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Stimulation of Peroxisome Proliferator-Activated Receptor-α by *N*-Palmitoylethanolamine Engages Allopregnanolone Biosynthesis to Modulate Emotional Behavior

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ABSTRACT

BACKGROUND: The endocannabinoid and neurosteroid systems regulate emotions and stress responses. Activation of peroxisome proliferator-activated receptor (PPAR)-α by the endocannabinoid congener *N*-palmitoylethanolamine (PEA) regulates pathophysiological systems (e.g., inflammation, oxidative stress) and induces peripheral biosynthesis of allopregnanolone, a gamma-aminobutyric acidergic neurosteroid implicated in mood disorders. However, effects of PPAR-α on emotional behavior are poorly understood.

METHODS: We studied the impact of PPAR-α activation on emotional behavior in a mouse model of posttraumatic stress disorder. Neurosteroid levels before and after PEA treatment were measured by gas chromatography–mass spectrometry in relevant brain regions of socially isolated versus group-housed mice exposed to the contextual fear conditioning test, elevated plus maze test, forced swim test, and tail suspension test. Neurosteroidogenic enzyme levels were quantified in hippocampus by Western blot.

RESULTS: PEA administered in a model of conditioned contextual fear reconsolidation blockade facilitated fear extinction and fear extinction retention and induced marked antidepressive- and anxiolytic-like effects in socially isolated mice with reduced brain allopregnanolone levels. These effects were mimicked by the PPAR-α synthetic agonists, fenofibrate and GW7647, and were prevented by PPAR-α deletion, PPAR-α antagonists, and neurosteroid-enzyme inhibitors. Behavioral improvements correlated with PEA-induced upregulation of PPAR-α, neurosteroidogenic enzyme expression, and normalization of corticolimbic allopregnanolone levels.

CONCLUSIONS: This evidence supports a previously unknown role for PPAR-α in behavior regulation and suggests new strategies for the treatment of neuropsychopathologies characterized by deficient neurosteroidogenesis, including posttraumatic stress disorder and major depressive disorder.

Keywords: Allopregnanolone, Biomarkers, Fenofibrate, *N*-palmitoylethanolamine, PPAR-α, PTSD

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N-palmitoylethanolamine (PEA) is an endogenous lipid neuromodulator belonging to the class of fatty acid ethanolamides (1). PEA is synthesized on demand from the phospholipid *N*-acyl-phosphatidyl-ethanolamines by *N*-acyl phosphatidyl-ethanolamine phospholipase D and is metabolized by the endocannabinoid degradative enzyme fatty acid amide hydrolase and, more selectively, by *N*-acylethanolamine acid amide hydrolase (1). PEA recently has been proposed to be part of the extended endocannabinoid (eCB) system that includes the classic eCBs, anandamide and 2-arachidonoylglycerol (2-AG), which act at cannabinoid receptor types 1 (CB1) and 2 (CB2) (2). PEA modulates several endogenous targets, such as the G-protein coupled receptors GPR55 and GPR119 (3,4). Importantly, it is widely recognized that PEA and synthetic ligands (e.g., fibrates) induce their main pharmacological effects by stimulating the ligand-activated nuclear receptor, peroxisome proliferator-activated receptor (PPAR)-α, which heterodimerizes with the retinoid X-receptor (RXR) before binding to the promoter regions of target genes (5). These include genes for lipid metabolism, synthesis of cholesterol, and catabolism of amino acids (5). PPAR-α is widely expressed in the brain and plays a role in suppressing neuroinflammation and oxidative stress (6). In support, targeting PPAR-α with selective agonists alleviates neurodegenerative disorders. In addition, PEA is used clinically as a neuroprotective, analgesic and anti-inflammatory agent (7).

The eCB system has been implicated in the neuropathophysiology of stress-related neuropsychiatric disorders, such as posttraumatic stress disorder (PTSD) and depression (8,9). Although PEA is detectable in the brain at physiologically relevant concentrations, and PEA levels are altered in neurodegenerative diseases (10), the potential role of PEA in psychiatric disorders is unclear. A single study showed that PTSD symptoms are inversely correlated with PEA levels in blood

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(11), and, interestingly, PEA levels were increased in rat corticolimbic regions by antidepressants (12).

Recently, an interaction between PPAR-α and the gamma-aminobutyric acidergic (GABAergic) neurosteroid system was suggested (13,14). Studies in astrocytes and spinal cord showed that PEA binding at PPAR-α stimulated the biosynthesis of allopregnanolone (Allo) and potentiated pentobarbital-evoked hypnosis (13,14).

Allo is a positive allosteric modulator of GABA action at $GABA_A$ receptors, and it potently induces anticonvulsant, anxiolytic, antidepressant, sedative, and analgesic effects (15). Importantly, Allo can be produced de novo from progesterone in glutamatergic neurons of the cortex, hippocampus, and basolateral amygdala by the dual action of 5α-reductase type I (5α-RI) and 3α-hydroxysteroid dehydrogenase (3α-HSD) (16,17). Given that PPAR-α is widely distributed in glutamatergic corticolimbic neurons (6), it is conceivable that PPAR-α activation by PEA or other PPAR-α agonists (e.g., fenofibrate) may induce Allo biosynthesis in corticolimbic neurons, which are involved in the regulation of emotion.

Compelling evidence indicates that endogenous Allo in the brain is downregulated in rodent stress models and in human mood disorders (18,19). Reduced Allo levels have been found in the serum, cerebrospinal fluid, and brain of individuals with depression and PTSD (18,20–23). Our previous studies showed that brain Allo levels fell markedly in rodents following protracted social isolation stress. Reduced brain Allo levels were, in turn, associated with PTSD-like behaviors, including increased aggression and anxiety-like responses, as well as the expression of exaggerated contextual fear, and decreased rates of extinction and impaired extinction retention after fear conditioning (24,25).

Hence, the socially isolated (SI) mouse exposed to fear conditioning offers a suitable stress model to study molecular mechanisms and behavioral deficits resulting from Allo biosynthesis downregulation. The SI mouse model also allows study of new agents that may mimic or stimulate Allo and thereby enhance GABAergic neurotransmission to improve behavior (26,27). Intriguingly, administration of Allo (at doses that elevate brain Allo levels) or Allo analogs has been shown to reduce the expression of contextual fear, as well as enhance extinction and extinction retention in SI mice with brain Allo deficits, but not group-housed (GH) mice with normal brain Allo levels (25,28). In addition, recent phase 3 clinical trials demonstrated the efficacy of intravenous Allo (brexanolone; Sage Therapeutics, Cambridge, MA) and an oral Allo analog (SAGE 217) in postpartum depression and major depressive disorder (29,30), respectively, conditions highly comorbid with PTSD. These studies have recently led to U.S. Food and Drug Administration approval of brexanolone as the first specific treatment for postpartum depression.

