Animal models of post-traumatic stress disorder and novel treatment targets

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Understanding the neurobiological basis of post-traumatic stress disorder (PTSD) is fundamental to accurately diagnose this neuropathology and offer appropriate treatment options to patients. The lack of pharmacological effects, too often observed with the most currently used drugs, the selective serotonin reuptake inhibitors (SSRIs), makes even more urgent the discovery of new pharmacological approaches. Reliable animal models of PTSD are difficult to establish because of the present limited understanding of the PTSD heterogeneity and of the influence of various environmental factors that trigger the disorder in humans. We summarize knowledge on the most frequently investigated animal models of PTSD, focusing on both their behavioral and neurobiological features. Most of them can reproduce not only behavioral endophenotypes, including anxiety-like behaviors or fear-related avoidance, but also neurobiological alterations, such as glucocorticoid receptor hypersensitivity or amygdala hyperactivity. Among the various models analyzed, we focus on the social isolation mouse model, which reproduces some deficits observed in humans with PTSD, such as abnormal neurosteroid biosynthesis, changes in GABA$_A$ receptor subunit expression and lack of pharmacological response

to benzodiazepines. Neurosteroid biosynthesis and its interaction with the endocannabinoid system are altered in PTSD and are promising neuronal targets to discover novel PTSD agents. In this regard, we discuss pharmacological interventions and we highlight exciting new developments in the fields of research for novel reliable PTSD biomarkers that may enable precise diagnosis of the disorder and more successful pharmacological treatments for PTSD patients. *Behavioural Pharmacology* 30:130–150 Copyright © 2019 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Stress and environmental factors play a fundamental role in developing maladaptation and behavioral abnormalities. Indeed, stressful events negatively affect several neuro-endocrine systems, which can cause deep repercussions on both cognitive and emotional processing (McEwen *et al.*, 2015; Pagliaccio *et al.*, 2015; Herman *et al.*, 2016).

In the general population, more than two-thirds of individuals experience a traumatic event at some point in their lifetime (Javidi and Yadollahie, 2012), but the majority of them develop the ability to adapt and develop resilience within the following 3–6 months (Bryant, 2003). However, after a traumatic event, a consistent proportion of subjects may develop severe psychiatric disorders, including generalized anxiety, major depressive disorder and/or post-traumatic stress disorder (PTSD). If not adequately treated, these conditions may progress into a more complex neuropathology with significant morbidity, prevalence, and comorbidity with other psychiatric disorders (Baldwin *et al.*, 2014; Roberts *et al.*, 2015; Yehuda *et al.*, 2015a, 2015b).

PTSD increases chronic disease, accelerates aging, and is associated with premature mortality (Koenen *et al.*, 2017).

It is a multifaceted disorder with four characterizing symptom clusters: intrusion, re-experiencing the traumatic event, increased arousal, and the constant avoidance of stimuli associated with the trauma (Brewin, 2001; Pai *et al.*, 2017). Its prevalence in the US population is 6.8% (Kessler *et al.*, 2005). Soldiers, abused children, and battered women are the most susceptible individuals affected by PTSD (Goldstein *et al.*, 2016). The nature of the traumatic event is important to predict the risk of developing PTSD. Repeated trauma (e.g. abuse) compared with a single traumatic exposure (e.g. car accident) increases the possibility of developing the disorder (Bichescu *et al.*, 2005). However, the duration or intensity of the trauma cannot completely explain the rates of the disorder among the general population (Smith *et al.*, 2016). Several factors can influence susceptibility to PTSD and sex plays an important role. Indeed, the number of women who develop PTSD is about double that of men (Shansky 2015; Yehuda *et al.*, 2015a, 2015b). Another important risk factor is a premorbid personality with preexisting anxiety and/or depressive disorders (Lassemo *et al.*, 2017; Gagne *et al.*, 2018). The high comorbidity with other psychiatric disorders makes it difficult to diagnose PTSD and to select an optimal treatment (Greene *et al.*, 2016). For example, in about 30–50% of individuals,

PTSD is complicated by major depressive disorders (Shalev, 2001). This impressive comorbidity rate can be partially explained by the presence of overlapping symptoms between the two disorders. Other disorders observed in PTSD patients are enhanced vulnerability to substance and/or alcohol abuse, generalized anxiety, or even attempted suicide (Spinhoven *et al.*, 2014; Gradus *et al.*, 2017; Lento *et al.*, 2018). The commonality of symptoms and behavioral phenotypes make it even more difficult to distinguish the different disorders or subpopulation of patients and to provide adequate medical assistance.

The selective serotonin reuptake inhibitors (SSRIs) are the first-line intervention to treat PTSD. Paroxetine and sertraline are currently the only Food and Drug Administration-approved drugs to treat PTSD symptoms (Friedman and Bernardy, 2017). Recent studies reveal a considerable variability in their efficacy, often with the resistant individuals, such as veterans (Bernardy and Friedman, 2015; Starke and Stein, 2017), who need augmentation or various combination of treatments (Stein *et al.*, 2009). As the response rate to SSRIs rarely exceeds 40–60%, research on discovering new treatments has become a priority. However, while adequate therapeutic approaches are needed, these firmly rely on reliable animal models and established biomarkers associated with PTSD. Regrettably, as of today, there are no biomarkers that have been assessed for PTSD and animal models only partially reproduce endophenotypes observed in the spectrum of PTSD neurobiology.

In this review, we discuss the most commonly studied animal models of PTSD. We critically address their ability to mimic the behavioral and biological dysfunctions observed in PTSD patients and focus on the pharmacological advantage of potential novel PTSD treatments. A specific focus has been given to the neurosteroid and endocannabinoid systems as promising fields to assess specific PTSD biomarkers and develop novel treatment options.

Animal models of post-traumatic stress disorder

The experimental manipulations that lead to a valid construct of PTSD-like psychopathology in animals are few and they are the topic of debates (Goswami *et al.*, 2013; Borghans and Homberg, 2015; Flandreau and Toth, 2018). Currently, there is no accepted animal model of PTSD, even if several protocols allow reproduction of some endophenotypes of the disorder. The principal challenge is to create an adequate rodent model of PTSD that closely reflects the complexity of PTSD, which leads patients to exhibit a variety of mood and cognitive symptoms (American Psychiatric Association, 2013). Furthermore, understanding the neurobiology of PTSD also means understanding the causes of individual susceptibility and resilience. PTSD is associated with exposure to different kinds of traumatic events and the diverse types of trauma could result in several subtypes of PTSD patients (Stein *et al.*,

2016). Given the heterogeneity of PTSD and the high comorbidity with other neuropsychiatric disorders, several animal models could be useful to investigate different aspects of the disorder (discussed in Locci and Pinna, 2019a). However, no results obtained from each of the models can be generalized to this neuropathology in its entirety but should be considered useful for a clearer understanding of a certain PTSD subpopulation. An alternative strategy to establish a valid animal model is to recognize symptom clusters that are actually shared between disorders and model these. PTSD symptoms include intrusion, avoidance, negative alterations in cognition and mood, and alterations in reactivity and arousal (American Psychiatric Association, 2013). Nevertheless, the possibility to distinguish between symptom clusters linked to PTSD or PTSD with comorbidities is not always so feasible. While, for example, emotional numbing symptoms may be associated with PTSD and comorbid alcohol abuse disorder (Jakupcak *et al.*, 2010), the symptom clusters of PTSD and comorbid depression are more difficult to identify (O'Donnell *et al.*, 2004). However, the overlap with other psychiatric disorder such as depression, anxiety disorder, and suicidal ideation, shows the need to identify a unique array of biomarkers associated with the disorder or with the comorbid conditions (Pinna and Izumi, 2018). For example, patients with PTSD show a specific correlation between decreased neurosteroid levels and changes in $GABA_A$ receptor subunits, resulting in decreased cortical and hippocampal benzodiazepine binding sites and lack of benzodiazepine-induced pharmacological effects. These alterations related to the $GABA_A$ receptor subunit changes form a biosignature exclusive for PTSD, which is different from that occurring in other disorders (reviewed in Pinna and Izumi, 2018).

An adequate preclinical model should recapitulate the specific signatures of the disorder and satisfy construct, face, and predictive validity criteria (Torok *et al.*, 2018). Construct validity allows assessment of the validity of the procedure. In the case of PTSD, the cause of the disorder is the exposure to a traumatic event; thus, an animal model should use a traumatic exposure to develop a similar construct. Face validity refers to the evaluation of the similarities between PTSD-like symptoms in animals and the symptoms of patients, listed in the *Diagnostic and Statistical Manual of Mental Disorder* version 5 (American Psychiatric Association, 2013; Torok *et al.*, 2018). Predictive validity is the ability to make predictions of human response on the basis of the model (e.g. the response to the use of newly developed drugs; Siegmund and Wotjak, 2006). Given that not all the individuals exposed to traumatic events develop PTSD, it is important to understand the risk factors that alter the ability to develop resilience or the vulnerability to develop the disorder in the population. Probably, a more appropriate animal model to reflect the human condition should consider applying chronic stress in combination with an acute traumatic event to precipitate PTSD-like behavior (Torok *et al.*, 2018). Furthermore, even if sex affects the response to

traumatic stressors, its effects were not thoroughly investigated in most of the animal models summarized below (Cohen and Yehuda, 2011). For example, only a few studies among over 200 that have used the single prolonged stress (SPS) model compared the behavioral response of the two sexes (Keller *et al.*, 2015). A recent study in rats subjected to the SPS paradigm shows that females are not more resilient to the consequences of traumatic events than males, but they simply respond differently to the trauma (Pooley *et al.*, 2018). Biological measures, such as the higher baseline and poststress corticosterone levels in females, confirmed differences due to a sex bias that leads to diverse behavioral outcomes in males and females (Seale *et al.*, 2004). These findings can help explain why, in humans, the two sexes show a different behavioral response to trauma: more externalizing symptoms are induced in men (aggression, impulsivity, and hyperarousal), while internalized symptoms are more prevalent in women (anhedonia, sadness, and depression) (Murphy *et al.*, 2018). The lack of sex-related knowledge on the understanding of the neurobiological mechanisms related to traumatic events makes it a priority to include females in preclinical research. Figure 1 summarizes the several rodent PTSD models described below.

