Drug Design, Development and Therapy

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REVIEW

Endocannabinoid Hydrolase Inhibitors: Potential Novel Anxiolytic Drugs

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Abstract: Over the past decade, the idea of targeting the endocannabinoid system to treat anxiety disorders has received increasing attention. Previous studies focused more on developing cannabinoid receptor agonists or supplementing exogenous cannabinoids, which are prone to various adverse effects due to their strong pharmacological activity and poor receptor selectivity, limiting their application in clinical research. Endocannabinoid hydrolase inhibitors are considered to be the most promising development strategies for the treatment of anxiety disorders. More recent efforts have emphasized that inhibition of two major endogenous cannabinoid hydrolases, monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH), indirectly activates cannabinoid receptors by increasing endogenous cannabinoid levels in the synaptic gap, circumventing receptor desensitization resulting from direct enhancement of endogenous cannabinoid signaling. In this review, we comprehensively summarize the anxiolytic effects of MAGL and FAAH inhibitors and their potential pharmacological mechanisms, highlight reported novel inhibitors or natural products, and provide an outlook on future directions in this field.

Keywords: endocannabinoid hydrolase inhibitors, endocannabinoid system, anxiety disorders, anxiolytic, MAGL, FAAH

Introduction

Anxiety disorders represent prevalent mental health conditions characterized by persistent psychogenic anxiety, somatic anxiety, and sleep disorders. According to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), the classification of anxiety disorders includes Generalized Anxiety Disorder, Phobias (including specific phobias and social anxiety disorder), Panic Disorder, as well as other anxiety-related conditions such as Separation Anxiety Disorder and Selective Mutism.[1] The global prevalence of anxiety disorders ranges from 7.3% to 28.0%, making them a significant concern worldwide.[2] Specifically, in China, anxiety disorders stand out as the most prevalent mental illness, with a lifetime incidence of 7.6%.[3]

The World Health Organization underscores the significance of anxiety disorders by ranking them as the sixth leading contributor to global disability, establishing it as a pressing health and wellness concern that demands attention.[4] Regrettably, the existing repertoire of anxiolytic medications faces challenges in adequately addressing the needs of the extensive population affected by anxiety disorders.[5] Benzodiazepines and selective serotonin reuptake inhibitors (SSRIs) antidepressants are the primary drugs employed clinically for the treatment of anxiety disorders.[6,7] However, their anxiolytic effects are secondary indications rather than primary, and the drugs, especially benzodiazepines, are subject to stringent controls and possess potential addictive properties.[8] Consequently, the development of novel drugs targeting anxiety disorders remains a focal point in the ongoing research and development of psychotropic medications.

Traditionally, anxiolytic drug development focused on neurotransmitter-based hypotheses, yielding medications like eszopiclone and buspirone.[9,10] Recent attention has turned to novel approaches, particularly targeting the endocannabinoid

Received: 3 February 2024
Accepted: 27 May 2024
Published: 11 June 2024

system (ECS). The ECS, integral to mood regulation, has been implicated in the pathogenesis of both anxiety and depression, underscoring its potential as a novel therapeutic frontier.[11] Novel drug designs that target the ECS are considered to be the most promising candidates for the treatment of anxiety disorders.[12]

Studies explore ECS modulation for anxiolysis, including increasing endogenous cannabinoids (eCBs) secretion and inhibiting their hydrolase enzymes. However, challenges arise due to the unique characteristics of endocannabinoids. Efforts to develop cannabinoid receptor agonists face obstacles like pharmacological intensity and adverse effects. A more promising strategy involves inhibiting eCB hydrolases, notably monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH), indirectly activating cannabinoid receptors. Clinical trials with FAAH inhibitors, such as JNJ-42165279, have shown positive anxiolytic potential.[13] This paper comprehensively summarizes the anxiolytic effects of eCB hydrolase inhibitors, focusing on underlying mechanisms and highlighting novel inhibitors or natural products. The insights from existing studies aim to provide new perspectives for the development of innovative anxiolytic drugs.

Endocannabinoid System

The ECS is one of the crucial regulatory systems in the central nervous system, and consists of cannabinoid receptor 1 (CB1R), cannabinoid receptor 2 (CB2R), eCBs, and their corresponding synthesizing and degrading enzymes. eCBs are classified into three categories according to chemical structure: 1) esters: such as 2-arachidonoylglycerol (2-AG), anandamide (AEA); 2) amides: such as palmitoylethanolamide (PEA), oleoylethanolamide (OEA); 3) ethers: 2-arachidonyl glyceryl ether (noladin ether or 2-AGE).[14,15] Among them, AEA and 2-AG are more abundant and widely distributed in the human body, while specific eCBs such as OEA and 2-AGE can only be detected in particular regions of brain tissue.[16] Notably, 2-AG is agonistic for both CB1R and CB2R, whereas AEA selectively has a high affinity for CB1R but almost no activity on CB2R.[17]

The ways of synthesis, transport and inactivation of 2-AG and AEA in their respective target tissues are also different. 2-AG is mainly produced by 1-oleoyl-2-arachidonoyl-sn-glycerol (OAG) and 1-stearoyl-2-arachidonoylglycerol (SAG) via diacylglycerol lipase α (DAGLα), whereas AEA produced by N-acylphosphatidylethanolamine (NAPE) catalysed by N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD).[11] Degradation and inactivation of eCBs mainly involve two pathways: hydrolysis and oxidation. MAGL, a specific hydrolase of 2-AG, is mainly distributed on the axons of presynaptic neurons and is able to terminate retrograde eCB signaling generated by postsynaptic neuronal activity. Approximately 85% of 2-AG in the brain is hydrolyzed and metabolized to arachidonic acid and glycerol by MAGL.[18] AEA was mainly degraded by FAAH into free arachidonic acid and ethanolamine. FAAH, a serine hydrolase mainly present in the endoplasmic membrane, is widely distributed in the central nervous system and hydrolyses various fatty acid amides, including AEA. In another oxidation pathway, the critical enzyme involved is cyclooxygenase-2 (COX-2), catalyzing the synthesis of prostaglandin ethanolamide (PG-EA) and prostaglandin glycerol (PG-G) from 2-AG and AEA.[11] Figure 1 briefly illustrates the composition of the endocannabinoid system, and the synthesis and degradation pathways of endocannabinoids.

Numerous studies have demonstrated that the anxiolytic effects could be exerted by increasing eCB secretion, applying exogenous cannabinoids, agonizing cannabinoid receptors, and decreasing eCB hydrolase activity.[19–21] However, unlike classical neurotransmitters and neuropeptides, eCBs are not stored within synaptic vesicles, but are produced on demand and immediately released from neurons. At the same time, the eCBs synthesis pathways are numerous and complex, and the rate-limiting enzymes are plentiful and poorly defined, leading to insignificant gains through the strategy of increasing the activity of eCB synthetases. In addition, drug development targeting cannabinoid receptor agonists or adding exogenous cannabinoids is susceptible to exclusion from clinical trials or even post-market withdrawal due to stronger pharmacological activity, irreversible binding, poor receptor selectivity, and increased risk of adverse effects.[22,23] Therefore, the most prominent strategies may be to search for inhibitors of eCB hydrolases MAGL and FAAH, especially reversible inhibitors that indirectly activate cannabinoid receptors by increasing synaptic gap levels of 2-AG and AEA, respectively.[24]

2-AG Hydrolase MAGL Inhibitors in Treatment for Anxiety

2-AG, an abundant brain eCB, surpasses AEA levels by 200 times.[25] Clinical studies revealed that serum 2-AG levels are significantly reduced in patients with post-traumatic stress disorder (PTSD), which was previously often categorized as an anxiety disorder.[26] Additionally, it has been found that increasing 2-AG levels through exercise can alleviate anxiety