The current preclinical study was therefore undertaken to investigate a possible role for PPAR-α agonists in the upregulation of Allo synthesis as a means for reducing PTSD- and depression- or anxiety-like behaviors in SI mice with Allo deficits. The results of the study demonstrate a previously unknown role for PPAR-α in regulating emotional behavior by elevation of corticolimbic neurosteroid biosynthesis. The results also suggest novel translational biomarker candidates and pharmacological agents potentially suitable for development of long-needed precision medicines for the treatment of PTSD and depression.

METHODS AND MATERIALS

Animals

Male Swiss-Webster mice (Harlan Breeders, Indianapolis, IN) or PPAR-α knockout (KO) mice (*Ppara*^{tm1Gonz}/J; Jackson Laboratory, Bar Harbor, ME) (21–23 days old at arrival) were maintained under a 12-hour dark/light cycle (light on at 6 AM and off at 6 PM) and provided food and water ad libitum. SI mice were housed individually in a cage (24 × 17 × 12 cm) for 5 weeks, while GH mice were housed in groups of 5. The vivarium temperature was 24°C and the humidity 65%. Depending on the experiment, the number of mice for each experimental group varied from 6 to 12 (for details on group number for each experiment, see figure legends). The total number of animals used was 436. Experimental protocols were approved by the Office and Animal Care and Institutional Biosafety Committee and the Office of the Vice Chancellor for Research of the University of Illinois at Chicago.

Drug Treatments

The PPAR-α agonists, PEA (5–20 mg/kg, intraperitoneally [i.p.], 5% Tween 80 in saline; Epitech Group, Milano, Italy) and GW7647 (G7) (5% 10 mg/kg, i.p., 5% Tween 80 in saline; Tocris, Minneapolis, MN) were given 1 hour before exposure of mice to the elevated plus maze test, forced swim test (FST), and tail suspension test (TST) or sacrifice. PEA, at the dose of 5 mg/kg, was given immediately after contextual fear reactivation sessions. The PPAR-α agonist fenofibrate (FFB) (7.5–120 mg/kg, subcutaneously, canola oil; Sigma, St. Louis, MO) was given 1.5 hours before the elevated plus maze test.

The PPAR-α antagonist GW6471 (3 mg/kg, i.p., 5% Tween 80 in saline; Tocris), the selective GPR55 antagonist, ML 193 (10 mg/kg, i.p., 5% Tween 80 in saline; Tocris), or the selective GPR119 antagonist, exendin (1 mg/kg, i.p., saline; MilliporeSigma, Burlington, MA), was injected 1.5 hours before testing in the elevated plus maze test or sacrifice (9,31). The 5α-RI inhibitor finasteride (50 mg/kg, subcutaneously, canola oil; Tocris) was given 2.5 hours before an elevated plus maze test or sacrifice.

Contextual Fear Conditioning

Contextual fear conditioning was performed as previously described (25). The conditioning and extinction chamber (25 × 18 × 21 cm), surrounded by 16 infrared photo beams, had a stainless steel rod floor connected to an electric shock generator (San Diego Instrument, Inc., San Diego, CA).

Conditioning Trial. Mice were placed in the chamber and exposed over 8 minutes to a conditioned stimulus paired with an electric foot-shock (unconditioned stimulus; magnitude 0.7 mV, duration 2 seconds), repeated three times every 2 minutes.

Reactivation. Twenty-four hours after fear conditioning, mice were reexposed for 5 minutes to the conditioning context to induce retrieval and/or reactivation of the fear-conditioned memory. The mice were treated by a single PEA injection immediately after the reactivation prior to return to the home cage.

Extinction and Extinction Retention. Beginning 24 hours after fear memory reactivation, the mice were reintroduced to the conditioning apparatus for 5 minutes once daily for 5 days. Total time spent freezing was used as an index of the contextual fear response. After an interval of 9 days, mice were reexposed to the chamber to assess extinction retention (25).

Elevated Plus Maze

Elevated plus maze tests were performed as previously described (28). Mice chose between entering open or closed arms that extend from a central platform. An arm entry was scored when four legs were within the arm. Behavior was recorded for 5 minutes. Total time spent and the number entries into the open arms were quantified as indices of anxiety-like behavior.

Locomotor Activity

To minimize the number of animals used for our experiments, mice were exposed to an open field to evaluate locomotor activity immediately after testing in the elevated plus maze. A computerized AccuScan 12 Animal Activity Monitoring System (Columbus Instruments, Columbus, OH) and VERSAMAX software (AccuScan Instruments, Columbus, OH) were used to evaluate locomotor activity (32). Horizontal sensor beam interruptions measured horizontal activity for a total of 10 minutes.

FST and TST

The FST or a TST was performed as previously described (33,34). FST sessions were video-recorded for 6 minutes, and the last 4 minutes were analyzed (30). TST duration was 6 minutes. Immobility time was an index of depressive-like behavior.

Brain Neurosteroid Measurements

Extraction, derivatization, and quantification of Allo, of its unconjugated isomer pregnanolone, and of its precursors, pregnenolone, progesterone, and 5α-dihydroprogesterone, were described previously (35). After addition of deuterium-labeled internal standards to tissue samples, steroids were extracted, purified, and separated by high-pressure liquid chromatography (25). The aqueous phase (2 mL) containing the sulfated neurosteroids pregnenolone sulfate, Allo sulfate (Allo-S), pregnanolone sulfate, and dehydroepiandrosterone sulfate underwent a solvolysis prior to extraction. After derivatization, gas chromatography–mass spectrometry analysis in the standard electron impact mode was performed (24,25).

Western Blot

The abundance of PPAR-α, steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (CYP11A1), and 5α-RI was determined in lysates of hippocampus as previously reported (32,36). Equal amounts of protein were subjected to gel electrophoresis (200 V for 75 minutes) on 4% to 12% gel (NuPAGE Novex; Thermo Fisher Scientific, Waltham, MA). Immunoblot with primary antibodies against PPAR-α, CYP11A1, and 5α-RI (1:500; Proteintech, Rosemont, IL), and StAR (1:500; Abcam, Cambridge, MA) were incubated with glyceraldehyde-3-phosphate dehydrogenase primary antibody (1:50,000; MilliporeSigma). Horseradish peroxidase–conjugated secondary anti-mouse and anti-rabbit antibodies were used. Membranes were developed and densitometric analysis was conducted with Image StudioTM (LI-COR Bioscience, Lincoln, NE).

