CANNABIDIOL TREATMENT IN NEUROTHERAPEUTIC INTERVENTIONS

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CANNABIDIOL TREATMENT IN NEUROTHERAPEUTIC INTERVENTIONS

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EDITORIAL
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Editorial: Cannabidiol Treatment in Neurotherapeutic Interventions

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Editorial on the Research Topic

Cannabidiol Treatment in Neurotherapeutic Interventions

Cannabidiol, usually referred to as CBD, is the second most abundant active ingredient in cannabis, one of the oldest medicinal plants in the world (Zuardi, 2006). Although CBD was first extracted from cannabis in 1940 (Adams et al., 1940), its chemical structure was not fully characterized until 1963 (Mechoulam and Shvo, 1963). In terms of its pharmacokinetic profile, CBD is highly lipophilic, have poor oral bioavailability (as low as 6%), and is well tolerated by humans with no signs of toxicity or serious side effects and drug interactions (Millar et al., 2018; Huestis et al., 2019). Pharmacodynamically, CBD acts on over 65 molecular targets, including transient receptor potential vanilloid (TRPV) channels, serotonin (5-HT_{1A}) receptors, and cannabinoid-related receptors such as G protein-coupled receptor 55 (GPR55). Interestingly, the actions of CBD on the two main endocannabinoid receptors are limited by its low affinity. At higher doses, it functions as a negative allosteric modulator for CB_1 receptors and an inverse agonist for CB_2 receptors. Indirectly, CBD also activates CB_1 receptors by inhibiting fatty acid amide hydrolase (FAAH), the enzyme that degrades anandamide (AEA), the endogenous ligand for CB_1 receptors (Britch et al., 2021). It is important to note that, unlike tetrahydrocannabinol (i.e., THC, the main psychoactive chemical in cannabis), CBD is non-addictive (Viudez-Martinez et al., 2019), which makes it an exceptional alternative to THC-derivative cannabinoid drugs.

Touted as a cure-all for many health conditions and disorders (e.g., anxiety, depression, schizophrenia, PTSD), CBD has become increasingly ubiquitous in the marketplace (Brown and Winterstein, 2019). Spurred by the increasing legality of the medical use of the Cannabis sativa plant, a number of medical benefits of CBD have been reported. In the U.S. specifically, CBD (Epidiolex®) is currently marketed for the treatment of Dravet and Lennox-Gastaut syndromes, pediatric epilepsies resistant to anticonvulsants, as well as for spasticity in multiple sclerosis (Sativex®, THC:CBD). Emerging evidence from basic and clinical research suggests a relevant role for CBD in treating a variety of neuropsychiatric disorders, including schizophrenia (Osborne et al., 2017), mood disorders (Pinto et al., 2020), PTSD (Bitencourt and Takahashi, 2018), and drug addiction (Gonzalez-Cuevas et al., 2018; Viudez-Martinez et al., 2018), among others. However, very little is still known regarding the precise neurobiological mechanisms, pharmacokinetics, drug interactions, and clinical consequences of CBD treatment in many of these psychiatric conditions.

Despite the current CBD "boom" in commercially available products, often at the edge of the law (Mead, 2017), it remains to be investigated if CBD is an effective medical treatment for a wide range of neuropsychiatric conditions. In this special issue "Cannabidiol Treatment in Neurotherapeutic Interventions," we present a series of reviews and research papers written by leading authors in the field of neuropsychopharmacology, providing a deep overview and analysis of scientifically sound evidence

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that evaluates the use of CBD alone or associated with another drug as a new therapeutic tool for the treatment of mental disorders.

Regarding the role of CBD in epilepsy, Dubois et al. evaluate a volumetric absorptive microsampling (VAMS) method combined with LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry) that allows quantification of CBD blood levels, offering valuable support for personalized therapy in refractory epilepsy, and Contin et al. report the first clinical pharmacokinetic study in patients with Dravet and Lennox-Gastaut syndrome. Furthermore, Raucci et al. demonstrate the role of the endocannabinoid system in epileptogenesis and alert about the need to conduct double-blinded placebo-controlled trials about CBD efficacy and safety. Exploring the role of CBD in Alzheimer's disease, Coles et al. find supporting evidence of CBD treatment potential for ameliorating cognitive impairments associated with this disease. Related to the potential treatment of schizophrenia with CBD, Leweke et al. show that CBD improves neurocognitive functioning in schizophrenics and Loss et al. point out to the CBD's beneficial potential for the neurodevelopmental disorders of schizophrenia as well as autism spectrum disorders. For CBD research on mood disorders, Gasparyan et al. report that the combination of CBD and sertraline attenuates PTSD-related behavioral disturbances in mice, while normalizing gene expression alterations. Finally, a number of articles review the potential treatment of CBD for neuropsychiatric interventions: Navarrete et al. summarize the key involvement of CBD in the therapeutic intervention for Substance Use Disorders, Batalla et al. provide an overview of the neuroimaging studies in which CBD modulate functional networks relevant for psychiatric disorders, Patricio et al. focus on the neurobiological mechanisms of the CBD actions in the treatment of Parkinson's disease and L-dopa-induced dyskinesias, Scarante et al. explore the contribution of glial cells to CBD effects in neuropsychiatric disorders, and Martinez-Orgado et al. assess the neuroprotective effects of CBD against Hypoxic-Ischemic Brain Injury (HIBI) in preclinical studies.

Neuropsychiatric illness, currently accounting a third of adult disability worldwide (Lake and Turner, 2017), will become the next major global health challenge unless new neurotherapeutics can be proven to provide clinical benefit (Insel, 2012). These are exciting times for research on CBD since it has demonstrated a wide range of promising therapeutic applications in preclinical studies, including the treatment of neuropsychiatric disorders. The current findings make this drug an attractive candidate for future clinical use and warrant further investigations. Only time, coupled with methodologically rigorous clinical trials, shall reveal the extent CBD interventions contribute to winning the fight against the mental pandemic of the 21st century.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Cannabidiol Treatment for Refractory Epilepsies in Pediatrics