The restraint stress model
An experimental manipulation to establish animal models for PTSD includes restraint stress, during which rodents are placed in a restraint device for a time varying from 15 to 120 min (Whitaker *et al.*, 2014). Animals can also be subjected to a chronic form of repeated restraint stress for

Fig. 1

PTSD MODEL	Stressor exposure	Behavioral phenotype	PFC/AMY disfunction	Increased HPA axis negative feedback	Altered hippocampal morphology	Inflammatory markers in plasma	Inflammatory markers in CNS	Sensitive to SSRIs	Findings in females
RESTRAINT STRESS	Immobilization for 30-120 min	Depression-like behavior and anhedonia (Chiba et al. 2012, Xu et al. 2017)	+ (Vyas et al. 2002)	+ (Risbrough et al. 2016)	−	Not reported	Not reported	+ (Chu et al. 2016)	Not reported
UNPREDICTABLE VARIABLE STRESS (UVS)	Daily exposure for 1-8 weeks to different kinds of stressors (predator scent, food/drink deprivation, overcrowding,etc.)	General avoidance (Wakizono et al. 2007)	Not reported	+ (Yehuda et al. 2015)	+ (Isgor et al. 2004)	+ (Tao et al. 2016)	+ (Tao et al. 2016)	+ (Garcia et al. 2009, Yin et al.2016)	Avoidance and GABAergic signaling changes in PFC (Shepard et al. 2016)
INESCAPABLE FOOT/TAIL SHOCK	Electric shocks (1-20 shocks of 0.3-1.5 mA for 0.5-10 s)	General avoidance, increased fear learning and deficits in fear extinction (Toth and Neumann 2013)	+ (Milad and Quirk 2002)	Not reported	+ (Golub et al. 2011)	+ (Cheng et al. 2015)	+ (Cheng et al. 2018)	+ (Bentefour et al. 2016)	Avoidance of trauma-specific cues (Diehl et al. 2007)
PREDATOR EXPOSURE	Direct predator exposure or to predator scent	General avoidance, exaggerated fear response and deficits in fear extinction (Seetharaman et al. 2016, Cohen et al. 2010)	+ (Wilson et al. 2010)	+ (Cohen et al. 2010)	+ (Wilson et al. 2010)	−	+ (Wilson et al. 2013, Deslauriers et al.2017)	−	Avoidance, memory deficits and enhanced basal CORT levels (Mazor et al. 2009)
SINGLE PROLONGED STRESS (SPS)	3 stressors in succession: - restraint (2h), - forced swimming (20min), - diethyl ether (until lost consciousness)	Avoidance, cue-induced fear (Le Dorze and Gisquet-Verrier 2016)	+ (Perrine et al. 2016)	+ (Liberzon et al. 1999)	+ (Han et al. 2013)	Not reported	+ (Lee et al. 2016)	−	Not reported
SOCIAL DEFEAT STRESS (SDS)	Resident-intruder interaction repeated for 5-21 consecutive days	General avoidance (Warren et al 2013)	+ (Patel et al 2018)	−	+ (Patel et al 2018)	+ (Reader et al 2015)	+ (Reader et al 2015)	−	Anxiety-related behaviors (Jacobson-Pick et al. 2013)
129S1/SvlmJ STRAIN	129 mouse substrain	Impaired fear extinction (Camp et al 2009)	+ (O'Connor et al 2009)	Not reported	+ (O'Connor et al 2009)	Not reported	Not reported	Not reported	Anxiety-related behaviors (Hadicke and Engelmann 2013)
SOCIAL ISOLATION	Almost 3-4 weeks of social isolation in individual cages	Avoidance, aggression, enhanced contextual fear and impaired fear extinction (Pinna et al 2003, Pibiri et al. 2008)	+ (Pinna et al. 2008)	+ (Malkesman et al. 2016)	+ (Agis-Balboa et al. 2007)	Not reported	Not reported	+ (Pinna et al. 2009)	Depression-like behavior and reduced contextual fear-conditioning (Weiss et al. 2004)

Behavioral and neurobiological phenotypes of rodent models of post-traumatic stress disorder (PTSD). A summary of the core neurobiological features that relate to the PTSD phenotype for each animal model. Plus (+) symbols indicate phenotypes reported for each PTSD rodent model, minus (−) symbols indicate that the results on the specific phenotype are still unclear or the results vary across the laboratories. 'Not reported' indicates that the phenotypes have not been yet investigated. In the last column are reported the main findings in females for each model. AMY, amygdala; CNS, central nervous system; CORT, corticosterone; HPA, hypothalamic–pituitary–adrenal; PFC, prefrontal cortex; SSRIs, selective serotonin reuptake inhibitors.

1 h/day repeated for 3 and up to 40 days (Gameiro *et al.*, 2006). The different protocols of restraint across laboratories (e.g. chronic or acute stress, repeated or single exposure) make it difficult to compare findings. This procedure induces a strong activation of the hypothalamic–pituitary–adrenal (HPA) axis, but the validity of mimicking PTSD-related behavior or neurobiology is limited and still unclear (Risbrough *et al.*, 2016). As in PTSD patients (Yehuda *et al.*, 2015a, 2015b), a 20-min reminder of restraint after 1, 7, or 13 days from the first restraint stress, induces a hypoactivation of the HPA axis with decreased levels of corticosterone and of adrenocorticotropic hormone (ACTH; Harvey *et al.*, 2006). This form of stress is more frequently used to induce depression-like behavior and anhedonia (Chiba *et al.*, 2012; Xu *et al.*, 2017). Interestingly, mice subjected to 24-h-restraint stress do not show anxiety, but only depression-like behavior, which can be improved by administering an SSRI (Chu *et al.*, 2016). The cannabinoid receptor type 1 (CB_1) activation using the selective agonist arachidonyl-2′-chloroethylamide had neuroprotective effects on restrained mice probably due to a modulation of the anti-inflammatory response (Zoppi *et al.*, 2011). Using the restraint stress model in conjunction with other stressful paradigms [e.g. forced swimming test (FST)], the first (restraint) enhances the sensitization to the second stressor (Liberzon *et al.*, 1997). This sensitization effect is intriguing because the restraint stress enhances the HPA negative feedback, which is a core feature of PTSD. Furthermore, this model causes opposite patterns of dendritic remodeling, enhancing dendritic arborization of the basolateral amygdala (BLA) pyramidal-like and stellate neurons and inducing dendritic atrophy in the hippocampus CA3 pyramidal neurons (Vyas *et al.*, 2002). The connectivity abnormalities in these two brain regions mimic the alteration reported in PTSD patients (Zhu *et al.*, 2018) and are responsible for the deficits in fear extinction, which are specific hallmark behavioral traits of PTSD (Risbrough *et al.*, 2016).

The unpredictable variable stress
A common model to mimic depression-like behavior, the unpredictable variable stress (UVS; Fig. 1), appears adequate to reproduce some forms of PTSD behavioral phenotypes and, in this model, behavioral dysfunctions can also be improved by SSRIs or ketamine treatment (Garcia *et al.*, 2009; Yin *et al.*, 2016). The experimental procedure consists in the exposure to various stressors over a period of 1–8 weeks and this is thought to reflect the unpredictable stress often experienced by members of the armed service (Wakizono *et al.*, 2007; Goswami *et al.*, 2013; Shepard *et al.*, 2016). Although it induces some degree of PTSD phenotypes, this model has low reproducibility and fails to induce the avoidance of trauma-specific cues which is a fundamental behavioral deficit related to PTSD, probably because of the use of several stressors. At a neurobiological level, this experimental procedure induces increased negative feedback of the HPA axis after stress (Yehuda *et al.*, 2015a, 2015b), mimicking the

alterations observed in PTSD patients, and produces volume deficits of the hippocampus (Isgor *et al.*, 2004). The chronic stress induced by this model down-regulates CB_1 expression and decreases the endocannabinoid, 2-arachidonoylglycerol (2-AG) in the hippocampus (Hill *et al.*, 2005). Furthermore, it has been reported that UVS enhanced levels of proinflammatory cytokines [interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF-α)], which are important biomarker candidates for PTSD and are strongly related to its symptoms (von Känel *et al.*, 2007; Tao *et al.*, 2016). However, after suspension of stress, the basal plasma glucocorticoid levels of animals subjected to UVS are increased, while, generally, PTSD patients show low to unchanged peripheral basal glucocorticoid levels (Algamal *et al.*, 2018). The elevated basal plasma glucocorticoid levels are features of other disorders, such as depression and generalized anxiety disorder (Pariante and Miller, 2001; Fischer and Cleare, 2017). Nevertheless, given the high comorbidity of PTSD with depression or generalized anxiety disorder, this model may be more appropriate to mimic deficits that encompass subpopulations of PTSD patients with high comorbidity with these disorders.

The inescapable shock model
One of the most common PTSD animal models, the inescapable shock (to the feet or tail), is based on an unpredictable and unexpected single stress exposure (Fig. 1). The electric foot or tailshock model, based on the administration of one or more electric shocks for few seconds (0.5–10 s), is widely used as a rodent fear paradigm to investigate stress responses and fear learning (Pryce *et al.*, 2011). The inescapable shock model is often used in combination with additional stressors, such as restraint (Nagata *et al.*, 2009) or corticosterone injection (Hui *et al.*, 2004). The use of various protocols complicates the comparison of the results of these studies. For example, the HPA axis adaptation to footshock is different if the intensities of the shock vary (Rabasa *et al.*, 2011). Furthermore, cell proliferation in the hippocampus failed to be affected by a single footshock exposure, while a chronic procedure changes the brain plasticity, suppressing hippocampal cell proliferation (Dagytė *et al.*, 2009). Thus, the characteristics of the procedure or the administration of additional stressors influence the results, leading to alterations that are not seen with other procedures, such as the adaptation to stress (Bali and Jaggi, 2015). Even with a single exposure, electric footshock triggers PTSD-like phenotypes, including hyperarousal, avoidance, and hippocampal-dependent memory deficits (Golub *et al.*, 2009; Philbert *et al.*, 2012; Toth and Neumann, 2013). Animals also show abnormalities of fear learning with deficits in fear extinction (Desmedt *et al.*, 2015). The use of SSRIs, including paroxetine, reduces some behavioral deficits, such as generalized avoidance and prevents their reactivation (Pryce *et al.*, 2011; Bentefour *et al.*, 2016). The administration of the CB1 agonist, WIN 55 212-22 (WIN) prevents fear extinction impairments following situational reminders, by preventing altered CB_1 expression in the hippocampus and prefrontal cortex (Korem and Akirav, 2014). Furthermore, this paradigm

induces a reduction of hippocampal volume and enhances neuronal activity in the prefrontal cortex (Milad and Quirk, 2002; Golub *et al.*, 2011). Mice subjected to foot shocks show a greater level of cytokines both in plasma and in the brain (Cheng *et al.*, 2015, 2018).