Statistical Analyses

Results are presented as mean ± SEM unless otherwise indicated. Two-tailed Student's t test, one-way analysis of variance, two-way analysis of variance, or two-way repeated measures analysis of variance followed by Tukey's post hoc tests were performed to analyze experimental data. Significance was set at $p < .05$.

RESULTS

PEA Improves Contextual Fear Responses and Facilitates Fear Extinction in SI Mice

SI mice exhibited enhanced contextual fear conditioning responses compared with the responses of GH-control mice (first 3 days of extinction trials: $p < .05$; recall day: +135%, +51%, $p < .05$) (Figure 1A). A single administration of PEA given immediately after the fear reactivation session facilitated fear extinction during the first 3 days of extinction trials (−75%; $p < .001$). It also prevented the spontaneous reexpression of contextual fear-related behavior on day 14 after completion of extinction training and the passage of time (−88%, $p < .001$; i.e., it facilitated fear extinction retention) (Figure 1A). Of note, PEA failed to affect contextual fear responses in both GH ($p = .54$) and PPAR-α KO ($p = .68$) mice (Figure 1A, B).

PEA Induces Anxiolytic Effects in SI Mice

SI mice, compared with GH mice, showed enhanced anxiety-like behavior consisting of less time spent (−42%, $t_{14} = 2.193$, $p < .05$) (Figure 1C) and a lower number of entries (−42%, $t_{12} = 2.488$, $p < .05$) (Figure 1D) in the open arms of the plus maze. PEA induced a dose-dependent anxiolytic effect represented by time spent and number of entries into the open arms, respectively (5 mg/kg: +113%, $t_{15} = 3.615$, $p < .01$, and +116%, $t_{10} = p < .001$; 10 mg/kg: +533%, $t_{18} = 4.53$, $p = .0003$, and +492%, $t_{18} = 5.598$, $p < .0001$) (Figure 1C, D). PEA treatment had no effect in GH mice (Figure 1C, D).

Antidepressant Action of PEA in SI Mice

When compared with GH-control animals, SI mice had significantly increased depressive-like behavior in the FST (time of immobility: +41%, $p < .05$) (Figure 1E) and TST (time of immobility: +43%, $p < .05$). Despite the lack of effect in GH-control animals, a median effective concentration dose of PEA, given to SI mice 1 hour before these tests, significantly decreased immobility in the FST (−40% vs. vehicle-treated SI mice, $p < .05$) (Figure 1E) and TST (−48% vs. vehicle-treated SI mice, $p < .01$; not shown). As expected, the PEA antidepressive-like effect was not observed in PPAR-α KO mice (FST: +5%, TST: +12% vs. vehicle-treated mice, not significant) (Figure 1F).

