



HHS Public Access

Author manuscript

Genes Brain Behav. Author manuscript; available in PMC 2017 January 01.

Published in final edited form as:

Genes Brain Behav. 2016 January ; 15(1): 108–124. doi:10.1111/gbb.12253.

Developmental regulation of fear learning and anxiety behavior by endocannabinoids

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Abstract

The developing brain undergoes substantial maturation into adulthood and the development of specific neural structures occurs on differing timelines. Transient imbalances between developmental trajectories of corticolimbic structures, which are known to contribute to regulation over fear learning and anxiety, can leave an individual susceptible to mental illness, particularly anxiety disorders. There is a substantial body of literature indicating that the endocannabinoid system critically regulates stress responsivity and emotional behavior throughout the life span, making this system a novel therapeutic target for stress- and anxiety-related disorders. During early life and adolescence, corticolimbic endocannabinoid signaling changes dynamically and coincides with different sensitive periods of fear learning, suggesting that endocannabinoid signaling underlies age-specific fear learning responses. Moreover, perturbations to these normative fluctuations in corticolimbic endocannabinoid signaling, such as stress or cannabinoid exposure, could serve as a neural substrate contributing to alterations to the normative developmental trajectory of neural structures governing emotional behavior and fear learning. In this review, we first introduce the components of the endocannabinoid system and discuss clinical and rodent models demonstrating endocannabinoid regulation of fear learning and anxiety in adulthood. Next, we highlight distinct fear learning and regulation profiles throughout development and discuss the ontogeny of the endocannabinoid system in the central nervous system, and models of pharmacological augmentation of endocannabinoid signaling during development in the context of fear learning and anxiety.

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The authors have no conflict of interest to declare.

Keywords

adolescence; neonatal; early life; juvenile; stress; anandamide; CB1 receptor; cannabinoid; development; emotional behaviour; 2-AG; fatty acid amide hydrolase; FAAH

In search of more effective therapeutic strategies for mood disorders, a substantial body of work has been devoted to understanding exaggerated or inappropriate fear responses within neurocircuitry implicated in stress and anxiety-related disorders in children and adolescents (e.g. Glenn *et al.*, 2012, Lau *et al.*, 2008, Swartz *et al.*, 2015). The shift towards identifying the relationship between development and anxiety and stress regulation is largely based on observations that there is a heightened incidence of mental illnesses, particularly for mood disorders, that emerges during the adolescent period. In fact, estimates indicate that one in five adolescents have a mental illness that will persist into adulthood (Paus *et al.*, 2008), and depression and anxiety disorders occur in as many as one in ten adolescents (Costello *et al.*, 2005, Kessler *et al.*, 2005). Anxiety disorders, specifically, are considered among the most prevalent psychopathologies affecting between 15-20% of youth (Kessler *et al.*, 2007, Kessler *et al.*, 2005, Merikangas *et al.*, 2010). Moreover, at least 75% of adults with a fear/anxiety-related disorder are reported to have met diagnostic criteria as children or adolescents (Kim-Cohen *et al.*, 2003). These statistics highlight the importance of characterizing physiological and neural mechanisms underlying anxiety and stress regulation throughout development (Pattwell *et al.*, 2013), and emphasize the need to enhance our understanding of how clinical treatments may be more or less effective based on age.

There is an extensive history of medicinal and recreational cannabis use in humans and in many cases, cannabis is utilized for its mood-enhancing, anxiolytic and stress-relieving properties (Cheung *et al.*, 2010, Green *et al.*, 2003, Hunault *et al.*, 2014, Temple *et al.*, 2014). This led to the subsequent identification of the major psychoactive constituents of cannabis known as Δ^9 -tetrahydrocannabinol (THC; Gaoni & Mechoulam, 1964) and the eventual characterization of the cannabinoid type 1 receptor (CB₁R) with which it interacts (Devane *et al.*, 1988). Although cannabis use has been reported to produce enhanced anxiety and paranoia in specific contexts and predisposed individuals (e.g. Moreira & Wotjak, 2010), studies indicate that THC and other natural and synthetic cannabinoids generally alleviate anxiety symptoms in recreational users (Green *et al.*, 2003, Hunault *et al.*, 2014, Temple *et al.*, 2014) and in patients with an anxiety disorder (Crippa *et al.*, 2011, Fabre & McLendon, 1981, Fraser, 2009, Jetly *et al.*, 2015, Nakano *et al.*, 1978, Roitman *et al.*, 2014). Similarly, the anxiolytic properties of THC and other cannabinoids in humans is consistent with the results from preclinical rodent studies (Gunduz-Cinar *et al.*, 2013a, Moreira & Wotjak, 2010).

Aside from being the biological target of THC from cannabis, the endocannabinoid (eCB) system has been widely studied for its ability to maintain homeostasis by exerting regulation over cognitive, behavioral, emotional, developmental and physiological processes (see review, Mechoulam & Parker, 2012). As natural and synthetic cannabinoids are reported to produce both anxiolytic, mood-enhancing effects as well as dysphoric, panic-like responses (Akirav, 2011), these biphasic effects of cannabinoids on anxiety suggest that the eCB

system is an important regulator of emotional homeostasis, in particular. Moreover, the eCB system is likely a major contributor to individual variation in anxiety levels, while dysregulated eCB signaling could serve as a risk for developing an anxiety disorder (Gunduz-Cinar *et al.*, 2013a). Moreover, there is preclinical evidence that eCB signaling in corticolimbic structures such as the prefrontal cortex, amygdala and hippocampus play a critical role in regulating adult stress responses by the hypothalamic-pituitary-adrenal (HPA) axis and emotional behavior such as anxiety (Campolongo *et al.*, 2011, Lee & Gorzalka, 2012, Morena *et al.*, in press, Rubino & Parolaro, 2008). Consequently, the eCB system has become a therapeutic target for a number of clinical conditions, including depression and anxiety-related disorders (Hill & Patel, 2013, Mangieri & Piomelli, 2007).

There is strong evidence indicating that the eCB system regulates both neurodevelopmental processes and adult conditioned and unconditioned anxiety behaviors: the eCB system modulates neuronal development and circuit connectivity (Harkany *et al.*, 2008, Maccarrone *et al.*, 2014), corticolimbic eCB signaling changes dynamically during development (Ellgren *et al.*, 2008, Heng *et al.*, 2011, Lee & Gorzalka, 2012, Rubino *et al.*, 2015, Wenger *et al.*, 2002) and deficiencies in central eCB ligand, N-arachidonylethanolamine (anandamide; AEA), are associated with greater anxiety behavior (Bluett *et al.*, 2014, Gray *et al.*, 2015). Moreover, there are preliminary indications reviewed here suggesting that corticolimbic eCB signaling is an important underlying mechanism mediating interactions between maturational stage (e.g., adolescence) and fear learning/anxiety behavior. Early life and adolescence are characterized by distinct maturational alterations in the eCB system that occurs on a similar timeline as sensitive periods in fear learning. Furthermore, studies employing cannabinoid agonists indicate that the eCB system regulates emotional behavior and HPA axis stress responses from early stages of development and also suggest that developmental cannabinoid exposure can contribute to long-term dysregulation of the brain and anxiety behavior, which is reminiscent of the results of extended glucocorticoid exposure (Campolongo *et al.*, 2007, D'asti *et al.*, 2010, Fride *et al.*, 2005). In this article, we first introduce the components of the eCB system and fear learning neurocircuitry, and then discuss clinical and rodent models demonstrating eCB regulation of fear learning and anxiety in adulthood. Next, we highlight distinct fear learning and regulation profiles throughout development and describe the ontogeny of the eCB system in the central nervous system. Lastly, we discuss findings from pharmacological studies of perinatal and periadolescent eCB signaling disruption and discuss their relevance in relation to our current understanding of anxiety and fear learning regulation by eCBs.

