a final ratio of 70:30 Methanol:Water. Sample (20 µl) was injected into a C-18 column (50 × 2 mm, 3 µm; Phenomenex or 50 x 2.1mm, 1.7 µm; Acquity) under either of the following two conditions:

1) 20% A (water with 80 μM silver acetate and 0.1% glacial acetic acid (v/v)) and 80% B (methanol with 80 μM silver acetate and 0.1% glacial acetic acid (v/v)) from 0 to 0.5 min, increased to 0% A and 100% B from 0.5 to 3.5 min and held for 1 min, and returned to 20% A and 80% B from 4.5 to 6.5. Analytes were detected via selective reaction monitoring (as [M+Ag]+ complexes except AA, which is ionized as [(M–H)+2Ag]+) in the positive ion mode using the following reactions (the mass in parentheses represents the mass of the deuterated internal standard): AA (*m/z* 519(527) → 409(417)); 2-AG (*m/z* 485(493) → 411(419)); and AEA (*m/z* 454(462) → 432(440)) using a Sciex QTrap 6500 mass spectrometer.
2) 65% A (water with 0.1% formic acid (v/v)) and 35% B (2:1 acetonitrile:methanol with 0.1% formic acid (v/v)) from 0 to 0.15 min, increased to 1% A and 99% B from 0.15 to 5 min and held for 1.8 min, and returned to 65% A and 35% B from 6.8-7.2. Analytes were detected via selective reaction monitoring in either the positive ion mode (2-AG and AEA as [MH]+ complexes) or negative ion mode (AA as [M-H]-) using the following reactions (the mass in parentheses represents the mass of the deuterated internal standard): 2-AG (*m/z* 379(384) → 287(287)); AEA (*m/z* 348(352) → 62 (66)); and AA (*m/z* 303(311) → 259(267)) using a Sciex QTrap 6500 mass spectrometer.

Quantification was achieved via stable-isotope dilution for AA, 2-AG and AEA.

Corticosterone ELISA

Trunk blood was collected in tubes containing 25 μL EDTA (128 μM) immediately following cervical dislocation and decapitation. Gentle inversion of tubes to promote mixing of the blood and the EDTA prevented coagulation. Samples were kept on ice. Plasma was then extracted via centrifugation and stored at -80°C. A corticosterone ELISA assay was performed

on the extracted plasma using a commercial kit (Enzo Life Science, Catalog No. ADI-900-097). The ELISA plate was read at 405nm. The online tool MyAssays (Cayman Chemicals) was used to calculate the corticosterone concentration of each sample.

Western blot

Tissue was dounce homogenized in lysis buffer containing 50 mM HEPES (pH 7.5), 0.5 mM TCEP, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mM PMSF, 2 mM EDTA and PhosStop tablets (Roche). Total protein was measured in lysates by Bradford protein assay, and equal protein was loaded and resolved by SDS-PAGE and transferred to nitrocellulose membranes for western blotting using rabbit anti-DGLa (1:6000), rabbit anti-CB1R (1:1000), and rabbit anti-MGL (1:750) (Keimpema et al. 2010). Densitometry was performed using Image J (National Institutes of Health, Bethesda, MD), and signals were normalized for protein loading by dividing the individual band area by Ponceau S staining. Full, uncropped blots are shown in Supplementary Fig. 13.

Immunohistochemistry

Following stereotaxic injections of AAV-GFP-Cre or AAV-GFP and behavioral analyses, mice were anesthetized with isoflurane and transcardially perfused with 10 ml of phosphate buffered saline (PBS, pH 7.4), followed immediately by 15 ml of 4% paraformaldehyde (PFA). Brains were dissected and post-fixed for 24 hours in 4% PFA, and then transferred to 30% sucrose solution for 4-5 days. Brains were cut at 40 µm using a Leica 3050-S cryostat (Leica Biosystems, Buffalo Grove, IL, USA). Slices were rinsed 3X for ten minutes in tris-buffered saline (TBS, pH 7.4) and subsequently washed for 30 minutes in TBS+ (4% horse serum, and 0.2% Triton X-100). The slices were then incubated overnight at room temperature in TBS+ solution containing rabbit anti-DAGLα primary antibody (Katona et al. 2006, Keimpema

et al. 2010) at 1:500 concentration. Slices were rinsed 3X for ten minutes in TBS+ and then incubated in TBS+ containing Alexa Fluor 546 donkey anti-rabbit secondary antibody (ThermoFisher Scientific, Waltham, MA, USA) at 1:1000 concentration for 2.5 hours. Slices were then rinsed 3X for ten minutes in TBS before being mounted onto slides with 0.15% gelatin solution. Slides were cover-slipped with DPX mountant, and imaged on an upright Zeiss Axio Imager M2 microscope (Zeiss, Oberkochen, Germany).

Ex vivo electrophysiological recordings

Mice used for electrophysiology were drug-naïve, but where noted, mice were foot-shock stressed 1 day prior to sacrifice for electrophysiological recordings. Mice were briefly anesthetized with isoflurane and transcardially perfused with ice-cold oxygenated (95% v/v O_2, 5% v/v CO_2) N-methyl-D-glucamine (NMDG) based ACSF (Ting et al. 2014) comprised of (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 Na-ascorbate, 3 Na-pyruvate, 5 N-acetylcyctine, 0.5 $CaCl_2{\cdot}4H_2O$ and 10 $MgSO_4{\cdot}7H_2O$. The brain was quickly removed and 250 μm coronal slices of the basolateral amygdala (BLA) were cut using a Leica VT1000S vibratome (Leica Microsystems, Bannockburn, IL, USA) in the NMDG solution. Slices were incubated for 8-10 minutes at 32°C oxygenated NMDG-ACSF and stored at 24°C until recording in HEPES-based ACSF containing (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 ascorbate, 3 Na-pyruvate, 5 N-acetylcyctine, 2 $CaCl_2{\cdot}4H_2O$ and 2 $MgSO_4{\cdot}7H_2O$. Slices from each animal were incubated for 2-3 hours in vehicle and Rimonabant (5 μM) or JZL-184 (1 μM) containing ACSF. The order of recording from each condition was alternated day by day to control for slice age and incubation time. Recordings were performed in a submerged recording chamber during continuous perfusion of oxygenated ACSF containing (in mM): 113 NaCl, 2.5 KCl, 1.2 $MgSO_4{\cdot}7H2O$, 2.5 $CaCl_2{\cdot}2H2O$, 1 NaH_2PO_4,

26 $NaHCO_3$, 1 ascorbate, 3 Na-pyruvate and 20 glucose; at a flow rate of 2.5 - 3 ml/min. For experiment in Supplementary Fig. 12, the CB1R agonist CP-55940 (5μM) was washed onto slices following acquisition of a stable baseline. For all experiments, and for drug solutions, 0.5 g/L of fatty acid free bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was also added to the ACSF to increase solubility of the lipophilic drugs, and to minimize nonspecific binding of these compounds.

Slices were visualized using a Nikon microscope equipped with differential interference contrast video microscopy. Whole-cell voltage-clamp recordings from BLA pyramidal cells held at -70mV were obtained under visual control using a 40x objective. 2 - 3 MΩ borosilicate glass pipettes were filled with high [K^+] based solution containing (in mM): 125 K^+-gluconate, 4 NaCl, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, and 10 Na-phosphocreatine. Only cells with access resistance <20 MΩ were included. For spontaneous EPSC (sEPSC) measurements ACSF was supplemented with the $GABA_A$ receptor antagonist picrotoxin (50 μM; Abcam, Cambridge, MA) to pharmacologically isolate glutamatergic transmission (Sumislawski et al. 2011, Ramikie et al. 2014, Shonesy et al. 2014), for optically induced EPSC (oEPSC) recordings picrotoxin (50 μM) was applied in the internal solution to avoid optically induced population activity. For optical stimulation 1-2 ms 480 nm blue light pulse was delivered by an LED (ThorLabs) directed through the objective. Optically evoked depolarization-induced suppression of excitation (oDSE) was examined under voltage-clamp conditions where cells were recorded at a holding potential of -70 mV. Excitatory postsynaptic currents (EPSCs) were elicited at a rate of 0.2 Hz. To induce DSE, a depolarizing pulse (-70 to +30 mV) was applied to the postsynaptic neuron for 10 seconds. Within each DSE trial, EPSC amplitudes were normalized to the averaged baseline response, and maximum DSE was classified as the first EPSC following the depolarizing pulse.

Recordings were performed using a MultiClamp 700B amplifier (Molecular Devices), and Clampex software (version 10.2; Molecular Devices).

Statistical analysis. All statistical analyses were performed with GraphPad Prism 6 (San Diego, CA, USA). Student's t-test, one-way ANOVA, and two-way ANOVA were used as appropriate. One- and two-way ANOVA were followed by post-hoc Sidak/Holm-Sidak multiple comparisons tests. Relevant F and P values for one- and two-way ANOVA are shown in individual figure panels. P values (and/or asterisks denoting significance as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$) for pairwise comparisons derived from post-hoc testing or from t-tests are shown in individual figure panels, with details in figure legends. R^2 and P values for linear regression analyses are shown in all correlation panels. Chi-squared analyses were performed to compare susceptibility ratios and the resultant p values are reported in each figure panel. Rout test for outlier identification was used. Testing was counterbalanced, but no randomization was performed, and sample sizes were derived empirically during the course of the experiments guided by our previous work using these assays (Gamble-George et al. 2013, Bluett et al. 2014, Shonesy et al. 2014). Experimenters were blinded to treatment condition during experimentation. Data are presented as mean ± SEM.

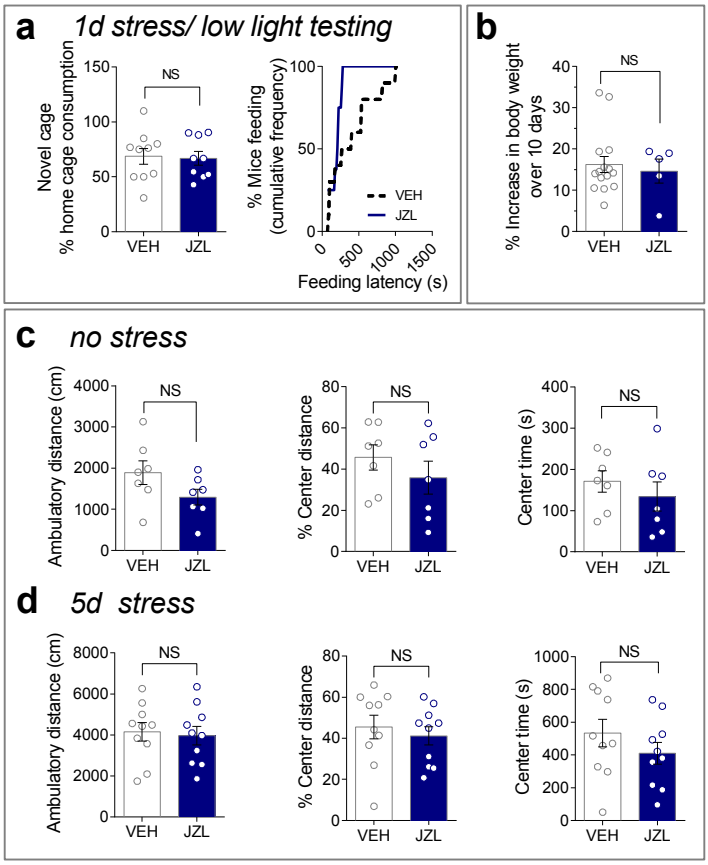

Figure S3.1 2-AG augmentation does not affect consummatory or exploratory drive. (a) Effect of acute JZL-184 on consumption and latency in a less aversive (low light) NIH novel-cage test 24h after foot-shock stress. **(b)** Effect of 10 days of JZL-184 treatment on body weight in unstressed mice. **(c)** Effects of acute JZL-184 on exploration in a novel open field under control conditions and **(d)** after 5 days of stress. P-values for unpaired two-tailed t-test reported in each panel. Data are presented as mean ± SEM. (RJB)