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Cannabis extracts in oil are becoming increasingly available, and, during the last years, there has been growing public and scientific interest about therapeutic properties of these compounds for the treatment of several neurologic diseases, not just epilepsy. The discovered role of the endocannabinoid system in epileptogenesis has provided the basis to investigate the pharmacological use of exogenously produced cannabinoids, to treat epilepsy. Although, physicians show reluctance to recommend Cannabis extracts given the lack of high-quality safety available data, from literature data cannabidiol (CBD) results to be a promising and safe anticonvulsant drug with low side-effect. In particular, according to early studies, CBD can reduce the frequency of seizures and lead to improvements in quality of life in children affected by refractory epilepsy. So, for these reasons, the detailed study of the interactions between CBD and anticonvulsant drugs (AEDs) administered simultaneously in polytherapy, is arousing increasing interest, to clarify and to assess the incidence of adverse effects and the relation between dose escalation and quality of life measures. To date, in pediatric age, CBD efficacy and safety is not supported by well-designed trials and strong scientific evidence are not available. These studies are either retrospective or small-scale observational and only during the last years Class I evidence data for a pure form of CBD have been available, as demonstrated in placebo-controlled RCTs for patients affected by Lennox-Gastaut syndrome and Dravet syndrome. It is necessary to investigate CBD safety, pharmacokinetics and interaction with other AEDs alongside performing double-blinded placebo-controlled trials to obtain conclusive data on its efficacy and safety in the most frequent epilepsies in children, not just in the epileptic encephalopathy. This review was aimed to revise the available data to describe the scientific evidence for CBD in Pediatric Epilepsies.

Keywords: CBD—cannabidiol, drug-drug interaction, drug-resistant epilepsy (DRE), children, epileptic encephalopathy, DRAVET syndrome, Lennox-Gastaut syndrome (LGS)

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INTRODUCTION

In the last two decades, public and scientific interest on the use of cannabis-derived products for therapeutic purpose in different disease has increased. More than 100 different phytocannabinoid compounds derived from the marijuana plant, *Cannabis sativa*, and *Cannabis indica* which contain up to 500 chemical species (Husni et al., 2014). Literature data have grown (Elliot et al., 2020) and one of the major fields of interest is on the anti-seizure role of the two main components of cannabis, Δ-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), for refractory epilepsy in the pediatric population (Paolino et al., 2016). THC is a psychoactive agent, and its role on seizure control is controversial because of its effect of exacerbating seizure activity; CBD is a non-psychoactive agent whose antiepileptic properties has been demonstrated by both anecdotal and scientific evidence (Friedman and Devinsky, 2015; O'Connell et al., 2017). Up to 30% of children with epilepsy were resistant to standard antiepileptic drugs (Kwan and Brodie, 2000; Kwan et al., 2010) and treatments options for these children are limited.

The discovered role of the endocannabinoid system in epileptogenesis has provided the basis to investigate the pharmacological use of exogenously produced cannabinoids, to treat epilepsy (Cheung et al., 2019; Huntsman et al., 2020).

Several studies, mainly retrospective or small-scale observational, have shown that CBD, both in isolation as a pharmaceutical-grade preparation or as part of a CBD-enriched cannabis herbal extract, is beneficial in decreasing seizure frequency in children with resistant epilepsy.

More studies are needed for physicians to be comfortable authorizing Cannabis-based therapies to children (Lattanzi et al., 2018; Huntsman et al., 2020).

EUROPEAN (EMA) AND ITALIAN LEGISLATION (AIFA), US (FDA)

The growing interest in the therapeutic potential of cannabis-related products is reflected in recent changes in legislation (Arzimanoglou et al., 2020). Laws regarding the use of raw herbal cannabis, cannabis extracts and cannabinoid-based drugs differ between countries (Specchio et al., 2020), and while the use of herbal cannabis for medicinal purposes is now authorized in different countries, cannabis and cannabis extracts have not been approved by the FDA or the European Medicines Agency (EMA).

In the European Union, in contrast to THC, CBD is not controlled and CBD products are approved it not containing more than 0.2% THC (Arzimanoglou et al., 2020).

In Italy from November 2015, can issue licenses for cultivation, production, possession, and use, and herbal cannabis may be prescribed with medical prescription. Italian legislation has recently approved medical use of Cannabis for some conditions such as pain control, chemotherapy- and radiotherapy-induced nausea and vomiting treatment, appetite stimulation in patients affected by cachexia, anorexia, cancer, or AIDS and for other pathologies, such as glaucoma and Tourette's syndrome (Baratta et al., 2019).

Physicians considering prescribing cannabis-related products should be fully aware of the relevant legislation since the situation can be complex. Guidelines from recognized national professional associations and or governmental bodies can be extremely helpful.

HISTORICAL REVIEW OF THE USE OF CANNABIS TO TREAT PEDIATRIC EPILEPSY

Therapeutic properties of cannabis plants have been known from ancient times with documented use for medical purposes in ancient Chinese books in the Middle East and India for at least 4000 years (Russo, 2017).

In 1840 Dr. William Brook O'Shaughnessy described his observation on the use of cannabis in India to treat infantile spasms in a 40-day old infant, and in 1942, he introduced the use of Cannabis Indical in Britain (O'Shaughnessy, 1843). Despite the Marijuana Tax act of 1937 and cannabis prohibition, several researchers and physicians continue the investigation on the medical use of its components. Finally, in 1990, after the discovery of endocannabinoid system and its role in epileptogenesis, and in neuromodulation with attenuation of brain activity, studies on both animal and human use of cannabinoids took place (Marsicano et al., 2003; Wallace et al., 2003; Russo et al., 2005; Englund et al., 2013; Mechoulam and Parker, 2013; Ibeas Bih et al., 2015; Todd and Arnold, 2016).

CBD has been shown to be effective against generalized tonic, clonic, tonic-clonic seizures and on drug-resistant epilepsy models, manifesting behavioral, EEG and neuroprotective effects in both acute and chronic protocols of experimental animal models (Lazarini-Lopes et al., 2020).

Efficacy in Epilepsy

The bioactive lipid system, their receptor targets, and the metabolic enzymes responsible for the synthesis and degradation of eCB constitute the so-called "endocannabinoid system" (ECS). Many studies have reported alterations of distinct components of the ECS in both animal models of epilepsy and in humans. Furthermore, compounds that act on the ECS have been shown to be effective against epilepsy. In particular, in several cases, the activation of the ECS seems to prevent seizures and reduce mortality, while the pharmacological block of the ECS exerts a proconvulsive action (Verrotti et al., 2016).

First data derived from anecdotal reports that have inspired families to seek CBD-related compounds for the treatment of their children's drug-resistant epilepsy (Filloux, 2015). The most well-known report is that of Charlotte, a 5-year-old girl in the US who was diagnosed in 2013 with *SCN1A*-confirmed Dravet syndrome who had more than 50 generalized tonic-clonic seizures. Following 3 months of treatment with high-CBD-strain cannabis extract (named "Charlotte's Web"), her seizures were reported to have reduced by more than 90% (Maa and Figi, 2014).

Other anecdotal reports suggesting that CBD may improve seizure control and alertness, mood and sleep have also been documented (Porter and Jacobson, 2013).