The endocannabinoid system

The eCB system includes two inhibitory G-protein coupled receptors, the CB₁ and CB₂ receptors. CB₁Rs are widely expressed in the brain on multiple neuronal populations such as GABA and glutamate, while CB₂ receptors are predominantly found in peripheral tissues. Although, CB₂ receptors have been detected in the central nervous system relatively recently (Onaivi *et al.*, 2006, Van Sickle *et al.*, 2005), the majority of these reports localize their presence in microglia rather than neurons (e.g. Cabral *et al.*, 2008). The eCB system also possesses two major endogenous ligands, N-arachidonylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) which are synthesized “on demand” and act as

retrograde messengers to regulate the release of other neurotransmitters (see reviews, Jutras-Aswad *et al.*, 2009, Katona & Freund, 2012) and contribute to both short- and long-term synaptic plasticity (Mackie, 2006). Both AEA and 2-AG are synthesized post-synaptically by activity-dependent cleavage of phospholipid head groups. The synthesis of AEA most commonly occurs through hydrolysis of N-arachidonoyl phosphatidylethanolamine (NAPE) by phospholipase D (NAPE-PLD), although it should be noted there are at least 3 other biosynthetic pathways by which AEA can be synthesized (Figure 1; Di Marzo, 2011, Liu *et al.*, 2008). There are two proposed pathways of 2-AG synthesis; however, the hydrolysis of inositol phospholipids containing arachidonic acid at the sn-2 position by phospholipase C into diacylglycerol, which is then further hydrolyzed to 2-AG by diacylglycerol lipase- α (DAGL α), is likely the most important (Figure 1; Hillard, 2000, Sugiura *et al.*, 2002, Ueda *et al.*, 2011). AEA is subject to rapid intracellular degradation primarily by hydrolytic enzyme, fatty acid amide hydrolase (FAAH; Di Marzo, 2011), resulting in arachidonic acid and ethanolamine (Ahn *et al.*, 2008). The pathways leading to breakdown of 2-AG are less clear, with at least 8 participating enzymes; however, 2-AG hydrolysis by monoacylglycerol lipase (MAGL) to arachidonic acid and glycerol is considered to be its major degradative pathway accounting for approximately 85% of 2-AG hydrolysis in the brain (Ueda *et al.*, 2011).

It is not entirely clear why there are two ligands for CB₁R; however, distinct biosynthetic and degradative pathways as well as pharmacokinetic differences between AEA and 2-AG facilitate differential patterns of signaling that could be contributing to the intricacies of regulation over a variety of complex processes with multiple targets, such as stress responsivity and emotionality (Hill & Tasker, 2012). AEA has a relatively high binding affinity for the CB₁R, yet induces somewhat poor intracellular signal transduction (i.e. partial agonist properties); in contrast, 2-AG has a relatively low binding affinity to the CB₁R, but produces a robust intracellular response (Hillard *et al.*, 1995). To add further complexity to this picture, AEA also binds to transient receptor potential vanilloid type (TRPV1) channels (Smart *et al.*, 2000, Zygmunt *et al.*, 1999), which is also known to modulate fear and anxiety responses (Moreira *et al.*, 2012, Moreira & Wotjak, 2010). Moreover, FAAH is found post-synaptically, whereas MAGL is mostly colocalized with CB₁R pre-synaptically (Haring *et al.*, 2012). It is currently believed that these differences in signaling properties implicates 2-AG as representing a phasic signal (stimulus-induced) in response to sustained depolarization and is involved in several forms of activity-dependent synaptic plasticity, particularly two models of retrograde neurotransmission known as depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE; Blankman & Cravatt, 2013). In contrast, AEA appears to represent a tonic signal that gates and maintains steady state conditions (Hill & Tasker, 2012). In support of this, blockade of neuronal firing in hippocampal slice preparations is found to facilitate AEA uptake and degradation, thereby reducing AEA tone, and resulting in a reduction of CB₁R-mediated suppression of GABA release (Kim & Alger, 2010). Moreover, *in vivo* studies of FAAH disruption suggest AEA pathways control select behavioral processes such as anxiety behavior (Bluett *et al.*, 2014); although, simultaneous increases in both AEA and 2-AG via inhibition of MAGL and FAAH are known to produce

some synergistic CB₁R-mediated effects, indicating cross-talk between the two pathways to regulate behavior (Blankman & Cravatt, 2013).

Neurocircuitry of fear learning and anxiety

Although the terms, “stress” and “anxiety” are often used together and interchangeably, they are distinct, yet related concepts. Stress is considered a state of strain elicited by a real or perceived threat to homeostatic functioning and initiates multiple mechanisms that facilitate adaptation and an organism's ability to deal with the threat at hand (e.g. McEwen, 2007). Fear or anxiety is often a component of this stress response, which includes anticipatory feelings of worry and unease to a stimulus or event (usually negative) that may or may not occur. Given the similarity between these concepts, it is not surprising that stress responsivity and anxiety are regulated by common corticolimbic structures including the amygdala, hippocampus and prefrontal cortex (PFC; Romeo & McEwen, 2006). Commonly used rodent tests of unconditioned anxiety include the elevated plus maze, open field, light/dark box, social interaction and novelty-induced hypophagia. These behavioral assessments are generally based on the rodent's innate conflict between fearfulness of being left vulnerable to prey and the exploratory drive to visit open, unprotected spaces or unfamiliar/familiar conspecifics (Lafenetre *et al.*, 2007).

In situations where fear responses are disproportionate to the risk and intensity of the real or perceived situation, this can lead to a maladaptive consequences such as developing an anxiety disorder (Akirav, 2011). Furthermore, a key maladaptive characteristic of anxiety disorders is the inability or difficulty in learning appropriate cues and contexts that signify safety and those that convey a threat (e.g. Akirav, 2011, Pine, 2007, Shin & Liberzon, 2010). Thus, experimental studies have also focused on understanding neural mechanisms underlying the human and rodent's ability to learn associations between previously experienced negative events and the cues and contexts that might predict their re-occurrence (i.e., fear learning) using a Pavlovian conditioning paradigm (Casey *et al.*, in press). This typically involves pairing a neutral cue (e.g. tone) with an innately aversive stimulus (unconditioned stimulus; US; e.g. a brief electric shock). Once an association between the neutral cue and aversive stimulus is established, the presentation of the neutral cue, now considered a conditioned stimulus (CS), produces similar physiological and behavioral responses (conditioned responses; CR) to the anticipated threat as presentation of the US itself. In rodents, freezing behavior is the most commonly assessed CR, while in humans, changes in skin conductance, startle responses and pupil dilation are measured. Despite the persistence of the learned fear memory, extinction learning can occur in which CRs (e.g. freezing behavior) to the CS (e.g. tone) can be inhibited by repeatedly presenting the CS in the absence of the US. In this modified paradigm, the organism learns the CS is now safe. One limitation of the extinction training paradigm is that the memory of the extinguished fear can re-emerge with the passage of time (i.e., spontaneous recovery; Bouton, 2004).

The amygdala is implicated in a variety of emotional functions including mood regulation, mediating fear and anxiety behavior, reward processing to reinforce or motivate behavior, and plays a fundamental role in fear learning acquisition, expression and extinction (Ledoux, 2007). The amygdala has been shown to be responsive to multiple types of fear-related

stimuli including pharmacological induction of fear, emotional stimuli, facial expressions and fear conditioning (Akirav, 2011). Furthermore, the amygdala can be divided into several nuclei that have divergent inputs and outputs and thus, functional contributions to fear learning and anxiety (see reviews, Ledoux, 2007, and Orsini & Maren, 2012). In particular, the basolateral complex of the amygdala (BLA) consists of the lateral nucleus, basolateral nucleus, and basomedial nucleus, which are collectively responsible for establishing a CS-US association (Orsini & Maren, 2012).

Specifically, the lateral nucleus of the amygdala receives inputs from sensory systems and in conjunction with the basolateral nucleus of the amygdala, serves to integrate relevant sensory information and relay it to the central nucleus of the amygdala. The central nucleus of the amygdala is a key output region for the expression of fear responses via downstream projections to hypothalamic and brain stem nuclei to produce autonomic responses (e.g. increased heart rate, freezing, stress response; Maren, 2001, Orsini & Maren, 2012).

