Acta Neuropsychiatrica

cambridge.org/neu

Interplay between endocannabinoid and endovanilloid mechanisms in fear conditioning

Rayssa C. Briânis, Julia P. Andreotti, Fabrício A. Moreira and Lia P. Iglesias

Department of Pharmacology, Institute of Biological Sciences; Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Review Article

Cite this article: Briânis RC, Andreotti JP, Moreira FA, and Iglesias LP. (2023) Interplay between endocannabinoid and endovanilloid mechanisms in fear conditioning. *Acta Neuropsychiatrica* 1–10. doi: 10.1017/neu.2023.54

Received: 25 April 2023
Revised: 10 November 2023
Accepted: 11 November 2023

Keywords:
Cannabinoids; anandamide; vanilloids; TRPV1; fear

Corresponding author:
L. P. Iglesias;
Email: liaparadaiglesias@gmail.com

Abstract

Objective: The transient receptor potential cation channel, subfamily V (vanilloid), member 1 (TRPV1) mediates pain perception to thermal and chemical stimuli in peripheral neurons. The cannabinoid receptor type 1 (CB_1), on the other hand, promotes analgesia in both the periphery and the brain. TRPV1 and CB_1 have also been implicated in learned fear, which involves the association of a previously neutral stimulus with an aversive event. In this review, we elaborate on the interplay between CB_1 receptors and TRPV1 channels in learned fear processing. *Methods:* We conducted a PubMed search for a narrative review on endocannabinoid and endovanilloid mechanisms on fear conditioning. *Results:* TRPV1 and CB_1 receptors are activated by a common endogenous agonist, arachidonoyl ethanolamide (anandamide), Moreover, they are expressed in common neuroanatomical structures and recruit converging cellular pathways, acting in concert to modulate fear learning. However, evidence suggests that TRPV1 exerts a facilitatory role, whereas CB_1 restrains fear responses. *Conclusion:* TRPV1 and CB_1 seem to mediate protective and aversive roles of anandamide, respectively. However, more research is needed to achieve a better understanding of how these receptors interact to modulate fear learning.

Summations

- The tripartite system, anandamide, TRPV1, and CB1, may be an important player in regulating fear responses.
- Anandamide either facilitates or restrains fear, by acting upon TRPV1 or CB1, respectively.
- The intensity of aversive stimulus and doses of cannabinoids or vanilloids ligands are major determinants in detecting anti-aversive effects.

Considerations

- This is a narrative review of the interactions among anandamide, TRPV1, and CB1 in the modulation of fear learning. We carefully selected studies contributing to the field.
- We recognize that some relevant work might not have been cited in this review.
- Here we suggest potential mechanisms and pathways involved in the regulation of fear by anandamide, TRPV1 and CB_1.

Introduction

Fear can be defined as a coordinated reaction, involving autonomic, behavioural and cognitive changes in response to innate or learned threatening stimuli (Fanselow and Pennington, 2018). Fear learning enables an individual to assign motivational content to cues previously paired with threatening stimuli, allowing the prediction of danger and the elaboration of an adaptive response (Krause & Domjan, 2017). Thus, conditioned fear is crucial for survival and well-being (Krause & Domjan, 2017). However, beyond certain limits, a mechanism sustaining health may become maladaptive and predispose to psychiatric disorders (Milton, 2019). Indeed, alterations in conditioned fear processing have been related to anxiety, depression and post-traumatic stress disorders (Foa *et al.*, 1989; Luyten *et al.*, 2011; Conoscenti and Fanselow, 2019; Bienvenu *et al.*, 2021).

In experimental animals, learned fear can be studied using protocols in which a neutral stimulus, such as a context or an auditory cue, is paired with an aversive one (unconditioned, such as a footshock). Thereafter, the formerly neutral stimulus, when subsequently presented, functions as a conditioned stimulus and triggers a conditioned response. Contextual fear conditioning involves the use of a contextual element as a neutral stimulus, while cue fear

conditioning often relies on auditore stimuli (a tone). The sound can terminate simultaneously or be delayed in relation to the duration of the aversive stimuli. Alternatively, the aversive stimuli can be delivered after a certain time, characterising the trace-auditory fear conditioning. One advantage of fear conditioning models is the possibility of dissecting the mechanisms underlying specific learning phases. The whole learning and memory process can comprise the following phases: The acquisition phase, when the two stimuli are presented simultaneously (e.g. shock + context, shock + tone) and the processing and initial encoding of memory takes place. This is immediately followed by memory consolidation, a series of complex molecular processes crucial for the duration of the memory (Asok *et al.*, 2019; de Oliveira and Do-Monte, 2021). Synaptic consolidation implies the stabilisation of the memory trace (Asok *et al.*, 2019; de Oliveira and Do-Monte, 2021), which is encoded by an assembly of neurones called engram (Josselyn *et al.*, 2015). Later, when the animals are re-exposed to the previously neutral stimulus, they will display the conditioned response, for which the underlying phenomenon is called memory retrieval, which involves the activation of the engram encoding the memory previously acquired (Josselyn *et al.*, 2015; Josselyn and Tonegawa, 2020). Likewise, the exposition to the conditioned stimulus can trigger different processes, depending on the duration of this exposition, among other factors (Auber *et al.*, 2013). Long or repeated expositions to the neutral stimuli can induce extinction, in which a new memory trace is formed, decreasing the intensity of the conditioned response. After extinguished, conditioned responses can be restored, by reinstatement, renewal or spontaneous recovery (Asok *et al.*, 2019; de Oliveira and Do-Monte, 2021).