Both the footshock and tailshock models have very limited data on females. The footshock model produces avoidance of trauma-related cues, but not general avoidance, and alterations of corticosterone level in the plasma in females (Diehl *et al.*, 2007). Moreover, only females exhibit enhanced negative feedback of the HPA axis response to stress, which is not observed in males (Louvart *et al.*, 2006). This finding shows that males subjected to the footshock procedure fail to mimic a core physiological alteration of PTSD, that is, the low cortisol levels associated with the increased negative feedback.

The predator-stress model

The predator-stress model comprises the exposure of the rodents to a potential predator or to a predator scent (Wilson *et al.*, 2014a, 2014b). Animals exposed to this stressor show enhanced negative feedback of the HPA axis, increased activity of the amygdala and altered levels of neurotransmitters, such as serotonin and norepinephrine, in both hippocampus and prefrontal cortex (Wilson *et al.*, 2014a, 2014b; Zoladz *et al.*, 2015; Dengler *et al.*, 2018). Moreover, the CB_1 receptor mRNA expression is downregulated in the frontal cortex and in the amygdaloid complex, promoting the anxiogenic effects (Campos *et al.*, 2013). This procedure also produces an increase in proinflammatory cytokines IL-1β, IL-6, and TNF-α, and a decrease in the anti-inflammatory cytokine IL-10 in the hippocampus and frontal cortex of the rodent brain (Wilson *et al.*, 2013; Deslauriers *et al.*, 2017). At a behavioral level, the exposure to predator-related stress evokes hyperarousal, avoidance, exaggerated fear responses, and reduces fear extinction (Cohen *et al.*, 2010; Zoladz *et al.*, 2015; Seetharaman *et al.*, 2016). The predator-stress model increases anxiety-like behavior measured by several behavioral tests, including the elevated plus maze, the light-dark box, and the social interaction (Adamec *et al.*, 2005). The response to stress appears to be graded, with more pronounced effects due to the direct exposure to a predator, and intermediate effects after exposure to predator scent or to the testing room context. This effect on anxiety is also found in individuals affected by PTSD, who exhibit graded responses to stress severity (Adamec *et al.*, 2004). This model is also sensitive to the SSRI, sertraline, and to the selective serotonin reuptake enhancer, tianeptine, which reduce anxiety-related behaviors and cue avoidance (Zoladz *et al.*, 2013; Wilson *et al.*, 2014a, 2014b).

This model is associated with weakness linked to poor reproducibility across laboratories, mostly as a result of the various paradigms used, including the severity of the protocol chosen (e.g. physical contact with predator vs. scent exposure only) or the species and the strain of rodent selected. Moreover, some protocols require a secondary exposure to the stressors to increase their efficacy, which, again, makes it more difficult to compare findings (Zoladz *et al.*, 2015; Kim *et al.*, 2017).

The predator exposure model is very limited in studies in females. The only few studies that compared the effects on sex differences showed divergent results on males and females. Females are more susceptible to the predator-stress paradigm than males, but only for the magnitude of the response to it, not for the prevalence of the behavioral alterations. Females show compromised memory performance in the Morris water-maze, avoidance and enhanced level of basal corticosterone (Mazor *et al.*, 2009; summarized in Fig. 1).

The single prolonged stress model

Liberzon *et al.* (1997) developed another widely used PTSD rodent model: the SPS (Fig. 1). This model consists of the exposure of the rodents to three stressors in succession: restraint-immobilization stress (2 h), forced swimming (20 min), and exposure to diethyl ether until loss of consciousness. This protocol is claimed to mimic the physiological and endocrine stress challenges of PTSD (Takahashi *et al.*, 2006). The administration of cortisol suppresses ACTH levels and enhances the HPA negative feedback (Liberzon *et al.*, 1999), which was attributed to an increased glucocorticoid receptor (GR) expression in the prefrontal cortex and hippocampus (George *et al.*, 2015). However, this effect on the HPA axis is evident only 7 days after the stress exposure, while it is not present 1 day poststress (Liberzon *et al.*, 1999). Cue-conditioned fear and extinction are unaffected by SPS after a fear conditioning test, but this model shows impairments in the retention of extinction (George *et al.*, 2015). Furthermore, other investigators found that the SPS procedure induces behavioral abnormalities that mimic PTSD symptoms, including hyperarousal and enhanced contextual freezing (Imanaka *et al.*, 2006; Yamamoto *et al.*, 2009). In the hippocampus, SPS enhanced the levels of TNF-α and IL-1β (Lee *et al.*, 2016). SPS also induces morphological changes in rodent brain, including apoptotic volume loss in the hippocampus, and in the dorsal raphe nucleus (Liu *et al.*, 2012a, 2012b; Han *et al.*, 2013), which may be comparable with the decreased volume of these regions in PTSD patients (Gilbertson *et al.*, 2002; Liu *et al.*, 2012a, 2012b).

The administration of WIN improves the impaired fear extinction of rats subjected to SPS (Ganon-Elazar and Akirav, 2012). The exposure to the stressors increases the cue-induced fear, which is attenuated by paroxetine (Perrine *et al.*, 2016). However, the results on SSRI effects are conflicting, because even the same SSRI, that is, paroxetine, improves behavioral deficits in some SPS models (Perrine *et al.*, 2016), but fails in others (Takahashi *et al.*, 2006). These discrepant results may be due to the differences in the design of the SPS, in the route of drug administration or in the behavioral test used in the studies (cue vs. contextual fear). However, SSRIs are ineffective in a portion of PTSD patients (Stein *et al.*,

2002) and they may be more successful in treating an individual with PTSD and comorbid anxiety or depression (Flandreau and Toth, 2018). This intriguing finding is supported by a recent discovery of Lin *et al.* (2016), who showed that the SSRI escitalopram reduced avoidance and depressive-like symptoms, but fails to improve fear extinction deficits. Importantly, animals exposed to SPS also show a different vulnerability, with more resistant or vulnerable rodents that differ in their reactivity to the trauma-related cues (Le Dorze and Gisquet-Verrier, 2016). The presence of these two subpopulations strengthens the validity of the SPS as an animal model of PTSD because it more closely reflects the human condition, with only a portion of the population developing the neuropathology as a maladaptive response to the trauma (Breslau, 2009).

The social defeat stress model
The social defeat stress (SDS) model is typically performed in male rodents using a resident-intruder protocol (Fig. 1). The resident-intruder interaction includes aggressive behavior and social stress to the intruder (Bjorkqvist, 2001; Hammels *et al.*, 2015). SDS induces an increase in social avoidance and produces some relevant behavioral outcomes linked to PTSD, such as hyperarousal, anhedonia, and impairment in the reward system (Warren *et al.*, 2013; Der-Avakian *et al.*, 2014). Repeated SDS leads to alterations of plasticity in the amygdala and hippocampus (Patel *et al.*, 2018). Moreover, the activation of the HPA axis induced by the experimental paradigm is linked to the enhanced levels of proinflammatory cytokines in both plasma and brain regions, such as the prefrontal cortex, the paraventricular nucleus of the hypothalamus and the amygdala (Reader *et al.*, 2015). The SDS model induces prolonged activation of the HPA axis, with increased plasma concentrations of ACTH and corticosterone, and enhanced CRH mRNA expression in the paraventricular nucleus (Keeney *et al.*, 2006). This HPA axis abnormality is relevant to the pathological alterations observed in other disorders, such as in clinical depression, but questions arise regarding the face validity of the model for PTSD. The administration of the CB_1 antagonist, rimonabant before the social stress sessions increased the freezing response of mice during cued fear recall tests, suggesting a fundamental role of the release of endocannabinoids during stress exposure (Dubreucq *et al.*, 2012). One important limitation of the repeated exposure to a predictable stressor is that it fails to induce a deficit in fear extinction, which is a core feature of PTSD (Page *et al.*, 2016).

Moreover, the inclusion of females in this paradigm is very problematic because of their less aggressive interactions with intruders in the home cage (Hollis and Kabbaj, 2014). In an attempt to solve this problem, older, lactating females have been used as residents to elicit aggressive behavior. As a result, pronounced anxiety-related behavioral effects appeared only 2 weeks after the stressor exposure (Jacobson-Pick *et al.*, 2013). Although several aggressive behavioral traits displayed by the two sexes are similar, the neural mechanisms underlying them may be different (Terranova *et al.*, 2017). Indeed, the aggression shown by lactating female residents toward intruders may not be of the same type as shown by resident males. Maternal aggression includes both offensive and defensive attack patterns, while resident males attack in defense of an individual's own integrity and territory, with only defensive aggression (Lonstein and Gammie, 2002). Moreover, the limitation due to the estrus cycle conditions and the inevitable presence of pups makes the procedure more demanding and complicates the feasibility of exposing females to the resident-intruder protocol (Palanza *et al.*, 2001).

The 129S1/SvlmJ strain model
Over the past decade, Holmes and colleagues observed the poor fear extinction profile of 129S1/SvlmJ mice in comparison to C57/B6 mice (Camp *et al.*, 2009). This mouse strain shows an impaired fear extinction, providing a valuable approach to modeling a 'genetic' preclinical model for several psychiatric disorders, including PTSD (Hefner *et al.*, 2008). Such a model provides an opportunity to investigate molecular and genetic mechanisms underlying fear extinction, focusing on the possible genetic contribution to individual vulnerability and predisposition to PTSD. The 129S1/SvlmJ mouse shows perturbations in autonomic and neuroendocrine parameters, with higher corticosterone levels and lower GR mRNA expression, besides behavioral abnormalities (Camp *et al.*, 2012). In the open field test and in the elevated plus maze, these animals show increased anxiety-like behaviors and reduced exploratory locomotor activity as compared with C57/B6 (Millstein and Holmes, 2007). Moreover, this mouse strain exhibits a tendency to overgeneralize fear of ambiguous environmental cues, and difficulties in inhibiting fear when safety signals are presented (Camp *et al.*, 2012). The behavioral abnormalities have been linked to HPA axis perturbations with a significant loss of GR-mediated negative feedback regulation. Furthermore, the characteristic impaired fear extinction has been associated with a perturbation in the activation of the prefrontal cortex–amygdala circuit, a significant trait of PTSD (Whittle *et al.*, 2010). This model also provides a suitable tool to study the effect of drugs to facilitate fear extinction (Holmes and Quirk, 2010). The SSRI antidepressant fluoxetine rescued the fear phenotype and decreased the contextual fear overgeneralization of the 129S1/SvlmJ mice (Camp *et al.*, 2012). This model can be useful to investigate the genetic predisposition to develop PTSD or other anxiety-related disorders, highlighting the possible role of the interaction between genes and environment.