Other studies have investigated the effect of oral cannabis extracts using parental reporting. Press et al. (2015) and Tzadok et al. (2016) in two different studies reported similar results, with a 50% seizure reduction in about 30% of patients. In a retrospective study by Porcari et al. (2018) of 108 children with epilepsy in the US, the addition of CBD oil over an average of 6 months resulted in >50% seizure reduction in 29% patients, with 10% becoming seizure-free.

A recent meta-analysis provides evidence for the therapeutic efficacy of high content CBD treatments (Pamplona et al., 2018). Overall, the studies on CBD-enriched oils indicate a 50% reduction in seizures in roughly 30–40% of patients (Gonzalez-Giraldo and Sullivan, 2020). However, it should be emphasized that these are uncontrolled studies with heterogeneous CBD preparations, the CBD content of which varied significantly and should be underlined the need for appropriately controlled studies.

In a Canadian prospective, open-label trial of a CBD/THC cannabis oil in DS, were treated 20 children affected by DS with a cannabis plant extract product, containing 100 mg/mL of CBD and 2 mg/mL of THC. After 20 weeks of therapy, a significant improvement in quality of life, reduction in EEG spike activity, and median motor seizure reduction of 70.6%, with 50% responder rate of 63%, were noticed. Adverse events, common during titration, included somnolence, anorexia, and diarrhea. Abnormalities of liver transaminases and platelets were observed with concomitant valproic acid therapy (McCoy et al., 2018).

PURIFIED CANNABIDIOL (EPIDIOLEX/ EPIDYOLEX®) EFFICACY IN EPILEPSY

In 2018, CBD was approved by the FDA as add-on antiepileptic drug in 2-year-old children with Dravet syndrome and Lennox-Gastaut syndrome. Later, it was approved also by the EMA in 2019. The purified preparation of CBD is available from GW Pharmaceuticals plc, named Epidiolex/Epidyolex. It has been shown to have good effects against a large spectrum of seizures from animal studies (Rosenberg et al., 2017a).

Data from an open-label, multicenter expanded access program in 214 patients with childhood-onset, drug-resistant epilepsy were reported in 2016 by Devinsky et al. (2016). Among them, 33 patients had a diagnosis of DS and 31 patients of LGS. An overall median reduction of motor seizures of 36.5% was reported (49.8% for DS patients), and five patients were free of all motor seizures (of the patients with motor and atonic seizures, and 39% and 56% showed a >50% reduction of seizures, respectively) (Devinsky et al., 2016).

A randomized, double-blind, placebo-controlled study was conducted to evaluate the use of Epidiolex®, a pharmaceutical-grade cannabidiol preparation, in Dravet's syndrome. The author demonstrated its efficacy showing a decrease in convulsive seizures frequency in the cannabidiol arm, with 5% of patients, compared with 0% in the placebo arm becoming seizure-free (p 1/4 0.08). The treatment was overall well-tolerated, but it is important to underline that in the cannabidiol arm, there were more serious adverse events such as elevated hepatic transaminases (Devinsky et al., 2017a).

One trial assessed its efficacy in atonic seizures in Lennox-Gastaut syndrome showed a median reduction of atonic seizures from baseline of 41.9% in participants treated with 20 mg of CBD/kg per day versus 17.2% in the placebo group (Thiele et al., 2018).

The efficacy of CBD in reducing seizures frequencies and in improving the quality of life in childhood epilepsy (QOLCE) scores was showed in a systematic review on 17 observational studies (Stockings et al., 2018). Moreover, four clinical trials in children with Dravet and Lennox-Gastaut syndromes showed a higher rate of seizure frequency reduction in CBD treated patients (Lattanzi et al., 2019). A study from Pietrafusa et al. (2019) on artisanal medical CBD oil in patients with developmental and epileptic encephalopathy (DEE) suggest that CBD may have beneficial effects in patients with DEE and an acceptable safety profile.

PHARMACODYNAMIC AND PHARMACOKINETICS OF CANNABIDIOL AND DRUG-DRUG INTERACTION

Although the precise mechanisms responsible for the antiseizure effects of CBD remain unclear, a multimodal mechanism of action of CBD in epilepsy had been proposed. Pharmacological data supporting the role of three targets, namely Transient receptor potential vanilloid-1 (TRPV1), the orphan G protein-coupled receptor-55 (GPR55) and the inhibition of adenosine reuptake. As TRPV1 agonist, CBD lead to a decrease of extracellular calcium influx through a TRPV1 channels desensitization, reducing, consequently, neuronal hyperactivity. CBD reduce neuronal hyperexcitability in epileptic tissue as GPR55 antagonist, inhibiting then intracellular calcium release. Finally, CBD depresses neuronal excitability, reducing adenosine uptake and increasing extracellular adenosine concentration, blocking the equilibrative nucleoside transporter ENT1. Other mechanics of action have been proposed: blockade of voltage-gated sodium channels, interactions with voltage-gated potassium channels, 5-HT1a receptors, and α3 and α1 glycine receptors, blockade of T-type calcium channels, modulation of voltage-dependent anion selective channel protein, and modulation of tumor necrosis factor alpha release (Alcorn et al., 2019; Franco and Perucca, 2019; Gray and Whalley, 2020).

Cannabidiol has a lipophilic structure, a variable absorption rate and extensive empathic first-pass metabolism by isozymes CYP2C19 and CYP3A4, explaining its poor oral bioavailability (Jiang et al., 2013). The pick plasma concentration after oil formula oral administration is at 2.5 hours with a biphasic elimination (initial half-life of 6 hours ant terminal half-life of 18-32 hours) related to its distributive process into different tissues (Devinsky et al., 2014; Devinsky et al., 2018a).

CBD may exhibit numerous interactions with AEDs (Johannessen and Landmark, 2010; Johannessen Landmark and Patsalos, 2010; Johannessen Landmark et al., 2012).

CBD has been found to inhibit at clinically relevant concentrations the activity of CYP2C8, CYP2C9, CYP2C19, and CYP2D6 (Franco and Perucca, 2019).

The most clinically significant interaction between CBD and other concomitantly used drugs, based on clinical trials, is with clobazam. CBD, *via* enzyme inhibition (CYP2C19), may lead to an increase (up to five-fold) in its less potent metabolite, N-desmethylclobazam (Geffrey et al., 2015), leading to toxicity principally manifesting as sedation (Gaston et al., 2017). Also, concurrent clobazam may lead to increased 7 hydroxy-cannabidiol (an active metabolite of CBD) (Morrison et al., 2019), which arguably may lead to better seizure control by boosting the effect of CBD; however, studies with and without clobazam are needed.