In terms of neural circuitry, fear learning requires an extensive network of central structures involved in cognition, emotion, and their corresponding autonomic and behavioural responses. The hippocampus (HPC) is responsible for encoding the contextual information associated with an aversive stimuli (Hennings *et al.*, 2022; Marks *et al.*, 2022; Lee and Kaang, 2023). Bidirectional projections between the HPC and the amygdala (AMG) are involved in sustaining the emotional valence of the conditioned response (Marks *et al.*, 2022). The various AMG nuclei process information related to conditioned cues (Li *et al.*, 2023) through neuronal connections with other structures, such as the peri-aqueductal grey (PAG) and the parabrachial nucleus and the thalamus (Marks *et al.*, 2022). In addition, cortical structures, especially the prefrontal cortex (PFC), receive substantial connections from subcortical brain regions such as the AMG and the HPC, integrating the fear-learning circuit (Thomas *et al.*, 2002; Alexandra Kredlow *et al.*, 2022). Other structures outside the classic circuit have also been investigated in the last years for their contribution to fear conditioning. For instance, the nucleus accumbens (NAC) core is involved in the evaluation of threat degree (Ray *et al.*, 2020). However, its role in fear associated with discrete cues remains uncertain (Thomas *et al.*, 2002).

Thus, different structures and pathways work in concert along the complex process of fear learning, in order to keep the balance between allostasis and allostatic overload (Milton, 2019). Various neurochemical mechanisms have been implicated in this process, including the endocannabinoid system (ECS) (Lutz *et al.*, 2015), which is briefly described in the following section.

The endocannabinoid system

The ECS comprises several molecular components, including the cannabinoid type-1 (CB_1) (Matsuda *et al.*, 1990) and type-2 (CB_2) (Munro *et al.*, 1993) receptors; the endocannabinoids (eCB), N-arachidonoylethanolamide (anandamide) (Devane *et al.*, 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995); the enzymes responsible for their synthesis, N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), respectively; and those responsible for their hydrolysis, fatty acid amide hydrolase (FAAH) (Deutsch & Chin, 1993) and monoacylglycerol lipase (Dinh *et al.*, 2002), respectively. One particularity of this system is that the eCBs are produced and released on demand; anandamide can be released by either the pre- or the postsynaptic neurones, while 2-AG seems to be released mostly from the postsynaptic neurones (Howlett *et al.*, 2002; Piomelli and Mabou Tagne, 2022). Once released, eCBs can act as retrograde messengers or as autocrine modulators (Uchigashima *et al.*, 2007; Busquets-Garcia *et al.*, 2018). CB_1 seems to be expressed mostly in presynaptic terminals, although their postsynaptic presence has also been suggested and described as regulating neuronal self-inhibition (Bacci *et al.*, 2004). Its functions seem to be related to the regulation of dendritic excitability mediating long-term potentiation (LTP), which is necessary for cognition and spatial memory (Maroso *et al.*, 2016; Busquets-Garcia *et al.*, 2018). In addition to these *classic* members, a more comprehensive description of the ECS may include other targets under the umbrella term of the expanded ECS (Cristino *et al.*, 2020). The list includes enzymes and receptors modulated directly by phytocannabinoids, eCB or other products of their biosynthetic pathways, such as the transient receptor potential cation channel subfamily V (vanilloid), member 1 (TRPV1) (Cristino *et al.*, 2020).

TRPV1 and CB_1 share some important features. Both were originally discovered as targets for phytochemicals. In the case of CB_1, its prototypical agonist is delta-9-tetrahydrocannabinol, the main psychoactive compound of *Cannabis sativa* (Devane *et al.*, 1988); as for TRPV1, its main agonist is capsaicin, a substance present in certain species of chilli peppers and responsible for the burning pain associated with their intake (Caterina *et al.*, 1997). After their discoveries as orphan receptors, both CB_1 and TRPV1 were found to share anandamide as a common endogenous agonist (Devane *et al.*, 1992; Zygmunt *et al.*, 1999). TRPV1 is preferentially activated at high temperatures (>42'C) (Caterina *et al.*, 1997), whereas anandamide binds to this channel with low affinity, as compared to CB_1 (Devane *et al.*, 1992; Ross, 2003). These particularities, however, do not refute the possibility that anandamide acts as an endogenous TRPV1 agonist. Indeed, TRPV1 activity is controlled by various other mechanisms in addition to temperature, including calmodulin (Numazaki *et al.*, 2003), ATP (Lishko *et al.*, 2007), calcineurin (Docherty *et al.*, 1996) and several kinases, such as PKA or PKC (Premkumar and Ahern, 2000; De Petrocellis *et al.*, 2001; Numazaki *et al.*, 2003). The action of these enzymes on TRPV1 may modify its response to its ligands (e.g. anandamide) and enable its activation at physiologic temperatures (Premkumar and Ahern, 2000; De Petrocellis *et al.*, 2001; Numazaki *et al.*, 2003). Therefore, it is not surprising that anandamide was initially described as a full or partial agonist (Zygmunt *et al.*, 1999; Ross, 2003) depending on the conditions determining TRPV1 conformation.