The social isolation model
By exposing rodents to a protracted and, probably, severe stressor, social isolation (SI) offers a putative animal model to investigate the development of vulnerability to PTSD. In rodents, SI can be considered a distressing event that induces behavioral deficits, even though the length of isolation varies among several laboratories. In our laboratory, this manipulation comprises the isolation of the animals in

individual cages for 3–4 weeks, which results in a time-dependent increase of anxiety-like and aggressive behavior (Pinna et al., 2003; Rau et al., 2005; Pibiri et al., 2008; discussed in Locci and Pinna, 2019a). Consistently, in humans, perceived SI or loneliness is associated with increased mortality and morbidity with physical, neurological, and psychological dysfunctions, including Alzheimer's disease, major depression, anxiety spectrum disorders, and suicidality (Cacioppo and Cacioppo, 2016). Several lines of evidence showed that social bonds and social supports contribute to regulation of emotions under conditions of traumatic stress, emphasizing the role of the emotional component as a risk factor for PTSD (Nemeroff et al., 2006; Charuvastra and Cloitre, 2008; Mehnert et al., 2010). The inability of an individual to manage emotional memories appropriately is seen as the leading cause of the development of symptoms of avoidance, re-experiencing, and hypervigilance (Cahill et al., 2003; Rothbaum and Davis, 2003; Pitman et al., 2006; Rauch et al., 2006). The individual housing of rodents is a chronic stressful condition that increases the vulnerability to behavioral deficits following stressful acute events, such as the electric shocks administered as part of the fear conditioning test. This highlights how social factors in rodents may alter coping abilities to overcome stress or, alternatively, the predisposition to develop PTSD (Charuvastra and Cloitre 2008; Pinna, 2010). However, some reports also contradict the appropriateness of SI in mice as a stressful environmental condition to induce behavioral dysfunction, including aggressive behavior (Brain, 1975).

In our laboratory, using a Pavlovian fear conditioning test, during which conditioned (i.e. acoustic tone) and unconditioned (i.e. footshock) stimuli are administered to the mice in a novel context, the animals show an enhanced freezing time 24 h after the training session (Pibiri et al., 2008; Pinna et al., 2008). In socially isolated mice, the response to the fear conditioning, as for aggressive behavior, increases during the 4 weeks of SI reaching a plateau after 4 weeks (Pibiri et al., 2008). Interestingly, during the contextual fear extinction, socially isolated mice failed to attain the low levels of group-housed control animals, suggesting an incomplete fear extinction due to the SI procedure (Pibiri et al., 2008). Thus, SI in rodents may mimic the chronic stress seen in patients before the exposure to a precipitating traumatic event, which leads to developing PTSD. This is further substantiated by the enhanced emotional reactivity induced by the protracted SI in rodents with an increased anxiety-like behavior, which is reminiscent of the behavioral traits observed in PTSD subjects after the re-exposure to events that remind the trauma (Grillon and Morgan, 1999; Rauch et al., 2006). Socially isolated rodents exhibit an HPA hyporesponsiveness, with lower levels of corticosterone and reduced release of CRH into the hypophyseal portal system (Sanchez et al., 1998; Chida et al., 2005; Malkesman et al., 2006). The hypofunction of the HPA axis is particularly evident after the exposure of socially isolated animals to an acute stressor, suggesting a reduced sensitization of the axis to stressful stimuli (Sanchez et al., 1998).

As in the PTSD models reviewed above, the SI model is mainly used to study PTSD-like behavior in male mice. The study of female mice exposed to SI is very limited and is mostly used to study depression-like behaviors. Furthermore, it is more commonly investigated in prairie voles and rats, rather than in mice (Weiss et al., 2004; Grippo et al., 2007).

Nevertheless, this model is of particular interest because, as in PTSD patients, the emotional alterations observed in the socially isolated rodent model can be associated with the down-regulation of GABAergic neurotransmission (Guidotti et al., 2001; Matsumoto et al., 2007). In the cortex and hippocampus, this prolonged stressor induces an alteration in the subunit expression of $GABA_A$ receptors, with a decrease in α_1, α_2, and γ_2 subunits expression and an increase in α_4, α_5, and δ subunits (Pinna et al., 2006; Serra et al., 2008). The α_4 and δ subunits are primarily expressed in the extrasynaptic $GABA_A$ receptors, which show an increased sensitivity for neuroactive steroids (Locci and Pinna, 2017). However, the $GABA_A$ receptor conformation including α_4, α_6, in combination with δ is benzodiazepine-insensitive. Thus, stress-induced remodeling of $GABA_A$ receptor subunit composition results in a lack of efficacy of pharmacological actions of benzodiazepines. The benzodiazepine inefficacy due to decreased benzodiazepine binding sites (Pinna et al., 2006) is in strong agreement with the dysfunctions observed in the cortex, hippocampus, and thalamus of PTSD patients (Geuze et al., 2008). The principal PTSD-like phenotypes recapitulated by SI are described in Fig. 1.

In socially isolated mice, the down-regulation of GABAergic neurotransmission is likely the result of decreased levels of allopregnanolone (Allo) in selective neuronal populations of the prefrontal cortex, hippocampus and BLA (Pinna et al., 2008; 2009; Agis-Balboa et al., 2007). Allo is an endogenous neurosteroid and a potent, positive, allosteric modulator of the GABA action at $GABA_A$ receptors (Belelli and Lambert, 2005; Belelli et al., 2018). Specifically, socially isolated mice show a decreased expression of Allo in the pyramidal glutamatergic neurons of frontal cortex layer V/VI and hippocampus, and in the pyramidal-like neurons of the BLA. These pyramidal neurons regulate the inhibitory function of intercalated inhibitory spiny GABAergic interneurons (ITC) of the central amygdaloid nucleus (CeA; Agis-Balboa et al., 2007). It is intriguing that PTSD patients show characteristic exaggerated amygdala hyperactivity because of deficits in the function of the prefrontal cortex and hippocampus (Akirav and Maroun, 2007). The glutamatergic neurons from the prefrontal cortex and hippocampus synapse on the amygdala GABAergic neurons, providing an inhibitory input to the amygdala. The GABAergic neurotransmission of the amygdala plays a fundamental role in the control of the emotional response to stress and can influence fear extinction learning.

During normal conditions, fear suppression is achieved by the activation of the prefrontal cortex and hippocampus that directly inhibit the hyperactivity of the amygdala. However, the exposure to a stressful experience can impair this cortical inhibitory activity on the amygdala, resulting in its inappropriate hyperactivity, and exaggerated fear response, as observed in PTSD patients (Liberzon and Sripada. 2008). The amygdala complex contains islands of GABAergic interneurons that inhibit the output of the central nucleus, the main output station of the amygdala that mediates conditioned fear responses (Fig. 2; Likhtik et al., 2008). Lesions of ITC neurons impaired fear extinction, while their activation facilitated it (Jungling et al., 2008; Likhtik et al., 2008). Moreover, stimulation of the infralimbic cortex increases the immediate-early gene expression in ITC neurons (Berretta et al., 2005), which reduces the excitability of CeA neurons