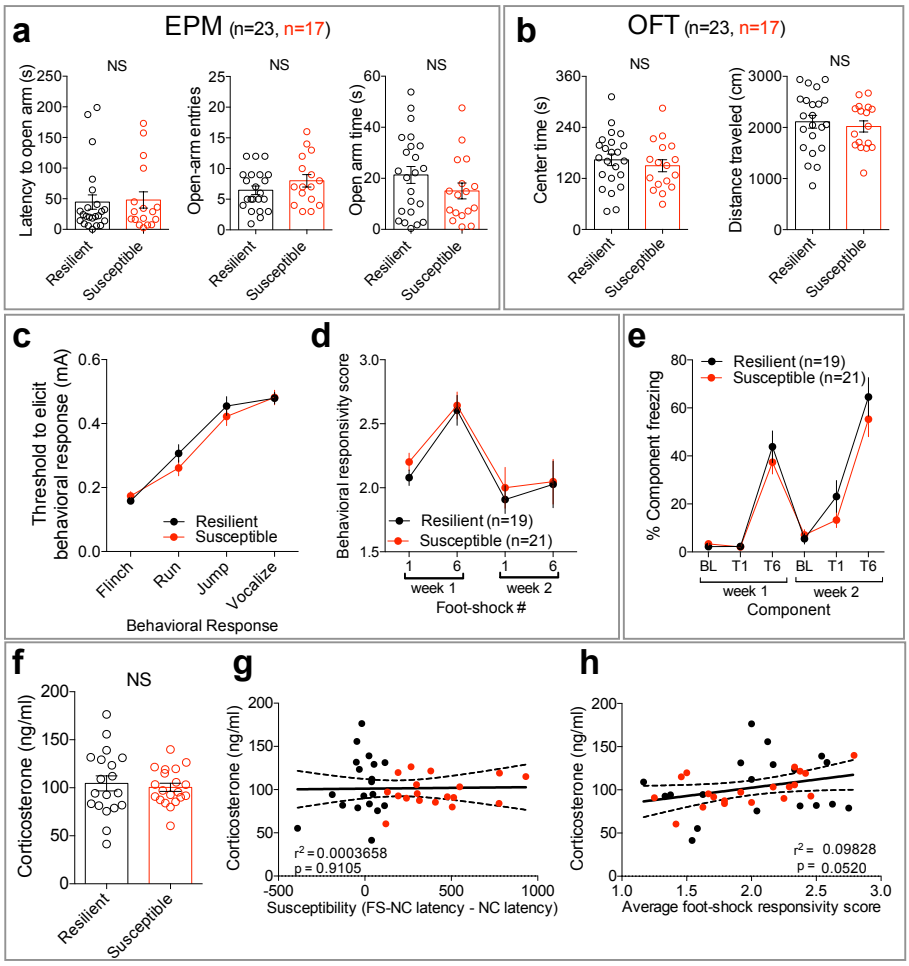

Figure S3.2 Baseline anxiety, foot-shock responsivity, and stress-induced corticosterone release do not differ between susceptible and resilient groups. (a) Resilient and susceptible pre-stress elevated plus maze (EPM) latency to open arm, open arm entries, and open arm time. **(b)** Resilient and susceptible open field test (OFT) center time and distance traveled. **(c)** Resilient and susceptible group average foot-shock current thresholds to elicit specified behavioral responses. Although group sizes were n=19 and n=21 for resilient and susceptible groups, not every individual exhibited every behavioral response. **(d)** Resilient and susceptible average behavioral response scores (1-flinch/walk, 2-run, 3-jump) to the first and 6th/last 2 second 0.7mA shocks for 2 stress exposures 1 week apart. **(e)** Resilient and susceptible percent freezing during baseline (BL), and the first tone (T1) and last tone (T6) of 2 foot-shock stress exposures 1 week apart. **(f)** Resilient and susceptible plasma corticosterone measurements 20 minutes after the initiation of foot-shock stress. **(g)** Correlation between plasma corticosterone levels from panel f and susceptibility as defined by the difference in latency between NIH foot-shock novel cage test (FS-NC) and baseline novel cage test (NC). **(h)** Correlation between plasma corticosterone levels in panel f and average foot-shock responsivity scores across all 6 shocks during week 2 (immediately prior to collection of plasma). Unpaired two-tailed t-test conducted on panels a-b, and f. Two-way ANOVA performed on panels c-e. R^2 and P value for linear regression reported in panels g and h. Data are presented as mean ± SEM. (a-b AH & RJB; c-e ADG & RJB; f-h AH, ADG, & RJB).

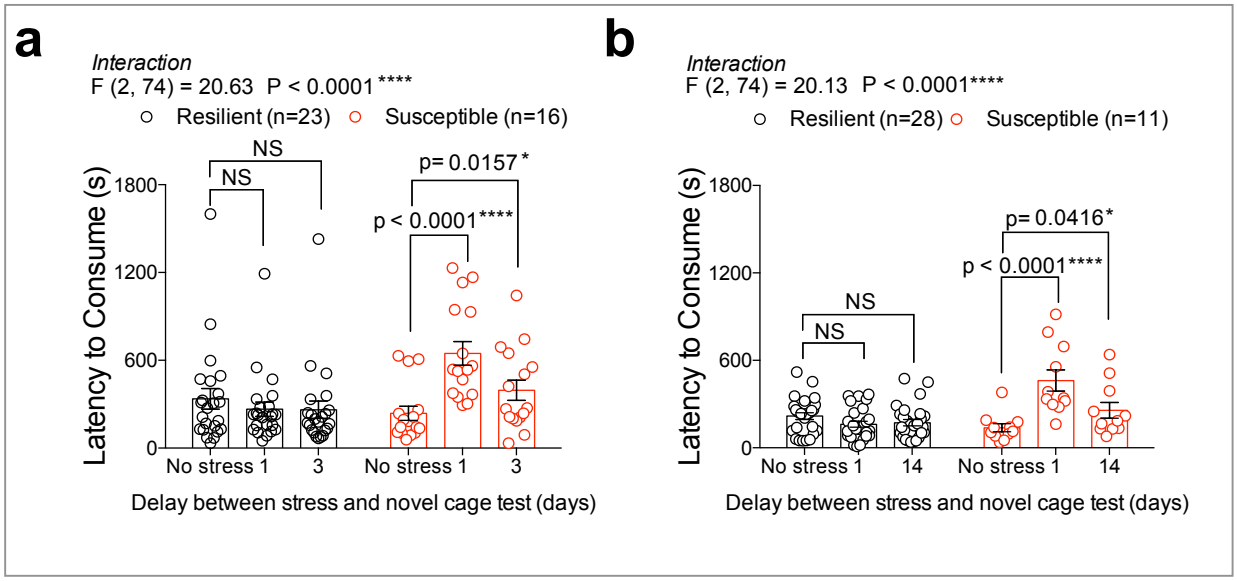

Figure S3.3 Anxiety-like behavior elicited by acute stress is long-lasting. (a) Resilient and susceptible novel cage test latencies in the repeated novelty-induced hypophagia paradigm at baseline (No stress), 1 day after stress, and 3 days or **(b)** 14 days after a second foot-shock stress exposure. F and P values for two-way ANOVA shown above individual panels. P values for pairwise comparisons derived from Holm-Sidak multiple comparisons test after ANOVA shown in panels. Data are presented as mean ± SEM. (AH, ADG, & RJB)

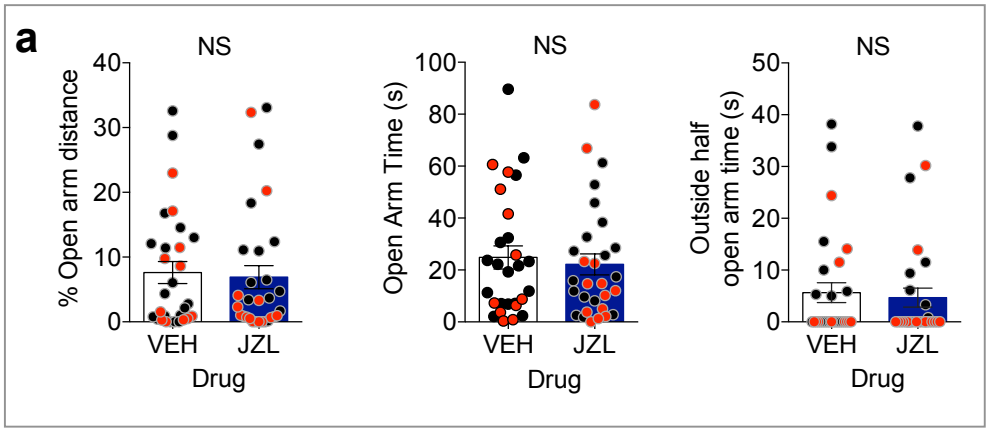

Figure S3.4 JZL-184 does not affect anxiety-like behavior in the elevated plus maze in repeated paradigm. (a) Elevated plus maze % open arm distance (left), open arm time (middle), and time on the outside half of the open arm (right) for resilient (black circles) and susceptible (red circles) individuals 24h after foot-shock stress at least 1 week after susceptibility categorization in the repeated NIH paradigm and treated with either vehicle (VEH) or JZL-184 (JZL; blue bar). Data combined from 2 independent cohorts. Unpaired two-tailed t-tests performed for each measure. Data are presented as mean ± SEM. (RJB)

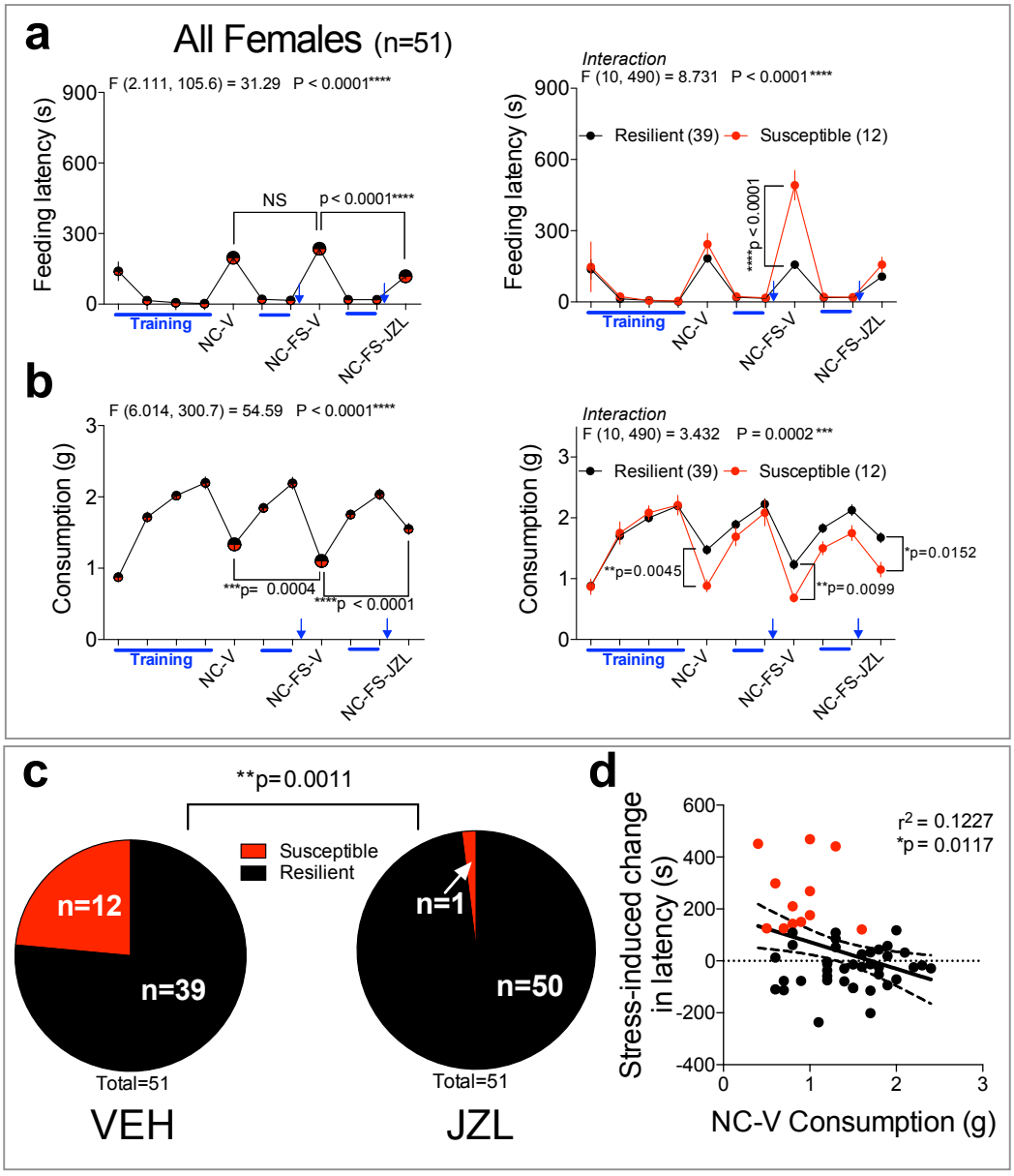

Figure S3.5 JZL-184 promotes resilience in female mice.
(**a**) Female home cage (blue lines) and novel cage (NC) latencies and (**b**) consumption at baseline (NC-V) and after stress with vehicle (NC-FS-V) or JZL (NC-FS-JZL). (**c**) Proportion of susceptible and resilient females after vehicle (VEH) and JZL-184 treatment. (**d**) Correlation between baseline novel cage consumption and stress-induced change in latency from baseline. Blue arrows indicate stress exposure. F and P values for one-way (left) or two-way (right) ANOVA shown above individual panels (a-b). P values for pairwise comparisons derived from Holm-Sidak multiple comparisons test after ANOVA shown in panels. R^2 and P value for linear regression reported in panel (d). P value from chi-squared test reported in panel (c). Data are presented as mean ± SEM. (AH & RJB)