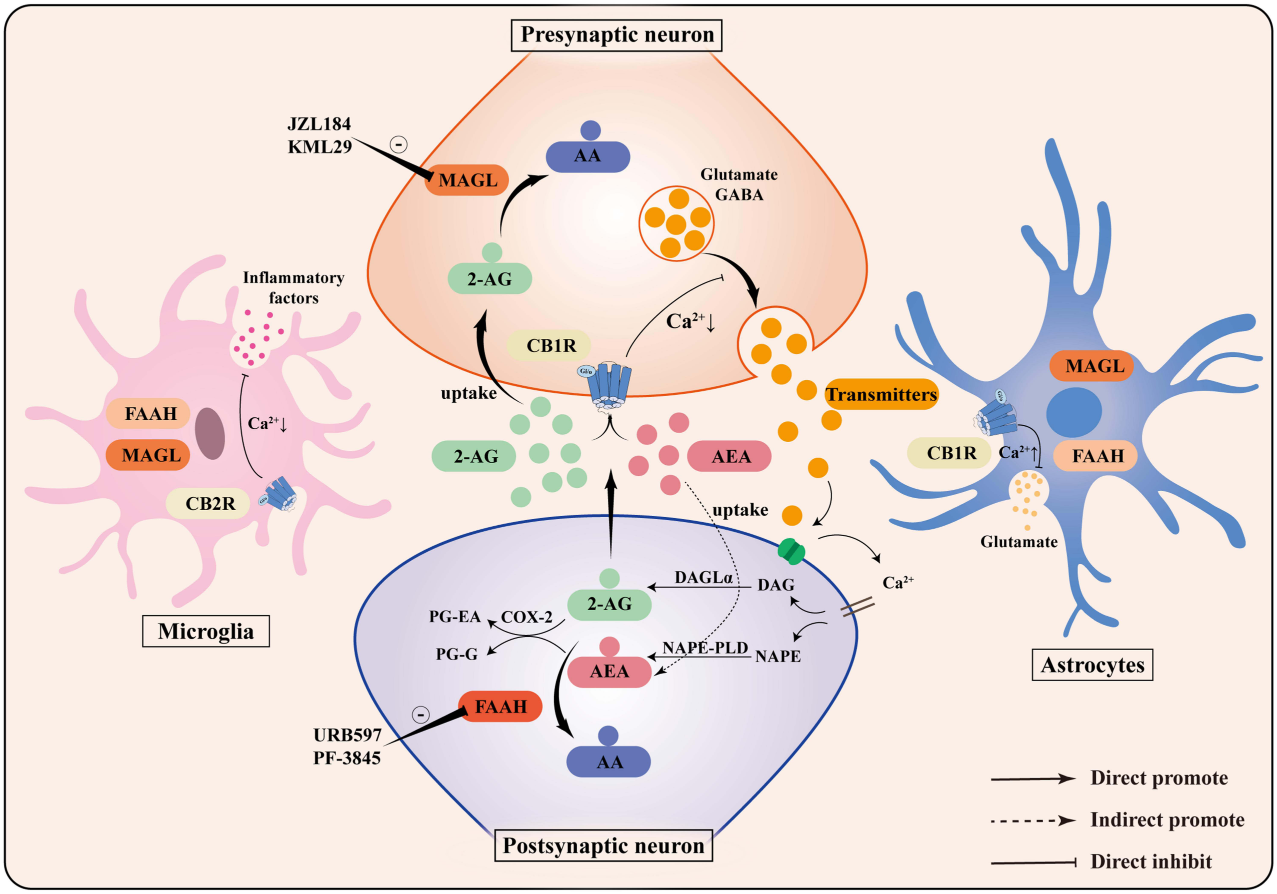

Figure 1 Composition of the endocannabinoid system. The endocannabinoid system consists of CB1R, CB2R, endocannabinoids, and their corresponding synthesizing and degrading enzymes. 2-AG and AEA, the primary endocannabinoids, are produced on demand and are synthesized from the postsynaptic terminals by DAGLα and NAPE-PLD, respectively, to activate presynaptic cannabinoid receptors. CB1R activation inhibits presynaptic neurotransmitter release and promotes astrocytic glutamate release. CB2R activation reduces microglial inflammatory factor production. 2-AG and AEA are enzymatically degraded to AA by MAGL and FAAH hydrolases, and can also be oxidatively degraded to PG-EA and PG-G by COX-2.

Abbreviations: CB1R, cannabinoid receptor 1; CB2R, cannabinoid receptor 2; 2-AG, 2-arachidonoylglycerol; AEA, anandamide; NAPE-PLD, N-acylphosphatidylethanolamine-specific phospholipase D; DAGLα, diacylglycerol lipase α; AA, arachidonic acid; MAGL, monoacylglycerol lipase; FAAH, fatty acid amide hydrolase; PG-EA, prostaglandin ethanolamide; PG-G, prostaglandin glycerol; COX-2, cyclooxygenase-2.

symptoms.[27] This findings are consistent with rodent studies,[28,29] where higher MAGL levels have been strongly linked to the production of anxious behaviors.[30] Inhibition of MAGL activity enhances central synaptic 2-AG-mediated phasic and tonic signaling, which has positive implications in anxiety relief, reduction of stress-induced anxiety susceptibility, and fear extinction.[31]

Anxiolytic Effects of MAGL Inhibitors

Due to MAGL's pivotal role in regulating 2-AG levels and synaptic transmission, efforts to enhance 2-AG signaling for therapeutic purposes have centered on inhibiting MAGL enzyme activity. Numerous pharmacological studies highlight that systemic or local administration of MAGL inhibitors can effectively reduce anxiety-like behaviors induced by acute or chronic stress (Table 1). For example, aberrant excitation of glutamatergic neurons in basolateral amygdala (BLA)-prelimbic prefrontal cortex (plPFC) neural circuit in mice subjected to chronic stress, accompanied by abnormal 2-AG-CB1R signal, and administration of a MAGL inhibitor reversed anxiety-like behavior.[32] Overexpression of MAGL in hippocampal glutamatergic neurons also increases anxiety-like behavior in animals.[33] In addition, anxiety-like behaviors induced by traumatic brain injury and alcohol withdrawal improved after administration with MAGL inhibitor.[34]

However, it is important to note that 2-AG levels do not consistently correlate negatively with anxiety disorders. Acute stress given to healthy people increased circulating concentrations of AEA in vivo but had no significant effect on 2-AG.[45] In

Table 1 Summary of the Anxiolytic Effects of MAGL Inhibitors in Preclinical Studies

Drug	Dose/Administration	Animal	Model	Test	Effects	References
JZL184	2, 10, 40 mg/kg, i.p	C57BL/6J mice, male	–	EPM; LDT	↑ percent time in open-arm; ↑ percent light time	[35]
	2 mg/kg, i.p	Sprague-Dawley rats, male and female	ELS	OFT	↑ activity level	[36]
	1, 3 mg/kg, i.p	C57BL/6J mice, male	ARS	EPM	↑ percent time in open-arm	[34]
	5, 8, 10, 40 mg/kg, i.p	ICR mice, male	ARS and Foot shock stress	LDT; NIH; EZM; OFT	↑ percent light time and distance; ↓ feeding latency and ↑ food consumption;↑ open arm entries and total distance, ↓ time immobile in open arm and exit latency; ↑ total distance and ↓ number of faeces	[37]
	3, 5, 10, 15 mg/kg, i.p	ICR mice, male	ARS	LDT	↑ percent light time, light distance, and total distance	[38]
	1 µg, intra-NAc microinjection	C57BL/6J mice, male	CSDS	OFT; LDT; EPM	↑ percent center time; ↑ time in light; ↑ percent time in open arm	[39]
	8 mg/kg, i.p	C57BL/6J mice, male	CUS	EPM; LDT	↑ duration and frequency in open arm; ↑ time and frequency in light compartment	[40]
	4, 8, 16 mg/kg, i.p	ICR mice, male	CRS	OFT; NIFS; EPM; MBT	No significance in OFT and EPM test; ↓ feeding latency; ↓ marble burying	[41]
	16 mg/kg, i.p	C57BL/6J mice, male	-	MBT	↓ marble burying	[42]
KML29	200 ng, intra-vmPFC microinjection	Fischer-344 rats, male	Tail shocks	Social exploration	↑ social exploration	[43]
	40 mg/kg, i.p		Tail shocks	Social exploration	↓ social exploration	
MJN110	5, 10 mg/kg, i.p	Wistar rats, male and female	-	NIH	↑ feeding consumption	[44]
	10, 20 mg/kg, i.p	Wistar rats, male	ARS	EPM	↑ percent time in open-arm	[34]

Abbreviations: ARS, acute restraint stress; CRS, chronic restraint stress; CSDS, chronic social defeat stress; CUS, chronic unpredictable stress; ELS, early life stress; EPM, elevated plus maze; EZM, elevated zero maze; i.p, intraperitoneal; LDT, light-dark box test; MBT, marble burying test; NAc, nucleus accumbens; NIFS, novelty-induced feeding suppression; NIH, novelty-induced hypophagia; OFT, open-field test.

chronic restraint stress mice, 2-AG levels in the cingulate cortex (ACC), caudate putamen (CP), nucleus accumbens (NAc), and piriform cortex (PIR) all increased.[46] Similarly, 2-AG content increased in the amygdala of chronic stress-induced anxiety mice.[41,47] The researchers explained that the above phenomenon was due to CB1R desensitization, as it did not affect the anxiolytic effect of applying JZL184. Bedse G's research also supports the idea that increased 2-AG is a compensatory response to counteract anxiety-like behaviors induced by stress, and that 2-AG pharmacological enhancers can augment this response to more effectively counteract the adverse effects of stress.[38] Therefore, the benefit of elevating 2-AG pharmacologically through MAGL inhibitors in the treatment of anxiety disorders is definitive.

Interestingly, instead of demonstrating anxiolytic effects, full knockout MAGL significantly reduced the duration in the light box in mice.[48] The study found that the CB1R receptor was significantly downregulated in MAGL knockout mice, possibly due to enhanced levels of innate endogenous cannabinoids leading to CB1R desensitization and the emergence of anxiety-like behavior. Consistent with these findings, CB1R density and functional responses were reduced in chronic MAGL inactivation and MAGL KO mice.[49] In conclusion, congenital and chronic MAGL inactivation may lead to CB1R desensitization and feedback down-regulation, which may inhibit the downstream anti-anxiety effect of CB1R, but more in-depth studies are needed.

Anxiolytic Mechanisms of MAGL Inhibitors

Although preclinical studies support the anxiolytic effects of MAGL inhibitors, their specific downstream molecular mechanisms remain poorly understood. As shown in Figure 2, we summarized the potential mechanisms of MAGL from the following three aspects based on the above pharmacological studies.