Fig. 2
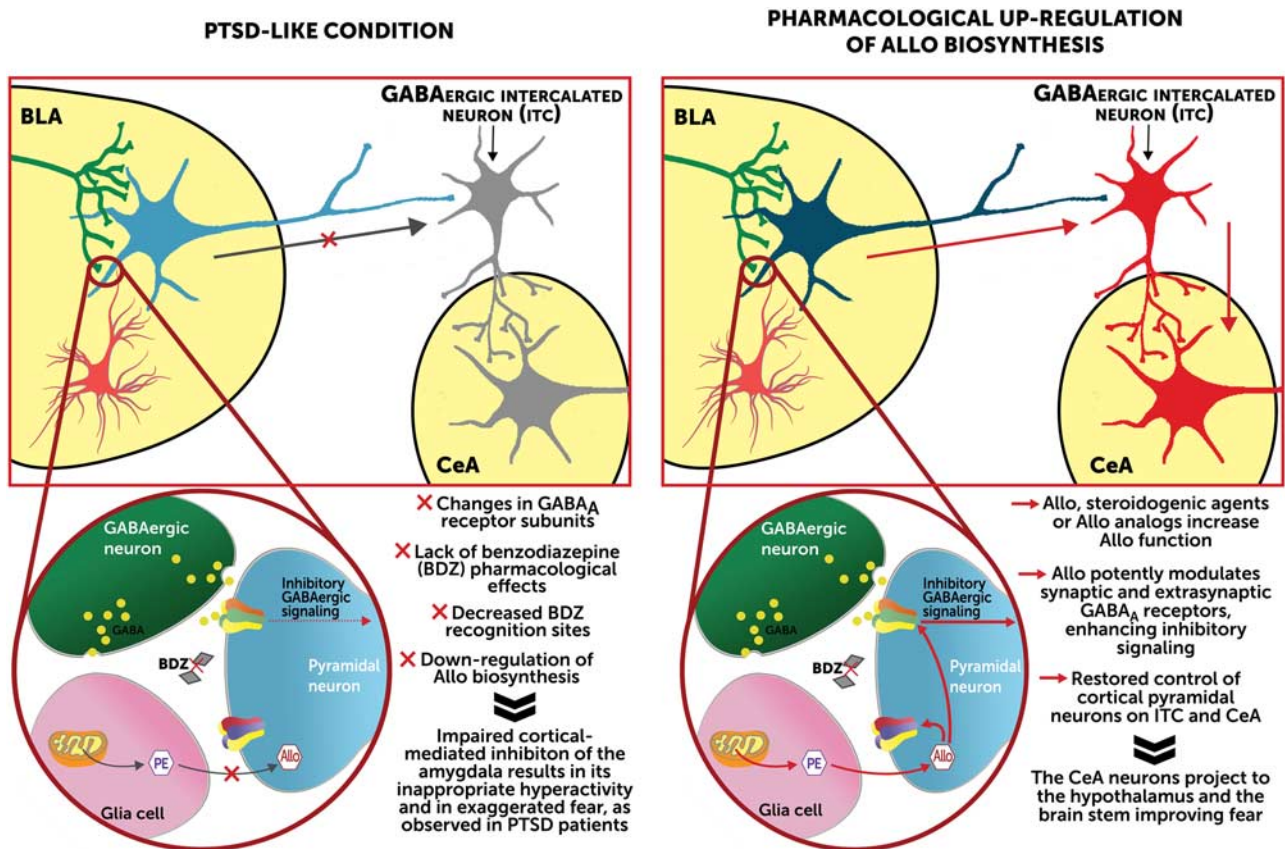
Regulation of GABAergic signaling by allopregnanolone (Allo) and effects on post-traumatic stress disorder (PTSD)-like behavior. Fear suppression is regulated by the prefrontal cortex and hippocampus, which project directly to the amygdaloid nuclei to inhibit their hyperactivity during stressful events (Herry et al., 2008). However, in susceptible individuals, the exposure to a stressful experience may impair this cortical inhibitory activity on the amygdala, resulting in its inappropriate hyperactivity and to exaggerated fear responses, as observed in PTSD patients (Akirav and Maroun, 2007). Indeed, in PTSD, the amygdala hyperactivity participates in the abnormal emotional processing that induces behavioral alterations after the exposure to a traumatic event. Patients with PTSD express a lower release of GABA (Vaiva et al., 2004), decreased plasma and cerebrospinal fluid Allo concentrations (Rasmusson et al., 2006, 2019), and changes in GABA$_A$ receptors subunit expressions, which is the cause of lower cortical and hippocampal benzodiazepine recognition sites and decreased benzodiazepine-induced pharmacological effects (Geuze et al., 2008). These alterations are important neurobiological underpinnings that may explain the appearance of PTSD symptoms in subjects who fail to develop resilience following exposure to traumatic events (Pinna, 2018; Pinna and Izumi, 2018). Animal studies, using socially isolated mice, a putative rodent stress model of PTSD-like behavioral deficits, have revealed that cortical and hippocampal projections to the basolateral amygdala (BLA) express decreased Allo biosynthesis, which is associated with behavioral deficits, including exaggerated fear responses and impaired fear extinction (depicted in the left panel) (Agis-Balboa et al., 2007; Pinna et al., 2009). In socially isolated mice, the decreased expression of Allo in the pyramidal glutamatergic neurons of frontal cortex, hippocampus, and in the pyramidal-like neurons of the BLA may represent the molecular mechanisms underlying an increased excitability of the pathway converging on the intercalated neurons and CeA GABAergic spiny neurons (Agis-Balboa et al., 2007; Pinna et al., 2008). This may result in an inhibition of the GABAergic output from the central amygdaloid nucleus to the hypothalamus and brainstem, brain regions involved in the expression of fear and aggression (reviewed in Pinna et al., 2008, 2009). Thus, a selective reduction in Allo levels in these glutamatergic neurons could impair the function of cortico-hippocampal-amygdaloid circuits and explain the excessive fear of socially isolated mice. Decreasing amygdala hyperactivity may be a primary effect of agents that reduce exaggerated fear responses (depicted in the right panel). Pharmacological interventions by acting on the enzymes that synthesize Allo or direct administration of Allo or its synthetic analogs enhance Allo function in cortical and hippocampal pyramidal neurons by stimulating synaptic, but mostly, extrasynaptic GABA$_A$ receptors, which, under stress, become hypersensitive to neurosteroids (Pinna, 2014; Locci and Pinna, 2017). The enhanced modulation of the inhibitory GABAergic signaling may regulate emotional processing, improving the behavioral deficits shown by PTSD patients (Locci and Pinna, 2017). Allo, allopregnanolone; CeA, central amygdaloid nucleus; ITC, interneuron.

(Quirk *et al.*, 2003) and decreases conditioned-induced freezing (Milad *et al.*, 2004). Thus, the prefrontal cortex regulates fear expression by directing the switching on and off of the ITC neurons in the amygdala (Pare *et al.*, 2004). Conversely, infralimbic and prelimbic cortex receive inputs from several regions that modulate fear responses, including the amygdala (Herry *et al.*, 2008). The amygdala itself may engage those brain regions to regulate the output of the amygdala (Laviolette *et al.*, 2005; Floresco and Tse, 2007). Furthermore, the ITC GABAergic neurons project to several brain regions, which include the brainstem and hypothalamus and influence the intensity of emotional responses to environmental stimuli (Pinna *et al.*, 2009). Thus, the corticolimbic circuits, involving prefrontal cortex, hippocampus, and amygdala, are responsible for the regulation of several emotional behaviors and encompass several circuits involved in the regulation of aggressiveness, fear responses, impulsivity, and anxiety disorders (Fig. 2; LeDoux, 2000; Milad *et al.*, 2007).

Collectively, the protracted SI model mimics some relevant neurobiological and behavioral alterations observed in PTSD patients (summarized in Fig. 2). Therefore, this model is valuable for testing the efficacy of new therapeutic approaches for PTSD.

The impact of neurosteroid biosynthesis on mood disorders

Neurosteroids are implicated in the regulation of several behavioral, neuroendocrine, and metabolic processes, such as food intake, impulsivity, and fear. Their biosynthesis, including the enzymes responsible for their production, are involved in the neuropathophysiology of mood disorders (Agis-Balboa *et al.*, 2014; Rasmusson *et al.*, 2017, 2018a, 2019). The cascade of enzymatic reactions that leads to their biosynthesis begins with the cleavage of cholesterol by the cytochrome P450$_{scc}$, a rate-limiting mitochondrial enzyme. Pregnenolone, the main precursor of neurosteroids, is further metabolized into progesterone, and its metabolites, which are produced and accumulate in the mammalian brain, are the best-known neurosteroids (Do Rego *et al.*, 2009). In the brain, progesterone is a substrate for the enzyme 5α-reductase type I (5α-RI), that converts it into 5α-dihydroprogesterone (5α-DHP), and this is further metabolized into Allo by the enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD; Wang, 2011).

In different regions of the brain, the concentrations of neurosteroids vary widely (Pibiri *et al.*, 2008). In particular, the glutamatergic neurons of the corticolimbic circuits, which include cortical and hippocampal pyramidal neurons and pyramidal-like neurons of the BLA, synthetize Allo and its equipotent GABAergic stereoisomer, pregnanolone (PA; Agis-Balboa *et al.*, 2006, 2007). These neuronal populations highly express GABA$_A$ receptors, and Allo and PA produced locally permit the fine-tuning of the receptor for GABAmimetics, agonists, and positive allosteric modulators (Pinna *et al.*, 2000; Belelli *et al.*, 2005). By

this mechanism, Allo and PA also regulate emotional behavior (Agis-Balboa *et al.*, 2007; Pinna *et al.*, 2008; Pinna, 2018). Importantly, the neurophysiological effects exerted by neurosteroids acting at the GABA$_A$ receptor are susceptible to variations in the receptor subunit composition (reviewed in Locci and Pinna, 2017), which influences the pharmacological profile of the receptor (Lambert *et al.*, 2001). For example, benzodiazepines have no pharmacological effect on a GABA$_A$ receptor subunit composition that fails to include γ$_2$ subunit, while changes in α$_4$ or δ subunit expression alter the sensitivity of the receptor to Allo (Turkmen *et al.*, 2011). Furthermore, the sulfated counterparts of these GABAergic neurosteroids, such as PA sulfate, are responsible for tonic inhibition of *N*-methyl-D-aspartic acid-mediated neurotransmission, which triggers important neuroprotective effects (Vyklicky *et al.*, 2016).

Several studies have reported lower levels of Allo and PA and/or their biosynthetic enzyme expression in serum, plasma, cerebrospinal fluid (CSF), and brain of patients with mood disorders (Romeo *et al.*, 1998, Uzunova *et al.*, 1998; van Broekhoven and Verkes, 2003). Allo concentrations are reduced in patients with unipolar major depression and premenopausal women with PTSD (Uzunova *et al.*, 1998). The comorbidity with depression is linked to a more severe decrease of Allo levels in PTSD patients (Rasmusson *et al.*, 2006). Moreover, comorbidity of depression and anxiety in patients with anorexia and obesity was also found to be associated with a reduction of blood Allo levels (Dichtel *et al.*, 2018). The levels of these neurosteroids are also altered in other neuropathologies, such as drug addiction and postpartum depression (Grobin *et al.*, 2005; Osborne *et al.*, 2017).

In PTSD and depression, the down-regulation of neurosteroid biosynthesis includes the reduction of Allo and PA concentrations, and most importantly, of the expression and, possibly, the function of their biosynthetic enzymes, 5α-RI, and 3α-HSD (Zorumski and Mennerick, 2013; Locci and Pinna, 2017; Agis-Balboa *et al.*, 2014). These deficits were pointed out as possible contributors to a GABAergic neurotransmission dysfunction that underlies the development of PTSD symptoms and depressive disorders (Rupprecht, 2003; Pinna *et al.*, 2006; Pinna, 2018). Indeed, in the CSF and serum of women with PTSD, a reduction of Allo levels was observed in absence of changes of progesterone or 5α-DHP concentrations, which suggests a possible deficit in the function of the enzyme 3α-HSD (Rasmusson *et al.*, 2006; Pineles *et al.*, 2017, 2018). In male patients with PTSD, by contrast, the reduction of Allo in the CSF appears to be caused by deficits in 5α-RI, which are associated with a strong negative correlation with PTSD symptoms determined by total CAPS (Rasmusson *et al.*, 2018a, 2019). In accordance with this observation, in male patients with depression, a down-regulation in the expression of 5α-RI was observed in the prefrontal cortex Broadman Area 9 (Agis-Balboa *et al.*, 2014). Importantly, the concentration of Allo, its ratio in

relation to its neuroactive steroid precursors, and the deficits in the function and/or expression of their enzymatic pathways, suggest that these neurochemical alterations may be valuable sex-related biomarkers for PTSD. Collectively, these findings provide insights into the development of new appropriate therapeutic strategies for PTSD patients. Of note, several studies showed that SSRI treatments in depressed patients with lower CSF or plasma Allo concentrations lead to recovery from depressive symptoms by normalizing Allo levels (Romeo et al., 1998; Uzunova et al., 1998).