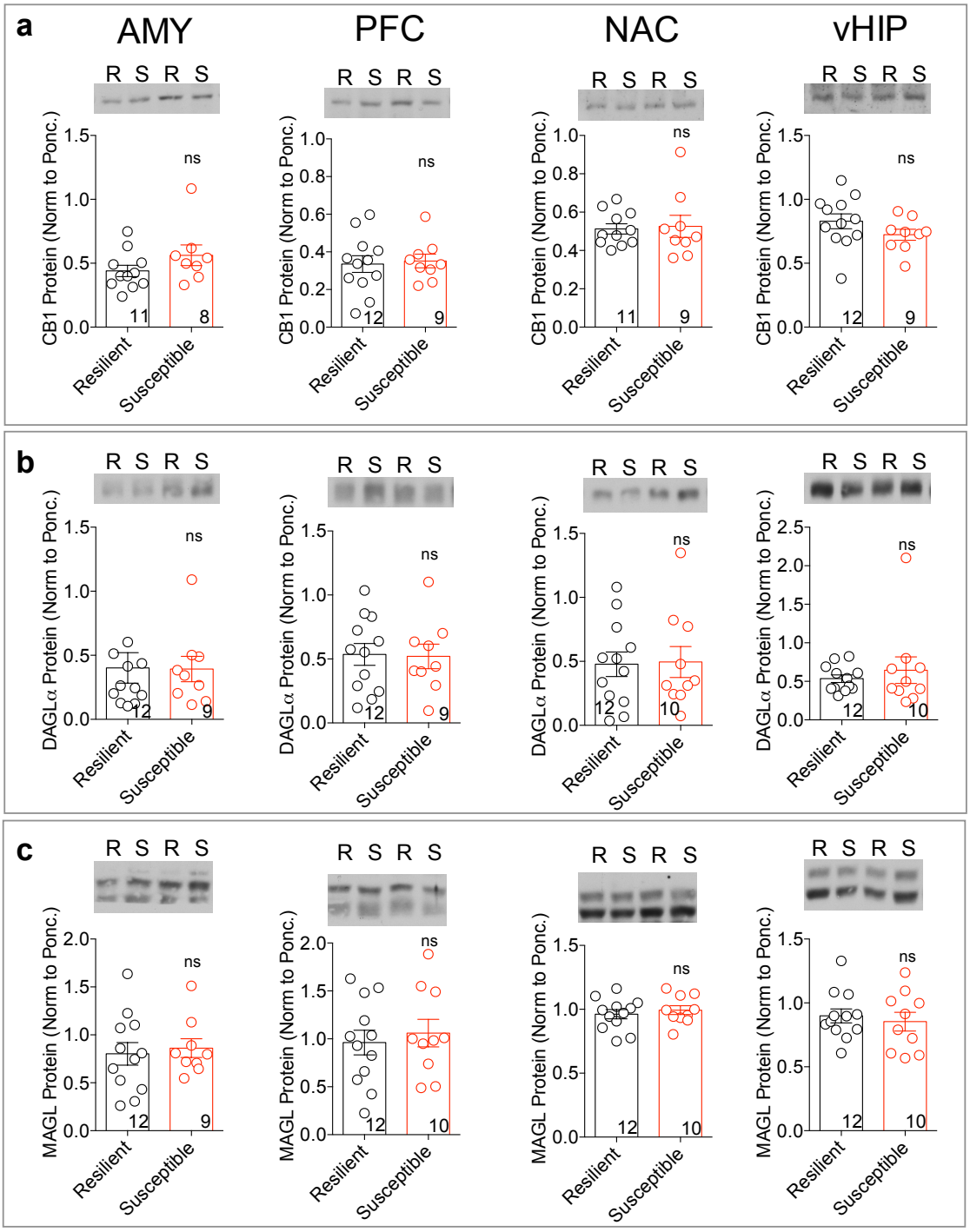

Figure S3.6 CB1 receptor, DAGLα, and MAGL protein levels do not differ between resilient and susceptible groups. (**a**) CB1 receptor protein levels as measured by western blot in the amygdala (AMY), prefrontal cortex (PFC), nucleus accumbens (NAC), and ventral hippocampus (vHIP) for resilient (R) and susceptible (S) mice. (**b**) DAGLα and (**c**) MAGL protein levels in each brain region as measured by western blot. Representative blots shown above each bar graph. Unpaired two-tailed t-tests were performed for each measure. Data are presented as mean ± SEM. (RJB, WPP, & BCS)

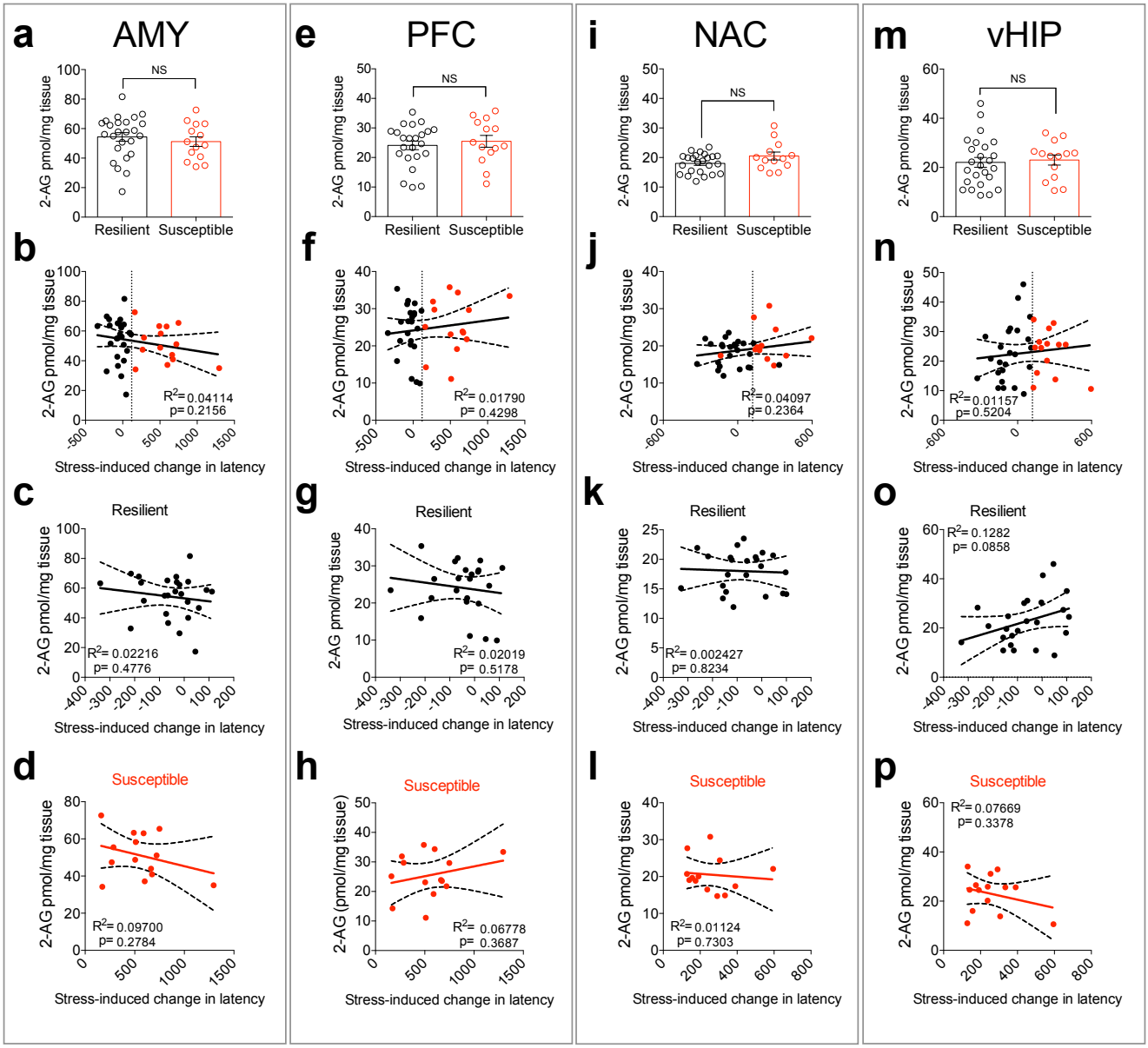

Figure S3.7 2-AG levels do not correlate with resilience. (**a**) Amygdala (AMY) 2-AG levels for resilient and susceptible groups. (**b**) Correlation between amygdala 2-AG and susceptibility as measured by stress-induced change in latency for the whole population and (**c**) resilient and (**d**) susceptible subpopulations. (**e**) Prefrontal cortex (PFC) 2-AG levels for resilient and susceptible groups. (**f**) Correlation between PFC 2-AG and susceptibility as measured by stress-induced change in latency for the whole population and (**g**) resilient and (**h**) susceptible subpopulations. (**i**) Nucleus accumbens (NAC) 2-AG levels for resilient and susceptible groups. (**j**) Correlation between NAC 2-AG and susceptibility as measured by stress-induced change in latency for the whole population and (**k**) resilient and (**l**) susceptible subpopulations. (**m**) Ventral hippocampus (vHIP) 2-AG levels for resilient and susceptible groups. (**n**) Correlation between vHIP 2-AG and susceptibility as measured by stress-induced change in latency for the whole population and (**o**) resilient and (**p**) susceptible subpopulations. Unpaired two-tailed t-tests were performed for panels a, e, i, and m. R^2 and P value for linear regressions reported in all other panels. Data are presented as mean ± SEM. (RJB)

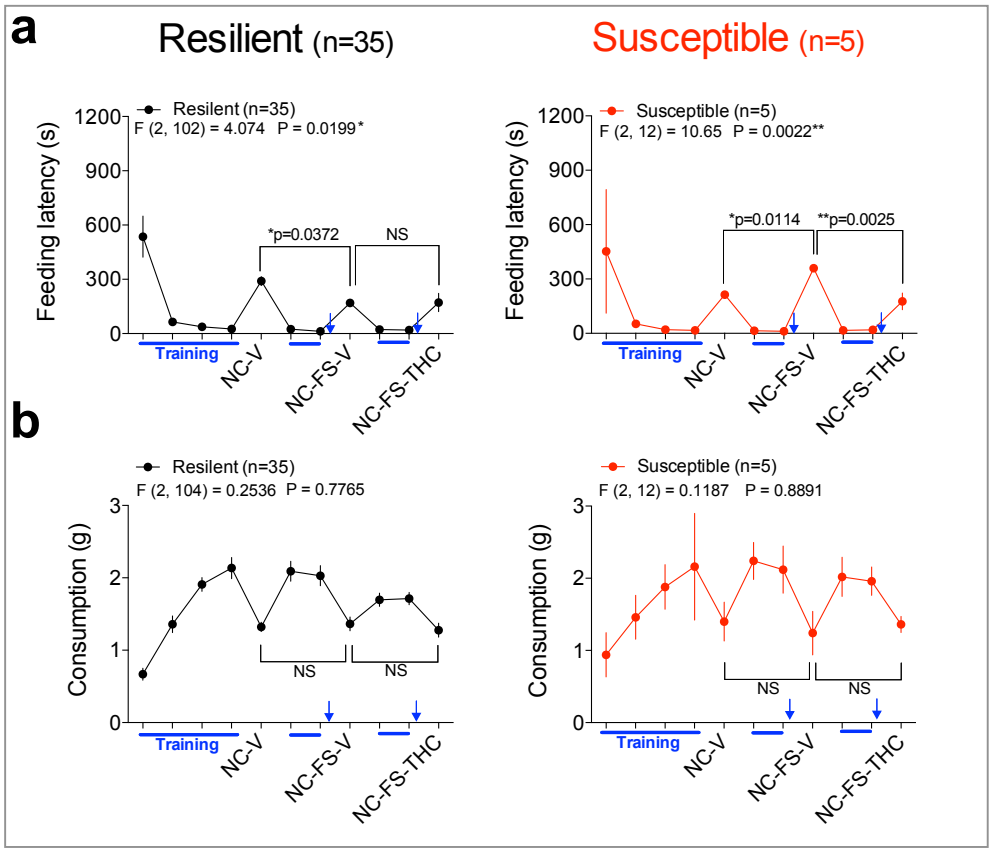

Figure S3.8 THC promotes resilience similarly to JZL-184. (**a**) Male resilient and susceptible home cage (blue lines) and novel cage (NC) latencies and (**b**) consumption at baseline (NC-V) and after stress with vehicle (FS-V) and delta-9-tetrahydrocannabinol (FS-THC). Blue arrows indicate stress exposure. F and P values for one-way ANOVA shown above individual panels. P values for pairwise comparisons derived from Sidak multiple comparisons test after ANOVA shown in panels. Data are presented as mean ± SEM. (RMB, ADG, & RJB)

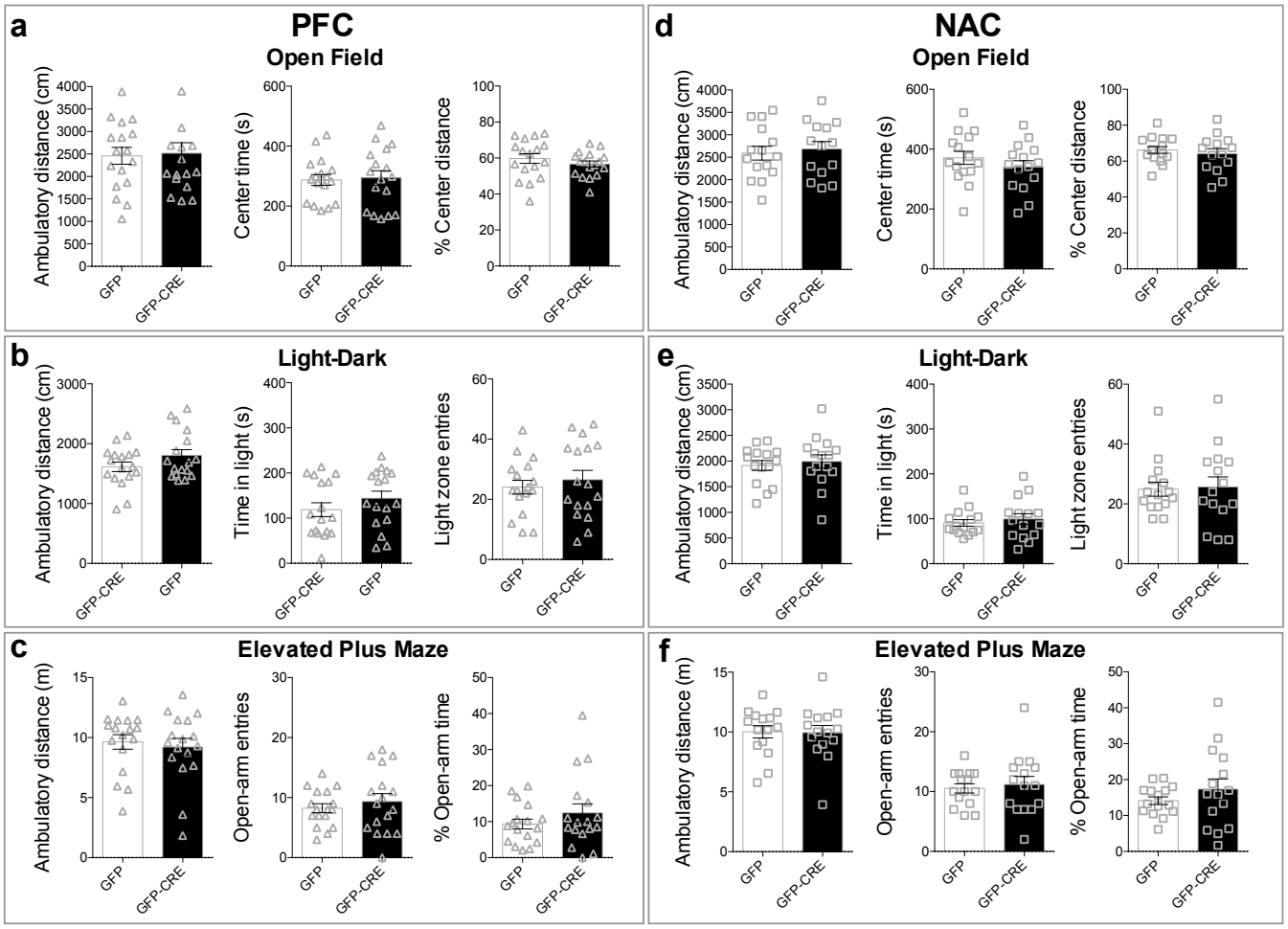

Figure S3.9 Basal anxiety is not significantly affected by loss of PFC or NAc 2-AG. (a) Open field test **(b)** light-dark and **(c)** elevated plus maze behavior of prefrontal cortex (PFC) GFP control versus GFP-CRE injected $DAGL^{fl/fl}$ mice. (**d)** Open field test **(e)** light-dark and **(f)** elevated plus maze behavior of nucleus accumbens (NAC) GFP control versus GFP-CRE injected $DAGL^{fl/fl}$ mice. Data for both brain regions was combined from two independent cohorts. Unpaired two-tailed t-tests were performed for each measure; no significant differences were found between groups for any measure. Data are presented as mean ± SEM. (ADG, AH, JB, & RJB)