The ventromedial PFC is important for an individual's ability to shift from fear expression to fear suppression (Milad & Quirk, 2002, Milad *et al.*, 2007, Santini *et al.*, 2004). Subregions within the ventromedial PFC are reported to differentially regulate the expression and extinction of conditioned fear responses; the prelimbic cortex is necessary for the expression of conditioned fear responses (Corcoran & Quirk, 2007) whereas the infralimbic region is likely involved in the suppression of these responses (Burgos-Robles *et al.*, 2009, Hefner *et al.*, 2008). It has been proposed that the infralimbic cortex is able to achieve this via projections to a cluster of GABAergic intercalated cells in the amygdala that suppress central nucleus output and thus, reduce the expression of the conditioned fear response (Likhtik *et al.*, 2008). However, more recent work indicates that the infralimbic cortex lacks direct functional innervation to intercalated neurons (Strobel *et al.*, 2015). Thus, it has been suggested that the infralimbic cortex guides amygdalar output by modulating BLA-related plasticity which could then recruit intercalated cells to suppress fear responses (see review, Maren & Holmes, 2015, Strobel *et al.*, 2015). This is consistent with findings of a recent optogenetic study indicating that the infralimbic cortex and its connections to the BLA are specifically necessary for facilitating the storage of extinction memory during extinction training, rather than the retrieval of the extinction memory (Do-Monte *et al.*, 2015). In line with this preclinical literature, patients diagnosed with post-traumatic stress disorder (PTSD) have reduced activity in the PFC coupled to high activity in the amygdala, when exposed to reminders of a traumatic event (Shin *et al.*, 2001). Taken together, it has been proposed that a deficiency in ventromedial PFC input to the amygdala is likely to result in a reduction of inhibitory tone within this structure, which could ultimately lead to the overexpression of fear responses and development of an anxiety disorder.

The hippocampus and amygdala are strongly interconnected, with projections from the ventral CA1 region to the basal nucleus of the amygdala and projections from the ventral subiculum to lateral nucleus, basal nucleus, basomedial nucleus, and central nucleus of the amygdala (Orsini & Maren, 2012). Reciprocal projections from the amygdala stem from the basal nucleus of the amygdala and terminate in the CA1, CA2, CA3 and ventral subiculum of the hippocampus (Orsini & Maren, 2012). The hippocampus plays a role in the acquisition and storage of contextual fear memory; however, the exact role of this neural

structure remains unclear (Orsini & Maren, 2012). From what is known, the high level of interconnectivity between the hippocampus and amygdala is important for fear extinction as the hippocampus provides information about the safety or threat of an environment based on contextual representations formed by previous experience (Fanselow & Dong, 2010, Orsini & Maren, 2012). Furthermore, the ventral subiculum and CA1 region have projections that terminate in the prelimbic and infralimbic cortex of the ventromedial PFC. Communication between the hippocampus and PFC is also known to be of high importance for fear extinction, which is supported by findings that a low frequency stimulation of the dorsal hippocampus impairs extinction recall and disrupts extinction-related long term potentiation (LTP) in the PFC whereas high frequency stimulation in the dorsal hippocampus facilitates extinction recall and LTP in the PFC (Farinelli *et al.*, 2006, Orsini & Maren, 2012).

Adult corticolimbic endocannabinoid signaling regulates adult unconditioned anxiety behavior

Cannabinoids are often reported to have both anxiolytic and anxiogenic properties (Campolongo *et al.*, 2011, Haring *et al.*, 2012, Hill & Gorzalka, 2009, Marco & Laviole, 2012). While cannabis use is generally associated with stress-relief and mood elevation, in some cases, it produces dysphoric responses that include panic or heightened anxiety (Hall & Solowij, 1998, Lafenetre *et al.*, 2007, Mechoulam & Parker, 2012, Micale *et al.*, 2013). These biphasic effects are consistent with rodent studies demonstrating dose-dependent effects of CB₁R agonists, such as THC, on anxiety behavior; moderate to high doses of CB₁R agonists enhance anxiety- and stress-responses, while low to moderate doses of CB₁R agonists exhibit anxiolytic effects in male and female rodents (Berrendero & Maldonado, 2002, Lutz, 2009, Patel & Hillard, 2006, Rey *et al.*, 2012, Rodríguez de Fonseca *et al.*, 1996). In contrast, pharmacological and genetic deletion of CB₁R in rodents are generally known to have anxiogenic effects (Blasio *et al.*, 2013, Patel & Hillard, 2006) and produce a behavioral and neural phenotype indicative of chronic stress exposure, including heightened emotional behavior (Haller *et al.*, 2002, Maccarrone *et al.*, 2002, Martin *et al.*, 2002, Urigüen *et al.*, 2004), hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Barna *et al.*, 2004, Cota *et al.*, 2007), and shorter, less complex pyramidal neurons in the medial PFC (Hill *et al.*, 2011, Lee *et al.*, 2014c). Correspondingly, CB₁R agonists directly infused into the hippocampus and medial PFC are known to exert anxiolytic effects (Lisboa *et al.*, 2015). However, there are known inconsistent results reported in the literature (e.g. no or opposing effects) which are at least partly due to differences between anxiety test used, strain, sex, drug, dose, age of testing and aversiveness of the environment (e.g. dim versus bright illumination in the testing room; Lafenetre *et al.*, 2007).

Adult corticolimbic endocannabinoid signaling regulates adult conditioned anxiety behavior

Pharmacological and genetic deletion of the CB₁R is generally found to have no effect on the acquisition of cued and contextual fear learning (Marsicano *et al.*, 2002, Suzuki *et al.*, 2004). Although, systemic injections of the CB₁R antagonist, AM-251, increases the acquisition and expression of freezing behavior in a trace (hippocampal-dependent) and

delay (amygdala-dependent) fear conditioning paradigm (Reich *et al.*, 2008). In contrast, low to moderate doses of CB₁R agonist, WIN55212-2, and FAAH inhibitor, URB597, injected directly into the medial PFC decreases conditioned fear as measured by cue-fear-potentiated startle reflexes (Lin *et al.*, 2008, Lin *et al.*, 2009). Local infusion of AEA or AEA transport inhibitor, AM-404, into the ventromedial PFC or dorsolateral column of the periaqueductal grey attenuated conditioned freezing behavior, which was blocked by pretreatment with a CB₁R antagonist (Lisboa *et al.*, 2010, Resstel *et al.*, 2008). Thus, the overall body of literature suggests that similar to unconditioned fear, moderate enhancement of eCB signaling reduces conditioned fear responses while inhibition of eCB signaling increases these fear responses (Akirav, 2011).

There is a wealth of evidence demonstrating that eCB signaling modulates extinction of conditioned fear. Inhibition of eCB signaling is found to attenuate or prolong fear extinction (Akirav, 2011, Chhatwal *et al.*, 2005, Lafenetre *et al.*, 2007, Lutz, 2007, Marsicano *et al.*, 2002, Pamplona *et al.*, 2006, Reich *et al.*, 2008, Simone *et al.*, 2015, Suzuki *et al.*, 2004), while an enhancement of eCB signaling is known to facilitate fear extinction (Abush & Akirav, 2010, Chhatwal *et al.*, 2005, Gunduz-Cinar *et al.*, 2013b, Pamplona *et al.*, 2008, Pamplona *et al.*, 2006, Rabinak *et al.*, 2013, Simone *et al.*, 2015, Suzuki *et al.*, 2004). Foundational work by Marsicano and colleagues (2002) demonstrated that CB₁R knockout mice exhibit no anxiety behavior differences during the acquisition and consolidation of conditioned fear, however CB₁R deficient mice display impaired fear extinction. Moreover, pharmacological blockade of CB₁R in rodents is reported to similarly impair fear extinction without affecting the acquisition or consolidation of the fear memory (Bitencourt *et al.*, 2008, Chhatwal *et al.*, 2005, Lafenetre *et al.*, 2007, Lin *et al.*, 2009, Lutz, 2007, Pamplona *et al.*, 2008). However, activation of the eCB system with CB₁R agonists, such as THC, during extinction learning is known to facilitate extinction recall by preventing the recovery of extinguished fear memories in rodents (Chhatwal *et al.*, 2005, Pamplona *et al.*, 2008, Pamplona *et al.*, 2006) and humans (Rabinak *et al.*, 2013).