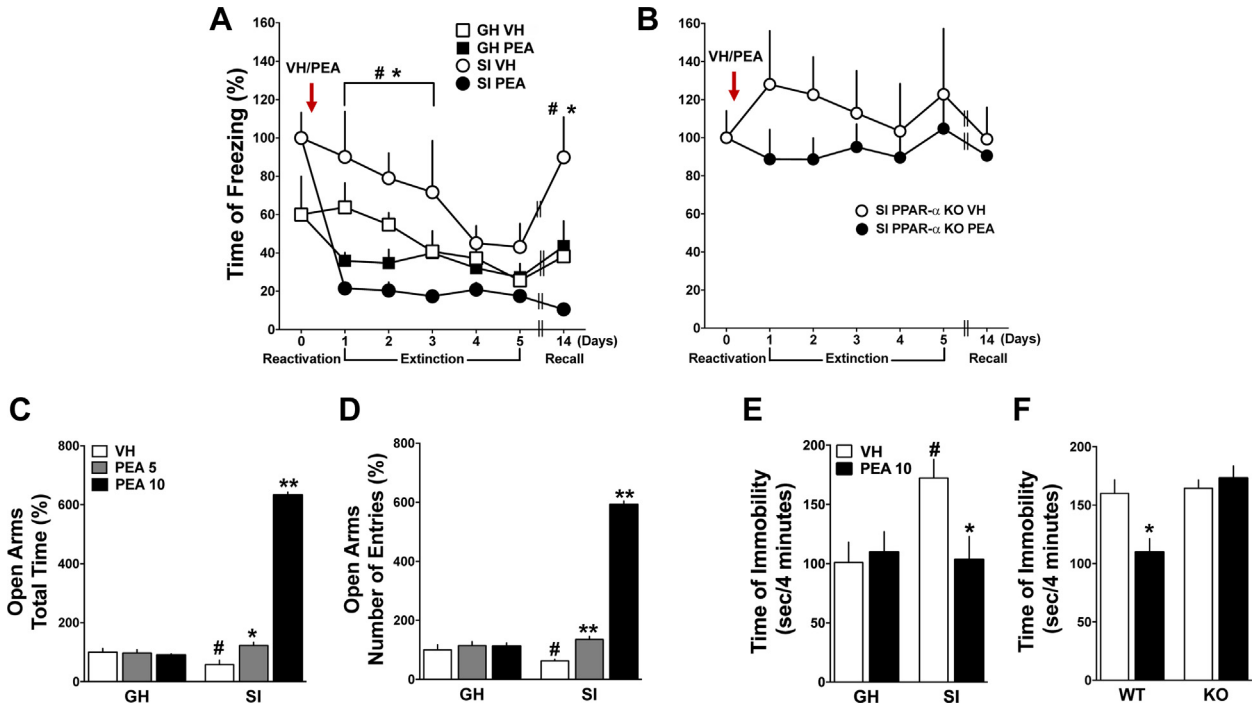

Figure 1. **(A, B)** *N*-palmitoylethanolamine (PEA) facilitates extinction of contextual fear memory and prevents spontaneous fear reinstatement in socially isolated (SI) mice. **(A)** A single administration of PEA (5 mg/kg, intraperitoneally) (black circles) or vehicle (VH) (white circles), given immediately after a contextual fear conditioning reactivation session, facilitates fear extinction (days 1–3) (treatment: $F_{3,32} = 10.01$, $p = .0002$; time: $F_{2,64} = 0.5$, $p = .61$; interaction: $F_{6,64} = 0.38$, $p = .89$), and fear extinction retention on recall (day 14) in SI mice (treatment: $F_{1,27} = 9.41$, $p < .005$; isolation: $F_{1,27} = 0.611$, $p = .441$; interaction: $F_{1,27} = 12.28$, $p = .002$). The same treatment failed to induce effects in group-housed (GH) control mice (squares). **(B)** Administration of a single dose of PEA (5 mg/kg, intraperitoneally) failed to affect fear responses in peroxisome proliferator-activated receptor (PPAR)-α knockout (KO) mice. Values are reported in percentage; average of vehicle-treated SI mice is considered as 100%. Data represent the mean ± SEM of 9 mice. $^{*}p < .001$, when compared with PEA-treated SI mice at the same time point; $^{\#}p < .05$, when compared with vehicle-treated GH mice at the same time point. Two-way repeated measures analysis of variance or two-way analysis of variance followed by Tukey's post hoc analysis. **(C, D)** PEA improves anxiety-like behavior in SI mice. Mice were exposed to an elevated plus maze test for 5 minutes. Bar graphs represent the effect induced by PEA or vehicle treatment on the percentage of total time spent in the open arms **(C)** and on the number of entries in the open arms **(D)** of the elevated plus maze in both GH and SI mice. Values are reported in percentage; average of vehicle-treated GH mice is considered as 100%. Data are reported as mean ± SEM of 8 to 12 mice. $^{*}p < .01$ and $^{**}p < .001$, when compared with vehicle-treated SI mice; $^{\#}p < .05$ when compared with vehicle-treated GH mice. Two-tailed Student's *t* test analysis. **(E, F)** PEA counteracts the social isolation–induced depressive-like behavior. Mice were exposed to the forced swim test and were recorded for 4 minutes. **(E)** Bars represent the effect induced by PEA on the time of immobility in SI mice exposed to the forced swim test (treatment: $F_{1,27} = 2.8$, $p = .11$; isolation: $F_{1,27} = 3.3$, $p = .08$; interaction: $F_{1,27} = 4.7$, $p = .004$). **(F)** PEA induces antidepressant-like effects in SI wild-type (WT) mice but not in SI PPAR-α KO mice. Data are expressed as mean ± SEM of 7 to 8 mice. $^{*}p < .05$, when compared with vehicle-treated SI or WT mice; $^{\#}p < .05$, when compared with vehicle-treated GH mice. Two-way analysis of variance followed by Tukey's post hoc analysis.

PEA Elevates Neurosteroid Levels in Corticolimbic Areas of SI Mice

Single dose administration of PEA (5–20 mg/kg, i.p.) increased Allo levels after 1 hour in the olfactory bulb ($F_{3,23} = 12.85$, $p < .0001$), prefrontal cortex ($F_{3,23} = 3.40$, $p = .0347$), hippocampus ($F_{3,21} = 10.45$, $p = .0002$), and amygdala ($F_{3,23} = 18.03$, $p < .0001$) of SI mice (Figure 2). This treatment did not affect Allo levels in the striatum (Figure 2).

PEA increased Allo precursor levels in several corticolimbic areas of SI mice. PEA (20 mg/kg) increased pregnenolone in the olfactory bulb ($F_{3,23} = 9.62$, $p = .0003$; +69%, $p < .05$) and progesterone levels in the hippocampus ($F_{3,15} = 7.42$, $p = .0028$; +3128%, $p < .01$), amygdala ($F_{3,22} = 5.90$, $p = .0041$; +223%, $p < .001$), and striatum ($F_{3,17} = 10.31$, $p = .0004$; +594%, $p < .001$). PEA also increased 5α-dihydroprogesterone in the hippocampus of SI mice ($F_{3,23} = 7.96$, $p = .0008$; 10 mg/kg: +64%, $p < .05$; 20 mg/kg: +87%, $p < .01$).

PEA-Induced Behavior and Allo Biosynthesis Are Mediated by PPAR-α

The specificity of PEA's effects at PPAR-α was further analyzed using a pharmacological approach. We selected the PEA median effective concentration dose (10 mg/kg, i.p.) that reduced aggression of SI mice toward a same-sex intruder (37). The PPAR-α synthetic agonist G7 improved anxiety-like behavior in SI mice (time spent in the open arms: +506%, $t_{10} = 2.281$, $p = .0457$; number of entries in the open arms: +247%, $t_{10} = 2.339$, $p = .0392$) (Figure 3A). In line with PEA and G7 effects, the PPAR-α agonist FFB also induced a dose-dependent anxiolytic-like effect in SI mice (time spent in the open arms: $F_{3,26} = 7.99$, $p = .0006$; number of entries in the open arms: $F_{3,26} = 7.448$, $p = .0009$) (Figure 3B). Importantly, the PEA anxiolytic effect was prevented by pretreatment with the selective PPAR-α antagonist GW6471 (time spent in the open arms: $F_{2,14} = 22.28$; $p < .0001$; number of entries in the

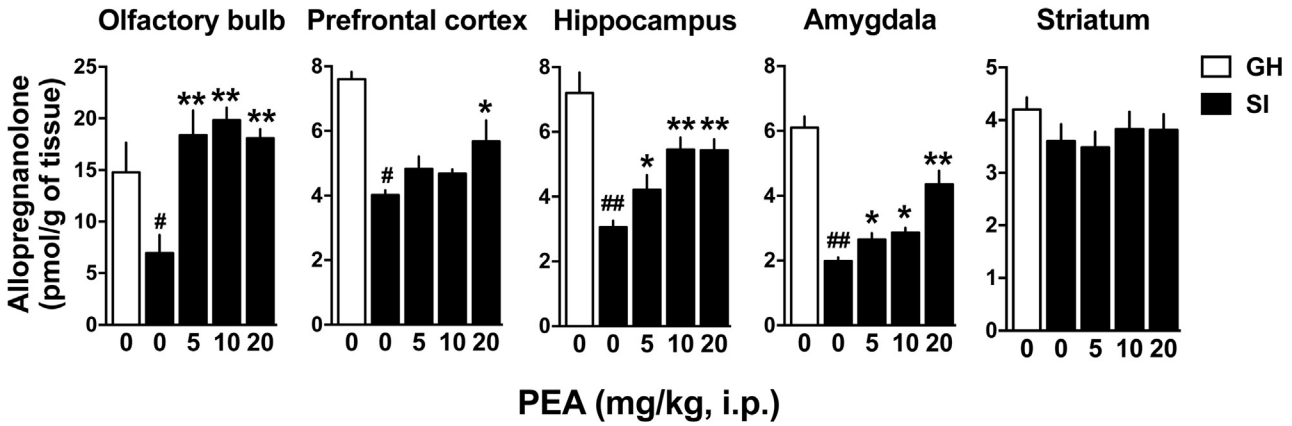

Figure 2. Neurosteroidogenic effect induced by *N*-palmitoylethanolamine (PEA) in corticolimbic areas of socially isolated (SI) mice. PEA increases allopregnanolone levels in the olfactory bulb (5 mg/kg: +164%, $p < .001$; 10 mg/kg: +185%, $p < .001$; 20 mg/kg: +160%, $p < .001$), hippocampus (5 mg/kg: +38%, $p < .05$; 10 mg/kg: +78%, $p < .001$; 20 mg/kg: +78%, $p < .001$), and amygdala (5 mg/kg: +34%, $p < .05$; 10 mg/kg: +42%, $p < .05$; 20 mg/kg: +120%, $p < .001$), and at the high dose in the frontal cortex (+41%, $p < .05$). However, PEA fails to change allopregnanolone levels in the striatum of SI mice. Data represent the mean ± SEM of 6 to 7 mice. $^{*}p < .05$ and $^{**}p < .001$, when compared with vehicle-treated SI mice; $^{\#}p < .05$ and $^{\#\#}p < .01$, when compared with vehicle-treated group-housed (GH) mice. Two-tailed Student's *t* test or one-way analysis of variance followed by Tukey's post hoc analysis. i.p., intraperitoneally.