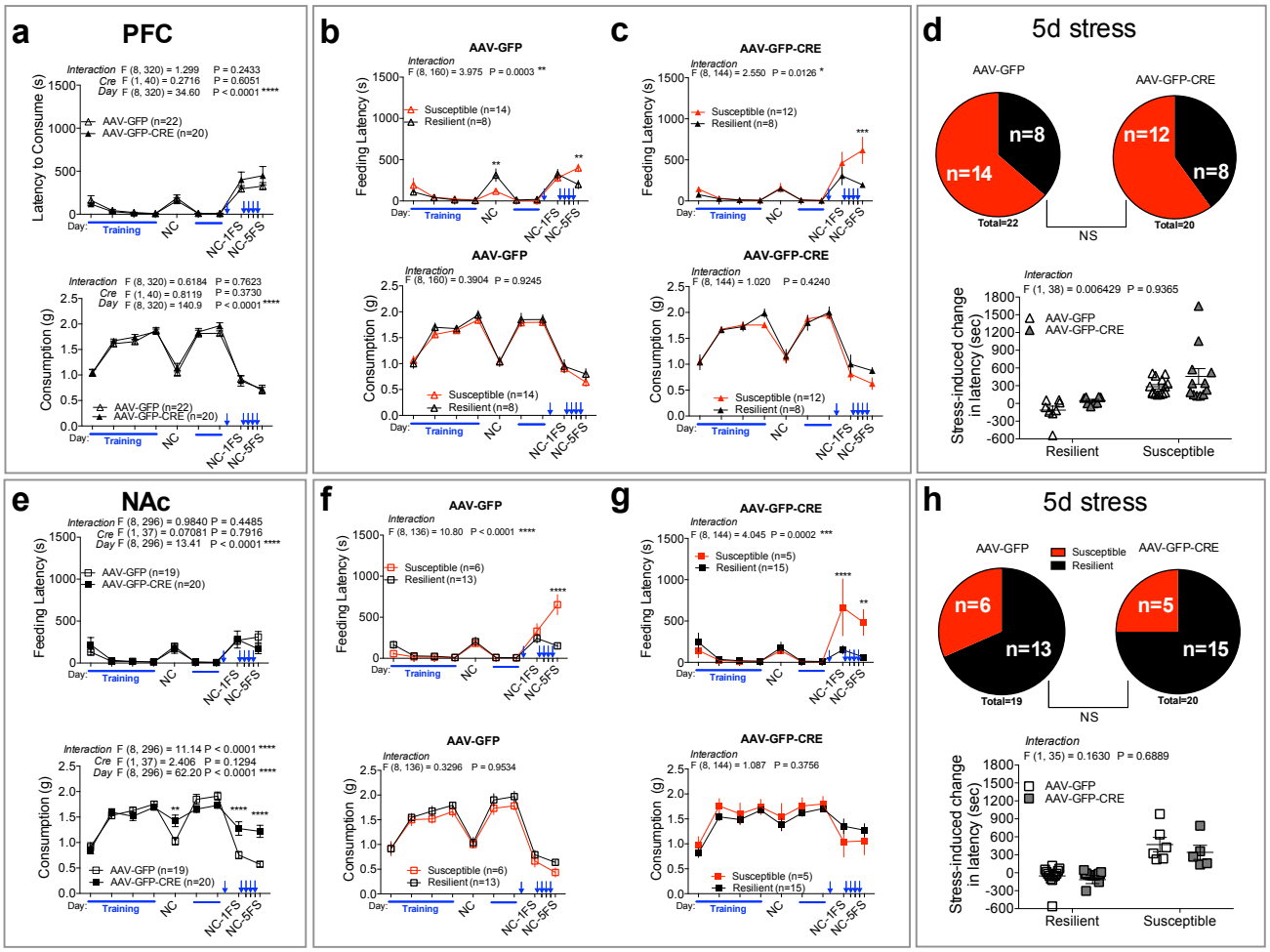

Figure S3.10 Stress susceptibility is not significantly affected by loss of PFC or NAC 2-AG. (**a**) Latency (top) and consumption (bottom) measurements for repeated novelty-induced hypophagia (rNIH) testing of prefrontal cortex (PFC) GFP control versus GFP-CRE injected DAGL$^{fl/fl}$ mice. (**b**) PFC AAV-GFP and (**c**) PFC AAV-GFP-CRE rNIH data from panel (a) split into resilient and susceptible subpopulations using the latency difference between novel cage testing 24h after 5 days of stress exposure (NC-5FS) and baseline (NC). (**d**) Susceptibility ratios for PFC AAV-GFP control versus AAV-GFP-CRE injected mice from panels (b) and (c). (**e**) Latency (top) and consumption (bottom) measurements for repeated novelty-induced hypophagia (rNIH) testing of nucleus accumbens (NAC) GFP control versus GFP-CRE injected DAGL$^{fl/fl}$ mice. (**f**) NAC AAV-GFP and (**g**) NAC AAV-GFP-CRE rNIH data from panel (a) split into resilient and susceptible subpopulations using the latency difference between novel cage testing 24h after 5 days of stress exposure (NC-5FS) and baseline (NC). (**h**) Susceptibility ratios for PFC AAV-GFP control versus AAV-GFP-CRE injected mice from panels (f) and (g). Blue arrows indicate stress exposure which occurred once per day for 5 consecutive days. Data for each brain region was combined from two independent cohorts. F and P values for two-way ANOVA shown in all panels. Asterisks indicate significance as determined by pairwise comparisons from Holm-Sidak multiple comparisons test after ANOVA. Chi-squared tests of susceptibility ratios were not significant (d,h). Data are presented as mean ± SEM. (ADG, AH,

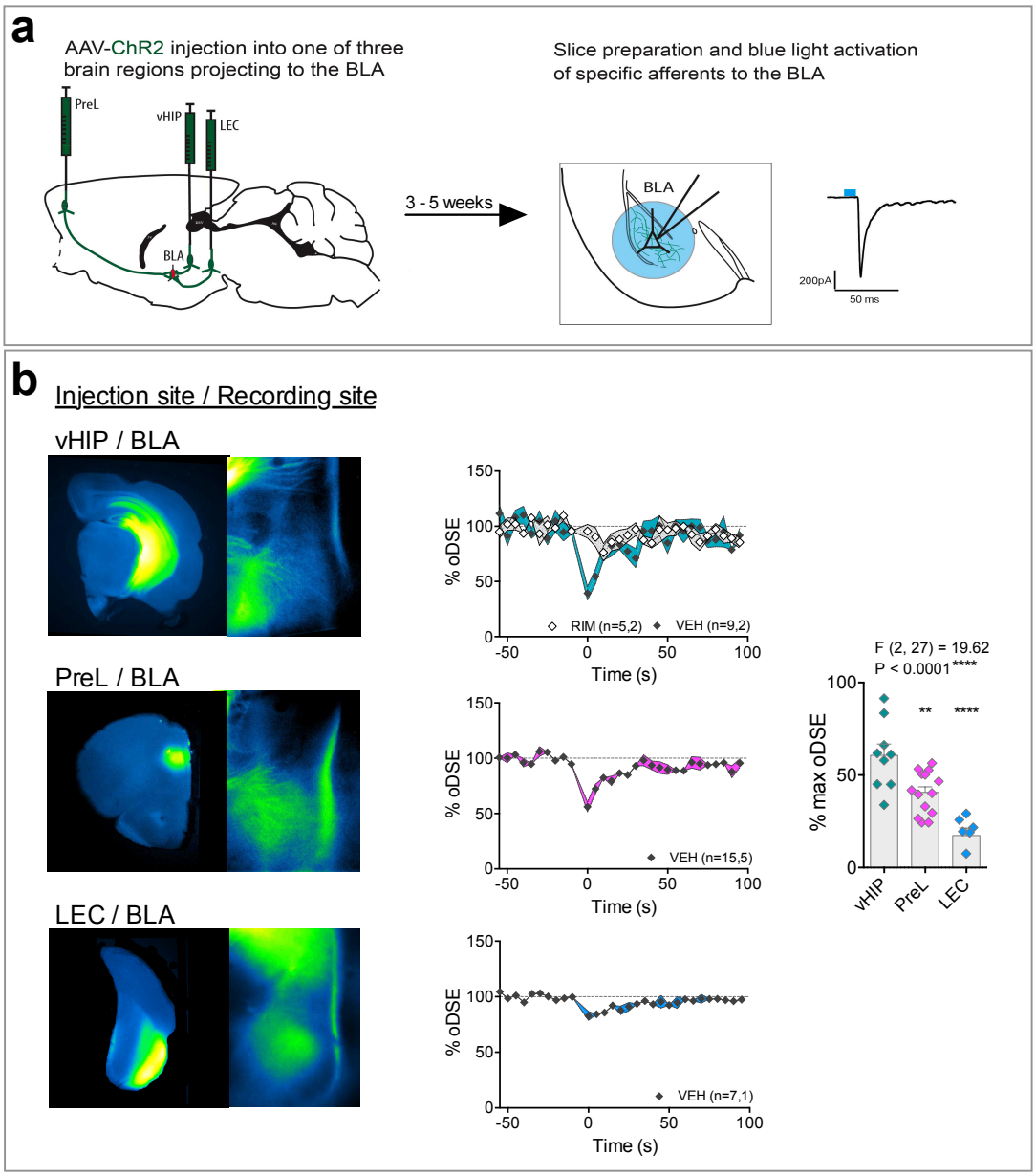

Figure S3.11 2-AG most strongly modulates ventral hippocampal inputs to the BLA. (a) Schematic figure for afferent-specific electrophysiological recordings in BLA. **(b)** Left: Representative images of YFP labeled injection and recording sites. Middle: Corresponding graphs of depolarization induced suppression of excitation (oDSE) of optically evoked currents from ventral hippocampal (vHIP), prelimbic prefrontal cortical (PreL), and lateral entorhinal cortical (LEC) inputs onto BLA pyramidal cells with vehicle (VEH) incubation. Rimonabant incubation verified that the vHIP-BLA oDSE is CB1R-dependent (RIM, gray trace, top middle). Right: comparison of the maximally evoked oDSE of the separate inputs. Number of (cells, animals) are shown for each group. F and P value for one-way ANOVA shown (d). Asterisks indicate significance of P value from Holm-Sidak multiple comparison test between PreL or LEC and vHIP. Data are presented as mean ± SEM. (RB)

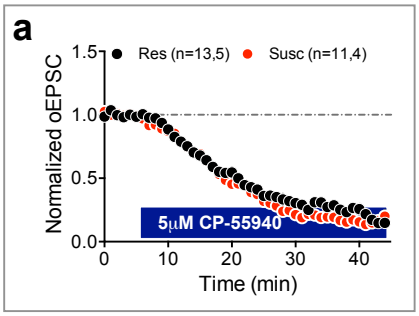

Figure S3.12 CB1 receptor agonist-induced depression in the BLA does not differ between resilient and susceptible mice. (**a**) CP-55940 induced depression of optically evoked EPSCs (oEPSC) from ventral hippocampal synapses in basolateral amygdala (BLA) pyramidal cells. Number of (cells, animals) are shown for each group. Data are presented as mean ± SEM. (RB)

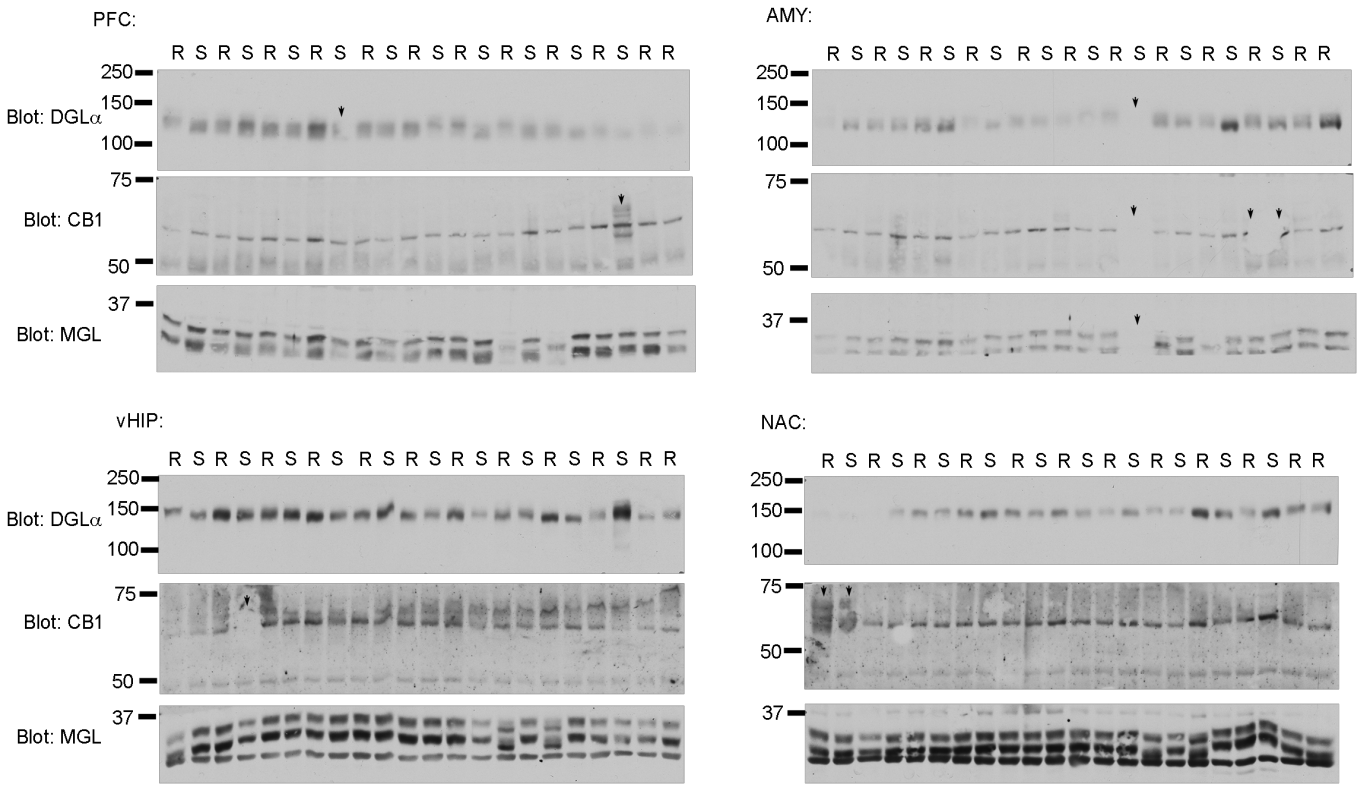

Figure S3.13 Full un-cropped blots are shown corresponding to Supplementary Figure 6. Arrows are shown marking samples that were not included in analysis due to western blot artifact. For MGL density measurements, several bands were present, presumably corresponding to multiple isoforms that were present in the tissue. For consistency, the density of the top two molecular weight isoforms were selected for analysis. (RJB, WPP, BCS)