There are still some unanswered questions regarding the pharmacology of CBD (Landmark and Brandl, 2020; Lattanzi S. M. et al., 2020b), and the clinical impact of its interactions with other drugs in the individual patient is difficult to predict.

In recent reports, addition of CBD increases the AUC of stiripentol by 55% and the plasma brivaracetam concentrations by 95–280%: This interaction could be related to inhibition of CYP2C19 by CBD (Franco and Perucca, 2019).

Only one retrospective, small cohort study, suggested that CBD may increase the plasma levels of topiramate, rufinamide, zonisamide, and eslicarbazepine (Gaston et al., 2017). The evidence of the effect of CBD on valproic acid are conflicting (Morrison et al., 2019).

Considering the effect of CBD on other type of drugs a possible elevation in plasma warfarin concentration should be considered, probably due to inhibition of CYP2C9 (Damkier et al., 2019).

On the other hand, the rule of enzyme-inducing AEDs (carbamazepine and phenitoine) need to be formally investigated. Stiripentol decreases in the levels of two CBD metabolites, 7-carboxy-CBD and 7-hydroxy-CBD (Morrison et al., 2019).

Finally, a rule of rifampicin as inductor and of ketoconazole as inhibitor had been demonstrated (Stott et al., 2013).

Based on the available studies, the difference in the pharmacokinetics of CBD in developmental age compared to adults is difficult to interpret. The pharmacokinetics of pure GW CBD have been evaluated in children with DS aged 4–11 years, who were randomized to different doses. CBD was administered twice daily in addition to background antiepileptic drugs (AEDs), represented mainly clobazam and valproate. Pharmacokinetic evaluations were based on sparse concentration data obtained on day 22, at the end of the maintenance period. Plasma CBD concentrations increased in an approximately dose-proportional manner across the three investigated dose groups (5, 10, and 20 mg/kg/day). Variability in CBD exposure among subjects was considerable, with coefficient of variation in AUC being in the order of 20–121%. 7-carboxy-CBD was the most abundant metabolite in plasma, with concentrations 13- to 17-fold higher than those of CBD. AUC values for 6-hydroxy-CBD were < 10% those of CBD, and those of 7-hydroxy-CBD were also lower respect CBD (Devinsky et al., 2018a).

CBD is related to some risk. While in animal models, CBD serious adverse events such as developmental toxicity, embryo-fetal mortality, central nervous system inhibition and neurotoxicity, hepatocellular injuries, spermatogenesis reduction, and hypotension have been demonstrated, they have been linked to the use of doses higher than human therapies. Human CBD studies reported only mild CBD adverse effects such as hepatic abnormalities, diarrhea, fatigue, vomiting, and somnolence (Huestis et al., 2019).

Patients should be systematically questioned about efficacy, tolerability, and adherence, and serum concentrations should be measured if possible and dosages adjusted accordingly to optimize each patient's treatment.

DRUG-RESISTANT PEDIATRIC EPILEPSY

Dravet Syndrome

FDA and EMA approved CBD use in patients suffering from DS based on the results of a randomized, double-blind, placebo-controlled trial performed on 120 DS subjects aged 2–18 years (GWP-CARE1 part B) (Devinsky et al., 2017b).

Patients were administered 20 mg/kg/day CBD over a 14-week titration plus maintenance period, and data were compared to the baseline period. The dose of 20 mg/kg/d was set by an independent drug safety monitoring committee based on pharmacokinetic and safety data from an initial part of this study (Part A). The median frequency of convulsive seizures per month decreased from 12.4 to 5.9 with CBD (from 14.9 to 14.1) and the 43% of patients with CBD had at least a 50% reduction in convulsive seizure frequency (27% in placebo group). In GWPCARE2 patients matched in three arms: patients received CBD at a dose of 10 mg/Kg/day, patients received 20 mg/Kg/day and patients receiving placebo. For CBD 10 group and CBD 20 group patients obtained respectively the 48.7% and 45.7% percentage reduction from baseline in convulsive seizure frequency. The conclusions were that adjunctive CBD at doses of 10 and 20 mg/kg/day led to similar convulsive seizure frequency; safety and tolerability profile was better in 10-mg/kg/day dose group (GWPCARE2; Devinsky et al., 2019).

Patients who completed GWPCARE1 part A or part B or GWPCARE2 were invited to enroll in a long-term open-label extension trial, GWPCARE5 (GWPCARE5; Devinsky et al., 2019). Data from an interim analysis were published. Two hundred and sixty out of 278 patients (95%) who had completed the original randomized trials were enrolled in the open-label extension. In patients from GWPCARE1 part B, over a 48-week periods, the median reduction in monthly seizure frequency ranged from 38 to 44% for convulsive seizures and 39 to 51% for total seizures. The 84% of patients/caregivers reported improvement in the patient's overall condition on the subject/caregiver GCI scale. The long-term effect of add-on CBD at up to 25–50 mg/kg/day over 144 weeks was reported for DS and LGS patients (Laux et al., 2019). Children and adults with LGS/DS

were included from 25 EAP sites across the United States. Motor seizures were reduced by 50% and total seizures by 44%, supporting CBD as a long-term treatment option (Laux et al., 2019).

In their recent economic analysis, Elliot and coworkers (2020) compared the cost effectiveness of cannabinoid oil as an adjunctive treatment (added to clobazam and valproate), with adjunctive stiripentol or with clobazam and valproate alone, for the treatment of DS in children, concluding that adjunctive cannabinoid oil may be a cost-effective treatment for DS.

Lennox-Gastaut Syndrome

In GWPCARE4 LGS double-blind placebo-controlled trials (Thiele et al., 2018) patients were administered CBD at 20 mg/kg/day, while in GWPCARE3 trial to 10 or 20 mg/kg/day (Devinsky et al., 2018b) over a 14-week treatment period compared relative to the baseline period.

In GWPCARE3 study, 171 patients were randomized (86 to CBD and 85 to placebo). During the titration plus maintenance period, patients on CBD achieved a 44% median reduction in drop seizure frequency vs. 22% in the placebo group. In the same treatment period, patients had a 49% median reduction in non-drop seizures vs. 23% in the placebo group. Regarding the response for both seizure types (drop and non-drop), patients on CBD had a 41.2% median reduction in seizure frequency compared to 13.7% in the placebo group (Thiele et al., 2018).

In GWPCARE4, a total of 225 patients were randomized; 76 to 20 mg/kg/day, 73 to 10 mg/kg/day, and 76 to placebo. The reduction in seizure frequency was 41.9% and 37.2% in the 20 and 10 mg/kg/d CBD group, respectively, vs. 17.2% in the placebo group, revealing a significant difference in both CBD arms relative to placebo (GWPCARE4) (Devinsky et al., 2018c).