open arms: $F_{2,14} = 13.79$; $p = .0005$) (Figure 3C). PEA also failed to induce anxiolysis in PPAR-α KO SI mice (time spent in the open arms: −1%; number of entries in the open arms: +6%) (Figure 3D). Inhibiting stimulation of Allo biosynthesis by pretreatment with the 5α-RI inhibitor finasteride also prevented the anxiolytic-like effect induced by PEA (time spent in the open arms: $F_{2,14} = 11.26$, $p = .001$; number of entries in the open arms: $F_{2,16} = 11.96$, $p = .0006$) (Figure 3C).

A single administration of the selective PPAR-α agonist G7 increased Allo levels in the olfactory bulb of SI mice (+89%, $t_{10} = 4.5$, $p = .0011$) (Figure 3E), while upregulation of olfactory bulb Allo levels by PEA was inhibited by the PPAR-α antagonist GW6471 ($F_{2,15} = 34.48$; $p < .0001$) (Figure 3F). PEA also failed to affect Allo levels in the olfactory bulb of PPAR-α KO mice (Figure 3G). Pretreatment with finasteride also prevented PEA-induced olfactory bulb Allo upregulation in SI mice ($F_{2,15} = 53.88$; $p < .0001$) (Figure 3F).

PEA Reverses the Social Isolation–Induced Hippocampal Downregulation of PPAR-α, StAR, CYP11A1, and 5α-RI Expression

We found a significant downregulation in the protein expression of PPAR-α (−49%, $p < .01$) in the hippocampus of SI mice (Figure 4A). To understand the mechanism by which PEA stimulates Allo biosynthesis, we studied the expression of proteins involved in neurosteroidogenesis, such as StAR, CYP11A1, and 5α-RI. The expression of these three steroidogenic proteins was found to be downregulated in the hippocampus of SI mice; specifically, StAR decreased by −80% ($p < .001$), CYP11A1 by −46% ($p < .01$), and 5α-RI by −40% ($p < .05$) (Figure 4B). Importantly, a single PEA median effective concentration dose normalized the expression of these proteins to the levels quantified in GH-control mice (PPAR-α: +109%, $p < .01$; StAR: +339%, $p < .001$; CYP11A1: +81%, $p < .01$; 5α-RI: +97%, $p < .01$, data are compared to SI vehicle-treated mice) (Figure 4A, B).

DISCUSSION

This study provides novel neurobiological evidence that stimulation of PPAR-α by PEA induces the corticolimbic biosynthesis of Allo, an effect that is directly associated with the improvement of a PTSD-like phenotype in SI mice. Specifically, PEA reduced the expression of conditioned contextual fear and facilitated extinction and extinction retention (Figure 1). PEA also induced anxiolytic- and antidepressant-like effects in SI mice. Furthermore, the behavioral effects of PEA in SI mice related to normalization of downregulated levels of Allo in hippocampus, amygdala, prefrontal cortex, and the olfactory bulb. PEA also elevated Allo-S in the hippocampus. In support of a PPAR-α dependent mechanism being involved in the salutary effects of PEA on behavior and brain Allo biosynthesis, the PPAR-α synthetic agonist G7 also normalized Allo's levels and improved behavior, whereas 1) antagonism of PPAR-α, 2) pharmacological inhibition of Allo biosynthetic enzymes, and 3) deletion of the PPAR-α gene prevented both the behavioral and neurosteroidogenic actions of PEA. Collectively, these pharmacological studies reveal mechanisms by which the therapeutic effects of PEA, PPAR-α, and Allo are linked.

In addition, the synthetic PPAR-α agonist FFB induces anxiolytic-like effects. Fibrates, such as FFB, ciprofibrate, and clofibrate, have been prescribed to treat dyslipidemia with a safe pharmacological profile (5). Moreover, preclinical studies suggest the promise of FFB for treatment of drug and alcohol addiction (38,39), which are highly comorbid with PTSD. FFB restored ventral tegmental area dopaminergic responses to appetitive stimuli, and sucrose preference in stressed rats, suggesting an antidepressant-like activity (31). FFB restored decreases in hippocampus brain-derived neurotrophic factor (BDNF) signaling and adult neurogenesis in a model of depression (40). These effects could be blocked by PPAR-α and BDNF signaling inhibitors (41). Remarkably, we previously showed that administration of Allo normalized corticolimbic BDNF expression downregulation in SI mice (42), suggesting