CB₁R signaling in the BLA appears to serve as a neural locus for gating behavioral and neuroendocrine responses to stress and fear responses (Bluett *et al.*, 2014, Gray *et al.*, 2015, Gunduz-Cinar *et al.*, 2013a, Hill *et al.*, 2009a). In support of this, CB₁R antagonism is found to increase neuronal activation within the BLA (Newsom *et al.*, 2012, Patel *et al.*, 2005), while also increasing HPA axis activity and anxiety behavior (Dono & Currie, 2012, Ganon-Elazar & Akirav, 2009, Hill *et al.*, 2009a). In mouse fear conditioning studies, extinction training produces an increase in BLA AEA, but not 2-AG levels (Marsicano *et al.*, 2002), and local administration of a FAAH inhibitor directly into the BLA enhances fear extinction (Gunduz-Cinar *et al.*, 2013b). Consistent with this, local administration of a CB₁R agonist into the BLA prevents the effects of stress on fear extinction (Ganon-Elazar & Akirav, 2009, Ganon-Elazar & Akirav, 2012), acoustic startle response (Ganon-Elazar & Akirav, 2012), and activation of CB₁R in the BLA after fear memory reactivation blocks fear memory reconsolidation (Lin *et al.*, 2006). Collectively, these data indicate that activation of eCB signaling within the BLA can reduce neurobehavioral indices of stress, anxiety and fear.

The role of endocannabinoid signaling in human fear learning

Human studies are consistent with preclinical work implicating a regulatory role for the eCB system in modulating emotional processing and fear learning. Acute THC administration reduces amygdalar reactivity to social signs of threat, without affecting activity in primary visual cortex and motor cortex (Phan *et al.*, 2008) and impairs recognition of facial fear and anger, but not sadness or happiness (Ballard *et al.*, 2012). Indeed, presentation of negative stimuli (pictures of fearful faces) reduces activity in neural structures such as the amygdala, orbital frontal gyrus, hippocampus, parietal gyrus, PFC, and regions in the occipital cortex following acute THC exposure, whereas presentation of positive stimuli (pictures of happy faces) increases activity within that network (Bossong *et al.*, 2013). Furthermore, THC facilitates extinction of learned fear in humans and increases activation in the ventromedial PFC and hippocampus upon presentation of the CS relative to subjects that received a placebo (Rabinak *et al.*, 2014, Rabinak *et al.*, 2013). Cannabidiol (CBD), a non-psychoactive cannabinoid found in cannabis, has also been found to enhance consolidation of fear extinction (Das *et al.*, 2013). In a study of genetic variability in human eCB signaling, fear learning was assessed in individuals that were genotyped for two single nucleotide polymorphisms (SNP) within the promotor region of the human CB₁R gene (known as CNR1). Carriers of either the rs2180619 or rs1049353 SNP demonstrated comparable acquisition and expression of fear potentiation of the eyeblink startle reflex; however, only homozygote (A/A) A-allele carriers of rs2180619 exhibited an absence of fear extinction in contrast to robust extinction of the fear potentiated eyeblink startle response in homozygote (G/G) and heterozygote (A/G) G-allele carriers (Heitland *et al.*, 2012). Through interaction with the 5HTTLPR SNP in the serotonin transporter gene, the rs2180619 SNP is also associated with individual differences in trait anxiety (Lazary *et al.*, 2009), however, the molecular mechanisms affecting CB₁R functionality and expression by this specific SNP remain to be determined (Heitland *et al.*, 2012).

In addition to playing a role in emotional learning in humans, there is also evidence that aberrant eCB signaling could contribute to mood and anxiety disorders in humans. Specifically, the results of clinical studies indicate that compromised eCB signaling could serve as a molecular underpinning for neuropsychiatric conditions such as depression and anxiety disorders. Basal serum concentrations of AEA and 2-AG are lower in female patients with major depression (Hill *et al.*, 2009b), suggesting that hypoactive eCB signaling could be involved in the etiology of depression (Hill & Patel, 2013). Similarly, lower circulating eCB concentrations are reported in human PTSD patients concomitant with an upregulation of CB₁Rs (Hill *et al.*, 2013, Neumeister *et al.*, 2013). Consistent with these findings, clinical trials using CB₁R antagonists (e.g. Rimonabant (Sanofi-Avantis) and Taranabant (Merck)) for the treatment of metabolic and cardiac conditions also yielded an increase in the incidence of depressed mood, anxiety and risk of suicide (Christensen *et al.*, 2007, Lutz, 2009). Collectively, the emerging picture indicates that the eCB system functions to reduce stress and anxiety responses primarily through activation of the CB₁R.

Therapeutic potential of FAAH and MAGL inhibition on fear learning and anxiety

Emerging research indicates that genetic and pharmacological enhancement of eCB signaling by augmentation of central AEA and 2-AG levels could ameliorate some of the neural and behavioral sequelae (e.g. HPA axis dysregulation and increased anxiety behavior) associated with depression and anxiety disorders. Preclinical work indicates that pharmacological inhibition of FAAH (e.g. URB-597, PF-3845; Hill *et al.*, 2007, Kathuria *et al.*, 2003) and AEA transport (AM-404; Bortolato *et al.*, 2006) has anxiolytic properties in several tests of anxiety, including: the elevated zero maze, elevated plus maze, defensive withdrawal and social isolation-induced ultrasonic vocalization tests. Moreover, transgenic mice with a FAAH deficiency exhibit increased central AEA levels and a less anxious phenotype in the elevated plus maze and light dark box relative to wild type controls, which is prevented by systemic injection of a CB₁R antagonist, Rimonabant (Moreira *et al.*, 2008).

In light of the preclinical literature, researchers have undertaken investigations of genetic models of natural variation in circulating AEA levels and identified a common SNP (C385; rs324420) in the human FAAH gene that results in elevated AEA levels due to substitution of an evolutionarily conserved proline at amino-acid position 129 with a threonine residue that renders the FAAH protein more vulnerable to proteolytic degradation (Chiang *et al.*, 2004, Dincheva *et al.*, 2015, Sipe *et al.*, 2002, Sipe *et al.*, 2010). Adults carrying this variant exhibit reduced amygdalar responses to threat stimuli of fearful faces and trait anxiety levels (Hariri *et al.*, 2009). Extending these findings, carriers of this variant are also found to exhibit accelerated habituation of amygdalar activity to repeated presentation of threat images and lower scores on the personality trait of stress-reactivity (Gunduz-Cinar *et al.*, 2013b). A parallel mouse study using FAAH inhibitors further demonstrate that this habituation is associated with enhanced extinction-related plasticity in the amygdala (Gunduz-Cinar *et al.*, 2013b). Lastly, we recently developed a knock-in mouse that biologically recapitulates the C385A SNP of the human FAAH gene. Adult humans and mice carrying the allele variant exhibit paralleled reductions in FAAH expression, elevated central AEA (Boileau *et al.*, in press, Dincheva *et al.*, 2015), enhanced fronto-amygdala connectivity and fear extinction learning as well as reduced anxiety behavior (Dincheva *et al.*, 2015). Collectively, these pharmacological, genetic and behavioral findings provide preliminary evidence that FAAH inhibition could be an effective treatment option specifically for stress-related neuropsychiatric disorders (Bluett *et al.*, 2014, Gunduz-Cinar *et al.*, 2013a).