Preclinical studies on PTSD models show striking analogies with the neurosteroid alterations found in PTSD patients. In the SPS model, rodents show a down-regulation of serum and cortical Allo levels (Qiu et al., 2015). Likewise, the SI mouse model shows a reduction of Allo concentrations in corticolimbic neurons in association with a down-regulation in the expression of 5α-RI (Dong et al., 2001; Guidotti et al., 2001). It is also important to underline that the deficits in Allo biosynthesis in these rodent models correlate with an increase of aggressive behavior, enhanced contextual fear responses and impaired fear extinction, and anxiety-like behavior (Pibiri et al., 2008; Nin et al., 2011; Qiu et al., 2015; Xu et al., 2018).

Altogether, neurosteroid biosynthesis offers a promising neuronal target for the development of new drugs as well as provides a rich selection of various biomarker candidates for PTSD (Kemp et al., 2008).

Neurosteroid biosynthesis down-regulation: a target for new therapeutic approaches

Currently, PTSD remains a disorder with no specific pharmacological treatment. The only drugs presently approved by the Food and Drug Administration, the SSRIs, sertraline, and paroxetine have low efficacy ($\sim$50%). Several studies show a strong correlation between specific traumas and the pharmacological response of patients to antidepressant treatment (Prigerson et al., 2001; Pinna, 2015). Moreover, a history of early-life trauma predicts a failure to respond to antidepressants later in life. Thus, traumas in specific and susceptible periods of the PTSD patient's life can predict if they will respond successfully to treatment with SSRIs. Furthermore, veterans who suffer from PTSD are particularly resistant to SSRI treatment (Bernardy and Friedman, 2015).

The high resistance to SSRI antidepressants underscores the relevance to explore new approaches for nonresponsive patients. Preclinical studies have recently exploited various neurosteroidogenic targets for promising new PTSD agents (Rupprecht et al., 2009, 2010; Pinna, 2014). For example, one of the most studied targets to develop new neurosteroidogenic drugs is the 18 kDa translocator protein (TSPO), which is a prominent starting point to activate the neurosteroidogenic cascade that ultimately results in enhanced brain Allo concentrations (Schule et al., 2011). TSPO forms a complex with the steroidogenic acute regulatory protein (StAR), which allows the entry of cholesterol from the cytosol into the inner mitochondrial membrane (Papadopoulos et al., 2006). In the mitochondria, the enzyme cytochrome P450$_{scc}$ converts cholesterol into pregnenolone, the precursor for all neurosteroids (Fig. 3). Thus, drugs that stimulate TSPO activity, and increase the corticolimbic Allo concentrations, are emerging as potential approaches to facilitate recovery from anxiety and PTSD (Rupprecht et al., 2009). Administration of YL-IPA08, a selective TSPO ligand, to SPS rodents, enhances Allo levels in mouse hippocampus and cortex and reverses depressive behaviors and anhedonia by enhancing locomotor activity and the sucrose preference (Zhang et al., 2017). The administration of YL-IPA08 in animal models of PTSD also induces a powerful suppression of contextual fear responses and reduces anxiety-like behaviors by decreasing contextual freezing time (Zhang et al., 2014). Another drug targeting the TSPO receptor is etifoxine. By this mechanism, etifoxine improves behavior by inducing neurosteroid biosynthesis. After its administration, the enhancement of Allo concentrations correlates with a reduction of anxiety-like behavior displayed by the rats during the Vogel conflict test (Verleye et al., 2005). The anxiolytic effect exerted by inducing neurosteroidogenesis was confirmed by studies in which pretreatment with finasteride, a 5α-reductase inhibitor, suppresses the pharmacological effect of etifoxine (Schule et al., 2011). Furthermore, etifoxine has proven to improve anxiety-related disorders in humans (Choi and Kim, 2015). In addition to increasing neurosteroid levels by stimulating TSPO, etifoxine may exert a positive allosteric modulation of GABA$_A$ receptor after binding selective sites that are not a target for benzodiazepines (Poisbeau et al., 2018). Indeed, etifoxine exerts anxiolytic effects also by binding β$_2$ and β$_3$ subunits of the GABA$_A$ receptor complex (Poisbeau et al., 2018).

Moreover, the treatment with the precursor of Allo, pregnenolone improves emotional behavior by enhancing brain Allo concentrations. Pregnenolone administration reduces neural activity in the circuits associated with negative emotions, in particular, the amygdala and the insula (Stein et al., 2007; Sripada et al., 2013). The serum levels of pregnenolone and Allo are negatively correlated with the activation levels of these two brain areas. Furthermore, pregnenolone administration also increases the activity of the dorsal medial prefrontal cortex, a region that impacts the regulatory control over emotions (Ochsner and Gross, 2005), as well as the connectivity between the amygdala and the dorsal medial prefrontal cortex, which is associated with a reduction in self-reported anxiety and improvement of performance in emotion regulation tasks. Thus, the increased Allo levels that result from administration of pregnenolone are associated with reduced neuronal activity in regions that regulate negative emotions (Sripada et al., 2013).

The use of SSRIs at low nonserotonergic doses in SI mice produces an up-regulation of Allo concentrations with a reduction of anxiety-like behavior, fear responses, and aggression (Pibiri et al., 2008; Pinna et al., 2008). Hence, SSRIs by acting as *selective brain steroidogenic stimulants* (Pinna et al., 2003, 2006) increase Allo

Fig. 3
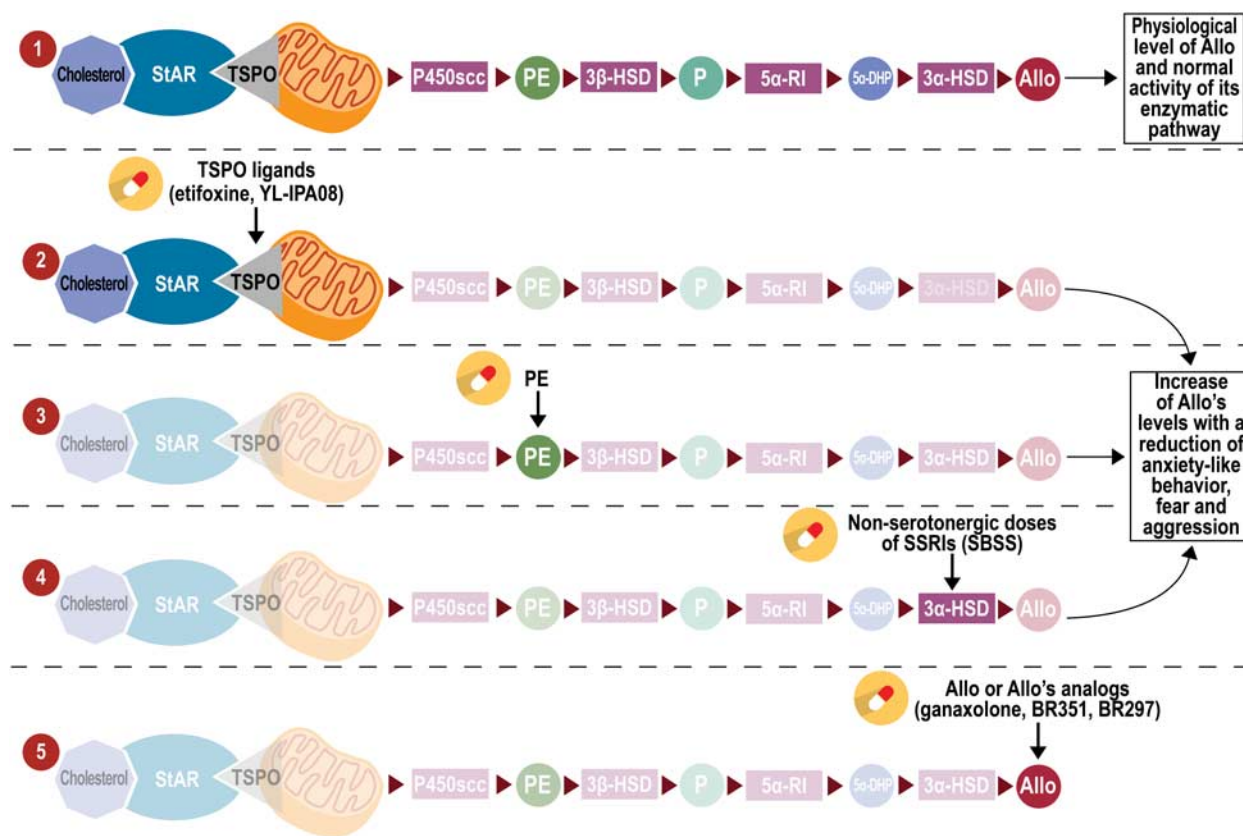
Pharmacological strategies to upregulate allopregnanolone (Allo) levels and improve emotional behavior. Allo biosynthesis is downregulated in association with behavioral deficits. (1) Schematic representation of the physiological pathway of Allo biosynthesis. (2) The reduced Allo levels are restored by administration of TSPO ligands (e.g. etifoxine), that, by enhancing cholesterol entry into the inner mitochondria membrane, stimulate neurosteroidogenesis. (3) The administration of the precursor of Allo, pregnenolone (PE) provides the substrate to restore the physiological concentrations of Allo levels and improve emotional behavior. (4) The use of the SSRIs at nonserotonergic doses increases Allo levels by acting as *selective brain steroidogenic stimulants* (SBSSs), acting on the enzyme 3α-HSD, which converts 5α-DHP into Allo. (5) The direct administration of Allo or of its analogs, such as ganaxolone, BR351 and BR297 recovers the behavioral deficits observed in stressed mice. Allo, allopregnanolone; CeA, central amygdaloid nucleus; 5α-DHP, 5α-dihydroprogesterone; 3α-HSD, 3α-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P, progesterone; P450scc, cytochrome P450 side-chain cleavage; PE, pregnenolone; StAR, steroidogenic acute regulatory protein; 5α-RI, 5α-reductase type I; SSRIs, selective serotonin reuptake inhibitors; TSPO, translocator protein 18 kDa.

concentrations and improve depression and anxiety symptoms in patients (Romeo *et al.*, 1998; Uzunova *et al.*, 1998). This treatment strategy employing SSRIs at low dosage could be useful to treat PTSD more efficaciously (discussed in Pinna, 2014, 2015; Locci and Pinna, 2017).