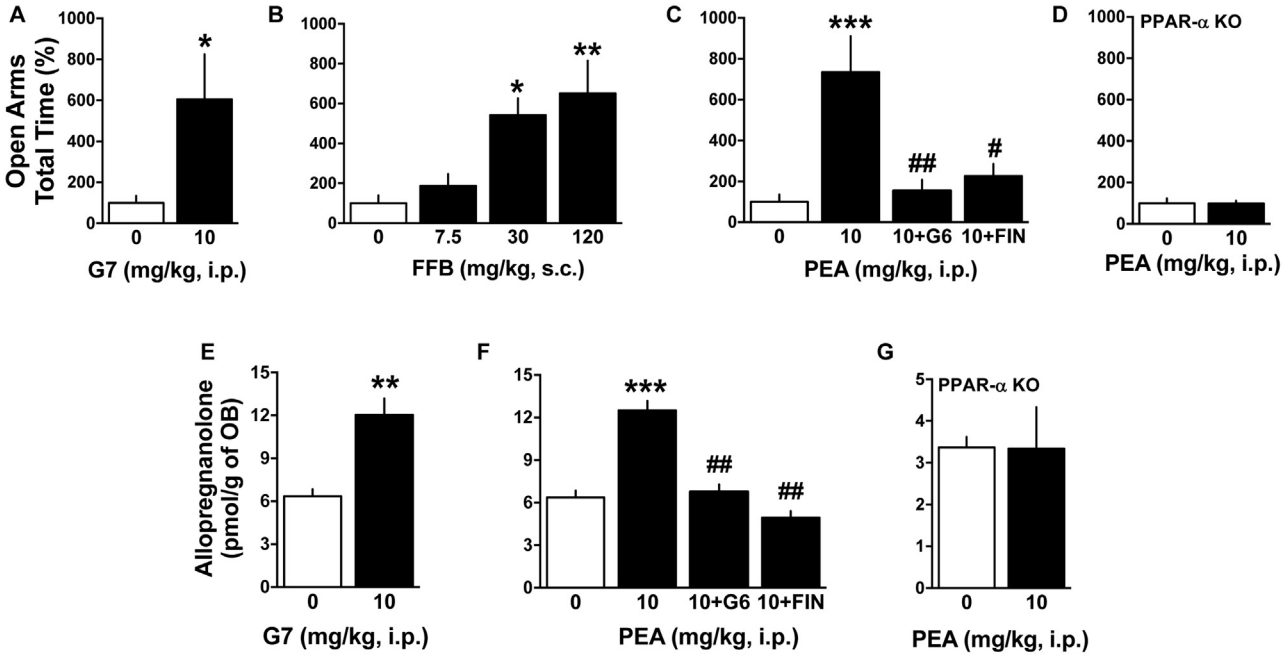

Figure 3. **(A–D)** Behavioral pharmacology of peroxisome proliferator-activated receptor (PPAR)-α in socially isolated (SI) mice. **(A)** Anxiolytic-like effect induced by the PPAR-α agonist GW7647 (G7) in SI mice. G7 increases the percentage of total time spent in the open arms. **(B)** The PPAR-α agonist fenofibrate (FFB) induces an anxiolytic-like effect in SI mice, as demonstrated by the dose-dependent increase of the time spent in the open arms (7.5 mg/kg: +88%, $p > .05$; 30 mg/kg: +442%, $p < .05$; 120 mg/kg: +552%, $p < .01$). **(C)** The selective PPAR-α antagonist GW6471 (G6) or the potent inhibitor of 5α-reductase type I, finasteride (FIN), prevents the anxiolytic effect of *N*-palmitoylethanolamine (PEA) in SI mice (G6 vs. PEA: −78%, $p < .01$; FIN vs. PEA: −68%, $p < .01$). **(D)** PEA fails to induce anxiolytic effects in SI PPAR-α knockout (KO) mice. Data represent the mean ± SEM of 6 to 8 mice. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$, when compared with vehicle-treated SI mice; $^{\#}p < .01$ and $^{\#\#}p < .01$, when compared with PEA. Two-tailed Student's *t* test or one-way analysis of variance followed by Tukey's post hoc analysis. **(E–G)** Pharmacology of PPAR-α-induced neurosteroidogenic effects. **(E)** G7 increases allopregnanolone levels in the olfactory bulb (OB) of SI mice. **(F)** Administration of G6, or the selective 5α-reductase type I inhibitor FIN blocked the neurosteroidogenic effect of PEA determined by the allopregnanolone content in the olfactory bulb of SI mice (G6 vs. PEA: −48%, $p < .001$; FIN vs. PEA: −60%, $p < .01$). **(G)** PEA fails to increase allopregnanolone levels in the olfactory bulb of SI PPAR-α KO mice. Data represent the mean ± SEM of 6 to 7 mice. $^{*}p < .01$ and $^{**}p < .001$, when compared with vehicle-treated SI mice; $^{\#}p < .001$, when compared with PEA. Two-tailed Student's *t* test or one-way analysis of variance followed by Tukey's post hoc analysis. i.p., intraperitoneally; s.c., subcutaneously.

that FFB-Allo-BDNF may be part of the same mechanism. Finally, FFB, like Allo, shows antiepileptic activity (43); thus, it is feasible that FFB's mechanism of action may include a neurosteroidogenic effect through PPAR-α activation. Remarkably, PEA and FFB are already approved by the U.S. Food and Drug Administration; hence, our findings entail immediate translational impact to treat neuropsychiatric disorders.

Improvement of fear extinction and anxiolytic-like effects in stressed rodents have been observed previously after treatment with natural and synthetic cannabinoids (e.g., anandamide, HU210, WIN55,212-2) that, in addition to binding CB1, activate PPAR-α (44). This observation poses the question as to whether improvements in stress-related behaviors credited to CB1 activation (8,45,46) are, at least in part, mediated by PPAR-α (and downstream activation of Allo). In support, raising levels of endogenous PPAR-α agonists (e.g., PEA, *N*-oleoylethanolamide [OEA]) by inhibiting their degradation or increasing their biosynthesis has antidepressant-like effects (47). Serum concentrations of PEA and OEA increase immediately after stress (48) before decreasing during recovery (47). Importantly, exposure to stressful stimuli evokes a fast induction of the enzyme fatty acid amide hydrolase, which reduces circulating anandamide and PEA levels (49). Exposure to predator stressors reduces peripheral PEA and OEA levels in rodents (50). An inverse correlation between PTSD symptoms and peripheral levels of PEA, OEA, and stearoylethanolamide has been shown in patients (11), and a recent study suggests that PEA administered adjunctively to citalopram improves depression (51). Finally, intense exercise may counteract depression and PTSD by increasing serum PEA and OEA levels (52), with possible downstream effects on Allo biosynthesis (53).

While extensive studies have showed that CB1 regulates emotions and stress responses (54), the new role for PPAR-α on emotions is just emerging. The impact of PPAR-α in behavior regulation is underscored by studies showing that, like CB1, PPAR-α is expressed in glutamatergic neurons of emotion-relevant areas (amygdala, hippocampus, frontal cortex) (6,55). However, unlike PPAR-α, targeting CB1 results in unwanted psychotomimetic effects and addition liabilities that limit its pharmacological potentials (56). Glutamatergic neurons in corticolimbic areas express the enzymatic machinery essential for Allo neosynthesis, which, similarly to the eCB system, regulates synaptic transmission and mediates