Notwithstanding these similarities, CB_1 and TRPV1 differ in several aspects. Firstly, although both CB_1 and TRPV1 are expressed in brain structures related to emotion and cognition, CB_1 is usually expressed in presynaptic neurones (Katona *et al.*, 1999), whereas TRPV1 is thought to predominate in postsynaptic neurones (Tóth *et al.*, 2005; Zhao and Tsang, 2017). Remarkably, despite different cellular locations, TRPV1 and CB_1

https://doi.org/10.1017/neu.2023.54 Published online by Cambridge University Press

are often co-expressed in the same synapsis. Regarding the mechanisms, CB_1 is one of the most highly expressed G-protein-coupled receptors in the brain (Tsou *et al.*, 1998; Busquets-Garcia, *et al.*, 2018), usually coupled to a Gαi/o protein (Busquets-Garcia *et al.*, 2018). Thus, when activated, CB_1 inhibits adenylate cyclase, activates inwardly rectifying K^+ channels and decreases neurotransmitters release (Howlett *et al.*, 2002), regulating depolarisation-induced suppression of both inhibition and excitation (Ohno-Shosaku *et al.*, 2001; Wilson and Nicoll, 2001; Uchigashima *et al.*, 2007). Conversely, TRPV1 is a non-selective cation channel, highly permeable to Ca^{2+} (Caterina *et al.*, 1997). Once activated, it promotes an increase in intracellular Na^+ and Ca^{2+}, with subsequent increase in neuronal activity (Marinelli *et al.*, 2005; Starowicz *et al.*, 2007). In addition, since eCB synthesis and release can be triggered by Ca^{2+} (Alger, 2002), TRPV1 activation may in turn increase eCB tonus (Maccarrone *et al.*, 2008). Finally, although both CB_1 and TRPV1 are activated by anandamide, this compound has at least twenty times more affinity for the former (Ross, 2003; van der Stelt *et al.*, 2005).

This body of evidence endorses the hypothesis that anandamide, CB_1 and TRPV1 configure a tripartite system regulating neuronal activity in the brain (Fig. 1). In synapsis expressing both receptors, low levels of anandamide activate presynaptic CB_1 receptors, decreasing neurotransmitter release, whereas higher levels of anandamide may also recruit TRPV1 receptors, increasing neuronal activity and counterbalancing CB_1-mediated effects. Here, we make the case for the possibility that the dual action of anandamide upon CB_1 and TRPV1 may also participate in the modulation of learned fear. Our hypothesis will be built upon pharmacological and genetic studies to investigate the role of each receptor in fear-conditioned paradigms, considering the various phases of fear-learning processing.

Role of CB_1 in fear conditioning

Studies using either genetic or pharmacological approaches support the involvement of CB_1 in the regulation of fear conditioning. Indeed, initial studies by Marsicano and colleagues revealed that CB_1-deficient mice presented impaired fear extinction when subjected to a tone previously paired with footshock (Marsicano *et al.*, 2002). Regarding the effects of pharmacological interventions, the modulation of fear by drugs targeting CB_1 and other molecular components of the ECS may differ depending on the specificity of the compound, dosage and intensity of conditioning. As elaborated below, it also varies according to the memory phase in which the intervention occurred (acquisition, consolidation, expression and extinction).

Acquisition of fear memory

Several studies, using pharmacological approaches, have implicated CB_1 in the acquisition of fear memory. The administration of CB_1 antagonists and inverse agonists, such as AM4113 (6mg/kg) and AM251 (4 and 8 mg/kg), respectively, impaired fear acquisition in an auditory fear conditioning task (Sink *et al.*, 2010). However, in another study, AM251 (5mg/kg) was found to enhance the subsequent fear responses in both trace (HPC-dependent) and delayed (amygdala-dependent) fear conditioning; these effects were further increased when the drugs were administered before both conditioning and expression (Reich *et al.*, 2008). Similarly, Sink and colleagues observed that AM251 enhanced the acquisition of fear conditioned to a context, while no effect was noticed after AM4113 treatment (Sink *et al.*, 2010). Regarding cannabinoid agonists, the non-selective CB_1, agonist WIN55,212-2 (2.5 and 5mg/kg), impaired contextual but not auditory fear conditioning (Pamplona and Takahashi, 2006). These effects were prevented by pre-treatment with selective CB_1 antagonists, such as SR141716A or SR147778 (Pamplona and Takahashi, 2006).

Consolidation

Studies focusing on the role of the ECS on fear memory consolidation observed that anandamide release restrained this process, an effect prevented by a subeffective concentration of AM251 (Scienza-Martin *et al.*, 2022). Also, the CB_1 agonists, HU-210 (Maćkowiak *et al.*, 2009) and ACPA (Nasehi *et al.*, 2016), impaired fear consolidation in both contextual and auditory fear conditioning. As expected, HU-210 effect was blocked when co-administered with AM251 (Maćkowiak *et al.*, 2009). Similarly, the phytocannabinoid cannabidiol impaired memory consolidation *via* CB_1, since its effect was prevented by AM251, although also by the CB_2 antagonist, AM630 (Stern *et al.*, 2017). This effect was observed either with systemic or local (dorsal HPC) administration (Stern *et al.*, 2017).