Based on the patient or caregiver Clinical Global Impression (CGI) scale, overall improvements were reported in patients of each trial: 58% patients (compared to 34% in the placebo group) in the study of Thiele et al. (2018), 57% and 66% in the 20 mg/kg/day and 10 mg/kg/day group, respectively (compared to 44% in the placebo group) in the study of Devinsky et al. (2018b) and 88% at 24 weeks (also similar at 38 and 48 weeks) in the open-label study of Savage et al. (2020).

Tuberous Sclerosis Complex

Eighteen patients with a diagnosis of tuberous sclerosis complex (TSC) were enrolled in an expanded-access study of CBD. The median weekly seizure frequency decreased to 13.3 compared to 22.0 baseline observation period after 3 months of treatment with CBD. Considering total weekly seizure frequency, the median percent change was -48.8% (Hess et al., 2016).

In GWPCARE6 clinical trial, Epidiolex was used as add-on treatment in patients with TSC (Thiele et al., 2019). Patients were randomized to 20 mg/kg/day, 50 mg/kg/day, and placebo. Two-hundred one patients completed the study, and percent change in total seizure frequency decreased, respectively, by 48%, 48% and 27%. Responders (>50% seizure reduction) were 36, 40, and 22%. An overall improvement, based on the caregiver CGI scale, was reported for 69, 62, and 40% in the three groups, respectively. The lower dose of 20 mg/kg/day give a similar efficacy compared to the higher dose of 50 mg/kg/day dose but with less AEs, making the former preferable (Thiele et al., 2019).

Infantile Spasms and Epileptic Spasms

Preliminary data, derived from a brief online survey, by Hussain et al. (2015), suggested that various formulations of CBD may show a potential efficacy through multiple resistant epilepsy syndromes, including infantile spasms (IS), but the authors concluded that the study "not represent compelling evidence of efficacy and safety" and because of the presence of limitations of paramount importance, with the suggestion of further controlled clinical trials.

Hussain et al. (2020), in a multicenter phase 2 study, enrolled 9 patients (median age, 23 months; range, 14–36 months) with resistant and long-standing IS (median duration, 17 months; range, 8–33 months), treated with synthetic pharmaceutical CBD. Eight of the nine patients had concomitant antiepileptic treatments upon entering the study, although none of them took clobazam. The results of efficacy demonstrated that only one patient had an immediate but temporary response, the other eight patients exhibited neither clinical nor electrographic response. The lack of a lasting response suggests that CBD is not highly effective in treating refractory IS. The Authors, despite the negative results in this small group of resistant IS, left the door open to new studies on younger patients with a shorter IS history.

Nevertheless, also in 2020, Herlopian et al. (2020) published an open-label study on CBD treatment of epileptic spasms (ES) in nine patients (average age, 9 years; range, 2–16 years) enrolled suffered from drug-resistant ES additionally to other types of seizures with an onset of ES at 4–21 months (average age, 8 months). Administration of CBD (10 to 50 mg/kg/day) in patients with ES corresponded to a positive clinical outcome in clinical and electrographic response with an adequate safety profile.

After six months of 18–84% reduction in seizures. Sixty-seven percent (6/9) of patients experienced a >95% reduction in seizure frequency in the first two weeks while at the end of the study, 67% (6/9) achieved >50% reduction in seizures frequency ES. CBD has also been effective in reducing the frequency of other types of seizures experienced by patients.

The seizure-free rate heightened from 33% at 2 months to 56% at 12 months. After nine months of treatment, only 22% experienced an increase in ES frequency after six months of 18-84% reduction in seizures. Sixty-seven percent (6/9) of subjects experienced a greater than 95% decrease in seizure frequency in the first two weeks while at the end of the study, 67% (6/9) had a greater than 50 reduction in seizures. CBD has also been effective in reducing the frequency of other types of seizures present in patients. Interestingly, eight of the nine (89%) patients had EEG studies prior to and after initiation of CBD. Three out of five patients (60%) had resolution in their hypsarrhythmia pattern.

In contrast to Hussain et al. (2020) results, pure CBD used in the last study (Herlopian et al., 2020) seems to be effective on clinical IS and EEG abnormalities. However, the small number of patient cohorts and the non-homogeneous clinical characteristics do not allow us to provide conclusive results on the different efficacy of pure CBD compared to synthetic CBD.

Despite 89% of the nine patients displaying adverse events such as drowsiness, diarrhea, ataxia, appetite loss, agitation, twitchiness, irritability, and elevated liver enzymes, none of the patients withdrew from the study.

CDKL5 Deficiency and Other Developmental Epilepsies

Severe early onset epilepsies such as CDKL5 deficiency disorder (CDD) and other developmental epilepsies are extremely debilitating, largely due to the early-onset and refractory nature of the seizures. Evidence for cannabinoids is limited but growing, with multiple anecdotal reports and an open-label trial showing cannabidiol to be associated with a significant reduction in seizure activity.

CDKL5 Deficiency

Dale et al. (2019), in a recent review, reported that while research on severe refractory epilepsy syndromes confers a role for medicinal cannabis, specific research in patients with CDD is primarily represented by unverified anecdotal reports, therefore still limited.

Pamplona et al. (2018) performed a meta-analysis on the role of CBD in various drug-resistant pediatric epilepsy describing a significant improvement in seizure control including some with CDD, however, these studies (Devinsky et al., 2016; Szafarky et al., 2018) do not specify the effects on the subset of CDD patient as a single entity. To date, only one promising open-label study performed a quantitative analysis of the efficacy of CBD in children with severe drug-resistant epilepsies and onset in childhood, including CDD, as well as Aicardi, Dup15q, and Doose (Devinsky et al., 2018d). In particular, in CDD patients, the monthly average frequency of seizure decreased from 66 (n = 17) to 41% at week 12 (n = 11), and from 60 to 36% at week 48 (n = 10).

However, this study, although promising, needs further confirmation to formally evaluate the safety and efficacy of CBD in patients with CDD, in particular using larger placebo-controlled randomized trials (Devinsky et al., 2018d).