Several studies on animal models confirmed a role of Allo in improving behavioral deficits. In SI mice, the administration of Allo or its analogs, such as ganaxolone, BR351 or BR297, reduce behavioral abnormalities (Pinna and Rasmusson, 2014; Locci *et al.*, 2017). Ganaxolone exerts a strong dose-dependent anxiolytic effect and decreases aggression in socially isolated mice subjected to a same-sex resident-intruder protocol. Importantly, ganaxolone reduces contextual fear responses by blocking the reconsolidation processes, which prevents the spontaneous reemergence of contextual fear a week after a successful extinction of fear memory (Pinna and Rasmusson, 2014). Of note, recent phase 3 clinical trials in patients affected

by postpartum depression and depression, confirmed that intravenous infusions of Allo (brexanolone: SAGE 547) or an oral Allo analog (SAGE 217) lead to a remission from the depressive symptoms in 70% of treated patients versus 10% for placebo (Kanes *et al.*, 2017a, 2017b).

Figure 3 shows TSPO ligands, selective brain steroidogenic stimulants, PE, or Allo analogs as promising therapeutic strategies for the treatment of mood disorders and potentially for PTSD.

The relevance of cannabinoids and their congeners in post-traumatic stress disorder

Recently, the endocannabinoids have been linked to the neuropathophysiology of depression, anxiety disorders and PTSD (Trezza and Campolongo, 2013; Coccaro *et al.*, 2018; Poleszak *et al.*, 2018). The endocannabinoid system affects connectivity between various regions of the brain

involved in stress regulation and relevant stress-related neurohormones and neurotransmitters, such as glucocorticoids and serotonin (Haring *et al.*, 2013; Morena *et al.*, 2016). By interacting with the glucocorticoids and their signaling, endocannabinoids suppress the activity of the HPA axis, through mechanisms that involve the prefrontal cortex, amygdala, and hypothalamus (Hill and Tasker, 2012).

The endocannabinoid system includes two G-protein coupled receptors, CB_1 and CB_2 (Lu and Mackie, 2016). While the CB_2 receptor is thought to have a more peripheral expression, CB_1 receptors are widely expressed throughout the brain (Howlett *et al.*, 2002). For this reason, most of the research investigating the relationship between endocannabinoids and responses to stress has focused on CB_1. Several studies have suggested that during environmental stress the CB_1 receptor plays a role in the regulation of HPA axis activity (Cota, 2008). Activation of the endocannabinoid system induces glucocorticoid release in both human and animal models (Wade *et al.*, 2006; Ranganathan *et al.*, 2009).

CB_1 receptors are strongly activated by $\Delta 9$-tetrahydrocannabinol (THC), the psychoactive constituent of *Cannabis sativa* (Pertwee, 2008), but their stimulation by several endocannabinoids is also relevant. *N*-arachidonoylethanolamine (anandamide, AEA; Devane *et al.*, 1992) and 2-AG (Sugiura *et al.*, 1995) are both synthetized and released postsynaptically and activate presynaptical CB_1 receptors located on glutamatergic and GABAergic axon terminals (Alger, 2002), resulting in inhibition of neurotransmitter release (Wilson and Nicoll, 2001; Ruehle *et al.*, 2012). The counterbalanced activity of GABAergic and glutamatergic neurons ensures a physiological emotional reactivity under basal conditions (Fig. 4; Hill, *et al.*, 2007; Ruehle *et al.*, 2012). However, the tuning of glutamatergic and GABAergic neurons by CB_1 receptors may influence the behavioral response in stressful conditions. Recently, the CB_1 receptor has received extensive attention for its implications in different mood disorders. The regulation of emotion processing, including anxiety and fear, is influenced by several neurotransmitters, among which glutamatergic and GABAergic systems play the main role (Hill *et al.*, 2007; Ruehle *et al.*, 2012). In case of stress, CB_1 receptors expressed on GABAergic neurons are downregulated, while those present on glutamatergic presynaptic neurons are strongly activated, which more potently inhibit glutamate release (Fig. 4; Rademacher *et al.*, 2008; Rossi *et al.*, 2008; Campos *et al.*, 2010; Ruehle *et al.*, 2012). The stronger inhibition of glutamate release after modulation of CB_1 receptors expressed in glutamatergic neurons versus a weaker inhibition of GABA release following activation of CB_1 on GABAergic neurons is regarded as the mechanism for the anxiolytic effects observed after cannabinoid administration (Ruehle *et al.*, 2012).

CB_1 receptor expression was found to be increased in individuals with PTSD (Neumeister *et al.*, 2013). This enhancement of expression has been correlated to a reduction of the peripheral levels of AEA, suggesting a neuroadaptation between lower concentrations of AEA and CB_1 expression (Neumeister *et al.*, 2013). Moreover, lower concentrations of both AEA and 2-AG have been found in the plasma of depressed women, suggesting a potential role of endocannabinoids in psychiatric disorder and in the development of novel drugs (Monteleone *et al.*, 2010). However, whereas peripheral AEA levels have been found to be decreased in PTSD patients in comparison to traumaexposed and healthy controls (Neumeister *et al.*, 2013). Hauer *et al.* (2013) reported enhanced AEA and 2-AG concentration in plasma of individual with PTSD in comparison to healthy controls only. While these conflicting results may result from different methodologies used to assess endocannabinoid concentrations (PET vs. HPLC-MS – MS), they highlight the need for a better investigation of the endocannabinoid system in PTSD patients.

Rodents exposed to chronic unpredictable stress showed an increase of CB_1 receptor binding sites and diminished AEA levels in prefrontal cortex, ventral striatum, and hippocampus (Hill *et al.*, 2008). In rats, the activation of cannabinoid CB_1 and CB_2 receptors through microinjection of the agonist WIN in the BLA reduces the stress-induced increase of corticosterone levels and reverses the effects of stressors on inhibitory avoidance conditioning and extinction (Ganon-Elazar and Akirav, 2009). The administration of WIN after SPS exposure prevents the disruption of extinction learning induced by the stressors (Ganon-Elazar and Akirav, 2012). Several studies have shown that the activation of CB_1 and the increase of AEA reduce HPA axis reactivity, reversing some of the PTSD-like behavioral effects induced by SPS in rodents (Gorzalka *et al.*, 2008; McLaughlin *et al.*, 2014).

AEA also plays a prominent role in the modulation of plastic changes in fear. This endocannabinoid is degraded by the catabolic enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase. Following administration of specific enzyme inhibitors, including URB597, the increase of AEA and 2-AG levels results in antidepressant and anxiolytic-like effects (Schlosburg *et al.*, 2010; Bambico *et al.*, 2016). Research on fear extinction in animal models has shown that enhanced AEA concentrations in the BLA facilitate fear extinction (Marsicano *et al.*, 2002). The endocannabinoid reuptake blocker AM404 increases AEA and 2-AG and facilitates fear extinction in rats (Bitencourt *et al.*, 2008). Moreover, a deletion in the FAAH gene in mice induces extinction of spatial reference memory (Varvel *et al.*, 2007). Thus, the inhibition of FAAH activity could lead to enhanced levels of AEA in corticolimbic circuits and to an improvement of fear extinction, suggesting a promising strategy to attenuate reconsolidation processes and ameliorate PTSD symptoms.

Interestingly, congeners of the endocannabinoids, including the ethanolamides, appear to be involved in PTSD. *N*-oleoyldopamine (OEA) and *N*-palmitoylethanolamine

Fig. 4
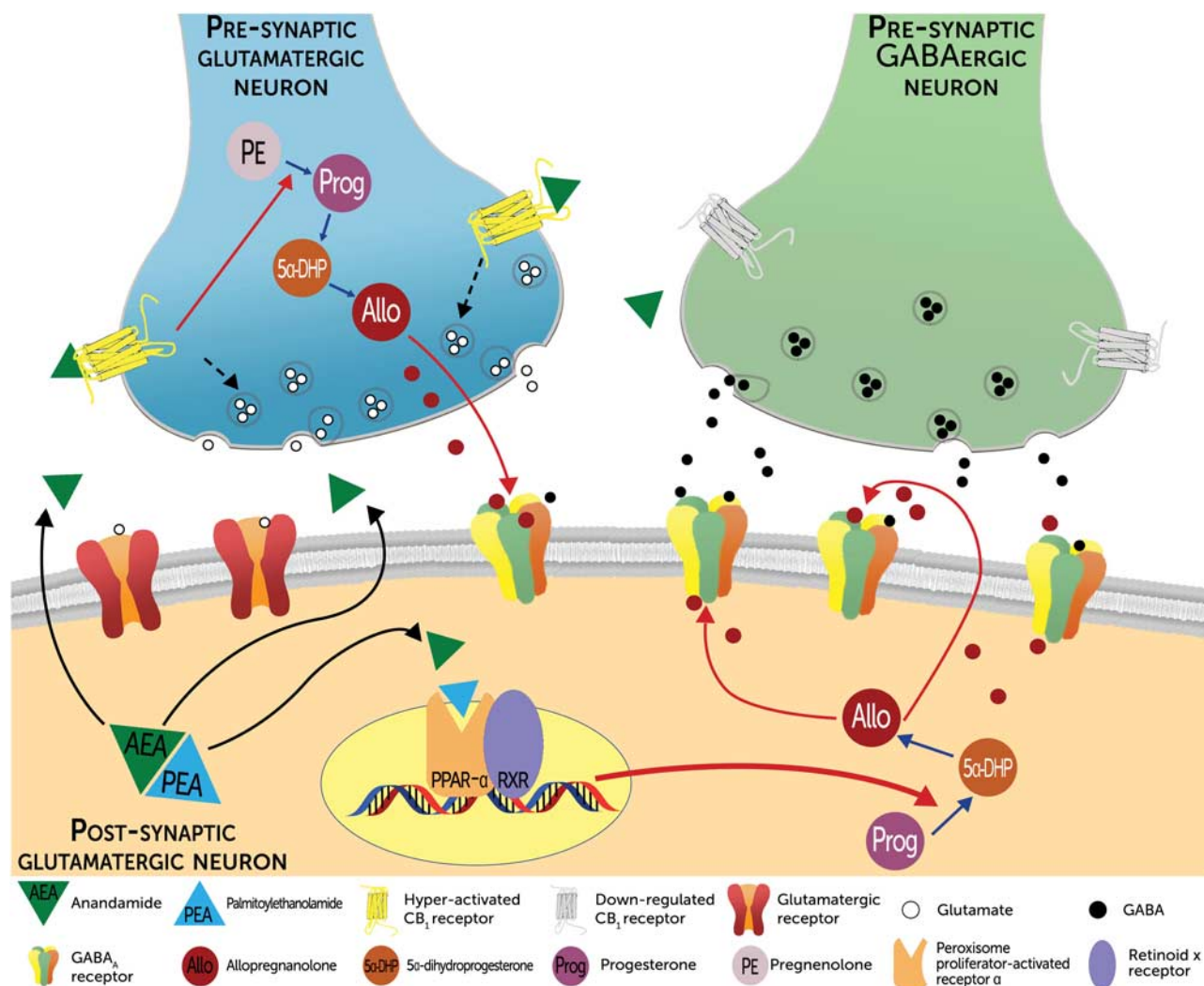
Endocannabinoid regulation of glutamatergic and GABAergic neurons and improvement of behavioral dysfunctions. The counterbalanced activity of GABAergic and glutamatergic neurons ensures a physiological emotional reactivity under basal conditions. Under the stressful condition, CB_1 receptors are hyperactivated in presynaptic glutamatergic neurons, whereas they are downregulated in presynaptic GABAergic terminals (Ruehle et al., 2012). Endocannabinoids, including anandamide (AEA), by acting at CB_1 receptors on glutamatergic terminals, greatly inhibit glutamate release (Wilson and Nicoll, 2001). However, the decreased activation of CB1 receptors in presynaptic GABAergic terminals fails to decrease GABA release in the synaptic cleft. This results in a potentiation of GABAergic inhibitory neurotransmission over a decreased glutamatergic excitatory neurotransmission, which is proposed as the molecular mechanism underlying the anxiolytic effects of cannabinoids (Rademacher et al., 2008; Ruehle et al., 2012). However, behavioral improvements may also be facilitated by the enhancement of Allo biosynthesis (Locci and Pinna, 2019a; reviewed in Pinna, 2018). Recent findings suggest that endocannabinoids, such as AEA or endocannabinoid-like molecules, including N-palmitoylethanolamine (PEA), may activate the nuclear PPAR-α receptor, which increases corticolimbic Allo concentration by stimulating the expression of enzymes involved in the neurosteroid biosynthetic pathway (Locci and Pinna 2019b). In turn, Allo, by binding $GABA_A$ receptors, potently and allosterically facilitates the inhibitory action of GABA (Pinna et al., 2000; Guidotti et al., 2001). Moreover, AEA may stimulate neurosteroid biosynthesis by acting directly at CB_1 receptors located presynaptically on glutamatergic neurons (Vallee et al., 2014). These mechanisms are consistent with an improvement of behavioral deficits, including exaggerated fear responses and impaired fear extinction, observed in animal models of post-traumatic stress disorder (PTSD) and in PTSD patients (Pinna and Rasmusson, 2014; Pinna, 2018; Rasmusson et al., 2019). Allo, allopregnanolone; 5α-DHP, 5α-dihydroprogesterone; PE, pregnenolone; PPAR-α, peroxisome proliferator-activated receptor-α; Prog, progesterone; RXR, retinoid X receptor.