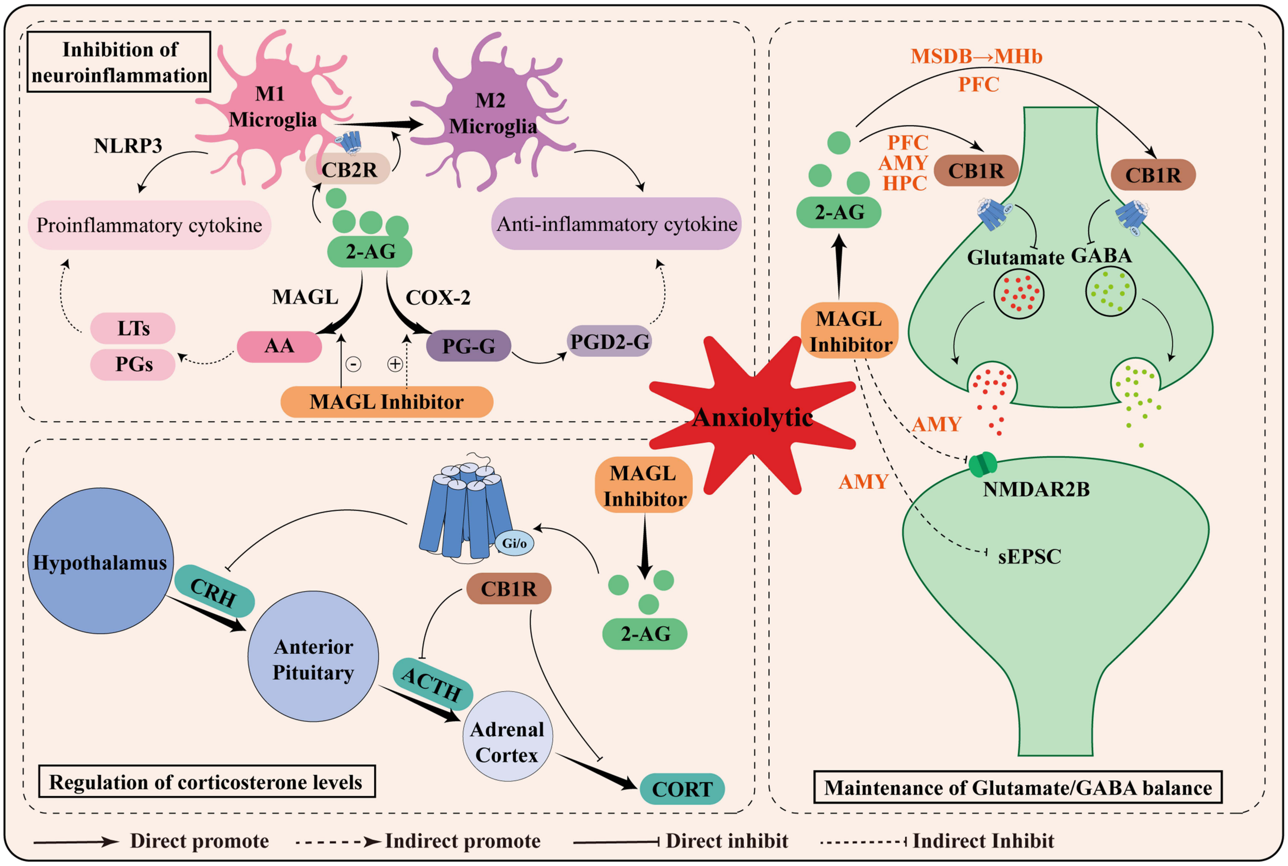

Figure 2 Schematic representation of the mechanism of anxiolytic action mediated by MAGL inhibitors. Briefly, the anxiolytic effects of MAGL are related to its maintenance of Glutamate/GABA balance, inhibition of neuroinflammation, and regulation of corticosterone levels.

Abbreviations: MAGL, monoacylglycerol lipase; CB1R, cannabinoid receptor 1; 2-AG, 2-arachidonoylglycerol; AMY, amygdala; PFC, prefrontal cortex; HPC, hippocampus; MSDB, medial septum and nucleus of the diagonal band; MHb, medial habenula; sEPSC, spontaneous excitatory postsynaptic currents; CB2R, cannabinoid receptor 2; COX-2, cyclooxygenase-2; AA, arachidonic acid; PGs, prostaglandins; LTs, leukotrienes; PG-G, prostaglandin glycerol; PGD2-G, prostaglandin D2-glyceryl ester; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; CORT, corticosterone.

Maintenance of Glutamate/GABA Balance

Glutamate and GABA are representative excitatory and inhibitory transmitters in the central nervous system, which cause neurons to generate corresponding excitatory and inhibitory currents through inter-synaptic transmitter transmission. Typically, neuronal excitation/inhibition (E/I) is in a dynamic balance. Once this balance is disturbed, especially when it tends to be excitatory, it can lead to the development of anxiety disorders. The E/I balance in the PFC, a brain region responsible for executive function, stress and emotion regulation, seems to play an important role in the anxiolytic effects of MAGL inhibitors.[50] PFC projection neurons are involved in the development of anxiety by modulating neural signaling in downstream brain regions during exposure to stress.[51] Systemic administration of the MAGL inhibitor, KML29, increased vmPFC excitability but could be blocked by CB1R or GABA receptor antagonist, which supports the conclusion that activation of the CB1R leads to transient inhibition of GABA release and long-term inhibition of inhibitory transmission.[43] Consistent with this finding, the CB1R agonist 5F-AMB attenuates glutamatergic and GABAergic synaptic transmission in mPFC L5 pyramidal neurons, leading to an E/I imbalance.[52]

Meanwhile, the amygdala, an essential part of the limbic system that controls emotion, plays a crucial role in the regulation of anxiety, and maintaining its E/I balance is of great significance.[53] MAGL inhibitors inhibit Glutaminergic neurotransmission in the amygdala region. It was found that increased expression of multiple glutamate receptors in the amygdala, including mGluR1, mGluR5, and NMDAR1, was accompanied by elevated expression of MAGL in an alcohol exposure-induced anxiety model,[30] and that JZL184 could exert anxiolytic effects by indirectly activating the CB1R to reduce NMDAR2B expression.[54] Bedse et al found that acute stress increased the frequency of spontaneous excitatory postsynaptic currents (sEPSC) in basolateral amygdala neurons and was positively correlated with anxiety-like behaviors, with the above reversed after administration with JZL184.[38]

Notably, 2-AG is recognized as a potent agonist of CB1R. CB1R is found in both glutamatergic and GABAergic neurons, but the relative abundance of glutamatergic and GABAergic neurons in various brain regions varies, potentially causing functional alterations in distinct brain regions. Lysine-specific demethylase 1 (LSD1) suppressed hippocampal MAGL transcript levels and activated CB1R to inhibit glutamate release in response to anxiety.[55] JZL184 enhanced the inhibitory effect of 2-AG on the release of GABA from the medial septum and nucleus of the diagonal band (MSDB) axons to the medial habenula (MHb) and produced anxiolytic effects.[56]

Inhibition of Neuroinflammation

Neuroinflammation is considered to be a trigger for behavioral changes and cognitive deficits in several psychiatric disorders, including anxiety disorders. Prolonged exposure to stress imbalances the central immune system and affects the secretion and function of immune cells and cytokines.[57] Currently, MAGL inhibitors play an important role in the treatment of central and peripheral inflammation.[58,59] The study showed that JZL184 reduced LPS-induced expression of IL-1β, IL-6, TNF-α, and IL-10 in the prefrontal cortex and spleen.[60]

Dual metabolism of 2-AG is closely relevant to the anti-inflammatory effects exerted by MAGL inhibitors. Arachidonic acid (AA), the main metabolite of 2-AG hydrolysis by MAGL, is a crucial precursor of proinflammatory prostaglandins and leukotrienes involved in inflammatory responses and immune initiation. The latest single-cell sequencing found that MAGL KO mice had significantly upregulated genes related to immunity and inflammation in microglia and astrocytes, which enable glial cells to react rapidly to insults.[61] Specifically knocking out MAGL in astrocytes reverses LPS-induced inflammatory activation and is not blocked by the CB1R agonist SR141716.[62] Similarly, CB1R/CB2R antagonists did not block JZL184 from inhibiting LPS-induced neuroinflammation.[63] This suggests that the anti-inflammatory effect produced by MAGL inhibition is a direct result of reduced prostaglandin, rather than a profile result of enhanced endocannabinoid signaling. In addition, enhanced inhibition of 2-AG hydrolysis can promote the oxidation pathway mediated by COX-2 to produce prostaglandin glycerides (PG-Gs), with PGD2-G exhibiting anti-inflammatory activation.[64]

CB2R, which can be fully activated by 2-AG, is mainly expressed in brain microglia and associated with neuroinflammation.[65] Many studies have confirmed the anxiolytic effect of CB2R agonists, and found that the use of CB2R agonist AM1241 inhibited the over-activation of PFC microglia by inhibiting the NLRP3 pathway, thereby improving anxiety-like behavior.[66] In addition, CB2R activation also promoted the transformation of microglia to M2 anti-inflammatory phenotype, creating positive feedback by releasing more 2-AG and AEA.[67,68] Thus, the effects of MAGL inhibitors in improving anxiety may be related to increasing the response of glial cells to external stimuli and promoting the polarization transformation of microglia.

Regulation of Corticosterone Levels

The dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, the main regulator of stress response, is one of the biological mechanisms of anxiety. Increased corticosterone is an important marker of HPA axis activation. Studies have shown that ECS bi-directionally regulates the function of the HPA axis. MAGL inhibitors reduced high corticosterone levels in mice 30 min after chronic restraint stress but restored higher corticosterone levels after 120 min, whereas these changes were not observed in CB1R knockout mice.[69] The reason for these phenomena was the predominant inhibition of HPA axis activation during the early phase of JZL184 injection, whereas the sustained activation of CB1R elevated circulating corticosterone levels with increasing 2-AG concentrations. Aliczki et al found that metiramone, a cortisol synthesis blocker, reversed the increased open-arm time in the EPM test in mice with JZL184, suggesting that the anxiolytic effect of JZL184 is accompanied by activation of the HPA axis.[70] Glucocorticoids released after exposure to stress activate CB1R signaling in the mPFC, inhibit GABA release, and act on the HPA axis negative feedback process to appropriately reduce corticosterone secretion.[71] Therefore, MAGL inhibitors may exert anxiolytic effects through CB1R-dependent glucocorticoid increases.