CHAPTER IV

CONCLUSIONS & FUTURE DIRECTIONS

Critical Comparison and Conclusions from Three Lines of Germline DAGLα KOs

The data presented in Chapter 2 demonstrate a clear necessity for DAGLα-mediated 2-AG production in the maintenance of low levels of basal anxiety-like behavior. This point has since been independently verified. Shortly after our studies using germline DAGLα KOs were published, two other groups reported similar findings in their own lines of germline DAGLα KO mice (Jenniches et al. 2015, Patel et al. 2015, Powell et al. 2015, Jenniches and Zimmer 2016). These groups reported a few phenotypic differences as well as additional phenotypes of DAGLα deletion. All three KO lines exhibited elevated levels of anxiety-like behavior and reduced bodyweight. The Zimmer group reported increased immobility in the forced swim test (FST) and a male-specific sucrose preference deficit (while our sucrose preference deficit was female-specific). We and the Lexicon Pharmaceuticals group showed no phenotype in the TST and, in contrast to the Zimmer group, the Lexicon group's KOs exhibited reduced immobility in the FST. These discrepancies in depressive-like phenotypes suggest that the role of 2-AG in regulating depressive phenotypes may be more complicated or environment-dependent than its clear and ubiquitous anxiety-reducing role. The Zimmer KOs also showed a reduction in hippocampal neurogenesis, maternal care, and impairment in fear extinction. Importantly their line was maintained using a homozygous by homozygous breeding scheme (introducing the possibility of early life parental and environmental differences contributing to their KO phenotypes) and exhibited reductions in AEA. This discrepancy in AEA levels could be

extremely problematic as AEA has been clearly shown to play similar but distinct roles in affective behaviors. Additionally, they showed much smaller increases in 2-AG (~33%) following administration of the same dose of JZL-184 in an overall aqueous solution (likely a suspension rather than a true solution) than we did using DMSO (~250%). This may be purely a bioavailability issue, or could be a true difference between the KO lines.

As mentioned above, most patients with affective disorders (and many others) who report use of cannabinoids cite stress and anxiety relief as their primary motivation for continued use (Reilly et al. 1998, Chabrol et al. 2005, Hyman and Sinha 2009, Fox et al. 2011, Bujarski et al. 2012). In addition, 2-AG deficits in patient cohorts with MDD and PTSD suggest the possibility that affective disorders may be partially caused by or exacerbated by deficient eCB signaling and that cannabis use within those patient populations may represent an attempt to self-medicate (Chabrol et al. 2005, Hyman and Sinha 2009, Bujarski et al. 2012, Bonn-Miller et al. 2014). Importantly, our reversal studies suggest that if eCB deficiency does contribute to the development of affective disorders in a subset of patients, acutely rescuing eCB levels may substantially ameliorate symptoms.

Interestingly, the Lexicon group made direct comparisons to their line of CB1R KOs throughout and found that DAGLα deletion largely recapitulated the metabolic and affective phenotypes of CB1R deletion. There were, however, a few minor distinctions that suggested 2-AG depletion may not be as aversive as direct CB1R antagonism which, they suggest, could mean that DAGLα inhibition might be better tolerated as a treatment for obesity. They also report that DAGLα KOs exhibited seizures which we observed anecdotally, and have been reported in CB1R KOs (von Ruden et al. 2015). The Zimmer KOs' deficit in hippocampal neurogenesis also mimics a known CB1R KO phenotype (Jin et al. 2004). It is likely that many

additional phenotypes that mirror those seen in CB1R knockout mice and mimic the effects of chronic stress will be discovered. This could indicate that loss of 2-AG signaling mediates many, if not most, of the obvious deficits of CB1R KOs although this will be difficult to confirm until an effective strategy for chronic reduction of AEA is developed. Since AEA levels are unchanged in our KOs and the anxiety phenotypes are largely consistent between all three KO lines we can at least conclude that the two eCBs are not redundant and that AEA does not normally compensate for 2-AG deficiency. However, given that partial normalization of 2-AG levels was able to acutely reverse some anxiety-like phenotypes it would be interesting to test if FAAH inhibition resulting in supraphysiological levels of AEA could also ameliorate the negative effects of 2-AG deficiency. Additionally, it would be informative to attempt to rescue the anxiety-phenotypes of DAGLα KOs using pharmacological or genetic methods to increase 2-AG in specific brain regions in order to determine where 2-AG signaling is required for the regulation of basal anxiety. A critical control in such studies would be the evaluation of CB1R expression and MAGL activity to be certain that DAGLα KOs do not exhibit compensatory changes that would confound the experiments.

While these germline DAGLα deletion studies have been informative, one major caveat is that others have shown clear and important roles for eCBs in the development of the nervous system meaning that it is possible these effects are mediated by altered CNS formation (Berghuis et al. 2007, Keimpema et al. 2013, Alpar et al. 2014). Particularly with that caveat, it is encouraging that rescuing 2-AG levels over a short period of time in adulthood is able to ameliorate some of the behavioral phenotypes. Future studies should also determine if long-term treatment is even more effective. Altogether these data strongly support the hypotheses that eCB-

deficiency may contribute to the development of affective dysfunction and eCB-enhancement may constitute an effective treatment strategy.

A New Model of Stress-Susceptibility

As discussed in the introductory chapter, most current models that are being used to characterize individual differences in susceptibility to the development of stress-induced affective dysfunction are primarily models of MDD. Animals qualify as susceptible based on increases in social avoidance, anhedonia, or failure to use active coping strategies to escape a shock. These chronic stress models generally produce equivalent levels of anxiety in both susceptible and resilient groups, although one group has used a clustering analysis to select for an anxiety phenotype after CSDS (Bosch-Bouju et al. 2016). While this is an important step forward and will absolutely produce additional important insights, there are a number of drawbacks to the CSDS model that limit its application in specific populations. For example, mice will rarely display aggression toward females – a group that, in humans, tends to have higher rates of mood and anxiety disorder diagnoses (Cyranowski et al. 2000). This is a particular problem in the field of affective dysfunction as many studies have demonstrated that fear and anxiety responses differ between males and females and are impacted by sex hormones (McHenry et al. 2014, Maeng and Milad 2015). Additionally, the use of chronic stress precludes many these models from being used to investigate how susceptibility differs over short periods of time like adolescence or over the course of the estrus cycle.

The rNIH model of stress-susceptibility is not only a reasonably simple paradigm both conceptually and procedurally but also exhibits face and construct validity as a model for stress-related anxiety. The etiological validity of the model is reasonably strong since evidence

discussed in the introduction indicates that stress causes or exacerbates trauma-related and other anxiety disorders. An extra layer of etiological validity is derived from the use of separation into susceptible and resilient individuals since only a subset of people who experience traumatic or repeated stress go on to develop affective disorders. In a line of outbred mice this mice model produces ~25-33% susceptibility which is much closer to the estimates of the percent of people who develop trauma-related disorders following traumatic life events than the ~50% of individuals susceptible to anxiety-like phenotypes reported in other models (Haagsma et al. 2012, Dorrington et al. 2014, Bosch-Bouju et al. 2016). This paradigm can be used in females and, in fact, my own experiments are suggestive of potentially interesting sex differences in susceptibility. The phenotypes produced by the rNIH paradigm are widely accepted as measures of anxiety-like behavior, namely increased latency and decreased consumption in the novel cage test of NIH and reduced exploration of the open arm and light compartment in the EPM and LD tests, respectively (Strekalova et al. 2004, Dulawa and Hen 2005, Griebel and Holmes 2013). These tests are conflict-based tests wherein the fear of unknown and potentially dangerous environments is pitted against the desire to explore and possibly discover novel sources of food etc. Anxiolytics like diazepam and chronic SSRIs reduce anxiety-like behavior in these tests (while acute SSRI treatment increases latency in the NIH test), suggesting they have predictive validity as well (Griebel and Holmes 2013).

I have additionally shown that the anxiety-like phenotype (increased latency in the NIH test) induced by foot-shock stress exposure remains significantly different from baseline in the stress-susceptible group at both 3 and 14 days post-stress and that the phenotype renews consistently with additional stress exposures. This allows for the comparison of intra-individual differences in stress susceptibility before and after an experimental manipulation. That said,

while on average the susceptible group stays susceptible and the resilient group stays resilient, there are crossovers week to week suggesting an additional level of complexity that needs to be investigated further. One additional caveat that is important to note is that the paradigm may require single housing of animals. All the rNIH studies reported herein were performed with singly housed animals and we have observed anecdotally that cohorts of group housed mice appear to respond less intensely to the foot-shock stress, perhaps due to socialization between stress exposure and anxiety testing (data not shown). This may present an interesting opportunity to study the effects of social support versus isolation on stress-responsivity and adaptation, but could also present an obstacle for the use of the rNIH model given the large cohort size required for producing an adequate group of susceptible individuals. It will also be important to test the ability of other types of stress to elicit similar behavioral changes in this paradigm. This may even provide an interesting way to compare the 'intensity' of various types of stress, which has previously only been possible by comparing the level of stress-induced glucocorticoid release.

Hopefully, this newly validated paradigm will be a useful additional tool for the field in examining individual differences in susceptibility to stress-induced anxiety-like phenotypes. A critically important approach for all of these stress-susceptibility models will be to utilize longitudinal *in vivo* electrophysiological and dialysis approaches in order to characterize potential baseline biomarkers that will predict susceptibility. Identification of factors that predict stress-resilience may produce the most powerful pool of strategies and targets for preventing and reversing the negative impact of traumatic stress exposure.

Region-specific 2-AG Manipulations and the Circuitry of Affective Disorders

In Chapter 3 of this work, I used the rNIH model to demonstrate first that global

manipulations of 2-AG content could promote or impair stress-resilience and then to demonstrate that 2-AG in the BLA specifically is critical for adaptation to repeated stress. This region-specific manipulation in adulthood suggests it is unlikely that all the effects of germline DAGLα deletion are due to disruptions in neural development. It remains possible that the overall elevations in basal anxiety are at least partly mediated by developmental effects, but it is clear that DAGLα is still required in adulthood at the very least for the maintenance of low levels of anxiety in the context of repeated stress exposure. I showed that 2-AG is not required in the mPFC or NAc for the maintenance of low levels of basal anxiety or for resilience or adaptation to foot-shock stress exposure. Other studies have clearly demonstrated a role for the PFC in regulating anxiety-like behavior, and shown that cells in the PFC produce 2-AG but our studies suggest that PFC 2-AG is regulating other aspects of fear and anxiety, which were not engaged here.

Interestingly, despite having a negligible effect on anxiety-like behavior, NAc 2-AG depletion almost completely eliminated hyponeophagia, with mice drinking nearly as much Ensure in the novel cage test as in their home cages even after stress. This suggests the possibility that while 2-AG in the NAc is not involved in regulating anxiety-like behavior in this paradigm, it may be critical for modifying feeding (perhaps specifically hedonic feeding) based on context. This effect is unlikely to be mediated purely by modulation of hunger because other studies have shown that *increasing* 2-AG specifically in the NAc stimulates feeding (Soria-Gomez et al. 2007, Parker et al. 2015). This failure of NAc 2-AG deficiency to impair stress-resilience is in stark contrast to the CSDS study in which individuals were characterized by anxiety-like behaviors. Their work partially complements the work presented herein in that eCB signaling deficiency is associated with susceptibility to stress-induced anxiety-like phenotypes

and increasing 2-AG normalizes both plasticity and behavior (Bosch-Bouju et al. 2016). Their study shows that spike-timing dependent eCB-mediated plasticity is impaired in the nucleus accumbens of individuals susceptible to the development of stress-induced anxiety, although they do not test if 2-AG signaling is impaired in other regions. They further show that direct infusion of JZL-184 into the NAc can normalize 2-AG mediated plasticity and ameliorate anxiety-like behavior. While my work indicates that 2-AG specifically in the NAc is not critical for resilience or adaptation to stress, it has been shown that different types of stress produce different patterns of activity in the brain (Pacák and Palkovits 2001, Figueiredo et al. 2003). It is possible, then, that this difference represents a distinction in the circuitry recruited for responses to either short (7.5 mins for 1 or 5 days) versus longer term (3h for 10 days) stress or for foot-shock versus social defeat stress. It is incredibly promising, given the procedural differences, that increasing 2-AG was able to reduce stress-induced anxiety-like behavior in both the rNIH and CSDS paradigms.