Retrieval

In studies investigating the role of CB_1 in fear memory retrieval, the CB_1 antagonist, SR141716 (1, 5 mg/kg), was ineffective (Mizuno *et al.*, 2022). However, the non-selective agonist WIN55,212-2 (0.25 mg/kg) decreased contextual fear responses when animals were subjected to a more intense, but shorter protocol (1 × of 1.5mA) (Pamplona *et al.*, 2008). When the intensity of the aversive stimulus was lower, but its duration was longer (3 × of 0.75 mA), WIN55,212-2 enhanced fear memory retrieval at both doses tested (0.075mg/kg and 0.75 mg/kg) in males, but just at the highest one in females (Mizuno *et al.*, 2022).

Regarding compounds that indirectly facilitated the ECS, JZL184, an inhibitor of 2-AG hydrolysis, increased freezing in females (8 mg/kg), but not in male mice, an effect mediated by CB_1, but not CB_2 receptors. The FAAH inhibitor URB597 (0.3, 1, 3 mg/kg) was ineffective (Mizuno *et al.*, 2022).

Concerning the brain regions involved, no effect was verified by administering AM251 into the ventromedial PFC (Lisboa *et al.*, 2010; Simone *et al.*, 2015), although this CB_1 antagonist did increase freezing in animals exposed to a less aversive conditioning protocol (Lisboa *et al.*, 2010). Local injection of anandamide (5 pmol/200 nl) or the anandamide transport inhibitor, AM404 (50 pmol/200 nl), into this region, attenuated the fear-conditioned responses, a result prevented by local pre-treatment with AM251 (100 pmol/200 nl). No effect was also reported in auditory fear conditioning after ACEA, another CB_1 agonist (Simone *et al.*, 2015). Finally, when administered locally into the PAG, 2-AG decreased freezing response, an effect prevented by AM251 (Brianis *et al.*, 2022).

Extinction

The importance of CB_1 in the extinction of aversive memories was observed through several strategies. For instance, CB_1-deficient mice showed impaired short- and long-term extinction, and this was mimicked by the administration of the CB_1 antagonist, SR141716A, to wild-type animals (Marsicano *et al.*, 2002). The extinction of the auditory fear conditioning was impaired by the

https://doi.org/10.1017/neu.2023.54 Published online by Cambridge University Press