Novel MAGL Inhibitors and Natural Product Development

Given the significant pharmacological effects and higher safety of MAGL inhibitors, there is growing attention towards the development of novel inhibitors, with substantial efforts underway to identify safer, more effective, and reversible MAGL inhibitors. Research has primarily focused on developing novel inhibitors through structural modification of lead compounds or the use of fluorescent probes. Finding MAGL inhibitors from natural products has also been a strategic approach. Encouragingly, the majority of natural products reported to have MAGL inhibitory activity are reversible, enhancing drug safety with their gentle effects.

Reversible MAGL Inhibitors

A total of 96 patents related to MAGL inhibitors were searched (https://patentscope.wipo.int/). Initially developed MAGL inhibitors were eliminated due to poor inhibition and lack of selectivity for MAGL. Later, Cravatt et al discovered JZL184, which was irreversible, inhibitory, and selective, was widely used in experimental studies. JZL184 is a potent tool compound, but it is difficult to use clinically due to the fact that it exhibits some cross-reactivity of FAAH at high doses (40 mg/kg) and some species differences in MAGL inhibitory potency.[72] Subsequently, KML29 was developed with better selectivity and good neuroprotection in a stroke model and good analgesic activity in a migraine model.[73,74]

However, JZL184, KML29, NAM, SAR629 and CK37 are all irreversible inhibitors of MAGL, which are prone to physical dependence, endocannabinoid dependent synaptic plasticity impairment and cross-tolerance to exogenous CB1 agonists.[75] JJKK048 is the first reported reversible, highly selective MAGL inhibitor with powerful analgesic effects and does not cause side effects similar to cannabis.[76] (R)-3t, a synthetic selective and reversible MAGL inhibitor, reduced arachidonic acid levels and increased 2-AG levels in the brain after gavage in mice.[77] Recently discovered M-18c, with an IC_{50} value of 662.6 nM for MAGL, attenuates LPS-induced acute kidney injury by inhibiting NLRP3-associated inflammation.[78] Compared with irreversible inhibitors, reversible inhibitors have better pharmacokinetic advantages and offer substantial advantages in drug safety, tolerability and efficacy, with better development prospects and clinical applications.

Structural Modifications Based on Lead Compounds

Virtual screening based on molecular docking was applied to discover novel reversible MAGL inhibitors. For example, Afzal O performed virtual docking of the ZINC database with 21 million compounds and screened seven potential activities, of which ZINC24092691 showed significant inhibitory activity.[79] DC630-8[80] and CL6a[81] also showed good inhibitory activity based on virtual docking. However, the screened compounds have poor drug-forming properties due to low screening efficacy. Therefore, many studies of structural modification based on lead compounds and functional group replacement have been added to the search for reversible inhibitors. Arylformylpiperidine derivatives developed by Zhi et al exhibit good reversible inhibitory properties and significantly ameliorate rifampicin-induced depressive-like behavior, providing support for MAGL as a potential therapeutic target for depression.[82] In addition, classical MAGL inhibitors such as CAY10499, JZL184, and ABX-1431 (also known as Lu AG06466) are classified as carbamate derivatives. Tiziano Tuccinardi's team discovered a series of new compounds with MAGL-inhibitory properties based on benzylpiperidine derivatives, such as compound 13 with antipancreatic cancer effects,[83] compounds 28 and 29 with low in vivo toxicity and high selectivity,[84] diphenylsulfide-benzoylpiperidine derivatives with anticancer activity.[85]

Looking for potential MAGL inhibitory activity in existing drugs is also a strategy. Disulfiram, an aldehyde dehydrogenase inhibitor used primarily in the treatment of chronic alcoholism, was shown to be a MAGL inhibitor that irreversibly inhibits MAGL through carbamoylation of Cys208 and Cys242 located near the MAGL active site.[86] However, due to the presence of some FAAH inhibitory activity, Omran synthesized compounds targeting MAGL but lacking anti-FAAH activity by replacing the two ethyl groups in the disulfide.[87] Recently, cetirizine and levetiracetam have also been found to have potential MAGL inhibitory activity, with IC_{50} values of 9.3931 µM and 3.0095 µM, respectively, and demonstrated some analgesic and anti-inflammatory activity.[88]

Consideration of pharmacokinetic distribution is crucial in developing MAGL inhibitors. LEI-515, a recently discovered peripherally restricted reversible MAGL inhibitor, interestingly increased 2-AG levels only in peripheral organs but not in the mouse brain, hinting at potential applications in peripheral diseases.[89] Additionally, the compound properties can be improved by structural modification or application of nanocarriers if the pharmacokinetics are not ideal. Muhammad Adeel's team developed the first nano-formulation of a MAGL inhibitor, MAGL23, which showed promising anti-tumour activity by using albumin-complexed nanocrystals that increased its solubility in water from less than 0.01 mg/mL to 0.82 mg/mL.[90]

Probe Development

Developing MAGL inhibitors based on active molecular probes is also a new strategy. Activity-based protein profiling (ABPP) technology, which uses active site-directed covalent probe molecules to detect the functional state of enzyme activities in complex proteomes, has been applied to various enzyme classes.[91] Cisar et al identified and optimised a highly effective, selective and centrally permeable oral MAGL inhibitor, ABX-1431 (Lu AG06466), from a carbamate library by ABPP technology, which has entered Phase II clinical trials.[92] In addition, some newly screened compounds such as quinoid diterpene and β-carbolines,[93] as well as newly synthesised structural modifiers based on benzylpiperidine and benzylpiperazine,[84] were confirmed for their potential MAGL inhibitory activity by ABPP. The ABPP technique was also applied in discovering and mapping the distribution of eCB hydrolase activity, and MAGL enzyme activity was found to be strongest in the PFC region.[94] In conclusion, the ABPP has made it possible to visualize the spatio-temporal release of eCB hydrolases with high spatial resolution.

In recent years, many target-labeled radioactive probes have been developed based on MAGL inhibitors, some of which have even entered clinical trials. This technology can be used to image the in vivo distribution of MAGL and provide a method for subsequent disease diagnosis and treatment. PET imaging using 18F-T-401 was the first to image and quantify the distribution of MAGL in the human brain and found that MAGL was highest in the cerebral cortex, intermediate in the thalamus and nucleus accumbens, and lowest in the white matter and brainstem.[95] He et al developed a modified compound 7 based on morpholin-3-one derivatives, which may be a potential MAGL PET tracer, and successfully mapped the MAGL distribution pattern on rodent brain in vitro radioautography using the fixation method of direct $^{11}CO_2$ synthesis.[96] Based on a unique 4-piperidinylazetidine diamide scaffold, Cheng et al developed a reversible and peripherally specific radiolucinated MAGL PET ligand, $[^{18}F]$FEPAD, which has excellent specificity and selectivity for MAGL in brown adipose tissue, a tissue known to be metabolically active.[97]

Natural Product Development

Chemically synthesized MAGL inhibitors face challenges in clinical use due to their potent pharmacological effects and safety concerns. The current focus is shifting towards natural products and botanicals to discover milder, safer, and more effective reversible MAGL inhibitors. Pritimerin and euphol were the first identified natural products with reversible MAGL inhibitory activity.[98] Four triterpenoid constituents, including pritimerin and euphol, have been reported to significantly inhibit human recombinant MAGL activity, of which pritimerin ameliorated mechanical pain in mice with a concentration-dependent manner.[99] Protium copal, commonly used as incense by the Maya, displayed significant MAGL inhibitory activity, alleviating anxiety-like behaviors in rats, and this effect was blocked by a CB2R blocker.[100] The compound 8-prenylnaringenin in *Humulus lupulus L.* reversibly inhibited MAGL and reduces neuroinflammation, promising for Alzheimer's disease.[101] Extracts from *Myristica fragrans* exhibited anxiolytic and antidepressant effects, with significant MAGL inhibitory activity.[102]

Taking a computer-aided drug design (CADD) approach, combined with molecular docking, accelerates the discovery of MAGL inhibitors from natural products. Through the establishment of the pharmacophore model Phar-MAGL, combined with molecular docking and Ligplot analysis, NP-2/8-PN ($IC_{50} = 9.5 \pm 1.2$ μM), NP-5 ($IC_{50} = 14.5 \pm 1.3$ μM), and NP-3 ($IC_{50} = 15.2 \pm 1.4$ μM) were successfully screened for their promising in vitro inhibitory activities of MAGL.[101] Interestingly, 8-PN also had a positive metamorphic modulatory effect on GABAA that was not mediated through a high-affinity benzodiazepine binding site, and its potential anxiolytic effect could be further investigated.[103] Phenylethanoid glycosides from *C. phelypeae*[104] and Jewenol A from *S. pseudorosmarinus*[105] also demonstrated good MAGL inhibitory activity through enzymatic assays and molecular docking. Screening based on existing efficacy can enhance hit rates, as demonstrated in the study by Mei et al, who screened the MAGL inhibitory activity of 12 Chinese herbal medicines commonly used for analgesia, identifying *Corydalis yanhusuo* as the most effective.[106] Forsythiaside, a phenolic acid glycoside in *Forsythia suspensa*, inhibits COX-2 and MAGL, demonstrating neuroprotective effects in Alzheimer's disease by increasing hippocampal 2-AG content.[107] Table 2 summarizes some reported natural products with MAGL inhibiting activity.