After our discovery that 2-AG in the BLA is critical for adaptation to repeated stress we next examined 2-AG-CB1R signaling and found that susceptibility is associated with a reduced capacity for 2-AG-CB1R signaling to regulate glutamatergic inputs to the BLA after stress. We next wondered if this represented a global deficit in signaling capacity or if 2-AG-CB1R signaling was impaired at specific BLA inputs. We found that neither prelimbic mPFC-BLA glutamatergic input strength nor regulation by 2-AG-CB1R signaling differed between resilient and susceptible mice. Conversely, the glutamatergic vHIP-BLA inputs onto pyramidal cells were significantly stronger and less regulated by 2-AG-CB1R signaling in susceptible individuals. It remains unclear if these signaling differences existed prior to stress exposure or if, for example, some component of the stress response was more effective in upregulating 2-AG signaling at

these synapses in resilient individuals. At least one study has demonstrated that innate PFC excitability predicted susceptibility in the CSDS paradigm suggesting the possibility that at least some differences may precede stress exposure and could potentially be used as biomarkers of resilience (Kumar et al. 2014).

While possible, it seems unlikely that differential strength and 2-AG-CB1R regulation of the vHIP-BLA projection is wholly responsible for producing the behavioral differences in susceptible and resilient groups. In particular, it is surprising that the mPFC input does not appear to differ. Our viral injections herein were primarily targeted to the prelimbic cortex, and since prelimbic and infralimbic PFC projections to the BLA have been shown to regulate emotional learning in opposite ways, examining the possibility of preferential regulation of these two pathways could be informative (Sierra-Mercado et al. 2011, Song et al. 2015, Cheriyan et al. 2016). It would also be interesting to examine the strength and 2-AG-CB1R sensitivity of additional pathways implicated in stress-susceptibility using other models, including the vHIP-NAc projection (Bagot et al. 2015). Overall, comparison of resilience and susceptibility factors in these models could provide important insights about the mechanistic differences between mood and anxiety (or trauma-related) disorders. Additionally, more studies designed to measure signaling prior to, during, and after stress exposure will be important to determine if individual differences in stress-induced affective dysfunction are driven by underlying differences which are amplified by stress exposure, inefficient stress-related resilience-promoting mechanisms, or both. Certainly, this complexity could explain why consistent genetic links to affective disorders have been so elusive.

Interestingly, there is very little in the literature regarding the role of the vHIP-BLA projection. The BLA-vHIP pathway has been clearly shown to be anxiogenic by optogenetic

studies (Felix-Ortiz et al. 2013). One study suggested that the vHIP preferentially targets the mPFC, rather than the amygdala, with anxiety-related information, with general task-performance information going to the amygdala and NAc (Ciocchi et al. 2015). Work from the Nestler group has suggested an important role for the vHIP-NAc pathway in regulating susceptibility in the CSDS model (Bagot et al. 2015). Their work is consistent with our work in that they found that resilience is associated with reduced vHIP activity and optogenetic *inhibition* of vHIP-NAc inputs enhanced resilience. Conversely, they also found that optogenetic *stimulation* of mPFC-NAc and BLA-NAc projections enhanced resilience. Together these studies suggest that the vHIP may be a driver of stress-related negative affect. Indeed, complete lesion of the vHIP is strongly anxiolytic (Bannerman et al. 2002). Other studies have shown that direct activation of dentate gyrus granule cells (glutamatergic projection neurons) strongly suppresses basal anxiety-like behavior, however the net output effect of granule cell excitation is strongly dependent on frequency due to their parallel activation of feedforward inhibitory mechanisms (Mori et al. 2004, Kheirbek et al. 2013). Interestingly, dentate gyrus neurons born in adulthood strongly activate the inhibition of older dentate gyrus granule cells, which could represent a mechanism whereby adult neurogenesis is critical for resilience in models of MDD (Drew et al. 2016). Clearly, the role of the vHIP in basal and stress-related anxiety is complex and a more detailed examination of how its internal microcircuitry is recruited during anxiety tasks and in response to stress will be required in order to build a strong understanding of how these data fit together. Additionally, *in vivo* local field potential recordings in the context of stress-induced anxiety may provide a better understanding of vHIP overall activation as well as functional coupling with other brain regions like the mPFC and BLA (Likhtik and Gordon 2014, Likhtik et al. 2014).

Potential Mechanisms for Functional eCB Signaling Differences

While repeated stress exposure has been shown to modulate expression of eCB system components, it remains unclear exactly how the functional difference in signaling at vHIP-BLA synapses is produced as 2-AG levels, DAGLα, MAGL, and CB1R expression do not differ between susceptible and resilient groups (Sumislawski et al. 2011). However, several post-translational mechanisms of control over 2-AG-CB1R signaling have been discovered. For example, the protein CB1 receptor interacting protein (CRIP1a) has been shown to reduce internalization of CB1Rs by blocking its β-arrestin binding domain thereby enhancing overall agonist-induced CB1R activation (Smith et al. 2015, Guggenhuber et al. 2016, Blume et al. 2017). This is, however, unlikely to be the primary mechanism underlying the differences in susceptible and resilient 2-AG-CB1R signaling since direct agonist-induced oEPSC depression did not significantly differ between groups. Other more likely possibilities include differences in DAGLα phosphorylation (Shonesy et al. 2013) or localization (Jung et al. 2012), or MAGL sulfenylation (Dotsey et al. 2015). DAGLα phosphorylation by calcium/calmodulin dependent protein II (CAMKII) inhibits 2-AG production and altered phosphorylation has been shown to affect DSE (Shonesy et al. 2013). It is possible, then that increased phosphorylation of DAGLα could mediate the impairment of 2-AG-CB1R signaling at vHIP-BLA synapses in susceptible mice. This should be tested using mass spectrometry or a DAGLα activity assay (Shonesy et al. 2013, Bisogno 2016). Several other proteins have been identified that interact with DAGLα, though their impact on its activity and localization are only just beginning to be elucidated (Shonesy et al. 2013). One of these proteins, Homer1a, is a scaffolding protein required for metabotropic glutamate receptor (mGluR) induced production of 2-AG (Jung et al. 2007). Disruption of this mGluR signaling complex by deletion of fragile x mental retardation protein

(FMRP) also impairs 2-AG production and signaling (Jung et al. 2012). However, all the vHIP-BLA oDSE herein was elicited purely by depolarization, not assisted by G_q-coupled receptor activation so this is unlikely to be the only mechanistic difference between susceptible and resilient groups. It has been suggested that two separate pools of DAGLα mediate 2-AG production in response to Ca^{2+} versus Gq-GPCR activation so a difference in one form of DSE cannot be assumed by a difference in the other (Zhang et al. 2011). However, it would be informative to test the difference in DSE with incubation of the mGluR agonist dihydroxyphenylglycine (DHPG) since mGluR activation may be contributing to 2-AG production during the rNIH procedure. Finally, hydrogen peroxide (whose production can be elicited by ionotropic glutamate receptor activation) can inhibit MAGL by sulfenylation, preventing the entry and degradation of 2-AG (Patel and Rice 2012, Dotsey et al. 2015, Scalvini et al. 2016). Some of these regulatory mechanisms targeting DAGL or MAGL would be expected to produce changes in overall 2-AG levels, but the extent to which tissue 2-AG levels are representative of 2-AG signaling capacity has been disputed. 2-AG measured by microdialysis is orders of magnitude lower than 2-AG measured by mass spectrometry of tissue homogenates, suggesting that while synaptically relevant 2-AG is synthesized on demand there may be pools of 2-AG elsewhere in the membrane that contribute to bulk tissue measurements (Buczynski and Parsons 2010).

Individual Differences in Psychiatric Disease

The experiments for validation of the rNIH paradigm were largely performed with a line of outbred mice. Given that the human population is so genetically diverse, it would be worthwhile to investigate potential consistent genetic differences between susceptible and

resilient groups in this outbred line of mice. Genome sequencing, however, would be a cumbersome approach to answer this question given that it is often difficult to predict how genetic mutations will affect the expression, localization, or function of gene products. Instead, RNA sequencing experiments using microdissections of the BLA subregion targeted by vHIP projections could be performed as an unbiased discovery approach. Separately, qRT-PCR could be used to quantify specific transcripts that might be expected to differ based on their involvement in the stress response, like CRH. Additional specificity could be gained by using transgenic mice that transiently express GFP under the promotor of the immediate early gene cfos to indicate recent activity. Laser capture microscopy could be used to specifically collect the cytoplasm and nucleus of BLA cells that are activated or not activated following stress exposure in stress-resilient versus -susceptible mice. An alternate approach could utilize Drop-seq to analyze differences in the overall distribution of particular cell types, identified by signature combinations of transcripts, within the BLA of susceptible and resilient individuals (Campbell et al. 2017, Ziegenhain et al. 2017). Any such experiments would necessarily include comparisons of stress-resilient and -susceptible mice to stress-naïve mice and transcripts which changed similarly in both stressed groups could be largely discounted.

Both stress and cannabinoids have been shown to produce changes in the epigenome and components of the eCB system can themselves be regulated by epigenetic changes. Many of the experiments herein were performed with outbred mice, but other models of stress-susceptibility discussed above have clearly demonstrated differences in stress-responsivity in inbred, or essentially genetically identical, lines of mice. This strongly suggests the involvement of epigenetics in shaping individual differences in stress-responsivity. As such, while the investigation of potential epigenetic differences between resilient and susceptible groups was

beyond the scope of this project, the field will likely largely move in that direction. For example, recent work shows that DNA methyl transferase 3a (DNMT3a) is induced in the NAc by chronic variable stress, particularly in females, an effect which may contribute to sex differences in stress responsivity (Hodes et al. 2015). Many additional studies suggest the strong likelihood that epigenetic changes are critically important for differential stress-responsivity and for sex differences in stress-responsivity (Papale et al. 2016). Although controversial, it has been suggested that some of these epigenetic differences are passed on to offspring, and even that some of them arise stochastically during development (Feinberg and Irizarry 2010, Czyz et al. 2012, Papale et al. 2016). Interestingly, by some definitions of epigenetics various micro- and long noncoding-RNAs qualify which may explain how epigenetic changes could be transmitted across generations despite nearly complete erasure of epigenetic markers on DNA during meiosis (Papale et al. 2016). If an epigenetic signature of stress-susceptibility (or perhaps, individual susceptibility signatures for different domains of stress-related dysfunction) could be identified it would be an incredible tool for diagnosis and discovery of treatments for stress-related psychiatric disease. Several studies have begun to build this kind of story by: examining hippocampal DNA methylation after acute and chronic stress; comparing chromatin regulation in the NAc of CSDS resilient mice to those produced by antidepressant treatment; and by engineering gene-targeted transcription factors to promote epigenetic modification and either amplifying or inhibiting stress-induced changes (Hunter et al. 2009, Wilkinson et al. 2009, Heller et al. 2014).

The study of epigenetics and the eCB system is in its early stages but several studies have suggested that CB1R transcription in both humans and mice is regulated by methylation (Franklin et al. 2010, D'Addario et al. 2013, Subbanna et al. 2014). In addition, eCB ligands have

been shown to induce epigenetic modification of various genes (Eljaschewitsch et al. 2006, Paradisi et al. 2008, Chioccarelli et al. 2010, Subbanna et al. 2014, Szutorisz and Hurd 2016). Whether these epigenetic effects of eCBs contribute to affective dysfunction has yet to be determined but will likely become an active area of investigation as the methods for investigation of epigenetics become more ubiquitous (Hunter and McEwen 2013). Regardless of whether eCB-modulation of epigenetic regulation is critical for affective disorders, it is extremely likely that epigenetic modifications of gene expression whether environmentally induced, inherited, or stochastically generated are involved in individual differences in stress-responsivity. Building a map of these mechanisms and the genes they regulate differentially in susceptibility and resilience in response to stress will allow for more intelligent targeting of pharmacotherapeutics to treat or prevent stress-induced affective dysfunction.