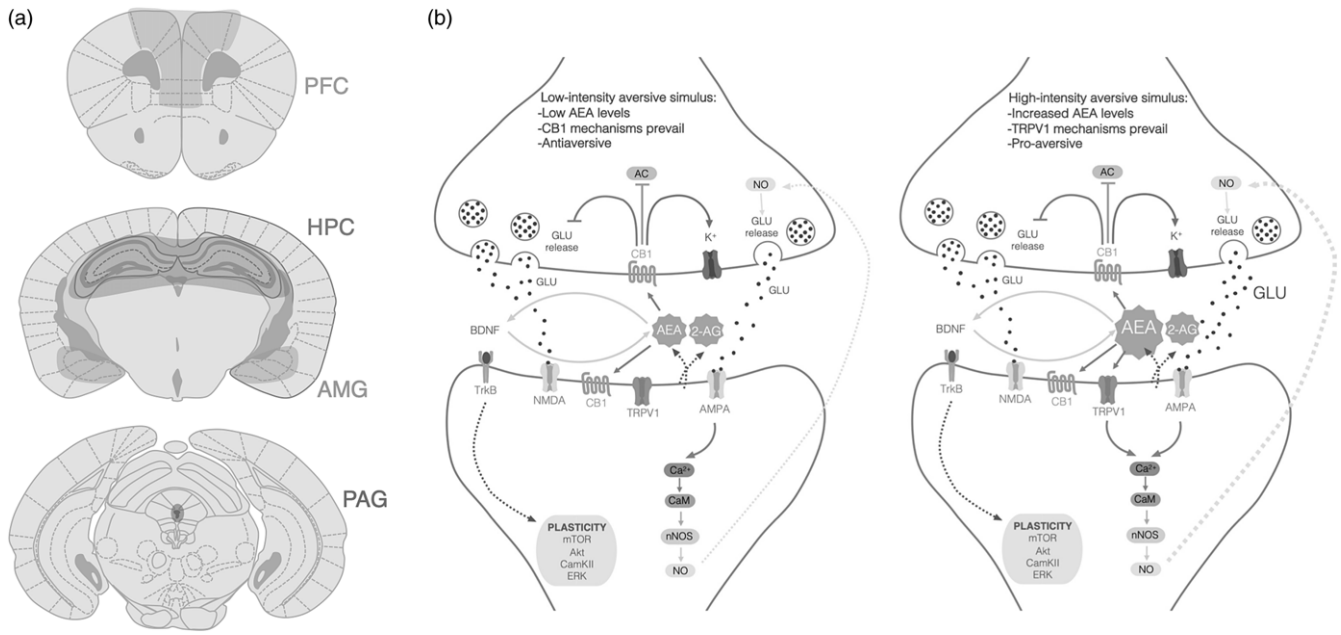

Figure 1. ***a***) CB_1 and TRPV1 receptors are co-localized in brain regions that modulate fear responses, such as the prefrontal cortex (PFC), hippocampus (HPC), amygdala (AMG) and periaqueductal grey (PAG). ***b***) Molecular pathways involved in fear memory modulated by CB_1 receptors and TRPV1 channels in response to low or high aversive stimuli. Under low-intensitiy, aversive stimuli (left panel) CB_1 activation by anandamide inhibits adenylate cyclase. (AC), reduces glutamate (GLU) release and activates rectifying potassium (K^+) channels. However, as the intensity of the aversive stimulus increases (right panel), anandamide binds to TRPV1 receptors and causes calcium (Ca^{+2}) influx. This leads to calmodulin (CaM) activation and neuronal nitric oxide synthase (nNOS) activity, resulting in nitric oxide (NO) production and its retrograde activity, increasing GLU release (2-AG, 2-arachidonoylglicerol; AC, adenylate cyclase; anandamide, N-arachidonoylehtanolamide or anandamide; Akt, protein kinase B; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid channel; BDNF, brain-derived neurotrophic factor; Ca^{+2}, calcium; CaM, calmodulin; CamKII, calcium–calmodulin (CaM)-dependent protein kinase II; CB_1, cannabinoid type 1 receptor; ERK, extracellular signal-regulated kinase; GLU, glutamate; K^+, potassium; mTOR, mammalian target of rapamycin; NMDA, N-mehtyl-d- aspartate channel; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; TrkB, receptor tyrosine kinase B; TRPV1, transient receptor vanilloid type-1 channel).

administration of another CB_1 antagonist/inverse agonist, AM251 (3 mg/kg), and facilitated by the selective agonist, ACEA (0,1 and 0,5 mg/kg) (Simone *et al.*, 2015).

Accordingly, a selective CB_1 antagonist disrupted extinction, while administration of WIN55,212–2 resulted in the opposite effect (Pamplona *et al.*, 2006). The facilitation of fear extinction was also observed in the extinction of remote memories, an effect prevented by a CB_1 antagonist (Pamplona *et al.*, 2006). In another study, however, the administration of WIN55,212-2 (0.075, 0.75 mg/kg) inhibited fear extinction in both sexes (Mizuno *et al.*, 2022). Regarding the direct modulation of endocannabinoid hydrolysis, extinction impairments were reported in animals treated with a DAGL inhibitor as well as in animals lacking DAGL ($DAGL\alpha^{-/-}$ mice) (Cavener *et al.*, 2018). In addition, 2-AG or anandamide facilitation, with JZL184 (4 or 8 mg/kg) and URB597 (0.3, 1, 3 mg/kg), respectively, inhibited fear extinction on day 5; however, such differences disappeared on the last day of extinction (day 28) for both sexes (Mizuno *et al.*, 2022).

Therefore, the predominant effect of facilitating endocannabinoid signalling is the inhibition of fear responses, whereas its blockade tends to induce the opposite response. These effects tend to occur particularly in protocols in which the animals are exposed to aversive stimuli of moderate intensity.

Role of TRPV1 in fear conditioning

The literature on TRPV1 and fear is scant, in comparison to the more robust evidence discussed for CB_1. One of the earliest studies implicating TRPV1 in fear memory reported phenotypic differences between TRPV1 KO and wild-type animals in the auditory fear conditioning (Marsch *et al.*, 2007); knock out animals displayed lower levels of freezing when evaluated shortly or remotely after conditioning, while unconditioned fear responses and pain threshold remained unchanged as compared to controls (Marsch *et al.*, 2007). The responses seemed to be dependent on the intensity of the conditioning (Marsch *et al.*, 2007). This early study suggested that TRPV1 may be involved in fear memory, but whether its role was relevant for acquisition, consolidation or retrieval was still unknown.

Acquisition

Pharmacological studies revealed no effect after the administration of a TRPV1 blocker, capsazepine, before conditioning, although administration of capsaicin, a TRPV1 agonist, had biphasic effects – low doses enhanced acquisition and high doses impaired it (Almeida *et al.*, 2019). A potential explanation for this biphasic profile is the fast desensitisation of TRPV1 induced by even single doses of capsaicin (Szallasi and Di Marzo, 2000; Almeida *et al.*, 2019).

Consolidation

In agreement with the results obtained from experiments in knockout mice, intra-hippocampal administration of capsazepine impaired memory consolidation when the animals were exposed to high intensities of the aversive stimulus, although no effect was observed after capsaicin administration (Genro *et al.*, 2012). Interestingly, this effect was replicated in a more recent study (Scienza-Martin *et al.*, 2022).

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Retrieval

Local administration of TRPV1 blockers (capsazepine or 6-iodonordihydrocapsaicin) into the ventromedial PFC decreased behavioural and autonomic responses to contextual fear learning (Terzian *et al.*, 2014). However, the effect of 6-iodonordihydrocapsaicin was not observed when the animals were exposed to aversive stimuli of low intensity (Terzian *et al.*, 2014). In spite of this, at this intensity, this compound was able to prevent the enhancement of conditioned responses induced by central administration of capsaicin (Terzian *et al.*, 2014). The augmentation of behavioural and autonomic responses induced by capsaicin administration into the ventromedial PFC was replicated in another study (Uliana *et al.*, 2020). Finally, TRPV1 blockers directly administered into the dorsal HPC impaired memory retrieval when the mice were exposed to footshocks of moderate-to-high, but not low, intensities (Iglesias *et al.*, 2023).