Table 2 Summary of MAGL Inhibitors Derived from Natural Products

Compounds	Category	Source	Pharmacological Effects	Experiment Type	References
Betulinic acid, Cucurbitacin B, Euphol, Pristimerin	Triterpenes	–	Analgesic	In vitro and ex vivo	[99]
Dehydrocorydaline	Alkaloid	*Corydalis yanhusuo*	Analgesic	In vitro or in vivo	[106]
8-prenylnaringenin	Flavonoid	*Humulus lupulus* L.	Promotion of neurogenesis and neurodifferentiation	In vitro	[101]
Lepidine B&E	Alkaloid	*Lepidium sativum* L. seeds	Inhibition of β-amyloid production and accumulation	In vitro	[108]
Forsythiaside	Phenolic acid glycosides	*Forsythia suspensa*	Inhibition of β-amyloid production and accumulation	Ex vivo	[107]
α-amyrins, β-amyrins, Lupeol, Protium copal resin	Triterpenes	*Protium copal*	Analgesic, anti-inflammatory, anxiolytic	In vivo and in vitro	[100,109]
Jewenol A	Diterpenes	*S.pseudorosmarinus* aerial parts	Anticancer	In vitro	[105]
–	Phenylethanoid glycosides	*C. phelypaea* aerial parts	Anticancer	In vitro	[104]
–	–	*Myristica fragrans* methanol extracts	Anxiolytic, Antidepressant	In vitro	[102]

AEA Hydrolase FAAH Inhibitors in Treatment for Anxiety

Human and rodent studies consistently show a negative correlation between anxiety levels and AEA concentrations.[45,110] Reduced AEA levels are strongly associated with PTSD severity, and moderate aerobic exercise has been found to increase AEA levels.[111] Stress-induced anxiety-like behaviors in rodents coincide with significant reductions in brain AEA levels, while microinjection of methanandamide (an AEA analogue) into the rat prefrontal cortex produces anxiolytic effects.[112] Alternatively, inhibition of AEA synthase promotes anxiety production. In NAPE-PLD-deficient mice, dorsal hippocampal AEA is significantly reduced and induces anxiety-like behavior.[113] FAAH is widely distributed in major neurons, including pyramidal cells in the BLA and hippocampus. Increased FAAH activity and decreased AEA levels have been found in mice subject to chronic restraint stress[114] and in Marchigian Sardinian alcohol-preferring rats with innate anxiety.[115] Therefore, enhancing AEA signaling by inhibiting FAAH activity is a potential strategy for the treatment of anxiety disorders.

Anxiolytic Effects of FAAH Inhibitors

Several new compounds developed for FAAH inhibitors have entered clinical trials with promising prospects (https://classic.clinicaltrials.gov/ct2/home). JNJ-42165279 produces central and peripheral FAAH inhibition, significantly increases AEA levels in cerebrospinal fluid and plasma, and has not been found to have any safety concerns.[116] In the latest clinical pilot study, attenuation of amygdala, bilateral anterior cingulate gyrus, and bilateral insula activation during an emotional face processing task was found after 4 days of administration of JNJ-42165279 (100 mg) to 43 subjects, which is in line with the effects previously observed with anxiolytics.[117] Meanwhile, in another clinical study, JNJ-42165279 (25 mg/d) was effective in improving anxiety-like symptoms after 12 weeks of administration to patients with social anxiety disorder.[13] For PF-04457845, it was well tolerated,[118] attenuated anxiety effects in healthy subjects facing stress,[119] and weakened cannabis withdrawal symptoms.[120]

Several preclinical studies have shown that pharmacological inhibition of FAAH activity exhibited remarkable anxiolytic effects in different animal models of anxiety (Table 3). Meanwhile, genetic evidence also supports the above results. It was found that FAAH whole genome knockout C57BL/6J mice are not induced with anxiety-like behavior by chronic restraint stress.[114] In contrast, increased FAAH expression leads to the development of anxious behaviors in animals. Specific overexpression of FAAH in hippocampal glutamatergic neurons using AAV vectors significantly reduced AEA and PEA levels and increased anxiety-like behaviors, which may be related to enhanced LTP in glutamatergic neurons, leading to increased glutamate release.[121] Similarly, specific overexpression of FAAH in PFC significantly reduced AEA levels and had anxiogenic effects.[112]

Table 3 Summary of the Anxiolytic Effects of FAAH Inhibitors in Preclinical Studies

Drug	Dose/ Administration	Animal	Model	Test	Effects	References
URB597	0.1, 0.3, 1 mg/kg, i.p	C57BL/6J mice, male	SDS	NSF	↓ latency to feed	[122]
	0.3384 ng, 3.384 ng, 33.84 ng, intra-BNST microinjection	Wistar rats, male	ARS	EPM; CFC	No significance in EPM test; ↓ percentage of freezing behavior	[123]
	0.3 mg/kg, i.p	Sprague-Dawley rats, male	TMT exposure	EPM	↑ open arm time and entries, ↓ anxiety index	[124]
	3 mg/kg, i.p	Sprague-Dawley rats, female	Induced by poly I:C	OFT; EPM	↑ number of transition to inner arena; ↑ duration in open arm	[125]
	1 μg, intra-CeA microinjection	Wistar rats, male	CRS	EPM	↑ open arm time and entries	[115]
	1 mg/kg, i.p	C57BL/6J mice, male	CUS	EPM; LDT	↑ duration and frequency in open arm; ↑ time and frequency in light compartment	[40]
PF-3845	10 mg/kg, i.p	Wistar rats, male	Cafeteria diet exposure	OFT; EPM	↑ center zone entries and distance; ↑ open arm time and ↓ closed arm time	[126]
	1, 3, 10 mg/kg, i.p	C57BL/6J mice, male	–	EPM; LDT	↑ percent time in open-arm; ↑ percent light time	[35]
	0.1, 1, 10 mg/kg, i.p	ICR mice, female	ARS and Foot-shock stress	LDT; NIH; EZM; OFT	↑ percent light time and distance, no significance in NIH test, EZM test and OFT	[37]
	10 mg/kg, i.p	ICR mice, male	Foot-shock stress	LDT	↑ percent light time and light entries	[127]
URB937	1, 3 mg/kg, i.p	Wistar rats and Sprague-Dawley rats, male	SDS, TMT exposure	EPM; SAA	↑ open arm time and entries, ↓ closed arm time; ↓ time spent in non-social compartment and latency to access the social compartment, ↑ time spent in social compartment	[128]
PF-04457845	100 ng, 1 μg, icv	Sprague-Dawley rats, male	Colitis-induced anxiety	EPM	↑ open arm time	[129]
ST4070	3, 10, 30 mg/kg, p.o	CD1 mice, male	–	EPM	↑ open arm time	[130]
	10, 30 mg/kg, p.o	Wistar rats, male	–	LDT	↑ light time	
JNJ5003	50 mg/kg, p.o	C57BL/6J mice, male	CRS	EPM	↑ time spent in the open arm, ↓ latency to enter the open arm	[114]

Abbreviations: ARS, acute restraint stress; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; CFC, contextual fear conditioning; CRS, chronic restraint stress, CUS-chronic unpredictable stress; EPM, elevated plus maze; EZM, Elevated zero maze; icv, intracerebroventricular; i.p, intraperitoneal; LDT, light-dark box test; NIH, novelty-induced hypophagia; NSF, novelty suppressed feeding; OFT, open-field test; SAA, social avoidance/approach; SDS, social defeat stress; TMT, 2,5-dihydro-2,4,5-trimethylthiazoline.

Single nucleotide polymorphism (SNP) in the FAAH gene, particularly the C385A (rs324420) allele, is noteworthy due to its significant association with FAAH expression. The polymorphism consists of the replacement of cytosine (C) by adenine (A) at nucleotide position 385, which translates into an amino acid exchange in which proline (Pro) replaces threonine (Thr) in codon 129. Mutations in the A allele result in decreased FAAH activity and increased AEA levels.[131] Healthy male adults carrying AC heterozygotes have higher levels of AEA and lower anxiety scores than CC homozygotes,[132] and carriers of the A allele have been found to have more robust neural network connectivity and lower anxiety levels in adolescents.[133] Similarly, knock-in of the FAAH C385A gene in mice significantly ameliorated anxiety-like behavior, which was associated with reduced FAAH expression, elevated AEA levels, and enhanced eCB signaling.[134] However, additional studies have shown that the FAAH C385A genetic variant in children increased the risk of anxiety.[135] Therefore, when FAAH inhibitor therapy is taken in the future, it may be necessary to consider the genotype of the patient's C385A to adopt a safer and more effective therapeutic strategy.

Anxiolytic Mechanisms of FAAH Inhibitors

As shown in Figure 3, FAAH inhibitors have partly the same anxiolytic mechanism as MAGL inhibitors due to similar functions. Except for maintaining Glutamate/GABA balance, suppressing neuroinflammation, and regulating the HPA