Summary and Overarching Conclusions

Herein I have presented data that support the hypothesis that 2-AG signaling may be a critical resilience factor protecting against stress-induced affective dysfunction and pharmacological strategies to elevate 2-AG may be effective for treating stress-relatedpsychiatric disorders (see Fig. 4.1). Germline DAGLα KOs exhibit basal anxiety along with various other phenotypes reminiscent of both CB1R KOs and chronically stressed mice. In order to directly test if 2-AG signaling controls resilience to stress-induced anxiety I then validated a novel paradigm for examination of individual differences in responsivity to acute stress-induced anxiety-like behavior. This model, which utilizes acute rather than chronic stress exposure andspecifically produces anxiety-like rather than depressive-like phenotypes (sucrose preference clearly shows no difference, though there is a trend for increased immobility in the susceptible

group in forced swim testing; data not shown), fills a critical gap in the field of stress responsivity. Using this model and bidirectional pharmacological manipulations, I show that increasing 2-AG can both reverse and protect against the development of stress-induced anxiety while decreasing 2-AG increases susceptibility to stress-induced anxiety-like behavior. In support of a long-standing hypothesis in the field, specifically reducing 2-AG in the BLA impairs adaptation to repeated stress. It is important to consider that stress-adaptation may involve some of the same molecular mechanisms and circuitry as resilience to acute stress and can conceptually be considered a form of resilience, but resilience and adaptation may also be

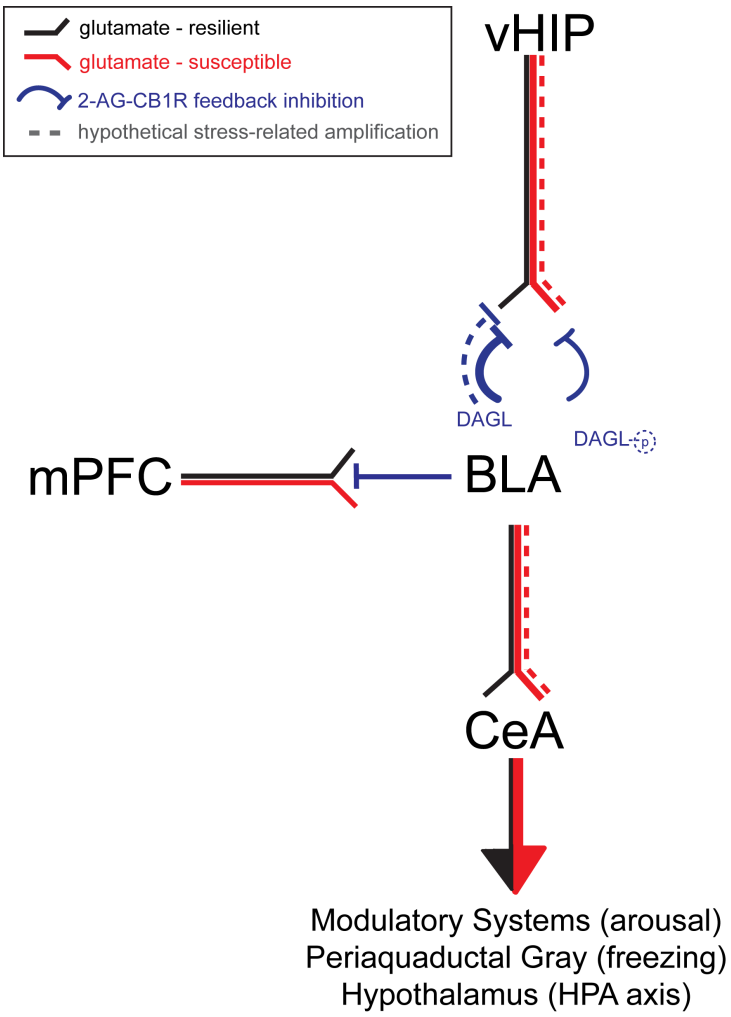

Figure 4.1 Schematic of suggested resilient and susceptible circuit differences. This schematic illustrates the overall hypothesis that increased vHIP-BLA glutamatergic drive (particularly after stress exposure) in susceptible individuals is less well inhibited by 2-AG-CB1R feedback inhibition. This relative hyperactivation of the BLA increases its output to the central amygdala (CeA) which has the overall effect of increasing anxiey-like behaviors through projections to the modulatory systems (norepinephrine, acetylcholine, etc.), the periaqueductal gray, and the hypothalamus. This deficit is at least partially specific to the vHIP-BLA projection as mPFC-BLA projection strength and sensitivity to phasic 2-AG-CB1R signaling does not differ between susceptible and resilient individuals.

mediated by at least partially distinct mechanisms. We then showed that stress-susceptibility in this model is associated with an overall reduction in the dynamic range over which 2-AG-CB1R signaling can regulate glutamatergic inputs to the BLA, as well as more specifically with a stronger vHIP-BLA glutamatergic projection that is less sensitive to phasic 2-AG mediated feedback inhibition (See **Figure 4.1**). It remains unclear if these differences are present prior to stress-exposure but this will be an interesting topic for future exploration using longitudinal *in vivo* electrophysiology techniques. Altogether these data strongly support the hypothesis that increasing 2-AG signaling could be an effective treatment approach for stress-related psychiatric disorders including mood, anxiety, and trauma-related disorders.

APPENDIX A

CENTRAL ANANDAMIDE DEFICIENCY PREDICTS STRESS-INDUCED ANXIETY: BEHAVIORAL REVERSAL THROUGH ENDOCANNABINOID AUGMENTATION

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Abstract

Stress is a major risk factor for the development of mood and anxiety disorders; elucidation of novel approaches to mitigate the deleterious effects of stress could have broad clinical applications. Pharmacological augmentation of central endogenous cannabinoid (eCB) signaling may be an effective therapeutic strategy to mitigate the adverse behavioral and physiological consequences of stress. Here we show that acute foot-shock stress induces a transient anxiety state measured 24 h later using the light–dark box assay and novelty-induced hypophagia test. Acute pharmacological inhibition of the anandamide-degrading enzyme, fatty acid amide hydrolase (FAAH), reverses the stress-induced anxiety state in a cannabinoid receptor-dependent manner. FAAH inhibition does not significantly affect anxiety-like behaviors in non-stressed mice. Moreover, whole brain anandamide levels are reduced 24 h after acute foot-shock stress and are negatively correlated with anxiety-like behavioral measures in the light–dark box test. These data indicate that central anandamide levels predict acute stress-induced anxiety, and that reversal of stress-induced anandamide deficiency is a key mechanism subserving the therapeutic effects of FAAH inhibition. These studies provide further support that eCB-augmentation is a viable pharmacological strategy for the treatment of stress-related neuropsychiatric disorders.

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Introduction

Stress is a major environmental risk factor for the development of mood and anxiety disorders (Caspi et al. 2003, Hunter and McEwen 2013). Understanding the neurobiological mechanisms by which stress is translated into psychopathology is essential to developing novel therapeutic approaches for the treatment of affective disorders. Current clinically utilized pharmacological treatments for affective disorders are primarily based on augmenting monoaminergic transmission, but there is an increasing appreciation of the role of neuropeptides, cytokines and bioactive lipids in the pathophysiology of mood and anxiety disorders (Griebel and Holmes 2013). These non-monoamine-based modulators of mood and anxiety are promising targets for novel therapeutic approaches to treating affective disorders. In particular, multiple studies have demonstrated that pharmacological augmentation of central endogenous cannabinoid (eCB) signaling represents a promising approach to the treatment of mood and anxiety disorders (Patel and Hillard 2009, Gamble-George et al. 2013, Gunduz-Cinar et al. 2013, Hermanson et al. 2013).

eCBs are lipid signaling molecules produced in the nervous system that exert biological actions primarily via the activation of cannabinoid receptors (CB1R and CB_2). Anandamide (N-arachidonylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) are the two most well-studied eCB ligands. Converging preclinical studies strongly suggest that stress inhibits AEA signaling, and that pharmacological augmentation of eCB signaling may be a viable strategy to treat mood and anxiety disorders (Kathuria et al. 2003, Gorzalka et al. 2008, Evanson et al. 2010, Wang et al. 2010, Bowles et al. 2012). Systemic treatment with a direct CB1 receptor agonist, however, produces both central and peripheral side effects (Bolla et al. 2002, Gomez et al. 2002, Moreira et al. 2009, Shrivastava et al. 2014). Pharmacological inhibition of the AEA degrading

enzyme, fatty acid amide hydrolase (FAAH), specifically augments AEA-mediated eCB signaling and has been shown to reduce unconditioned anxiety and despair behaviors in animal models in a CB1 receptor-dependent manner (Moreira et al. 2008, Kinsey et al. 2011, Gunduz-Cinar et al. 2013). More recently, genetic deletion and chronic pharmacological inhibition of FAAH have been shown to prevent emergence of some of the adverse behavioral effects of chronic stress (Bortolato et al. 2007, Gunduz-Cinar et al. 2013, Hill et al. 2013). Importantly, stress exposure decreases AEA levels in several limbic brain regions (Patel et al. 2005, Rademacher et al. 2008, Gunduz-Cinar et al. 2013). This suggests that deficits in AEA signaling contribute to stress-induced anxiety-like behavior, and that normalization of stress-induced AEA deficiency could be the mechanism subserving the anxiolytic effects of FAAH inhibition.

Here we sought to explicitly test the role of AEA signaling on acute stress-induced anxiety states. We first tested the effect of acute FAAH inhibition after the onset of stress-induced anxiety in two preclinical models of anxiety, the light–dark box test and the novelty-induced hypophagia (NIH) assay, which we have recently shown to be highly sensitive to eCB signaling (Gamble-George et al. 2013). Results from both models indicate that acute FAAH inhibition reverses the expression of anxiety-like behaviors induced by stress. We then analyzed the association between stress-induced AEA deficiency and anxiety state and found that stress-induced AEA deficiency significantly correlated with anxiety-like behavior. These data provide further support for the potential utility of FAAH inhibitors in the treatment of stress-related neuropsychiatric disorders.

Results

To examine the effects of AEA augmentation on stress-induced anxiety we first determined the effects of the FAAH inhibitor PF-3845 on anxiety-like behavior in control mice and mice exposed to foot-shock stress 24 h before behavioral testing using the light–dark box. Stress induced anxiogenic effects in the light–dark box test. Two-way ANOVA revealed a significant effect of stress exposure on distance traveled in the light zone (Fig. A.1a, $F_{(1,35)}$=4.54; P=0.04) and number of light-zone entries (Fig. A.1c, $F_{(1,35)}$=28.27; P<0.0001), although its effect on light-zone time was not significant (Fig. A.1b). Conversely, ANOVA revealed PF-3845 treatment was anxiolytic in the light–dark box as shown by a significant increase in percent total distance traveled in the light zone (Fig. A.1a, $F_{(1,35)}$=16.9; P=0.0002), total light-zone time (Fig. A.1b, $F_{(1,35)}$=16.9; P=0.0002) and light-zone entries (Fig. A.1c, $F_{(1,35)}$=15.33; P=0.0004), but not total distance traveled (Fig. A.1d, $F_{(1,35)}$=0.23; P=0.64). *Post-hoc* analyses revealed that PF-3845 significantly increased percent light-zone distance (P<0.01) and light-zone time (P<0.001) in stressed mice. PF-3845 also significantly increased the number of light-zone entries in both stressed (P<0.01) and control (P<0.05) mice. No significant stress by PF-3845 interaction was observed for any anxiety measure in the light–dark box assay.

We then evaluated the effects of PF-3845 in control and stressed mice in a second preclinical model of anxiety, the NIH assay, which we have recently shown to be highly sensitive to tonic eCB signaling (Gamble-George et al. 2013).Two-way ANOVA revealed significant effects of stress (Fig. A.1e, $F_{(1,39)}$=7.6; P=0.009) and PF-3845 treatment (Fig. A.1e, $F_{(1,39)}$=13.77; P=0.0006) on feeding latency. Although there was no significant interaction between stress and PF-3845 treatment by ANOVA, *post hoc* analyses revealed that PF-3845

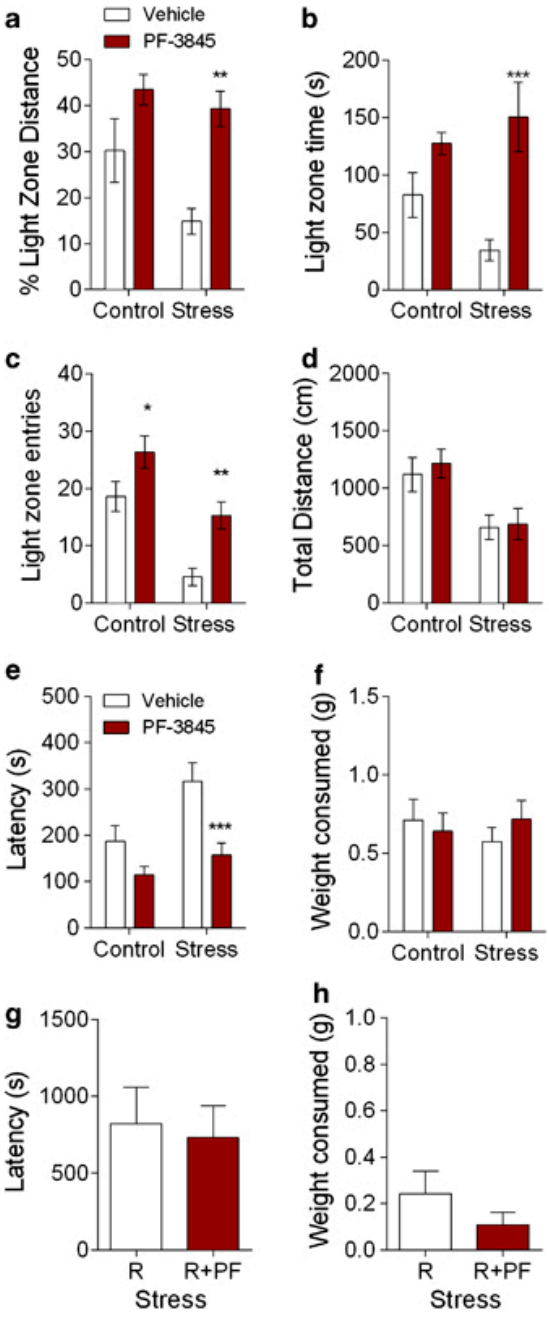

Figure A.1 PF-3845 treatment reduces anxiety-like behaviors in the light–dark box and NIH assay. PF-3845 (maroon bars) reverses stress-induced anxiety in the light–dark box as compared with vehicle treatment (white bars) as measured by (a) distance traveled in the light zone as a percent of total distance traveled, (b) time in the light zone and (c) total number of light-zone entries. (d) PF-3845 also reverses the stress-induced increase in latency to first drink in the NIH assay (e) without affecting total food consumption (f). (g) Pharmacological blockade of CB1 receptors via rimonabant treatment (R, white bars) prevents PF-3845 (maroon bars) from reversing stress effects on latency in the NIH assay. (h) PF-3845 does not significantly alter consumption in the NIH assay with rimonabant co-treatment. **P*<0.05, ***P*<0.01, ****P*<0.001 significantly different from vehicle. NIH, novelty-induced hypophagia.

significantly reduced feeding latency in stressed (P<0.001) but not control mice. Neither stress nor PF-3845 affected total food consumption in the NIH assay (Fig. A.1f).

To determine the mechanism responsible for the anxiolytic effect of PF-3845, we tested the effect of PF-3845 on stressed mice co-treated with rimonabant, a selective CB1 receptor antagonist. Stressed mice were utilized due to the significant reduction in feeding latency produced by PF-3845 in stressed, but not control mice. Stressed mice co-treated with rimonabant and PF-3845 did not differ significantly in either latency or consumption from stressed mice treated with rimonabant alone (Fig. A.1g and h), confirming that PF-3845's anxiolytic effects in the NIH assay are mediated by CB1 receptor activation. Although we did not directly test the effect of rimonabant relative to vehicle treatment here, we have previously demonstrated an anxiogenic effect of rimonabant in unstressed animals in the NIH assay (Gamble-George et al. 2013). Consistent with our previous work, comparison of feeding latencies between vehicle-treated stressed mice (~300 seconds; Fig. A.1g) and rimonabant-treated stressed mice (~800 seconds; Fig. A.1e) suggests that rimonabant maintains this anxiogenic effect after exposure to 1 day of foot-shock stress.