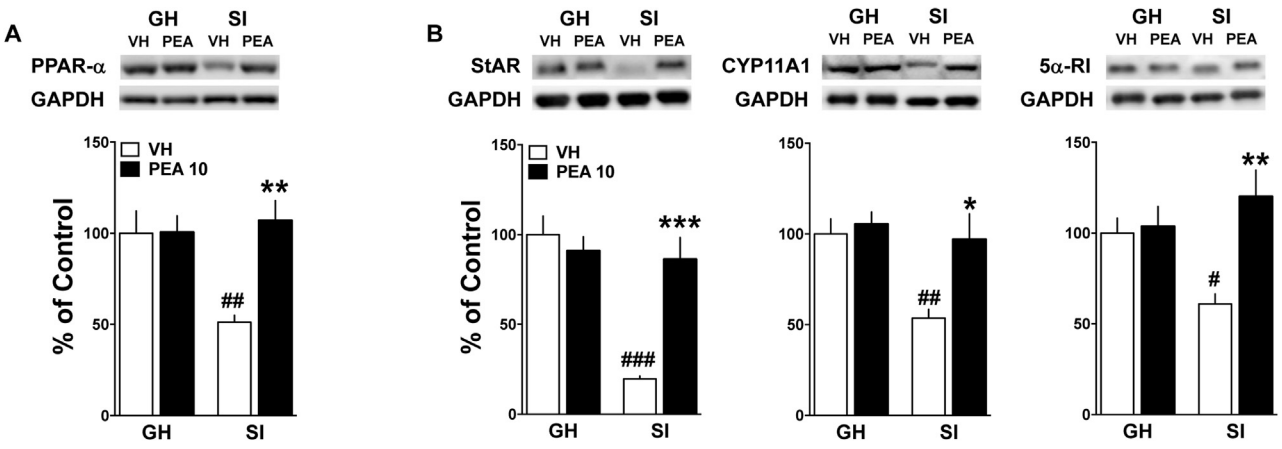

Figure 4. *N*-palmitoylethanolamine (PEA) affects expression of peroxisome proliferator-activated receptor (PPAR)-α and neurosteroidogenic proteins in the hippocampus of socially isolated (SI) mice. A single administration of PEA (black bars) reverses the downregulation of **(A)** PPAR-α (treatment: $F_{1,24} = 8.76$, $p = .007$; isolation: $F_{1,24} = 4.94$, $p = .036$; interaction: $F_{1,24} = 8.38$, $p = .008$), as well as **(B)** steroidogenic acute regulatory protein (StAR) (treatment: $F_{1,24} = 10.39$, $p = .0036$; isolation: $F_{1,24} = 22.77$, $p < .0001$; interaction: $F_{1,24} = 17.97$, $p = .0003$), cholesterol side-chain cleavage enzyme (CYP11A1) (treatment: $F_{1,24} = 7.22$, $p = .013$; isolation: $F_{1,24} = 9.06$, $p = .006$; interaction: $F_{1,24} = 4.34$, $p = .048$), and 5α-reductase type I (5α-RI) (treatment: $F_{1,23} = 8.59$, $p = .008$; isolation: $F_{1,23} = 1.09$, $p = .31$; interaction: $F_{1,23} = 6.74$, $p = .016$). Values are reported in percentage; average of vehicle (VH)-treated group-housed (GH) mice is considered as 100%. Data represent the mean ± SEM of 7 mice. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$, when compared with vehicle-treated SI mice; $^{\#}p < .05$, $^{\#\#}p < .01$, and $^{\#\#\#}p < .001$, when compared with vehicle-treated GH mice. Two-way analysis of variance followed by Tukey's post hoc analysis. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

plasticity, influencing synaptic formation and neurogenesis in response to stress (16,17). Intriguingly, in mouse models of stress, decreased Allo levels in corticolimbic neurons have been linked to anxiety-like behavior and increased fear responses, while supplementing Allo or giving neurosteroidogenic agents, including PPAR-α agonists, rescues behavioral deficits (25,28,57).

The relevance of the PEA-induced increase of Allo-S, in addition to enhancing Allo levels, is twofold. Sulfated neurosteroids, such as Allo-S and its isomer, pregnanolone-sulfate, negatively regulate tonic *N*-methyl-D-aspartate (NMDA) neurotransmission (58). Targeting NMDA receptors in circuitry with relevance for mood disorders is becoming a strategy in novel antidepressant treatment (59). This therapeutic trend is highlighted by growing evidence of rapid antidepressant action of molecules (e.g., ketamine) that function as NMDA antagonists (60). Hence, it is conceivable that PEA-induced Allo and Allo-S upregulation may act in concert, on one hand by potentiating $GABA_A$ receptor–mediated inhibition, and on the other by inhibiting NMDA-mediated excitatory neurotransmission. In kind, recent findings have shown that PEA improves neurological (e.g., normalization of astrocytic function and glutamatergic transmission) and behavioral abnormalities in animal models of PTSD, traumatic brain injury, and Alzheimer's disease (36,61,62).

In SI mice, decreased Allo levels correlate with the downregulation of the rate-limiting step enzyme 5α-RI in cortical and hippocampal pyramidal neurons (17,63). This conforms to human studies that show that 5α-RI is downregulated in the prefrontal cortex of depressed male patients (23). In PTSD, a gender-dependent disruption in the Allo biosynthetic enzyme pathway was found at the level of 3α-hydroxysteroid dehydrogenase in women and 5α-R (5α-RI and II could not be differentiated) in men (22,64). Our study shows that PEA normalizes the decreased 5α-RI hippocampal expression in SI mice; we did not, however, investigate possible parallel effects on 5α-RII—a potential focus for future studies.

Furthermore, studies conducted in rodent and human liver suggest that PPAR-α regulates the expression of genes, including lipid/hormone and cholesterol transport (65). Among the mechanisms connecting PPAR-α with Allo, PEA upregulates StAR, which participates in the translocation of cholesterol into the inner mitochondrial membrane, and CYP11A1, which is the first-step enzyme-converting cholesterol into pregnenolone (the precursor of all neurosteroids), in the hippocampus of SI mice. Future studies will have to address whether PEA induces StAR, CYP11A1, and 5α-RI gene expression through direct or indirect mechanisms, including activation of cofactors implicated in the regulation of gene expression or epigenetic modifications (66).

Importantly, we also show that social isolation downregulates the hippocampal PPAR-α expression and that administering PEA reverses this effect. Mechanistically, it is conceivable that PEA-induced upregulation of PPAR-α may normalize behavior by enhancing PPAR-α binding at the consensus region, PPAR response element, on the promoter of neurosteroidogenic genes to initiate transcription (Figure 5). In support, the downregulation of PPAR-α expression can be normalized by application of PEA in primary astrocytes (67). Furthermore, topical administration of PEA and the PPAR-α synthetic agonists G7 and Wy-14643 increased PPAR-α expression in mouse skin (68). Remarkably, in our studies, the effects of PPAR-α agonists on neurosteroidogenesis appeared 1 hour after administration. This relatively fast effect could be supported by the evidence that activation of PPAR-α by agonists induces the upregulation of "early response genes," including *Fos, Junb, Jun, Jund,* and *Myc*, which can be activated and transcribed within minutes, without requiring de