Doose Syndrome

In a study by Porter and Jacobson (2013) the parents of 4 patients with Doose Syndrome reported clinical improvement in 3 patients with more than 80% decrease of seizures (in two of these complete seizure freedom) after a follow-up di 2-4 months while 1 patient was unresponsive after 2 weeks of CBD. Press et al. (2015), in 75 patients with drug-resistant epilepsies reported that all three patients with Doose Syndrome were unresponsive to CBD. Nevertheless, albeit considering the small number, Devinsky et al. (2018d) based on an open-label trial of a drug-resistant form of epilepsy in which seven patients had Doose syndrome had promising results. These patients presented a reduction of seizure frequency passing from a median convulsive seizure frequency pre-CBD of 60.8 and a total seizure frequency of 64.7 to a median reduction of convulsive seizures from baseline of 58.6% by week 12 and 28.8% by week 48 after CBD.

Dup15q Syndrome

15q duplication syndrome and related disorders (dup15q) are caused by at least one extra maternally derived copy of the Prader-Willi/Angelman critical region (PWACR) within chromosome 15q11.2-q13.1. Clinically, Dup15q is characterized by hypotonia, motor delays, intellectual disability, autism spectrum disorder (ASD), and epilepsy including drug-resistant form (Finucane et al., 1993).

Devinsky et al. (2018d) based on an open-label trial of a drug-resistant form of epilepsy in which eight patients with had Dup15q variant a reported median convulsive seizure baseline frequency of 118.5 (n = 8, IQR: 32–231) and a total seizure frequency of 149.1 (n = 8, IQR: 57–313). In the Dup15q subgroup, the median number of seizures decreased from baseline (118.5 [n = 8], IQR: 18–241) to week 12 (48.8 [n = 7], IQR: 5–99), with no change from week 12 to week 48 (53.02 [n = 6], IQR: 7–207) ($\chi 2(2) = 3.00$, $p = 0.223$). Those with the Dup15qmutation have a reported median convulsive seizure decrease from baseline [n = 8] of 25% by week 12 (n = 7; IQR: -10–71) and 38.4% by week 48 (n = 6, IQR: -13–88). The Dup15q subgroup had a 38% responder rate, which persisted through week 48.

Sturge-Weber Syndrome

Sturge-Weber syndrome is characterized by leptomeningeal vascular malformations, refractory epilepsy, stroke (s) and cognitive disabilities. In preclinical models, CBD has been shown to have a possible anticonvulsant, antioxidant and neuroprotective action (Kaplan et al., 2017).

Kaplan et al. (2017) suggested that CBD may be well tolerated and provides initial data as an adjunctive medication for resistant epilepsy of Sturge-Weber syndrome. Three out of five subjects reported mild side effects considered related to CBD. Three out of five patients, demonstrating a better CBD response had bilateral brain involvement, were treated with two or more anticonvulsants and low-dose aspirin at the entry and had significant cognitive, neurological, behavioral or mood issues; the remaining two patients were removed from the study for lack of efficacy.

Migrating Focal Seizures Associated With KCNT1 Mutations

Epilepsy of Infancy with Migrating Focal Seizures (EIMFS) is a rare, developmental and epileptic encephalopathy most commonly associated with mutations in KCNT1, a potassium channel (Coppola, 2013; Auvin et al., 2016). Saade and Joshi (2015) described the beneficial effect of CBD in sustained seizure reduction with the addition of CBD to the antiepileptic regimen in an infant with EIMFS (tested only for mutations in the SCN1A gene, while not for KCNT1).

Recently, Poisson et al. (2020) evaluated CBD response in three patients with EIMFS secondary to KCNT1 mutations; two subjects showed no benefit and voluntarily discontinued CBD. One patient showed an overall decrease in seizure frequency, however, had significant decrease in seizure intensity with the possible progression of development.

ADVERSE EFFECTS OF CANNABIS EXTRACT AND CBD

The most frequent side effects reported using Cannabis extract are sleepiness, fatigue, nausea diarrhea ad decrease appetite. There are concerns about exposition to THC and its effect on brain development and long-term data moreover indicate a possible negative effect on cognitive and behavioral performance (Lagae, 2020). However, no conclusive data can be derived from available studies, given the methodological limitation, the unknown dosage of THC in artisanal products, the different duration of exposure, genetic factor, the combined use of other antiepileptic drugs, and the seizure control.

Considering pure CBD, 86% of patients in CBD groups versus 76% in placebo groups reported AEs in RCTs. However, the vast majority of AEs were mild and most of them appeared within the first two weeks of treatment.

The most frequent are somnolence, decreased appetite, pyrexia, and diarrhea, followed by other less frequent AEs such as vomiting, fatigue, and upper respiratory infections.

Serious AEs, such as somnolence, pyrexia, convulsion, rash, lethargy, and elevated transaminases (>3 times), were far less common, affecting 19% of CBD groups and 9% of placebo groups.

Elevated transaminases occurred in 16% of patients in the CBD groups and 1% in the placebo groups. The majority of the cases with elevated transaminases were patients concomitantly taking valproate. No seizure worsening, suicidal ideation, or deaths related to the treatment were reported (Devinsky et al., 2017a; Thiele et al., 2018; Devinsky et al., 2018c).

The long-term AEs are currently unknown.

In the TSC trial with the higher dose of 50 mg/kg/day CBD (Thiele et al., 2019), the most common AEs were diarrhea, decreased appetite, and somnolence, and treatment discontinuation due to AEs occurred in 11, 14, and 3%, respectively. Elevated liver enzymes were reported in 12% (n = 9) and 25% (n = 18) in the 25 and 50 mg/kg/day, respectively (of those, 81% were also taking valproate).

DIRECTIONS FOR USE AND FUTURE RESEARCH

Drug-resistant epilepsies in children represent a challenge, both for efficacy and safety aspects. In the landscape of the pediatric drug-resistant epilepsy responsive to CBD treatment, few conditions, such as Dravet and Lennox-Gastaut syndromes, have given good scientific evidence showing a good therapeutic response. Nevertheless, in this paper, we summarized the current state of evidence and indications for CBD therapy in the most frequent epileptic syndromes in childhood, not just in Dravet and Lennox-Gastaut syndromes.

Support for CBD use in pediatric epilepsies should take into account the CBD mechanisms of action and the knowledge of the epileptogenic mechanisms in the single specific epileptic syndrome, and increasing into the knowledge of the pharmacogenomic profile of CBD-AEDs interactions, starting from animal models studies.

Personalized medicine, through the study of pharmacogenomics, could provide useful information for the therapeutic choice and recognition of the patients (and specific epileptic syndromes) most responsive to CBD therapy; also to provide information on the best association between CBD and other AEDs in the specific individual affected by epilepsy.

In the case of weak pathophysiological hypotheses, the clinical studies should identify a specific subpopulation, affected by specific epileptic syndromes, which may benefit from the CBD treatment.