Extinction

The potent TRPV1 inhibitor iodoresiniferatoxin administered before conditioning, retrieval and extinction enhanced fear extinction without affecting other phases (Laricchiuta *et al.*, 2013). However, in the auditory fear conditioning, an acute administration of SB366791 had no effect (Llorente-Berzal *et al.*, 2015).

In summary, the scarce literature suggests that activation of TRPV1 channels exacerbates fear memory with a biphasic profile, possibly associated with fast desensitisation of TRPV1 channels at certain doses of agonists (Szallasi and Di Marzo, 2000; Almeida *et al.*, 2019). On the contrary, genetic deletion as well as pharmacological blockade of TRPV1 promotes anti-aversive responses. This effect depends on the intensity of the aversive experience, probably due to the tonus of the ECS, as will be discussed below.

TRPV1 and (endo)-cannabinoid interactions

Although the aforementioned studies focused on CB_1 and TRPV1 separately, we argue that these receptors function in concert to mediate opposite functions of anandamide. In this section, we built on the hypothesis that anandamide, CB_1 and TRPV1 form a tripartite system modulating fear memory based on three sets of evidence: First, CB_1 and TRPV1 are co-localized in fear-related brain regions; second, they interfere with common downstream pathways involved in fear memory. Finally, they depend on each other to mediate the effects of selective pharmacological interventions.

CB_1 and TRPV1 are co-localized in fear-related structures

The presence of CB_1 in brain circuits modulating fear learning is supported by an enormous and consistent body of evidence (Tsou *et al.*, 1998; Wilson-Poe *et al.*, 2012; Lazenka *et al.*, 2013; Gomes-de-Souza *et al.*, 2021). Moreover, histological studies with specific antibodies found CB_1 to be co-expressed with TRPV1 in several synapses of fear-related regions (Cristino *et al.*, 2006). Additional studies in specific brain regions observed TRPV1 and CB_1 colocalization in the PFC (Fogaça *et al.*, 2012; Diniz *et al.*, 2019), the PAG (Casarotto *et al.*, 2012) and the dorsomedial hypothalamus (Dos Anjos-Garcia & Coimbra, 2019). In the dorsal HPC, the use of high-resolution confocal microscopy and z-stack three-dimensional analysis also revealed co-expression of CB_1 and TRPV1 (Iglesias *et al.*, 2023). Therefore, studies applying histological immuno-histochemical techniques, along with microscopy analysis, support the possibility that CB_1 and TRPV1 can be simultaneously activated by anandamide in fear-related brain regions, provided the local synaptic concentration of these endocannabinoids reaches levels high enough to bind both receptors.

CB_1 and TRPV1 functions modulate common fear memory-related pathways

Neurotrophic signalling exerts several functions in the brain, including modulation of fear memory (Notaras & van den Buuse, 2020). Activation of tyrosine receptor kinase B (TrkB) by brain-derived neurotrophic factor (BDNF) leads to the regulation of several downstream pathways involved in plasticity, such mTOR, Akt, CamKII or ERK, all of them crucial for the consolidation of fear memory (Minichiello, 2009). The link between neurotrophic and anandamide-CB_1 signalling has been extensively explored in recent years. On the one hand, BDNF and TrkB activation enhances endocannabinoids release (Yeh *et al.*, 2017; Wu *et al.*, 2020); on the other hand, certain effects derived from CB_1 activation are mediated by BDNF (Blázquez *et al.*, 2015; Navabpour *et al.*, 2021). As for TRPV1, its relation with BDNF remains unknown. However, one study showed that TRPV1-mediated synaptogenesis in the HPC seems to require BDNF (Hurtado-Zavala *et al.*, 2017). Despite the scarcity of data, one could hypothesise that, if neurotrophic signalling increases endocannabinoid tonus, this may facilitate the recruitment of TRPV1 and its involvement in some of the BDNF-related effects. Indeed, an in vitro study showed that anandamide enhances TrkB phosphorylation (Diniz *et al.*, 2019). Interestingly, the mechanism underlying this effect is dose-dependent, with low doses of anandamide acting through CB_1, whereas at higher doses, anandamide action occurs through TRPV1 activation (Diniz *et al.*, 2019).

Another important player in fear and memory is the nitric oxide (NO) pathway (Susswein *et al.*, 2004; Medeiros *et al.*, 2022). In postsynaptic neurones, calcium influx and calmodulin activation promote neuronal nitric oxide synthase (nNOS) activity, with the subsequent retrograde effects of NO and enhancement in neurotransmitter release (Huang, 1997). Several pieces of evidence point to the involvement of nNOS/NO in plasticity and specifically in the modulation of certain fear memory phases (Sadeghi *et al.*, 2022). More important for the scope of this review, the ECS and the nitric oxide pathway seem to interact to modulate learned fear. For instance, the enhancement of the endocannabinoid tonus, by means of FAAH inhibition, prevented the extinction deficits in mice with genetic deletion of inducible NOS, iNOS (Lisboa *et al.*, 2015), suggesting that the ECS may be a downstream effector of NO or at least able to compensate for some of its effects. Moreover, facilitation of fear retrieval induced by TRPV1 agonism or CB_1 antagonism was prevented by a subeffective dose of a NO scavenger, NO inhibitors and a soluble guanylate cyclase inhibitor (Uliana *et al.*, 2016). Similar results were observed in the ventromedial PFC (Uliana *et al.*, 2020). In addition, NOS inhibition blocked the LTP induced by a TRPV1 agonist in the AMG, the same effect being elicited by a CB_1 antagonist (Zschenderlein *et al.*, 2011). These data suggest that TRPV1 and CB_1 act in opposite directions upon the NO pathway, which may partially explain their contrasting role in modulating fear memory.