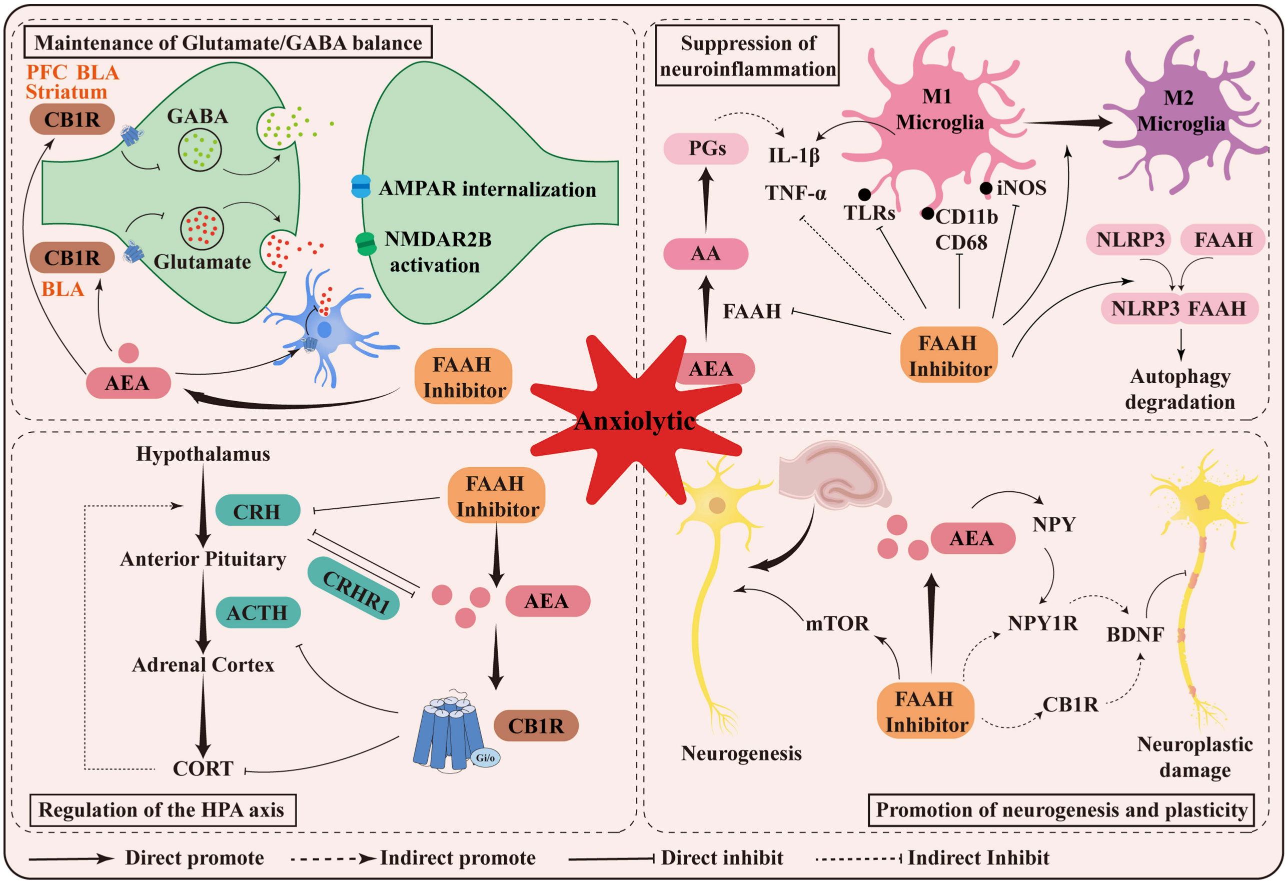

Figure 3 Schematic representation of the mechanism of anxiolytic action mediated by FAAH inhibitors. Briefly, the anxiolytic effects of FAAH are related to its maintenance of Glutamate/GABA balance, suppression of neuroinflammation, regulation of HPA axis, and promotion of neurogenesis and plasticity.

Abbreviations: FAAH, fatty acid amide hydrolase; CB1R, cannabinoid receptor 1; AEA, anandamide; AMY, amygdala; PFC, prefrontal cortex; AA, arachidonic acid; PGs, prostaglandins; IL-1β, Interleukin-1β; TNF-α, Tumor Necrosis Factor-α; iNOS, inducible nitric oxide synthase; TLRs, Toll-like receptors; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; CORT, corticosterone; corticotropin-releasing hormone receptor 1; NPY, neuropeptide Y; BDNF, brain-derived neurotrophic factor.

axis, the promotion of neurogenesis and plasticity has also been suggested to be related to the anxiolytic effects of FAAH inhibitors.

Maintenance of Glutamate/GABA Balance

Similar to MAGL inhibitors, FAAH inhibitors also exert anxiolytic effects by regulating the E/I balance in various brain regions. As we mentioned, AEA selectively exhibits a high affinity for CB1R. CB1R exists in high density in the axonal terminals of Glutaminergic neurons and GABAergic neurons. When activated by AEA signals, G protein-mediated signal cascades are activated, thereby inhibiting the opening of voltage-gated calcium channels and membrane hyperpolarization caused by increased potassium channel opening.[136] Thus, FAAH inhibition restores the E/I balance and exerts anxiolytic effects primarily through CB1R alteration of brain Glutamatergic and GABAergic signaling.

Within the amygdala, constitutive signaling of AEA is present at the glutamatergic terminal CB1R, which limits excitatory transmission in the BLA and central amygdala (CeA).[137] Expression of FAAH in the postsynaptic terminal is higher in the BLA compared to the CeA.[138] The loss of AEA signaling in the amygdala leads to increased glutamate release, which increases the activity of postsynaptic output neurons, triggering anxious behaviors and stress responses.[139] Inhibition of FAAH prevented the rapid loss of AEA signalling caused by stress, which countered the effects of different types of stress, including elevated anxiety.[124] PF-04457845 similarly reversed the dysregulation of amygdala E/I balance in rats, reducing anxiety-like behaviors and increasing social behaviors.[140,141] Interestingly, in contrast to activating neuronal CB1R to inhibit glutamate release, AEA could activate astrocytic CB1R to promote glutamate release, leading to NMDAR2B activation and AMPAR internalization, which induced long-term depression (LTD) and inhibited BLA pyramidal neurons to produce anxiolytic effects.[142] Therefore, the excitatory balance of BLA may depend on the level of glutamate release in neurons and astrocytes.

The striatum is also rich in CB1R and is involved in the regulation of anxiety. FAAH inhibition of accumulated AEA modulates excitatory and inhibitory neurotransmission in the striatum.[143] Social defeat stress induction decreased the sensitivity of striatal GABAergic synapses, but not glutamatergic, to CB1R activation and was restored by UBR597.[144] URB597 also prevented quinolinic acid-induced neuroexcitotoxic damage and preserved striatal structural integrity.[145]

Suppression of Neuroinflammation

FAAH inhibitors have also been reported to have robust anti-inflammatory effects. For example, URB937 reversed increased plasma inflammatory factor levels in social failure stress rats.[128] URB597 also reversed the expression of peripheral and cerebral proinflammatory cytokines under stress induced by LPS.[146] Studies have additionally shown that increased FAAH activity in LPS induced social behavioral deficits in adolescent rats and that imposition of PF04457845 reversed social behavioral changes.[147]

The central anti-inflammatory effects of FAAH inhibitors have been linked to the modulation of microglia polarisation phenotype.[148] AEA is an important signalling molecule in regulating microglia function, promoting their anti-inflammatory gene expression and inhibiting pro-inflammatory cytokine release.[67,149] The FAAH inhibitors PF3845 and URB597 both inhibited the production of prostaglandin E2 and pro-inflammatory gene expression in the BV2 microglial cell line, and the inhibitory effect of PF3845 was more pronounced.[150] URB597 improved the morphological characteristics of rat hippocampal microglia and promoted the transformation of microglia into an anti-inflammatory phenotype.[151] In addition, PF3845 also inhibits the expression of inducible nitric oxide synthase and COX-2 and promotes the shift of M1 pro-inflammatory phenotype to M2 anti-inflammatory phenotype.[152]

Modulation of Toll-like receptors (TLRs) may be another pathway. TLRs are intimately involved in the nervous system's immune response and are thought to underlie and exacerbate neurological disorders. Systemic administration of URB597 or PF3845 both inhibited TLR3/4-induced associated neuroinflammation in the PFC and hippocampus.[153] URB597 also reduced the TLR3-mediated increase in the expression of microglia/macrophage activation markers CD11b/CD68 and significantly alleviated anxiety-like behavior in rats.[125] Furthermore, recent studies revealed that FAAH has membrane anchoring and stabilising effects on NLRP3, and that URB597 and PF-04457845 inhibit NLRP3-FAAH interactions and induce autophagic NLRP3 degradation, suppressing the inflammatory phenotype.[154]

Regulation of the HPA Axis

Patients with anxiety disorders were accompanied by high levels of adrenocorticotropic hormone (ACTH), glucocorticoids in the blood, and overall hyperactivity of the HPA axis.[155] The level of AEA is negatively correlated with the activation of the HPA axis. It has been found that reduced homeostasis of AEA after repeated stressor exposure leads to basal overproduction of corticosterone.[156] The use of the hydrolase inhibitor URB937 reversed high plasma corticosterone levels 24 hours after social failure.[128]

The underlying mechanisms of FAAH inhibitors suppress HPA axis activation may be mediated by AEA/CB1R. In stressed rats, AEA levels in the amygdala were negatively correlated with serum corticosterone concentrations, and repeated corticosterone injections also resulted in a stress response. Meanwhile, injection of UBR597 into the BLA reduced stress-induced corticosterone secretion, and this effect was blocked by the CB1R antagonist AM251.[157] The administration of AM251 to rats similarly increased their plasma concentrations of ACTH and CORT.[158]

Alternatively, corticotropin-releasing hormone (CRH) may be a candidate for linking the AEA to the HPA axis. The FAAH inhibitor URB597 reduced HPA axis hyperactivation and anxiety-like responses to stress[159] and dose-dependently down-regulated stress-induced CRH mRNA expression in the paraventricular nucleus of the hypothalamus.[160] CRH administration rapidly reduced AEA levels in the amygdala but not 2-AG, and induced anxiety-like behavior and HPA activation, both of which were reversed by URB597.[161] Interestingly, the increase in FAAH activity induced by the administration of CRH was not accompanied by an increase in FAAH protein levels or mRNA levels, which may be related to dynamic changes in enzyme activity or consistent with the specific coupling of the CRHR1 and FAAH regions.

Up-regulation of CRH was associated with CORT, with CRH mRNA significantly elevated in the rat PFC after CORT administration and dependent on CRHR1 signaling to regulate amygdala AEA content but not 2-AG.[162] Therefore, the relationship between CRH, AEA and HPA axis is not completely linear, indicating a potential crosstalk that requires further exploration.