Increase in genetic knowledge that underlies and regulates the pharmacodynamics and pharmacokinetics of CBD and drug-drug interaction will significantly improve the choice of the therapeutic CBD prescriptions, both as monotherapy and polytherapy, in children with epilepsy.

CONCLUSIONS

CBD has been used as an anticonvulsant for at least 4000 years (Russo, 2017). Its use for medicinal purposes is now authorized in many different countries around the world. THC is a controlled substance and according to EU law, CBD products must not contain more than 0.2% THC (Arzimanoglou et al., 2020). In Italy from November 2015, cannabis may be prescribed with medical prescription. Italian legislation has approved regulations regarding the administration of medical Cannabis for specific medical conditions (pain therapy, chemotherapy/radiotherapy-induced nausea and vomiting, cachexia, anorexia, cancer patients, AIDS, glaucoma, and Tourette's syndrome (Baratta et al., 2019).

There are concerns about exposure to THC and its effect on brain development. Frequent side effects reported were sleepiness, fatigue, nausea diarrhea and decreased appetite, somnolence, pyrexia, and diarrhea, followed by other less frequent events such as fatigue, upper respiratory infections, convulsion, rash, lethargy, and elevated transaminases (>3 times); developmental regression abnormal movements and status epilepticus have also been described. Long-term data indicate a possible negative effect on cognitive and behavioral performance (Lagae, 2020). Unfortunately, appropriate pediatric dose and pharmacokinetics continue to make the authorization of cannabis-based therapies to children a challenge (Huntsman et al., 2020).

Nonetheless, the role that the endocannabinoid system plays in epileptogenesis, encourages to investigate the use of exogenously cannabinoids to treat epileptic children (Cheung et al., 2019; Huntsman et al., 2020).

CBD, both in isolation as a pharmaceutical-grade preparation or as part of a CBD-enriched cannabis herbal extract, shows beneficial effects in decreasing seizure frequency in children with drug-resistant epilepsy. Recently, in patients with Lennox-Gastaut syndrome and Dravet syndrome (Devinsky et al., 2016; Devinsky et al., 2017a; Devinsky et al., 2017b; Rosenberg et al., 2017b; Thiele et al., 2018; Stockings et al., 2018; Lattanzi et al., 2019; Lattanzi S. et al., 2020) have been conducted a

placebo-controlled RCTs with a pure form of CBD, which gave good results (Class I evidence). Later, CBD was approved by the FDA as an add-on antiepileptic drug in 2 years old children with Dravet syndrome and Lennox-Gastaut syndrome. Subsequently, it was approved also by the EMA in 2019. The purified preparation of CBD is available from GW Pharmaceuticals plc, named Epidiolex/Epidyolex. It has been shown to have positive effects against a wide spectrum of seizures from experimental studies (Rosenberg et al., 2017a).

Although to date just preliminary results and weak scientific evidence are available for many other epileptic conditions, we reported above also every specific pediatric epileptic condition in which CDB was be tried, with more or less encouraging data. CBD investigations in pediatric age, better evaluation of the incidence and the prevalence of epileptic syndromes age-related, together with increased knowledge of their natural course, and the development of new end points could provide some suggestions for future improvements for the therapeutic utilization of CBD therapy in epileptic children.

AUTHOR CONTRIBUTIONS

UR and PaP directed the review and were responsible for the overall guidance. All authors contributed to the article and approved the submitted version. The article's content has been made by consensus among all the authors.

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frontiers in Pharmacology

BRIEF RESEARCH REPORT
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A Volumetric Absorptive Microsampling Technique to Monitor Cannabidiol Levels in Epilepsy Patients

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Purpose: Interest in cannabis-based therapies has recently increased, due to the availability of cannabidiol (CBD) for the treatment of epilepsy without psychoactive effects. Therapeutic drug monitoring can prevent drug interactions and minimize drug toxicity. We evaluated a volumetric absorptive microsampling (VAMS) method combined with LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry) for the quantification of CBD blood levels in patients with refractory epilepsy.

Methods: Prospective observation of patients with Dravet syndrome receiving open-label, add-on GW-purified CBD (Epidyolex®) at different doses. CBD plasma samples were obtained from venipuncture and LC-MS/MS was used to measure CBD in venous and capillary blood samples collected by VAMS.

Results: We enrolled five patients with a mean age of 13 (range: 4–27) years. CBD levels measured by VAMS on capillary blood did not differ from CBD levels measured in plasma by venipuncture ($R^2 > 0.93$).

Conclusion: This proof-of-concept study suggests that VAMS allows monitoring of CBD plasma levels and can offer valuable support for personalized therapy in refractory epilepsy.

Keywords: epilepsy, therapy, cannabidiol, therapeutic drug monitoring, volumetric absorptive microsampling, refractory seizures

INTRODUCTION

Epilepsy is one of the most common brain chronic disorders, affecting around 70 million people of all ages worldwide (Hirtz et al., 2007; Zaccara and Schmidt, 2017). The identification of the appropriate treatment allows in most patients a medium and long-term remission in seizures control (Striano and Striano, 2009; Striano et al., 2016; Lattanzi et al., 2019). Despite the use of numerous therapeutic options, including third-generation antiseizure medications (ASMs), neuromodulation, surgical and

dietary interventions, 30% of patients continue to have seizures (Striano and Striano, 2009; Zaccara and Schmidt, 2017).

The interest in cannabis-based therapies has increased, in particular in the two main phytocannabinoids: cannabidiol (CBD) and ∆9-tetrahydrocannabinol (THC). CBD stimulates interest because of its anti-convulsive properties in absence of psychoactive effects and abuse liability, unlike THC (Devinsky et al., 2014; Arzimanoglou et al., 2020). The therapeutic potential of galenic preparations marketed to contain CBD/THC was found to depend on preparation procedures, components concentration, and presence of other constituents (De Caro et al., 2017; Carcieri et al., 2018; Lattanzi et al., 2018; Lattanzi et al., 2019). Purified CBD produced by GW pharma (EPIDYOLEX®) is the first of a new class of ASMs. (Lattanzi et al., 2019; Lattanzi et al., 2020). The approval in July 2019 by the European Medicines Agency to use CBD as an additional treatment with clobazam in two forms of childhood refractory epilepsy (Dravet syndrome and Lennox-Gastaut syndrome) is a milestone in the medical use of phytocannabinoids for the treatment of epileptic disorders. Due to the heterogeneity of epilepsy clinical manifestations and interindividual response to old and new antiepileptic drugs, therapeutic drug monitoring (TDM) is a valuable clinical support in patients' treatment.