Acute pharmacological MAGL inhibition (resulting in elevated 2-AG levels) has also been reported to generally reduce rodent anxiety behavior in the elevated plus maze (Aliczki *et al.*, 2012, Aliczki *et al.*, 2013, Almeida-Santos *et al.*, 2013, Busquets-Garcia *et al.*, 2011, Sciolino *et al.*, 2011), elevated zero maze (Busquets-Garcia *et al.*, 2011) and marble burying tasks (Kinsey *et al.*, 2011). Consistent with these findings, genetic knockout studies indicate that deficient 2-AG levels result in enhanced anxiety behavior. In one model, DAGL α knock out mice were generated in which, central, but not circulating 2-AG levels are reduced and associated with impaired eCB-mediated retrograde synaptic signaling at excitatory synapses

in the BLA as well as greater anxiety behavior and anhedonia than wild-type mice (Shonesy *et al.*, 2014). Interestingly, this behavioral and neural phenotype is reversed by pharmacological normalization of 2-AG levels by MAGL inhibitor, JZL-184 (Shonesy *et al.*, 2014). In similar fashion, another DAGL α knock out mouse model indicates mice had an 80% reduction of central 2-AG levels and reductions in cortical and amygdalar AEA that corresponded with a behavioral and neural phenotype reminiscent of pharmacological and genetic deletion of CB $_1$ Rs: enhanced anxiety behavior in the open field and light/dark box, impaired fear extinction, maternal neglect behavior, reduced hippocampal neurogenesis, and reduced stress coping behavior in the forced swim test (Jenniches *et al.*, in press). Adeno-associated virus-mediated overexpression of MAGL in hippocampal glutamatergic neurons also results in a 50% reduction in 2-AG levels, impairs DSE (but not DSI) and enhanced anxiety behavior in the elevated plus maze and open field tests (Guggenhuber *et al.*, in press). In contrast, genetic deletion of MAGL (resulting in elevated 2-AG levels) was found to enhance anxiety behavior in the marble burying test, accompanied with impaired CB $_1$ R signaling and enhanced excitatory drive in mPFC/BLA circuitry (Imperatore *et al.*, in press).

In summary, the evidence indicates that elevated levels of AEA and 2-AG by FAAH and MAGL generally have an anxiolytic effect while reductions in these eCB ligands are associated with enhanced anxiety. Further research determining the exact role of the endogenous ligands of the eCB system in fear learning and anxiety behavior is clearly warranted.

Fear learning and regulation across development

The neurocircuitry underlying fear learning and extinction in adulthood has been relatively well characterized. However, far less is known about the developmental processes shaping maturation of this neurocircuitry and how it may modulate fear learning and extinction by age (Casey *et al.*, in press). Indeed, prefrontal and sub-cortical circuitry that is implicated in the regulation of fear learning and extinction is known to undergo substantial change from childhood to adulthood (Gogtay *et al.*, 2004).

Rodent studies indicate that fear learning is linked to the maturation of the amygdala, which begins early in life and is tightly linked to maternal presence (Figure 2; Landers & Sullivan, 2012). Early postnatal life is considered a sensitive period for attachment learning in which rodent pups generally exhibit a profile characterized by suppressed fear responding, presumably to promote infant-caregiver attachment, even in the face of diminished quality of care (Landers & Sullivan, 2012, Raineke *et al.*, 2010, Roth & Sullivan, 2005). During this period, odor-shock conditioning produces a paradoxical odor preference despite being paired with a shock that produces a pain response (Landers & Sullivan, 2012, Moriceau & Sullivan, 2006, Rudy & Cheatele, 1977). Post natal day (PND) 10 marks the end of this sensitive period in that amygdalar GABAergic-related synaptic plasticity begins to emerge (Thompson *et al.*, 2008) and rodent pups can demonstrate adult-like cued fear learning to odor-shock conditioning (Moriceau & Sullivan, 2006); however, maternal presence, which maintains relatively low levels of circulating corticosterone in the pup (Sullivan & Holman, 2011), can provide social buffering and modulate the pup's learning to reinstate odor preference instead of aversion (Landers & Sullivan, 2012). Although able to learn fear

associations, infant rodents (Figure 2) demonstrate a functional “infantile amnesia” in which fear memories are not as persistent as in adulthood (Akers *et al.*, 2014, Campbell & Spear, 1972, Kim & Richardson, 2007). In support of this, animals conditioned on PND 18 appear to forget the fear memory within 10 days (Kim & Richardson, 2007). This may be related to the infant's lack of ability to associate environmental cues with the conditioned fear memory. In contrast to cued fear learning, contextual fear conditioning is thought to emerge by PND 24, coinciding with maturation of amygdala-hippocampus connectivity (Rainekei *et al.*, 2010) and developmental-related increases in hippocampal neurogenesis that have been shown to interfere with infant contextual fear memory persistence (Akers *et al.*, 2014).

However, by PND 23, a new sensitive period for extinction learning emerges (Pattwell *et al.*, 2013). As juveniles, fear memories are persistently attenuated following extinction training, an effect not observed in adult rodents (Pattwell *et al.*, 2012). That is, unlike adults, juvenile rodents do not display spontaneous recovery of the fear memory that typically follows extinction training (Kim & Richardson, 2007, Pattwell *et al.*, 2012, Yap & Richardson, 2007). Consistent with this behavioral pattern, extinction training in the juvenile rodent is found to be amygdala-dependent and does not require infralimbic cortical engagement at PND 18 (Kim *et al.*, 2009, Kim & Richardson, 2008). In contrast, we have previously found that by PND 23, the juvenile rodent exhibits persistent attenuation of fear memory; however, the fear memory appears more susceptible to interference by paralleled potentiation of the infralimbic cortex during extinction training, rather than degrading on its own (Pattwell *et al.*, 2012).

In non-linear fashion, both fear extinction learning and retention are attenuated during adolescence relative to younger and older mice and humans (Casey *et al.*, in press, Kim *et al.*, 2011, McCallum *et al.*, 2010, Pattwell *et al.*, 2012, Pattwell *et al.*, 2013). Adolescent mice display blunted fear extinction learning that is paralleled by an absence of infralimbic extinction learning-induced plasticity (Kim *et al.*, 2011, Pattwell *et al.*, 2012). Reminiscent of risky behavior in adolescent humans, a second behavioral pattern that emerges in the adolescent rodent is diminished hippocampal-dependent contextual fear expression (Pattwell *et al.*, 2011) or “fearlessness” in contexts that have been previously paired with negative consequences. Unlike juveniles and adults, adolescent mice do not display a fear response when returned to the context in which they experienced the aversive event (shock). In this case, the suppression of contextual fear is a result of contextual fear retrieval failure, rather than acquisition, given that the same animals that exhibited diminished contextual fear as adolescents, demonstrate a fear response to the context when tested as young adults (Pattwell *et al.*, 2011).

The adolescent period is characterized by increased exploration and a transition to independence, making specific danger cues particularly relevant in this novelty-seeking period. Thus, diminished fear extinction and contextual fear during this period does hold some benefits in this context. Furthermore, the differential rates of development in adolescent subcortical-prefrontal connections (e.g. myelination, synaptic pruning) are also likely to contribute to these shifts in fear regulation (Somerville & Casey, 2010). For example, in a human fMRI study examining developmental changes in connectivity between the medial PFC and the amygdala, blood oxygen level-dependent (BOLD) activity within

the ventromedial PFC and the amygdala shifts from a positive to negative correlation from childhood to adolescence and is then stabilized during adulthood (Gee *et al.*, 2013). Thus, it is possible that changes in connectivity between the amygdala, hippocampus and ventromedial PFC during the adolescent period could mediate a shift from restricted subcortical circuitry governing fear learning in juvenile stages, towards a more flexible and expansive circuit for fear regulation that is more evident in adulthood.

It is clear there are multiple sensitive periods for fear learning and its underlying neurocircuitry during normative development from early life to adulthood. Furthermore, the developmental course of fear learning and its associated neural correlates occurs in a highly ordered pattern. While these sensitive periods appear to facilitate greater flexibility in some situations, they may also confer greater susceptibility to external events (e.g. stress exposure) that could initiate a cascade of negative consequences, possibly leading to risk for mental illness. Much of our understanding regarding fear learning and regulation during development is based on descriptive work, leaving the exact molecular mechanisms of action and conditions by which risk for mental illness is conferred largely unexplored.