Promotion of Neurogenesis and Plasticity

Impaired neurogenesis as well as altered neuroplasticity are commonly seen in anxiety disorders caused by chronic stress. Studies have shown that neural progenitor cell division is affected by eCB signaling[163] and enhanced neurogenesis is always associated with elevated eCBs.[164] Repeated injections of cannabidiol, an exogenous cannabinoid, attenuated anxiety-like behavior by inhibiting FAAH activity and also promoted hippocampal neurogenesis and dendritic remodeling.[165] Moreover, HIV-1 Gp120-mediated impaired neurogenesis was rescued by FAAH gene deletion.[166] URB597 increased neuroplasticity by modulating long-term potentiation in the hippocampal CA1 region and the amygdala, attenuating fear memory.[167] JNJ5003 significantly reversed chronic restraint stress-induced dendritic expansion and increased spine density in BLA, promoting synaptic remodeling and reducing anxiety-like behavior.[114,168] URB532 and URB597 similarly prevented the reduction in AEA, the dendritic hypertrophy of the BLA, and the increase in anxiety-like behavior induced by stress.[168] The above studies illustrate that the use of FAAH inhibitors enhances brain neurogenesis as well as interneuronal transmission.

mTOR and neuropeptide Y (NPY) signaling may be associated with enhanced neurogenesis and neuroplasticity by FAAH inhibitors. mTOR signaling is essential for maintaining hippocampal neurogenesis and protecting against stress-induced impairment of neuroplasticity, and inhibition of this signaling increased anxiety-like behaviors in mice.[169] Recent studies have found that URB597 has an inverted U-shaped anxiolytic quantity-effect relationship in mice exposed to social defeat stress, and that its anxiolytic effect can be blocked by rapamycin (an mTOR antagonist), while its anxiogenic dose reduces the number of newborn neurons.[122] NPY is widely distributed in the nervous system and is particularly highly expressed in NAc and BLA.[170] Numerous studies have demonstrated that NPY levels are negatively correlated with anxiety,[171] which may be related to neuroprotective effects by promoting neurogenesis and neuroplasticity,[172] and decreasing amygdala excitability.[173] NPY has been reported to be involved in the neuroplastic protective effects of AEA, and inhibition of its expression antagonizes the effects of URB597 in suppressing PTSD behavior.[174]

Novel FAAH Inhibitors and Natural Product Development

Unlike the urgent need to find reversible inhibitors of MAGL, irreversible FAAH inhibitors, such as URB597, PF3845, and PF04457845, do not exhibit significant toxic side effects and are well tolerated clinically. Therefore, the structural modification and optimization of FAAH inhibitors are more directed towards improving the drug-forming properties like solubility and central permeability for better bioavailability. ARN14633 and ARN14280 are novel analogues of URB597 with improved solubility and bioavailability effectively alleviating anxiety-like behavior in rats exposed to predator-evoked fear models.[175,176] Structural modifications based on lead compounds, as well as extractions from natural products, are equally pivotal in the development of novel FAAH inhibitors.

Structural Modifications Based on Lead Compounds

In recent years, the development of novel FAAH inhibitors is mainly based on the skeletons of piperazine, isatin, oxazole and carbamate for structural modification and performance optimisation. Among them, piperazine-based FAAH inhibitors account for a relatively large proportion. Compound 4i (IC_{50}= 0.12 μM) developed on the basis of indole-2-carbonyl piperazinourea derivatives possessed desirable antidepressant, analgesic and anti-inflammatory effects.[177] Among the piperazinourea derivatives with thiadiazole portion, the compounds with 4-chlorobenzyl (19) and 4-fluorobenzyl (20) tails on the piperazine side were found to be the most effective in inhibiting FAAH, with IC_{50} of 0.13 and 0.22 μM, respectively.[178] Heteroaryl ureas with a thickened bicyclic diamine core exhibited better FAAH inhibitory activity compared to compounds constructed with a piperazine core.[179]

Jaiswal et al designed a series of isatin derivatives using the contemporary scaffold hopping approach in which compound 8c possessed antidepressant and anxiolytic effects without any neurotoxicity[180] and used the Dihydroindole-

2,3-dione derivatives as lead compounds to further search for FAAH inhibitors with good pharmacological properties.[181] JZP327A (IC_{50} = 11 nM), synthesized using 1,3,4-oxadiazol-2-ones as a scaffold, is a highly selective, slowly reversible FAAH inhibitor[182] with good analgesic effects.[183] FAAH inhibitors developed on the basis of carbamates also exhibit good selectivity, reversibility, water solubility and some neuroprotective effects.[184]

Three-dimensional quantitative structure-activity relationship (3D-QSAR) model, which can better represent the structure-activity relationship of ligand-enzyme interaction and help to develop more effective compounds, has been widely used in FAAH inhibitor development. Zięba et al constructed two 3D-QSAR models based on 31 FAAH inhibitors containing the 1,3,4-oxadiazol-2-one structure, which contribute to the design of novel, more potent, and more indicative FAAH inhibitors.[185] Lorca et al also constructed a similar model based on piperazine-carboxamide scaffold and designed 10 new compounds with highly predicted FAAH inhibitory activity.[186] Application of QSAR model and molecular docking technology to screen potential FAAH inhibitors in current clinical drugs also deserves attention. Montelukast, Repaglinide, Refenacin, Raloxifene and Buclizine are considered to have potential FAAH inhibitory activity, but further in vivo and in vitro validation is required.[187]

Dual-Target FAAH Inhibitors

Dual-target FAAH inhibition increases disease specificity. For example, Ibu-AM68[188] and Flu-AM4[189] both have dual FAAH and COX-2 inhibition, which circumvents the gastrointestinal response to NSAIDs, and have anti-inflammatory or analgesic effects. Development of dual FAAH/ChE inhibitors targeting the neuroprotective effects of FAAH inhibitors as promising candidates for the treatment of Alzheimer's disease.[190,191] Dual FAAH/sEH inhibitors with the piperidinyl-sulfonamide portion as the pharmacophore have been shown to have good inhibitory effects on neuropathic pain and inflammation.[192] In addition to dual inhibition, inhibition/excitation can also exist simultaneously. UCM1341 inhibits FAAH while activating melatonin receptors, producing anti-inflammatory effects to provide neuroprotection.[193] Compounds designed to target FAAH inhibition/activation of CB2R also have potent neuroinflammatory inhibitory effects.[194]

Furthermore, FAAH inhibitors also have central and peripheral inhibition selectivity. For example, ASP3652, which entered clinical trials, is a well-tolerated peripheral reversible FAAH inhibitor that reduces lower urinary tract symptoms but no efficacy in the improvement of patients' pain symptoms, possibly related to its lack of central inhibition.[195] Surprisingly, URB937, a peripherally restricted FAAH inhibitor, was unable to cross the blood-brain barrier but also possessed central activities such as anxiolysis[128] and analgesia.[196] This result suggests the presence of peripheral and central crosstalk, with anxiolysis possibly related to sympathetic efferents and analgesia possibly related to reduced afferents for injurious pain, requiring further experimental verification.

Off-Target Effects of FAAH Inhibitors

However, although FAAH inhibitors show relatively excellent pharmacological activity, their off-target effects should not be overlooked. FAAH is a serine hydrolase that uses highly conserved serine residues in its active site as nucleophilic reagents to catalyse the hydrolysis of its substrate. Most FAAH inhibitors exert their inhibitory effects by binding and modifying catalytic serine residues, which can also inhibit other serine hydrolases.[197] For example, URB597, BMS-1, OL-135 and LY2077855 all have low selectivity and show a variety of off-target effects, the main off-target being carboxylesterases.[197] Unfortunately, the off-target effects of FAAH inhibitors have been disregarded and even carried over into clinical trials, resulting in a tragic lesson. BIA 10–2474, an irreversible FAAH inhibitor entered Phase I clinical trials in 2016, was urgently called off due to resulting in the death of one volunteer and mild to severe neurological symptoms in four volunteers.[198] Subsequent studies showed that the cause of the clinical incident of BIA 10–2474 may be that its off-target protein PNPLA6 is strongly associated with organophosphorus neurotoxicity.[199] Another irreversible FAAH inhibitor, PF04457845, has entered a Phase 2 trial with no serious adverse events.[120] Therefore, the off-target effects must be evaluated to ensure clinical safety when developing novel FAAH inhibitors.

As mentioned above, ABPP technology can also be used to screen FAAH enzyme inhibitors. Otrubova et al assessed the FAAH inhibition performance of a series of N-acyl pyrazole derivatives by ABPP, minimizing off-target activity.[200] Lamani et al demonstrated that FAAH inhibitors 9 and 31 were highly selective for brain FAAH and protective against kainic acid-induced excitotoxicity by ABPP method.[201] JZP-327A, a slowly reversible FAAH inhibitor with over 900-fold selectivity for MAGL and COX isozymes, was shown by ABPP to have higher FAAH selectivity compared to other serine hydrolases.[182] Generally, evaluating the potential pharmacological activity of novel inhibitors by ABPP technology enables timely circumvention of off-target effects and minimization of compound side effects.