Furthermore, CB_1 and TRPV1 interact in the modulation of electrophysiological processes underlying memory and neuronal plasticity. For instance, in the neocortex and striatum, spike

https://doi.org/10.1017/neu.2023.54 Published online by Cambridge University Press

time-dependent LTP was blocked by both TRPV1 and CB_1 antagonism (Cui *et al.*, 2015, 2018). Similarly, anandamide induced long-term depression (LTD) through both CB_1 and TRPV1 in the Nac (Grueter *et al.*, 2010). In the dentate gyrus, CB_1 and TRPV1 agonists seem to modulate excitatory postsynaptic field potentials and LTP in opposite directions (Tahmasebi *et al.*, 2015). However, this intersection between CB_1 and TRPV1 remains controversial. For example, anandamide facilitated LTD through TRPV1 but not CB_1 (Yang *et al.*, 2014), while other reports indicated that anandamide rescued impaired hippocampal LTP through CB_1 activation (Basavarajappa *et al.*, 2014).

Some of these processes may be explained by the capacity of TRPV1 and CB_1 to modulate glutamatergic neurotransmission. For example, CB_1 activation in the hypothalamus decreases glutamate release, while the opposite goes for TRPV1 (Jamieson *et al.*, 2022). Similarly, CB_1 and TRPV1 presented opposite effects on NMDA-induced autonomic responses (Lagatta *et al.*, 2018) and plasticity (Back & Carobrez, 2018). These examples suggest that TRPV1 and CB_1 may act upon common mechanism in the regulation of fear memory, usually leading to opposite outcomes.

Anandamide, CB1 and TRPV1 interact to modulate fear responses

Complementing the histological and neurochemical evidence, the last section will focus on studies using pharmacological interventions in animals exposed to fear conditioning. Administration of anandamide itself, compounds that inhibit its hydrolysis (by inhibiting FAAH blockers, such as URB597) or compounds that exert dual TRPV1 and FAAH blockade (e.g., AA-5-HT) provide evidence of opposite functions for CB_1 and TRPV1 in mediating the actions of anandamide in different phases of fear responses.

Acquisition

The systemic administration of the FAAH inhibitor URB597 had no effect on the acquisition of fear memory (Laricchiuta *et al.*, 2013; Balogh *et al.*, 2019). Similarly, local administration of this drug into the ventral HPC, prelimbic PFC or AMG had no effects on memory retrieval (Balogh *et al.*, 2019). However, direct administration of anandamide into the NAC core impaired the acquisition of contextual, but not auditory, fear (Pedroza-Llinás *et al.*, 2013). Likewise, after systemic FAAH inhibition by the compound OL-135, an impairment in the acquisition of the contextual fear conditioning was observed (Burman *et al.*, 2016), but no effect was detected in the auditory fear conditioning (Burman *et al.*, 2016). Contextual and auditory fear conditioning rely on different brain structures, specifically, the dorsal portion of the HPC seems involved in contextual but not in auditory fear conditioning (Phillips and LeDoux, 1992). Even though FAAH is expressed in the amygdala, which is involved in both tasks (Gulyas *et al.*, 2004), its inhibition might not be relevant to the acquisition of the auditory fear conditioning (Burman *et al.*, 2016). Instead, the disruption observed in the contextual task after increasing endocannabinoid tonus (Burman *et al.*, 2016) may be related to the action of this drug in structures such as the dorsal HPC, which is not involved in auditory fear conditioning. However, this effect seems to depend on the type of modulation, since the impairment in fear acquisition was observed with OL-135 (Burman *et al.*, 2016), but not with URB597 (Laricchiuta *et al.*, 2013; Balogh *et al.*, 2019). This may be related to differences in the way how these compounds inhibit FAAH (Naidu *et al.*, 2007) or off target enzymes (Zhang *et al.*, 2007).

Resembling URB597 effects, the dual FAAH/TRPV1 blocker, AA-5-HT, had no effect on the acquisition of contextual fear conditioning (Gobira *et al.*, 2017). However, the non-selective anandamide reuptake blocker and TRPV1 agonist, AM404, impaired fear acquisition, an effect dependent on both TRPV1 and CB_1, since it was prevented by capsazepine and by rimonabant (Almeida *et al.*, 2019). Similarly, intra-HPC administration of AM404 prevented memory acquisition *via* CB_1 activation (Lin *et al.*, 2011).

The discrepancies between the administration of FAAH dual blockers and AM404 may rely on two different but complementary hypotheses. First, the levels of anandamide depend on intensity/aversiveness of the experience (Morena *et al.*, 2014; Iglesias *et al.*, 2023), thus low intensities may not promote enough release of anandamide and its hydrolysis inhibition will not reach a substantial effect. Indeed, Gobira (2017) showed no effects of AA-5-HT, while Almeida and colleagues (2019) did observe fear inhibition after AM404 administration. However, the intensity of the conditioning was much higher in the latter study. Alternatively, AM404 may act as a partial agonist at TRPV1 (Ross, 2003).