Dynamic changes in corticolimbic endocannabinoid signaling during different developmental periods

Numerous human and rodent studies indicate that the eCB system is significantly involved in the regulation of adult fear learning and unconditioned anxiety behavior (Riebe *et al.*, 2012). Together with *in vitro* and *in vivo* studies that demonstrate regulation of neurodevelopmental processes by eCBs, it is reasonable to suggest that corticolimbic eCB signaling is involved in the developmental course of fear learning and anxiety behavior described above. Previous work has established that eCB signaling plays a multifaceted role in structural and functional neurodevelopment (see review Harkany *et al.*, 2008, Maccarrone *et al.*, 2014), regulating proliferation of neural progenitors and cell lineage commitment (Mulder *et al.*, 2008), immature neuronal migration and axonal path finding (Berghuis *et al.*, 2005, Berghuis *et al.*, 2007, Harkany *et al.*, 2008, Mulder *et al.*, 2008), as well as initiation of synaptic communication of neural networks in perinatal rat tissue (Berghuis *et al.*, 2007, Bernard *et al.*, 2005). Moreover, studies investigating the ontogeny of eCB signaling indicate that this system changes dynamically and in a temporal-specific fashion throughout development, particularly in adolescence (Figure 3). Distinct patterns of eCB activity during development coincide with distinct fear learning sensitive periods, suggesting that corticolimbic eCB signaling could serve as an underlying mechanism mediating age-specific sensitive periods in fear learning and regulation outlined above. The presence of the eCB system has been detected during early embryonic stages in chicken and mice (Psychoyos *et al.*, 2012). Furthermore, the eCB system is found to be functional in the rat central nervous system as early as gestational days 11-14 (Berrendero *et al.*, 1999, Harkany *et al.*, 2008, Rodriguez de Fonseca *et al.*, 1993). Rodent central 2-AG levels peak around birth then decrease to PND 5 concentrations, which are comparable to those found in adults (Berrendero *et al.*, 1999, Fernandez-Ruiz *et al.*, 2000). Conversely, AEA is reported to steadily increase from early life to adulthood (Berrendero *et al.*, 1999, Fernandez-Ruiz *et al.*, 2000). However, no late neonatal, juvenile, or adolescent time points were included in these

studies (i.e., no sampling between PND 5 – 56), leaving an existing gap in our understanding of the ontogeny of central AEA and 2-AG during early life to juvenile ages and highlighting the need for a comprehensive determination of the trajectory of AEA and 2-AG signaling throughout this period.

Accumulating evidence suggests there is considerable variation in the developmental course of adolescent corticolimbic AEA and 2-AG levels within neural structures regulating anxiety and fear learning. A comparison of cortical and hippocampal AEA and 2-AG levels in wild type and CB₁R knockout male mice at PND 28 and 120 revealed no significant differences between wild type groups; however, elevated hippocampal FAAH activity and reduced AEA levels were observed only in adult knockout mice (Maccarrone *et al.*, 2002). Behaviorally, young (PND 28) CB₁R knockout mice exhibited moderately elevated anxiety behavior relative to older knockout mice, yet no age-specific differences in anxiety behavior in the open field or light-dark box were detected in the wild type mice (Maccarrone *et al.*, 2002). In the male rat PFC, AEA was found to exhibit a gradual and progressive increase (i.e., PND 29-50) to adult levels while 2-AG levels were highest in early adolescence (PND 29), decreasing in mid-adolescence (PND 38) before increasing again in late adolescence (PND 50; Ellgren *et al.*, 2008). Conversely, in the female rat PFC, AEA concentration increases from PND 46-60, but decreases from PND 60-75, with no differences in 2-AG concentrations as well as FAAH and MAGL activity (Rubino *et al.*, 2015). Furthermore, hypothalamic AEA content is observed to increase immediately preceding vaginal opening (as a physical marker of pubertal onset; Wenger *et al.*, 2002). We have also previously reported that corticolimbic AEA concentrations consistently fluctuate in the same pattern across corticolimbic structures: amygdala, PFC, hippocampus and hypothalamus, throughout the male rodent adolescent period (PND 25-70; Lee *et al.*, 2013). These fluctuations in AEA were found to be at least partially due to corresponding changes in FAAH activity (Lee *et al.*, 2013).

CB₁Rs are detected and functional in human fetal tissue as early as gestational week 9 (Zurolo *et al.*, 2010) and gestational days 11-14 in rats (Berrendero *et al.*, 1999, Fernandez-Ruiz *et al.*, 2000), with high CB₁R expression limited to the amygdala and hippocampus and low distributions in striatum, thalamus and cerebral cortex during early to mid-gestation in the human fetus (gestational weeks 17-22) and rat neonate (Rodriguez de Fonseca *et al.*, 1993). In contrast, higher and wider density of these ubiquitous receptors is reported in the adult rat (Herkenham *et al.*, 1991) and human brain (Jutras-Aswad *et al.*, 2009). Furthermore, from gestation into the first few days after birth, CB₁R expression is localized to white matter areas of the midbrain, commissural tracts and brainstem; however, by adulthood, this phenomenon is no longer observable and CB₁Rs are primarily expressed within grey matter (Berrendero *et al.*, 1998, Berrendero *et al.*, 1999, Fernandez-Ruiz *et al.*, 2000, Romero *et al.*, 1997). By PND 10, limbic, striatal and midbrain structures are reported to exhibit low concentrations of CB₁R expression (Rodriguez de Fonseca *et al.*, 1993) and the ability of eCBs to regulate synaptic transmission begins to emerge and increases throughout development to adulthood (Liang *et al.*, 2014, Zhu & Lovinger, 2010).

The onset of adolescence at PND 30-40 marks a peak in limbic, striatal and midbrain CB₁R expression, which then decreases to reach adult levels of expression by PND 70 in male and

female rats (Rodriguez de Fonseca *et al.*, 1993). In contrast, CB₁R binding from birth to PND 60 is also reported to increase gradually to adult levels in male and female whole rat brains, although there are no adolescent time points included in this study (Belue *et al.*, 1995). Nonetheless, CB₁R expression in the rat is most consistently reported to be highest with the onset of adolescence (PND 25-30), followed by a general linear decline to adult levels within the PFC (Ellgren *et al.*, 2008, Heng *et al.*, 2011), limbic, striatal and cortical structures (Rodriguez de Fonseca *et al.*, 1993). Furthermore, more recent work has demonstrated differential rates at which these CB₁R declines occur, with declines in limbic/associative regions happening gradually and throughout adolescence whereas major changes in sensorimotor regions are not exhibited until mid- to late- adolescence (Heng *et al.*, 2011). Functionality of these receptors, as measured by DSE, also follows the same developmental pattern (Heng *et al.*, 2011). In summary, despite somewhat inconsistent findings that are at least partially due to strain and sex differences between studies, the rodent literature indicates that corticolimbic eCB ligand concentrations, particularly with respect to AEA, fluctuate throughout adolescence while CB₁R expression peaks at the onset of adolescence before declining to adult levels (Ellgren *et al.*, 2008, Lee & Gorzalka, in press, Lee *et al.*, 2013, Rubino *et al.*, 2015, Wenger *et al.*, 2002).

In humans, CB₁R expression is reported to gradually increase to adult levels in post-mortem brain tissue (Mato *et al.*, 2003). However, a more recent study reports that CB₁R expression peaks during infancy (<1 year old) to toddler-age (1.5 – 4.5 years old), then gradually decreases to adult levels in human PFC tissue samples (Choi *et al.*, 2012, Long *et al.*, 2012). Moreover, expression of enzymes that contribute to AEA synthesis (i.e., NAPE-PLD) and degradation (i.e., FAAH) both increase from infancy to adulthood, suggesting greater AEA regulation in adulthood than earlier in life (Long *et al.*, 2012). In terms of 2-AG, synthetic enzyme, DAGL α , peaks between school age and young adulthood, while MAGL follows a similar pattern as CB₁R expression (i.e., expression peaks around toddler age; Long *et al.*, 2012). However, there is a critical gap in our current understanding of eCB signaling development in humans; to date, there is no information regarding the ontogeny of AEA or 2-AG concentration in humans, even in the periphery. Interestingly, although a similar general pattern of CB₁R expression occurs in humans and rodents, the maturational trajectories of these receptors exhibit a peak around toddler-age in humans whereas this peak occurs closer to pubertal onset/early adolescence in the rodent. While the functional consequences of these developmental differences in eCB signaling require further investigation, it is possible that the dynamic changes in eCB signaling reflect a general structural and functional instability compared to that of adults. For example, adolescent AEA fluctuations and declining CB₁R expression could underlie adolescent behavioral profiles that are regulated by corticolimbic circuits, such as relatively low anxiety, high responsivity to rewards and reduced inhibitory control (Casey & Jones, 2010). Given that this behavioral profile is maximal during adolescence and coincides with changes in AEA/CB₁R expression and activity, corticolimbic eCB signaling may represent a neural substrate of the adolescent phenotype.