Natural Product Development

Various active ingredients with FAAH inhibitory activity were also found in natural products (Table 4). Among them, flavonoids inhibited the activity of FAAH significantly. Daidzein, silybin and chickpea bractein A showed high FAAH inhibitory activity both in vivo and in vitro.[202] Kaempferol inhibited FAAH activity in a concentration-dependent manner in vitro, while in vivo experiments further confirmed that acting on FAAH reduced anxiety-like behavior.[203] In addition, isoflavonoids are thought to target the ECS by modulating eCB metabolism. Three isoflavonoids, 7-hydroxyflavone, biochanin-A, and genistein, all bind to the FAAH active site and dose-dependently inhibit FAAH activity, increasing AEA levels in the PFC and decreasing blood corticosterone concentrations.[204]

Terpenoids and phenolic compounds also showed positive FAAH inhibitory activity. All 17 triterpenoids isolated from *Ganoderma lucidum* exhibited some inhibitory activity against FAAH without cytotoxicity, and FAAH may be a potential target for anti-neuroinflammation.[209,220] β-stigmasterol and eugenol extracted from *Harpagophytum procumbens*, a sesquiterpene and monopostane constituent, respectively, exerted an anti-arthritic effects by inhibiting FAAH expression,[214] with β-stigmasterol also being a selective CB2R agonist.[221,222] Some phenolic compounds, such as cannabidiol extracted from *Cannabis sativa* L. and 5′-methoxylicarin A extracted from *Myristica fragrans* Houtt., have also been reported to inhibit FAAH activity and have anxiolytic efficacy.[216]

Some other types of natural products also have FAAH inhibition effects. Macamides, a unique series of non-polar long-chain fatty acids N-benzamide isolated from Maca, are mainly alkaloids with neuroprotective properties,[223] of which N-Benzyloctadeca-9Z,12Z-dienamide exhibits the best FAAH inhibitory activity and attenuates ischaemic stroke injury,[224] but this inhibition is an irreversible inhibitor that exhibits time-dependent inhibition.[205] Lavender essential oil, an over-The-counter herbal medicine approved by the European Medicines Agency for the relief of anxiety, displayed

Table 4 Summary of FAAH Inhibitors Derived from Natural Products

Compounds	Category	Source	Pharmacological Effects	Experiment Type	References
Macamides	Alkaloid	*Lepidium meyenii Walp*	Relief of ischemic injury, analgesic, anti-inflammatory, neuroprotective	In vitro	[205, 206]
Silybin, isosilybin	Flavonolignans	*Silybum marianum*	Alleviate peripheral neuropathy	In vitro	[207]
–	–	Ashwagandha	Antioxidant	In vivo	[208]
–	Lanostane triterpenoids	*Ganoderma lucidum*	Suppress neuroinflammation	In vitro	[209, 210]
7-Hydroxyflavone, biochanin A, genistein	Isoflavone	*Glycine max* Merrill. germ, *Trifolium pratense* L.	Antidepressant, analgesic	In vivo and in vitro	[204, 211]
Isorhamnetin, kaempferol, quercetin	Flavonoid	*Moricandia sinaica* aerial parts	Analgesic, antipyretic, anti-inflammatory	In vivo and in vitro	[212, 213]
Kaempferol	Flavonoid	–	Anxiolytic	In vivo and in vitro	[203]
Eugenol, β-caryophyllene	Terpenoid	*Harpagophytum procumbens root*	Anti-arthritic	In vitro	[214]
Citral	Monoterpene	*Cymbopogon citratus*	Anti-inflammatory, analgesic	In vivo and in vitro	[215]
Licarin A, 5′-methoxylicarin A, malabaricone C	Phenol	*Myristica fragrans* Houtt.	Anxiolytic	In vivo and in vitro	[216]
Linalool, Linalyl acetate	Essential oil	*Lavandula angustifolia* Mill.	Relief of neuropathic pain, anxiolytic, antidepressant	In vivo and in vitro	[217]
Cannabidiol, tetrahydrocannabinol, cannabigerol	Phenol	*Cannabis sativa* L.	Antioxidant, inhibition of inflammatory bowel disease-associated hypermobility, anxiolytic	In vivo and in vitro	[218, 219]

FAAH inhibitory activity in vitro and produced effects comparable to diazepam in mice.[217] In addition, Xiaoyao Pills, a proprietary Chinese medicine widely used in China for the treatment of depression, was shown to alleviate depression-like behavior in rats by inhibiting FAAH levels in the brain.[225]

Techniques such as fluorescent probes and molecular docking have been used in recent years to screen potential FAAH inhibitors from natural products. Ginkgolide, the main active ingredient in *Ginkgo biloba*, was confirmed to bind well to FAAH by molecular docking.[226] The aqueous extracts and fatty oils of *Platycladi Semen* are also predicted to exert anxiolytic effects through FAAH.[227] The FAAH-activated fluorescent probe named THPO developed by Tian et al identified a natural inhibitor, neobavaisoflavone, from 68 traditional herbs.[228] Similarly, DAND, a FAAH-activated near-infrared fluorescent probe, screened piperine as a novel inhibitor of FAAH and presented excellent anti-inflammatory activity in the vitro experiments.[229]

Summary and Prospect

The high prevalence of anxiety disorders and socio-economic burden highlights the pressing need for effective treatments, as current options remain limited. Although named with a plant possessing psychoactive properties, ECS is actually one of the crucial regulatory systems of the organism. In recent years, the idea of targeting the ECS for the treatment of anxiety disorders has received increasing attention. Inhibition of hydrolase activity, particularly targeting the dominant enzymes MAGL and FAAH, offers a promising avenue by indirectly increasing eCB levels, thereby avoiding the addictive properties and adverse effects linked to direct exogenous cannabinoid supplementation.

The anxiolytic efficacy of FAAH and MAGL inhibitors has been supported by both clinical and preclinical studies, indicating their potential as promising treatments for anxiety disorders. The pharmacological mechanisms of these inhibitors are primarily associated with maintaining Glutamate/GABA balance, suppressing neuroinflammation, modulating the HPA axis, and promoting neurogenesis. Given the crucial role of neuronal excitatory/inhibitory (E/I) imbalance in the pathology of anxiety disorders, the maintenance of Glutamate/GABA homeostasis is particularly vital. CB1R, the primary mediator of the biological effects of 2-AG and AEA, is extensively expressed in glutamatergic and GABAergic terminals, offering the potential to reverse this imbalance. The clarification of the above mechanisms is not only crucial for drug development and clinical application of endocannabinoid hydrolase inhibitors, but also aids in achieving precision treatment for anxiety disorders. Similarly, more research is necessary to support or further clarify the precise mechanisms of FAAH and MAGL inhibitors in treating anxiety disorders.

Numerous biochemical techniques, including ABPP, fluorescence probes, molecular docking, and 3D-QSAR, have been used to generate novel MAGL and FAAH inhibitors. These techniques have greatly improved the screening efficiency and facilitated the discovery of new compounds. Notably, most of these new compounds are mainly developed for anticancer, analgesic, and anti-inflammatory purposes, and their potential anxiolytic activity remains to be further evaluated. Meanwhile, while significantly reducing the adverse effects associated with direct supplementation of cannabinoids or activation of cannabinoid receptors, the potential cardiotoxicity of some MAGL inhibitors and off-target effects of FAAH inhibitors should not be overlooked. In addition, given the polymorphism in the FAAH C385A gene, the genotype of patients must be thoroughly considered when applying FAAH inhibitors for treatment to achieve precision medicine. In contrast, some natural products isolated from plants have been shown to exert anxiolytic activity by inhibiting FAAH or MAGL, mostly reversibly, which greatly improves the tolerability and safety of the drugs, and thus active compounds derived from natural products would be a good source of novel anxiolytic drugs.

The development of dual MAGL/FAAH inhibitors has also come into the limelight. The newly developed AKU-005 significantly enhanced 2-AG and AEA levels and inhibited neural excitability in rat and human meninges, which is expected to be a new treatment for migraine.[230] JZL195, a classical dual MAGL/FAAH inhibitor, embodies an antidepressant activity,[231] potent neuroleptic activity,[232] weak antihypertensive effect[233] and anti-inflammatory activity.[234] Regrettably, the anxiolytic effects of JZL195 remain a subject of controversy. Some studies suggested an improvement in anxiety-like behavior in EPM test in mice.[35] On the contrary, other research have shown that it fails to reverse restraint stress-induced anxiety-like behaviors or even promotes anxiety-like behaviors.[37,235] These discrepancies may stem from variations in experimental conditions and animal models. However, it's crucial to acknowledge that the potential anxiolytic effects of MAGL/FAAH dual inhibition cannot be conclusively denied.

Furthermore, consistent with the inhibition of hydrolase activity, the levels of synaptic interstitial 2-AG and AEA can also be increased by inhibiting the activity of eCB transporter proteins, and intracellular transport is necessary for eCB hydrolysis. Current studies focus on AEA transporter proteins, which mainly include FABP5, HSP70, and FLAT1.[236,237] Recent studies have found that the use of a FABP5 inhibitor, SBFI-103, in either the amygdala or the PFC produces significant anxiolytic effects.[238] WOBE437, a natural product-derived inhibitor of AEA reuptake, irreversibly blocks 2-AG, AEA membrane transport, producing anxiolytic effects.[239] Similarly, AM404, also an AEA reuptake inhibitor, has been reported to exhibit reliable anxiolytic activity.[240,241] Although the above studies demonstrated the anxiolytic promise of targeted transporter proteins, it should not be neglected that they are still in their infancy, and need to be confirmed by further experimental studies.

In summary, there exists great potential to develop a clinically effective, safe, and well-tolerated novel anxiolytic drug from MAGL and FAAH inhibitors compared to direct cannabinoid receptor agonists. This can be achieved by screening and synthesizing new hydrolase inhibitors using advanced technologies, or by exploring active ingredients with robust inhibitory activity from natural products. Structural optimization and modification may further enhance the selectivity and biological activity of these potential drugs.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by National Natural Science Foundation of China (No. 82104836), Natural Science Foundation of Hunan Province (No. 2023JJ60482), Scientific Research Project of Hunan Provincial Department of Education (No. 23B0391), and Science & Technology talent promotion Project of Hunan Province (No. 2023TJ-N22).

Disclosure

The authors declare no conflicts of interest in this work.

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