(PEA) act by stimulating the peroxisome proliferator-activated receptor-α (PPAR-α), which is involved in neuroinflammatory processes (Racke and Drew, 2008, Esmaeili et al., 2016). These two PPAR-α endogenous agonists are not strictly considered as part of the endocannabinoid system because of their lack of affinity for the classic CB_1 and CB_2 receptors. However, they exhibit cannabimimetic actions for an 'entourage' effect, potentiating the AEA response (Smart et al., 2002; Ho et al., 2008) by the inhibition of FAAH, for which PEA is also a substrate (Di Marzo et al., 2001).

Recent studies have shown that PEA and OEA are decreased in the hair of patients with PTSD (Wilker et al., 2016).

Although it is important that this initial study should be replicated in serum and, possibly, CSF of PTSD patients, this and other findings in the field have suggested a role for PPAR-α and its endogenous modulators in mood disorders. For example, in preclinical studies, the predator-stress model shows a reduction of PEA and OEA levels (Holman *et al.*, 2014). PEA has antidepressant and anxiolytic effects, reducing immobility of mice exposed to the FST (Yu *et al.*, 2011, Crupi *et al.*, 2013). In their study, Yu *et al.* (2011) compared the behavioral improvement obtained by administering PEA with that after giving fluoxetine. They observed that, in the tail suspension test, PEA is more effective at a lower dosage than fluoxetine, whereas in the FST their effects are comparable (Yu *et al.*, 2011). Furthermore, PEA is a safe adjuvant to the SSRI citalopram in male patients with major depressive disorder (Ghazizadeh-Hashemi *et al.*, 2018). The exact mechanism of action of PEA in depression is still not clear, but its anti-inflammatory effects might be beneficial to improve the depressive symptoms (Kohler *et al.*, 2014). By binding PPAR-α, PEA reduces inflammation and the expression of proinflammatory cytokines such as IL-6 (Lo Verme *et al.*, 2005). It has also been reported that the administration of the PPAR-α synthetic agonist, fenofibrate reduces behavioral abnormalities linked to impulsivity in a rat model of schizophrenia (Rolland *et al.*, 2012). Fenofibrate has shown neuroprotective action in an animal model of Huntington's disease and of stroke (Deplanque *et al.*, 2003). The activation of PPAR-α by fenofibrate promotes mitochondrial stability and leads to neuroprotection by reducing neuroinflammation (Esmaeili *et al.*, 2016).

Importantly, given that PEA shares the same catabolic enzymes with AEA, the enzyme FAAH, an intriguing hypothesis is that behavioral effects observed following administration of FAAH inhibitors, in addition to the enhancement of AEA levels and activation of CB1 receptor signaling, may also include the activation of PPAR-α by elevated brain PEA levels. Furthermore, AEA may also bind and act at PPAR-α (O'Sullivan, 2007). While this hypothesis remains to be corroborated by experimental findings, it is noteworthy that recent observations have shown that the activation of PPAR-α by PEA, by increasing biosynthesis of Allo in hippocampus, amygdala, and frontal cortex improves fear extinction and retention of fear extinction and decreases depressive-like and anxiety-like behavior, and aggression in socially isolated mice (Locci and Pinna, 2017; Locci *et al.*, 2017; Locci and Pinna, 2019b). Also, the behavioral effects of PEA in socially isolated mice are blocked by the use of PPAR-α antagonists and in PPAR-α KO mice. Moreover, the administration of finasteride, by reducing the activity of 5α-RI and, consequently, of Allo levels, reverts the behavioral effects of PEA. This evidence provides mechanistic insights into the role of Allo and PPAR-α in the pharmacological effects of PEA. The ability to induce de novo synthesis of Allo has been previously observed in the spinal cord in association with potentiation of pentobarbital-induced sedation (Sasso *et al.*, 2010). This evidence highlights an interesting novel role of PPAR-α in neuropsychiatric disorders (Locci and Pinna, 2019b).

In addition to THC, one of the primary psychoactive component of cannabis, cannabidiol (CBD) has recently received growing attention for its pharmacological properties. CBD is devoid of the psychotomimetic effects observed following THC administration, and it fails to induce withdrawal and tolerance liabilities (Bergamaschi *et al.*, 2011). Several preclinical studies that focused on the effects of THC, CBD, or their analogs on fear memory have suggested that by their action, mediated by CB_1 receptors, these herbal extracts facilitate extinction of fear memory (Pamplona *et al.*, 2006; Stern *et al.*, 2012; Do Monte *et al.*, 2013). In rodents, CBD affects emotional behavior, including a reduction of depressive-like behaviors and of anhedonia and improving fear responses (Stern *et al.*, 2012; Linge *et al.*, 2016; Sartim *et al.*, 2016).

CBD may act through different mechanisms: It has low affinity for CB_1 and CB_2 receptors but increases cannabinoid neurotransmission by reducing the activity of FAAH (Bisogno *et al.*, 2001). Besides these mechanisms, CBD may act at the $5HT_{1A}$ and at the PPAR-γ which exerts anti-inflammatory effects (Russo *et al.*, 2005; Esposito *et al.*, 2011). Interestingly, this receptor can be activated not only by CBD, but also by cannabinoids, AEA, 2-AG, and THC (Muñoz *et al.*, 2017). The exact mechanisms through which activation of PPAR receptor-γ by CBD produces anxiolytic effects is still elusive, but the release of cytokines or the restoration of brain-derived neurotrophic factor levels associated with an enhanced neurogenesis rate are all mechanisms that could be implicated in the pharmacological action of CBD (Angelucci *et al.*, 2000). These observations, collectively, increase our understanding of the complex interactions of the endocannabinoid system, endocannabinoid-like and endocannabinoid congeners, and their interaction with the neurosteroid system in the regulation of emotional behavior. The interface between the endocannabinoid and neurosteroid systems may unveil a useful and exclusive biomarker axis for PTSD (reviewed in Aspesi and Pinna, 2018; Pinna, 2018; Pinna and Izumi, 2018). The use of a biomarker axis that reflects the synergistic relation of several neurobiological alterations for one disorder may enhance diagnostic accuracy and improve drug selection to treat PTSD more efficiently. In PTSD, for example, the altered peripheral endocannabinoid concentrations together with the down-regulation of Allo levels, the changes in $GABA_A$ receptor subunits, and the lack of benzodiazepine pharmacological effects may represent a specific biomarker profile that can be considered discriminative for the disorder (Fig. 2). The identification of a panel of altered biomarkers, which are specific for a disorder or even for a subpopulation of that disorder, will be instrumental in establishing an objective diagnosis and providing a more appropriate therapeutic approach.

Conclusion

PTSD is complex and debilitating neuropathology with a frequent overlap of symptoms and comorbidity with other disorders, including depression, anxiety disorders, drug abuse, and suicidal ideation. Taking into account its heterogeneity, the hypothesis that single alterations might be found that are responsible for the multifaceted aspects of the disorder is not conceivable. For this reason, it is also challenging to establish an appropriate animal model that recapitulates the several behavioral and biochemical abnormalities observed in PTSD patients. Among the preclinical models proposed, we have focused on the SI mouse model, which offers a valuable experimental tool to explore new pharmacological approaches by enhancing GABAergic neurotransmission. SI leads to both behavioral and neurobiological alterations that are reminiscent of deficits observed in PTSD patients. These include an enhanced level of aggression and impaired fear extinction, in association with altered neurosteroid biosynthesis and changes in the GABA$_A$ receptor subunit composition (reviewed in Locci and Pinna, 2017).

Unveiling a biomarker axis for PTSD could be a useful strategy to assess a relevant biosignature to improve diagnosis and treatment options for PTSD patients (Pinna and Izumi, 2018; Aspesi and Pinna, 2018). The fact that there are several subpopulations of PTSD patients with specific impairments or comorbidity with other psychiatric disorders makes it clear that only a few biomarkers or only one treatment option are insufficient to help all PTSD patients.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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