Long-term consequences of developmental exposure to cannabinoids

To date, there are no studies investigating the role of eCB signaling on fear learning during development. Despite this fundamental gap in our understanding of eCB regulation over fear learning during development, there are several studies that have investigated the impact of developmental cannabinoid exposure on unconditioned anxiety behavior. The majority of these studies indicate that cannabinoid exposure during development, whether by maternal use or during adolescence, can contribute to long-term dysregulation of the brain, the HPA axis and emotionality, which is reminiscent of the results of extended glucocorticoid exposure (Lee & Gorzalka, in press, Lee & Gorzalka, 2012). Early life and adolescence also appear to be periods of susceptibility to the effects of chronic cannabinoid exposure as adult rats exposed to the same treatment do not appear to be as greatly affected as adolescents (e.g. Bambico *et al.*, 2010, Zamberletti *et al.*, 2014).

There is clear evidence that the eCB system is involved in the regulation of emotional behavior from early developmental stages (Trezza *et al.*, 2008) and perinatal cannabinoid exposure is found to induce a variety of long-term neural, cognitive and emotional alterations in adulthood (see review, Higuera-Matas *et al.*, 2015). This is demonstrated in a longitudinal rodent study that found perinatal THC exposure increases ultrasonic vocalizations following removal from the nest during early life, reduces social interactions and play during adolescence, and increases anxiety-like behavior in the elevated plus maze with no impairment to general locomotor activity in adulthood (Trezza *et al.*, 2008, Trezza & Vanderschuren, 2008). This treatment regimen was also found to impair inhibitory avoidance task (as a measure of long-term aversive memory) performance in adulthood, paralleled with reductions in extracellular norepinephrine and glutamate concentrations in the adult male rat PFC (Campolongo *et al.*, 2007). Similarly, perinatal THC and synthetic cannabinoid agonist, CP-55,940, treatment increases adult anxiety behavior in the open field (Newsom & Kelly, 2008), social interaction and emergence tests (O'Shea *et al.*, 2006). However, perinatal exposure to FAAH inhibitor, URB-597, moderately reduces stress-coping behavior in the forced swim test without affecting anxiety behavior in the elevated plus maze in adulthood (Wu *et al.*, 2014).

Adolescent CB₁R agonist exposure generally produces enduring increases in anxiety behavior in a number of rodent behavioral tests (Rubino & Parolaro, 2008, Schneider *et al.*, 2005) and results in a multitude of other long-term, sex-dependent neural and behavioral consequences associated with stress-induced HPA axis dysregulation (Rubino *et al.*, 2009a, Rubino *et al.*, 2009b, Rubino *et al.*, 2008). On a behavioral level, adolescent THC exposure reduces stress coping behavior in the forced swim test in adult female rats, impairs spatial working memory in adult males, and leads to anhedonia in both adult males and females as measured by the sucrose preference test (Rubino *et al.*, 2008). Neural consequences of adolescent cannabinoid exposure include differential reductions in amygdalar and hippocampal CB₁R binding and signaling capacity in adult males and females (Rubino *et al.*, 2008), decreases in markers of neuroplasticity such as synaptophysin and PSD 95 in the PFC of females (Rubino *et al.*, 2009a), dendritic atrophy and reduced number of spines in hippocampal neurons in males (Rubino *et al.*, 2009b), and reductions in cell proliferation (Realini *et al.*, 2011) and survival (Lee *et al.*, 2014b) in the dentate gyrus of the

hippocampus in adult female and male rats. Chronic administration of the FAAH inhibitor URB-597 in adulthood reverses effects induced by adolescent THC administration such as reductions in stress-coping and increases in anxiety behaviors in female rats (Realini *et al.*, 2011) and decreases hippocampal CB₁R expression in male rats (Marco *et al.*, 2009). These findings suggest that a persistent deficit in AEA signaling in adulthood from adolescent cannabinoid exposure may mediate many of the sustained detrimental effects of this treatment. Adolescent exposure to CB₁R agonist, WIN55,212-2, produces similar results as adolescent THC exposure (reduced stress coping and increased anhedonia) as well as increased anxiety responses in the novelty-suppressed feeding test coupled to attenuated serotonergic and heightened noradrenergic activity (Bambico *et al.*, 2010). Consistent with these studies, sustained CB₁R blockade by CB₁R antagonist/inverse agonist, AM-251, treatment during adolescence increases stress-coping behavior in the forced swim test and moderately increases risk assessment behavior in the elevated plus maze in adult male rats (Lee *et al.*, in press).

Recent work has demonstrated that the detrimental impact of adolescent THC treatment is due, at least in part, to disruption of normative adolescent eCB signaling that regulates PFC maturation (Rubino *et al.*, 2015). Moreover, adolescent THC exposure results in impaired eCB-mediated signaling and plasticity in glutamatergic synapses in the PFC of adult female mice, which is reversible with MAGL inhibitor, JZL184 (Lovelace *et al.*, in press). In line with this, administration of CB₁R agonist, CP 55,940, during PND 28-43 increases CB₁R activity in the PFC of adult male but not female rats (Mateos *et al.*, 2011). Moreover, multiple studies (Rubino *et al.*, 2015, Rubino *et al.*, 2009a, Rubino *et al.*, 2009b, Rubino *et al.*, 2008, Zamberletti *et al.*, 2014) have demonstrated that chronic adolescent THC exposure results in long term modulation of corticolimbic CB₁R density and binding as well as other long term neural and behavioral changes similar to those produced by chronic stress (via glucocorticoid hypersecretion; e.g. McEwen, 2005) and prolonged glucocorticoid exposure (Hill *et al.*, 2008). Together, these studies suggest that eCB signaling during adolescence impacts the normative development of circuits that regulate stress and emotionality in adulthood. As such, perturbations of the eCB system during adolescence, due to either stress or exogenous cannabinoid exposure, may have long-lasting effects on emotional stability and flexibility in adulthood. However, there are some contrasting reports that chronic adolescent exposure to the CB₁R agonist, CP 55,940, either decreases (Wegener & Koch, 2009) or has no effect on anxiety behavior as measured in the open field test, elevated plus maze (Biscaia *et al.*, 2003) and social interaction test (O'Shea *et al.*, 2006). Similar to the adult literature, the results of these developmental cannabinoid exposure studies likely vary based on sex, strain/species, drug, dose and age of exposure (Rubino & Parolaro, 2008, Schneider, 2008).