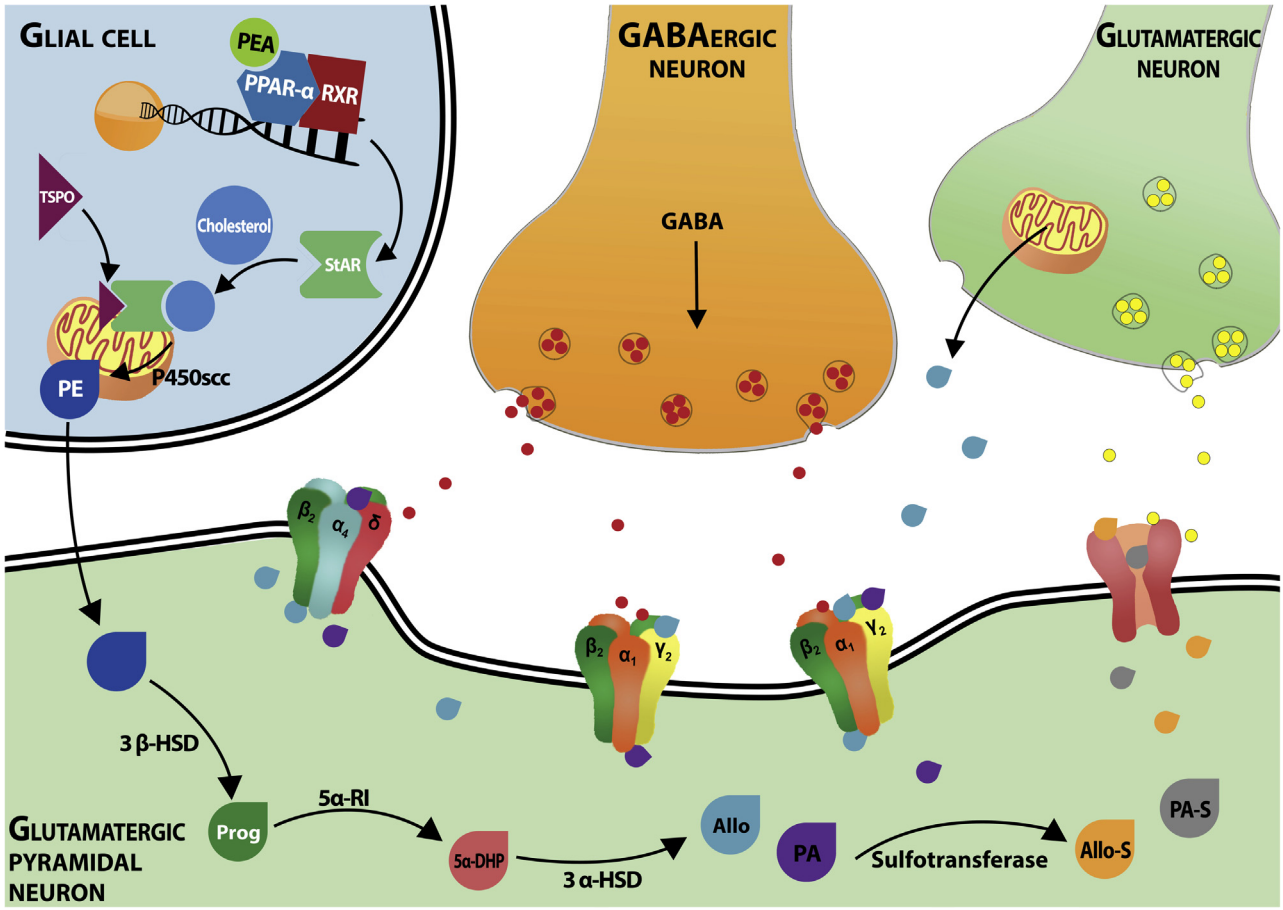

Figure 5. Schematic representation of the peroxisome proliferator-activated receptor (PPAR)-α-allopregnanolone (Allo) biomarker axis. When PPAR-α is activated by an endogenous (*N*-palmitoylethanolamine [PEA]) or a synthetic (e.g., GW7647, fenofibrate) agonist, it heterodimerizes with a PPAR-α-specific retinoid X-receptor (RXR). The PPAR–RXR dimer then recognizes and binds the PPAR response element, which is located in the promoter region to regulate gene expression. Our findings suggest that PPAR-α-activation reverses corticolimbic social isolation–induced downregulation of steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme (P450scc), which are proteins that are crucial to the translocation and metabolism of cholesterol into pregnenolone (PE) (the precursors of all neurosteroids) in the inner mitochondrial membrane (18). PE can be converted to Allo and its equipotent isomer, pregnanolone (PA), which potently, allosterically, and positively potentiate the action of gamma-aminobutyric acid (GABA) at $GABA_A$ receptors (15) in pyramidal neurons (16). Normalization of 5α-reductase type I (5α-RI) expression induced by activation of PPAR-α is an additional contribute to reverse the reduction of brain Allo concentrations induced by social isolation. Allo and PA can also be sulfated and can inhibit tonic-mediated *N*-methyl-D-aspartate receptor neurotransmission, which may have additional important effects in improving emotions and cognition. 5α-DHP, 5α-dihydroprogesterone; Allo-S, allopregnanolone sulfate; HSD, hydroxysteroid dehydrogenase; PA-S, pregnanolone sulfate; Prog, progesterone; TSPO, translocator protein.

novo protein synthesis (69). Future studies should elucidate in better detail the molecular mechanisms underlying PEA activation of PPAR-α and related downstream effects.

While PEA acts at PPAR-α and shows no affinity for PPAR-δ or PPAR-γ (4), some of the effects of PEA could be mediated by GPR55 and GPR119 (3). Recent findings suggest that PEA modulates striatal inhibitory synaptic currents via GPR55 activation, promoting a transient increase of GABAergic spontaneous inhibitory postsynaptic current frequency (2). However, we found that PEA's anxiolytic-like effects were not related to GPR55 or GPR119 activation in SI mice (Supplemental Figure S3), which supports the idea that PPAR-α's effects on behavior are mediated through PPAR-α on brain neurosteroidogenesis.

Future experiments in SI mice should evaluate 1) brain PEA levels and correlations among eCBs and neurosteroids, and 2) the corticolimbic expression of PEA biosynthesis and metabolic enzymes. Moreover, tissue-specific (cerebrospinal fluid, saliva, serum) correlations among alterations in specific eCBs, their congeners, neurosteroids, and their interactions could unveil biomarkers and improve treatment of PTSD [discussed in (70–72)].

Conclusions

The demonstration that PPAR-α activation by endogenous (PEA) or synthetic (G7, FFB) ligands improves PTSD-related behaviors by stimulating corticolimbic Allo biosynthesis may lead to novel strategies to diagnose, prevent, or treat PTSD.

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GP and AL have a patent pending application on PEA and PPAR-α agonists in the treatment of neuropsychiatric disorders.

ARTICLE INFORMATION

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