Consolidation

During the consolidation of fear memory, increased levels of eCB were observed in the basolateral AMG and the HPC, but not in the PFC (Marsicano *et al.*, 2002; Morena *et al.*, 2014). In the HPC, anandamide levels seem to depend on intensity of aversive stimuli (Morena *et al.*, 2014). However, post-training administration of OL-135 (Burman *et al.*, 2016) or anandamide (intra-NAc) (Pedroza-Llinás *et al.*, 2013) did not impair contextual or auditory fear conditioning. On the other hand, similarly to the acquisition, the administration of AM404, an inhibitor of anandamide reuptake, disrupted contextual fear consolidation when administered into the CA1 area of the dorsal HPC (Scienza-Martin *et al.*, 2022).

Retrieval

Anandamide levels increase in the basolateral AMG after retrieval of fear memory (Gaspar *et al.*, 2022). The same was observed in the HPC, where anandamide levels increase as a function of fear intensity (Iglesias *et al.*, 2023). Moreover, the direct administration of anandamide into the medial PFC (Lisboa *et al.*, 2010) or the PAG (Resstel *et al.*, 2008) reduced freezing. Furthermore, administration of AM404 systemically (Pamplona *et al.*, 2008), into the PFC (Lisboa *et al.*, 2010), PAG (Resstel *et al.*, 2008) or into the HPC (Scienza-Martin *et al.*, 2022), mimicked anandamide effects. AM404 effects on retrieval were prevented by a CB_1 antagonists (Lisboa *et al.*, 2010; Llorente-Berzal *et al.*, 2015) and by a TRPV1 blocker (Llorente-Berzal *et al.*, 2015). In addition, AA-5-HT (a dual FAAH/TRPV1 blocker) administered systemically or into the HPC impaired fear memory retrieval, an effect prevented by pre-treatment with AM251 (Gobira *et al.*, 2017). Remarkably, AA-5-HT effects were mimicked by co-administration of subeffective doses of a FAAH inhibitor with a TRPV1 blocker (Gobira *et al.*, 2017), supporting the hypothesis of opposite roles for CB_1 and TRPV1. In agreement with this possibility 1) the administration of a CB_1 antagonist or a TRPV1 agonist into the dorsolateral PAG induced the same effect on fear expression (Uliana *et al.*, 2016); 2) a CB_1 antagonist prevented the retrieval deficits induced by TRPV1 blockers in the HPC (Iglesias *et al.*, 2023) and 3) a TRPV1 blocker prevented the enhancement of memory retrieval induced by CB_1 antagonists in the PAG (Uliana

https://doi.org/10.1017/neu.2023.54 Published online by Cambridge University Press

et al., 2016). Altogether, these data support our proposal that CB_1 and TRPV1 act in concert to mediate opposite functions of anandamide in the control of fear responses.

Extinction

Anandamide infusion into the dorsal HPC facilitates extinction, an effect prevented by pre-treatment with AM251 (de Oliveira et al., 2008). In addition, administration of AM404 systemically (Pamplona *et al*., 2008), *via* intracerebroventricular (Bitencourt *et al*., 2008) or directly into the dorsal HPC (Abush and Akirav, 2010), facilitates fear extinction. These effects seem to depend on the activation of CB_1, but not TRPV1 (Bitencourt *et al*., 2008). However, TRPV1 involvement in AM404 effects on extinction was observed in auditory fear conditioning (Llorente-Berzal *et al*., 2015).

Conclusion and future directions

The evidence reviewed here supports our hypothesis that anandamide, CB_1 and TRPV1 act in concert as a neurochemical system regulating fear memory. Low-intensity aversive stimuli could promote moderate anandamide release, which activates CB_1 receptors and decreases the release of glutamatergic neurotransmission, with subsequent inhibition of fear. However, in response to highly aversive stimuli, anandamide levels would further increase; as a result, TRPV1 channels would be activated to promote Ca^{+2} influx, increase neuronal firing and, finally, activate the neuronal mechanisms promoting fear.

However, some limitations should be considered. For instance, most studies have focused on male rodents as experimental subjects. Since only recently has sex been included as an experimental variable, little is known regarding differences in anandamide/CB_1/TRPV1 interactions between males and females. A recent study showed that eCB signalling facilitation had no effect on fear extinction in males, while extinction was impaired in females, probably *via* TRPV1 activation (Morena *et al*., 2021). Another biological variable to be taken into consideration is development, since TRPV1 (Huang *et al*., 2014) and CB_1 (Liu *et al*., 2003) expression change along the lifespan. Future research should address the impact of these variables in anandamide/CB_1/TRPV1 interactions in specific brain regions. Finally, a remaining question is how this tripartite system could be targeted for developing new drugs for the treatment of certain psychiatric disorders, particularly those resulting from exacerbated responses to aversive stimuli.

Acknowledgements. FAM thanks Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG; project APQ-00741-21); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; research productivity fellowship, level 2); and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES): 'O presente trabalho foi realizado com o apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001' – ' This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001'.

Author contribution. LPA was the main responsible for designing the review. All the authors have contributed to this study by participating in the literature search and writing the article. All authors have agreed on the final version of the manuscript.

Competing interests. None.

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