Concluding Remarks

eCB signaling has been established to be involved in the regulation of unconditioned and conditioned anxiety behavior in adulthood, making it a current therapeutic target for the treatment of stress- and anxiety-related disorders. However, with the emergence of mental illness peaking in adolescence (Paus *et al.*, 2008), it is critical that we gain a more comprehensive understanding of normative developmental processes that confer both

plasticity and vulnerability to pathogenic experiences. Previous work has established there are distinct sensitive periods for fear learning during normative development. At the same time, there are multiple temporal-specific alterations in corticolimbic eCB signaling (Figure 3) that may facilitate the fear responses characterizing each of these sensitive periods. During the infantile sensitive period (suppressed fear responding), 2-AG levels are high while AEA and CB₁R gradually increase (Berrendero *et al.*, 1999, Fernandez-Ruiz *et al.*, 2000). Moreover, during the juvenile sensitive period for fear extinction (absence of spontaneous recovery or reinstatement of fear memory), AEA (Lee *et al.*, 2013, Wenger *et al.*, 2002) and CB₁R expression (Heng *et al.*, 2011, Rodriguez de Fonseca *et al.*, 1993) peak between the end of the juvenile period and onset of adolescence. Lastly, the adolescent sensitive period for fear extinction and contextual fear conditioning (blunted fear extinction and contextual fear memory) coincides with fluctuating corticolimbic AEA levels (Ellgren *et al.*, 2008, Lee *et al.*, 2013, Rubino *et al.*, 2015) and declining CB₁R expression (Rodriguez de Fonseca *et al.*, 1993). Research has also revealed that developmental exposure to cannabinoid agonists is associated with a multitude of long-term behavioral and neural consequences on unconditioned anxiety behavior (Campolongo *et al.*, 2007, Rubino *et al.*, 2015). Given that each sensitive period of fear learning also coincides with a distinct eCB signaling profile, it is tempting to speculate that the eCB system is a neural correlate for fear learning throughout the life span and accordingly, can facilitate the plasticity associated with each sensitive period as well as serve as a neural substrate by which external factors can lead to the development of an anxiety disorder. However, there is a significant gap in the literature investigating the role of normative eCB signaling in the corticolimbic circuit and regulation of fear learning during different developmental ages. Future research determining the exact role of eCB signaling in fear learning during development will contribute to our understanding of vulnerability to mental illness and may inform which treatments are more effective based on age.

Acknowledgments

The authors wish to thank Caitlin Riebe for her technical assistance generating figures for this article. This work was supported in part by the Canadian Institutes of Health Research (TTYL, MNH), Natural Sciences and Engineering Research Council of Canada (MNH) and the National Institute of Mental Health Grant P50 MH079513 (FSL).

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Major pathways of endocannabinoid synthesis and degradation

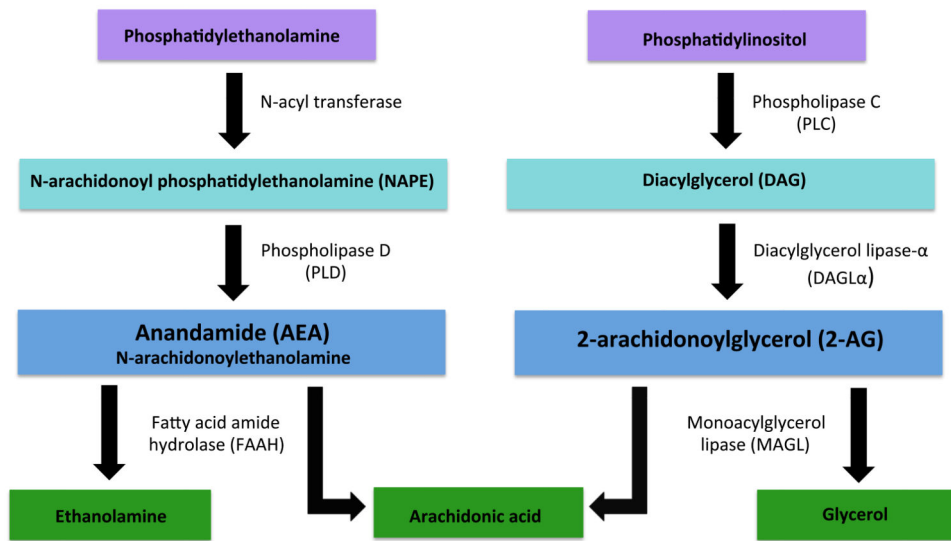


Figure 1. Major pathways of anandamide and 2-arachidonoylglycerol (2-AG) synthesis and degradation

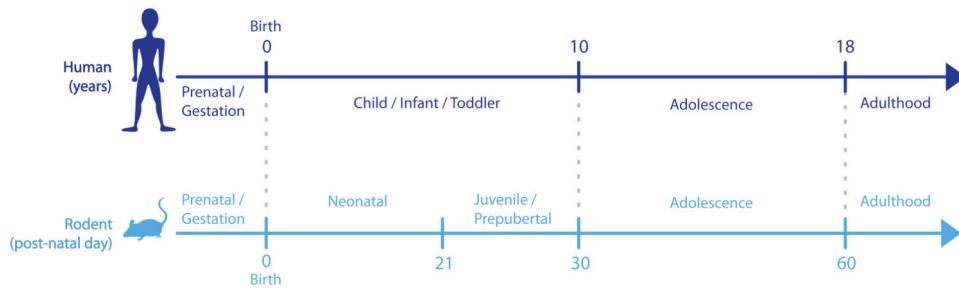


Figure 2. Human and rodent development timelines with commonly used terms for referring to those stages of development (adapted from Lee & Gorzalka, 2012)

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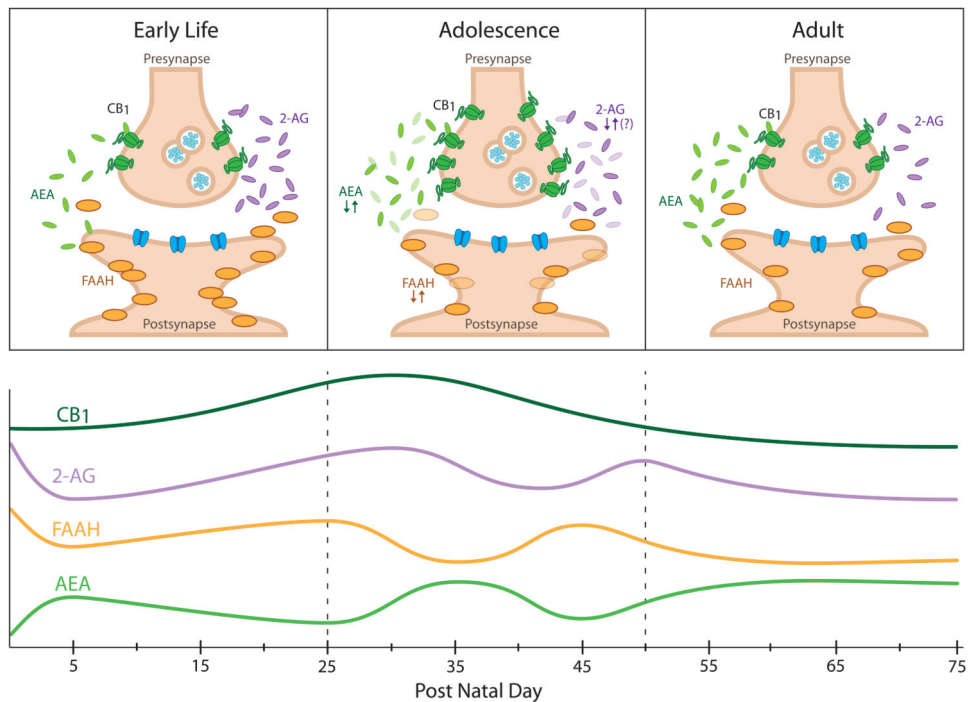


Figure 3. Corticolimbic endocannabinoid signaling changes dynamically across rodent development

(A) Distinct endocannabinoid signaling profiles in early life, adolescence and adulthood. Components of the endocannabinoid system are represented schematically within a synapse (adapted from Long *et al.*, 2012). (B) Developmental trajectories of the components of the endocannabinoid system. CB₁ receptor (CB₁) expression peaks with the onset of adolescence. 2-arachidonoylglycerol (2-AG) is highest around birth and may fluctuate throughout adolescence. N-arachidonylethanolamine (anandamide; AEA) gradually increases during early life and fluctuates during adolescence. Fatty acid amide hydrolase (FAAH) activity fluctuates in reciprocal fashion to AEA during adolescence. Based on data from (Berrendero *et al.*, 1999, Ellgren *et al.*, 2008, Fernandez-Ruiz *et al.*, 2000, Heng *et al.*, 2011, Lee *et al.*, 2013, Rodriguez de Fonseca *et al.*, 1993, Rubino *et al.*, 2015, Wenger *et al.*, 2002).