To investigate associations between stress-induced changes in eCB levels and anxiety-like behaviors, we analyzed whole brain eCB levels in control and stressed mice after completion of the light–dark box assay (~24 h after foot-shock stress) by mass spectrometry. Given that behavioral testing is itself a mild stressor, it is possible that exposure to the light–dark box test may have altered overall eCB content across all groups; however, this design allowed for direct analyses of the correlation between eCB levels and behavior in individual mice. Foot-shock stress reduced brain AEA levels in vehicle-treated (Fig. A.2a), P<0.05 but not PF-3845 treated mice. In contrast, stress did not affect the levels of oleoylethananolamide or 2-AG (Fig. A.2b and

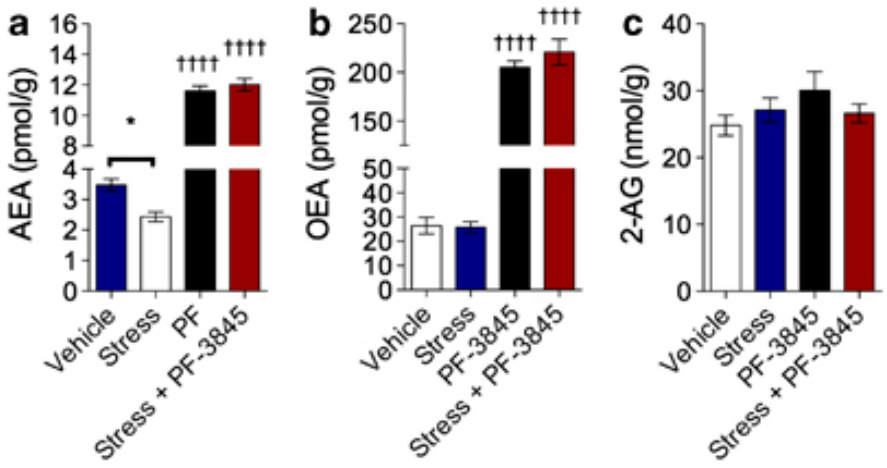

Figure A.2 Stress reduces whole brain AEA levels while PF-3845 treatment increases AEA and OEA. Whole brain (a) AEA, (b) OEA and (c) 2-AG levels from mice immediately after completion of the light–dark box test (24 h after acute foot-shock stress and 2 h after drug treatment) demonstrate that stress decreases AEA and PF-3845 increases AEA and OEA, whereas neither stress nor PF-3845 treatment affects 2-AG. *P<0.05, $^{††††}P$<0.001 significantly different from corresponding vehicle control. AEA, N-arachidonylethanolamine; 2-AG, 2-arachidonoylglycerol; OEA, oleoylethananolamide.

c). As expected, PF-3845 treatment robustly increased AEA and oleoylethananolamide, but not 2-AG, relative to corresponding vehicle treatments in both control and stressed groups (Fig. A.2a-c, P<0.0001 for all). We then performed a linear regression analysis of whole brain AEA levels and anxiety-like behaviors in the light–dark box of both control and stressed mice. This analysis revealed significant positive correlations between AEA levels and light-zone entries (Fig. A.3a), r^2=0.0562; P=0.0003, percent light-zone distance (Fig. A.3c), r^2=0.257; P=0.031, light-zone time (Fig. A.3e), r^2=0.3; P=0.018, and total distance (Fig. A.3g), r^2=0.3; P=0.02, indicating that higher levels of AEA are associated with reduced anxiety-like behaviors. Linear regression of AEA levels and anxiety-like behaviors in control and stressed mice treated with PF-3845 did not reveal any significant correlations, suggesting a saturation of AEA effects on anxiety behaviors at supraphysiological levels (Fig. A.3b, d, f, and h).

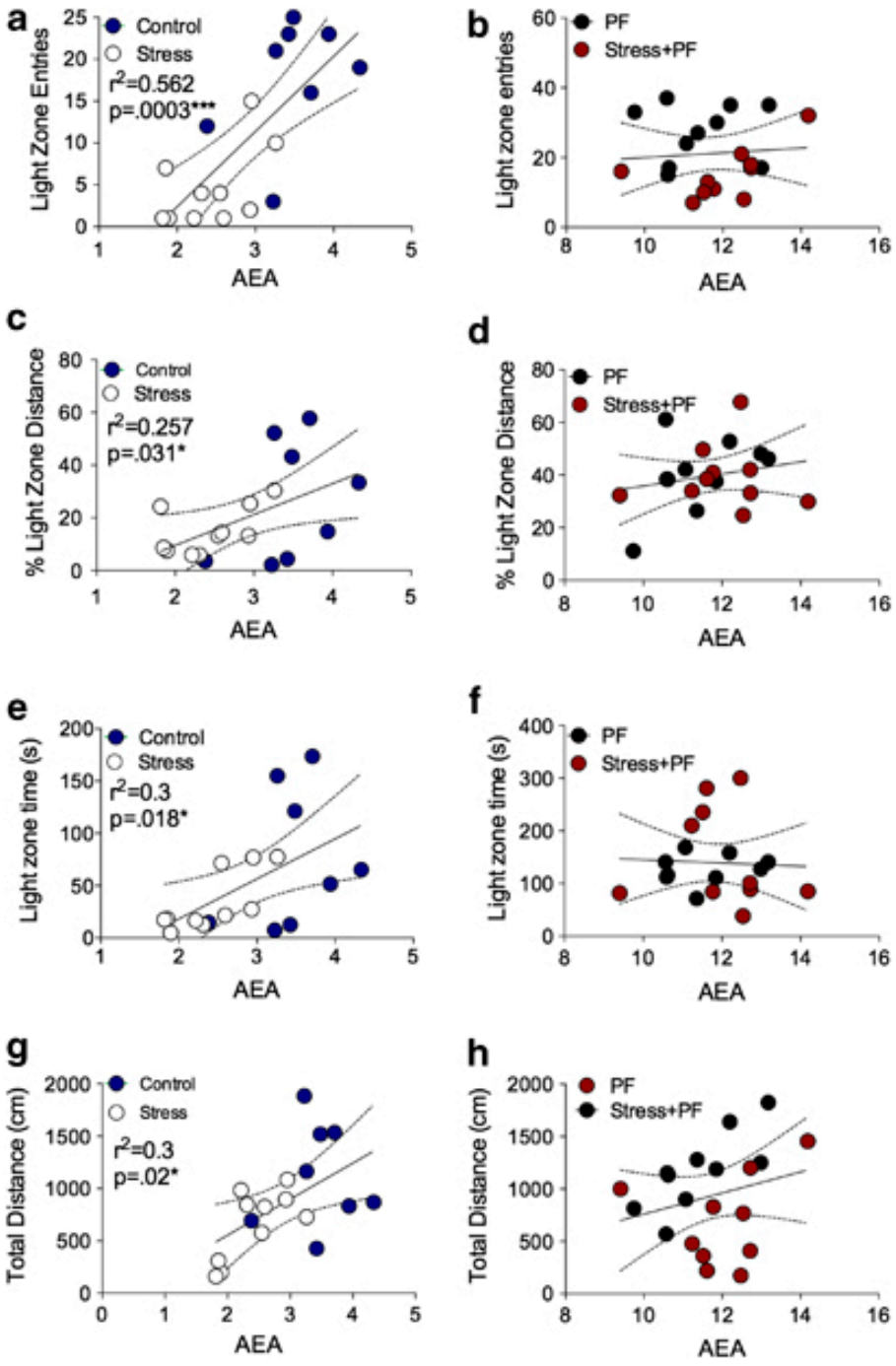

Figure A.3 Whole brain decreases in AEA correlate with increased anxiety in the light–dark box test. Linear regression analyses reveal significant correlations between whole brain AEA levels in control and stressed mice and (a) light-zone entries, (c) light-zone distance traveled as a percent of total distance traveled, (e) light-zone time and (g) total distance traveled. After PF-3845 treatment, however, the same measures no longer correlate with AEA levels (b, d, f and h), suggesting a saturation of AEA effects on anxiety at supraphysiological levels. Linear regression (solid line) with 95% confidence intervals (dashed lines) shown in figures. AEA, N-arachidonylethanolamine.

Discussion

These data demonstrate that pharmacological elevation of AEA after stress, via acute FAAH inhibition with PF-3845, is able to reverse the anxiety-like behaviors typically exhibited after exposure to an intense, acute stressor in both the light–dark box and NIH assays. Interestingly, FAAH inhibition had little effect under control, non-stressed conditions, which is generally consistent with previous studies indicating that the anxiolytic efficacy of eCB-augmentation **is** enhanced by anxiogenic or aversive environmental contexts (Patel and Hillard 2006, Haller et al. 2009, Rossi et al. 2010, Sciolino et al. 2011).These studies are also consistent with previous data demonstrating that genetic deletion or pharmacological inhibition of FAAH during chronic stress exposure is able to prevent some of the adverse physiological and behavioral effects of stress (Bortolato et al. 2007, Gunduz-Cinar et al. 2013, Hill et al. 2013). Our studies extend these data to suggest that elevation of AEA could also be an effective treatment not only as a preventative measure, but also after stress-related psychopathology has begun to manifest. This ability to reverse already established psychopathologies is an essential component of potential novel therapeutics for clinical use.

Importantly, these studies demonstrate that whole brain levels of AEA are predictive of anxiety state, with decreased AEA corresponding to increased anxiety-like behavior in the light–dark test. Although it has been previously shown that stress decreases AEA, this is the first direct evidence that lower levels of AEA in the central nervous system are correlated with greater expression of anxiety-like behaviors (Hill et al. 2005, Hill et al. 2013). This finding parallels recent studies showing that baseline anxiety inversely correlates with peripheral AEA content in human subjects and that among individuals with posttraumatic stress disorder, those with lower peripheral AEA content exhibit more intrusive symptoms (Hill et al. 2008, Dlugos et al. 2012,

Hill et al. 2013). Further studies will be required to determine the validity of peripheral AEA levels as a biomarker for anxiety states. Importantly, our study also demonstrates that pharmacological augmentation of AEA signaling after stress exposure can reverse stress-induced anxiety, which is a necessary feature of novel therapeutics for stress-related psychopathology. Taken together, our findings strongly support the utility of AEA augmentation as a therapeutic approach for stress-related affective and anxiety disorders.

Methods

Animals, foot-shock stress and drug treatments

All studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Vanderbilt University Institutional Animal Care and Use Committee. Male ICR mice (4–7 weeks old) were housed on a 12:12 light–dark cycle with lights on at 0600 (Harlan, Indianapolis, IN, USA). All experiments were conducted during the light phase. Food and water were available *ad libitum*.

Foot-shock stress occurred 24 h before behavioral testing and consisted of six 0.7 mA foot-shocks delivered 1 min apart using a MED Associates fear-conditioning chamber (St. Albans, VT, USA). Each 2-s shock was preceded by a 30-s auditory tone. Drugs were administered 2 h before behavioral testing. The FAAH inhibitor PF-3845 (10 mg kg^{-1}, Cayman Chemical, Ann Arbor, MI, USA) and the CB1 receptor antagonist rimonabant (3 mg kg^{-1}, NIMH Drug Supply Program) were dissolved in dimethylsulfoxide (Sigma Aldrich, Milwaukee, WI, USA) and intraperitoneally injected at a volume of 1 μl g^{-1}.

Behavioral assays

Light–dark box: Mice were individually placed into sound-attenuating chambers (27.9 × 27.9 cm; MED-OFA-510; MED Associates, St. Albans, VT, USA) containing dark box inserts that split the chamber into light (~25 lux) and dark (<5 lux) halves (Med Associates ENV-511). Beam breaks from 16 infrared beams were recorded by Activity Monitor v5.10 (MED Associates) to monitor position and behavior during the 5-min testing period. Mice were stressed and drugs were administered as described above.

Novelty-induced hypophagia: The NIH test consisted of four training days in the home cage and one test day in a novel cage. Mice were singly housed for 4 days before training; cages were not changed for the duration of the experiment. Mice were habituated to testing rooms illuminated by red light (<50 lux) for at least 30 min. After habituation, water was replaced with a highly palatable substance (liquid vanilla Ensure, Abbott Laboratories, Abbott Park, IL, USA) for 30 min during which latency to first consumption and total consumption were recorded. Foot-shock stress was administered immediately following the fourth training session and novel cage testing occurred 24 h later. Mice were habituated in red light for 60 min and then transferred to new, empty cages in a brightly lit room (~300 lux) with 30 min access to Ensure during which latency to drink and total consumption were recorded.

Mass spectrometry

One cohort of mice was killed by cervical dislocation and decapitation immediately following completion of the light–dark box test. Brains were then rapidly removed, frozen on a metal block in dry ice and stored at −80 °C until lipid extraction. Lipids from brain tissue were extracted via homogenization and sonication in 2 ml of acetonitrile containing 1000 pmol 2-AG-d_8 and 20 pmol AEA-d_8. The homogenate was centrifuged and the supernatant was removed and

dried under nitrogen. Samples were then resuspended in 200 µl of methanol:water (50:50). Analytes were quantified using LC-MS/MS on a Quantum triple-quadrupole mass spectrometer in positive-ion mode using selected reaction monitoring. Detection of fatty acids was performed as previously described (Kingsley and Marnett 2003, Hermanson et al. 2013).

Data analysis

Behavioral data were analyzed by *t*-test or analysis of variance (ANOVA) followed by *post hoc* Sidak's multiple comparison test unless otherwise specified. AEA levels and behavioral data were correlated by linear regression. All statistical analyses were conducted with Prism GraphPad 6 (San Diego, CA, USA). Results are shown as mean±s.e.m. Statistical significance was set at $P<0.05$.

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