In refractory epilepsy, the relationship between the dose administered and CBD blood levels demonstrated in some studies (Geffrey et al., 2015; Landmark and Brandl, 2020) has provided a starting point for the use of TDM in the wide variability of CBD pharmacokinetics (Ocque et al., 2019). TDM is useful in clinical practice as it allows to obtain the ideal dose of cannabis-based therapy based on the identification of the individual concentration associated with an optimal response. Moreover, in polypharmacy TDM can prevent drug interactions by guiding dose adjustments and minimizing toxicity (Striano et al., 2008; Patsalos et al., 2018; Brandt, 2019). Microsampling techniques based on dried blood spots allow a reliable and non-invasive collection of small blood volumes. Recently, the novel device VAMS (Volumetric Absorptive Microsampling) has been introduced in the market, commercial name MITRA®, successfully applied to several quantitative TDM methods. This device allows the collection of a fixed volume of blood (10 or 30 µl) avoiding the effect of hematocrit (HCT) on the analytical performances (Mano et al., 2015; Barco et al., 2017; Kok and Fillet, 2018; D'Urso et al., 2019). We evaluated VAMS in combination with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for the quantification of CBD blood levels to be used in clinical practice to personalize the cannabis-based treatment of refractory epilepsy. In particular, we determined CBD concentrations in capillary and venous blood obtained by micro-sampling and compared them with CBD concentration in plasma, which is the matrix most frequently used for TDM in epilepsy patients.

METHODS

Participants

We investigated five subjects with Dravet syndrome treated with CBD oral solution (Epidyolex®) given for compassionate use. All participants were taking a stable dose of ASMs and were followed-up prospectively through medical charts and parents/caregivers' information.

Study Design

Patients received Epidyolex as compassionate use approved by the Regional Ethics Committee. Written informed consent was signed by parents, caregivers, or legal representatives. CBD was administered at the initial dose of 2.5 mg/kg two times per day (5 mg/kg/day) to be increased after 1 week to a maintenance dosage of 5 mg/kg twice daily (10 mg/kg/day). The CBD dose could be increased in weekly increments of 2.5 mg/kg twice daily according to clinical response. Physical examination and laboratory tests (FBC, serum sodium, potassium, chloride, creatinine, ALT, AST, total bilirubin, INR, and glucose) were performed at baseline (within 2 weeks after initiation of CBD treatment) and after 1 month, 3 months, and 6 months of treatment. Patients' parameters, i.e., weight, height, and body mass index, were recorded at each scheduled visit and a safety check was carried out by monitoring CBD plasma levels by venipuncture. CBD blood levels were evaluated at least 3 months after the start of treatment. During the monitoring study of the different cannabis-based therapies, the doses of concomitant ASMs administered to patients were not modified, establishing an appropriate observation condition.

Quantification of Cannabidiol in Plasma by Volumetric Absorptive Microsampling-Liquid Chromatography Coupled to Tandem Mass Spectrometry

Blood samples were obtained in the morning before the first daily medication. Venous blood was collected by venipuncture on tubes containing ethylenediaminetetraacetic acid and plasma was separated by centrifugation at 2,000 *g* for 5 min. The 30 µl VAMS devices (MITRA®, Neoteryx, Torrance, CA, United States) were used to collect venous and capillary blood. The venous VAMS samples were obtained from blood collected by ethylenediaminetetraacetic acid tubes, as described (Barco et al., 2018; Pigliasco et al., 2020). Capillary VAMS were obtained following the manufacturer's instructions: before pricking the patient's finger with a microneedle, the area was disinfected and after the first drop of blood was removed, the VAMS tip was placed in contact with the surface of the second drop to adsorb the matrix.

Statistical Analysis

The correlation between CBD venous and capillary VAMS and CBD plasma levels was assessed by linear regression analysis ("Medcalc," Software Ltd., Ostend, Belgium).

TABLE 1 | Demographic and clinical features of the patients.

Patient/Age (years)	BMI (kg/m^2)	Total dose (mg/day)	Fraction (mg/day)	Concomitant antiseizure medications
#1/(4–6)	19.6	385 (17.5 mg/kg/day)	192 mg × 2 days	Valproic acid (378 mg/kg/day), stiripentol (750 mg/kg/day), clobazam (10 mg/kg/day), topiramate (100 mg/kg/day)
#2/(10–12)	28.1	650 (10 mg/kg/day)	325 mg × 2 days	Valproic acid (1,000 mg/kg/day), clobazam (15 mg/kg/day), levetiracetam (16 mg/kg/day)
#3/(16–18)	13.5	665 (17.5 mg/kg/day)	332 mg × 2 days	Valproic acid (650 mg/kg/day), stiripentol (1,500 mg/kg/day), topiramate (100 mg/kg/day)
#4/(25–27)	22.5	1,360 (20 mg/kg/day)	680 mg × 2 days	Valproic acid (600 mg/kg/day), stiripentol (1,500 mg/kg/day), clobazam (10 mg/kg/day), topiramate (200 mg/kg/day)
#5/(7–9)	14.7	230 (10 mg/kg/day)	115 mg × 2 days	Valproic acid (500 mg/kg/day), stiripentol (500 mg/kg/day)

TABLE 2 | CBD therapeutic monitoring by VAMS and venipuncture including formulation and dose cannabis-based treatment.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
CBD plasma (ng/ml)	356	119	144	169	64
CBD venous VAMS (ng/ml)	447	163	141	190	72
CBD capillary VAMS (ng/ml)	405	153	112	122	52

CBD, cannabidiol; VAMS, volumetric absorptive microsampling.

RESULTS

Demographic Characteristics and Compliance

The demographic and clinical features of the participants are summarized in **Table 1**. Four subjects were males and the mean age of the five patients was 13 (range: 4–27) years. At enrollment, all participants had failed from two to four ASMs and were on stable treatment (mean: three concomitant drugs) for at least 3 months before CBD add-on. **Table 1** also shows the dose and amount of CBD provided to each subject. The mean dose of Epidyolex administered was 658 mg/day (15 mg/kg/day).

Outcome Therapeutic Monitoring Cannabidiol Levels by Venipuncture and Volumetric Absorptive Microsampling

The results achieved from the analysis of plasma and venous and capillary VAMS are illustrated in **Table 2**. The highest CBD plasma levels, ranging from 356 to 64 ng/ml (mean CBD level 175 ± 102 ng/ml), were related to Epidyolex administered at a mean dosage of 15 mg/kg/day. Linear regression analysis (**Figure 1**) showed a correlation between CBD concentrations measured on capillary blood sampled by VAMS did not differ from those measured by venous VAMS ($R^2 > 0.98$) and plasma from venipuncture ($R^2 > 0.93$).

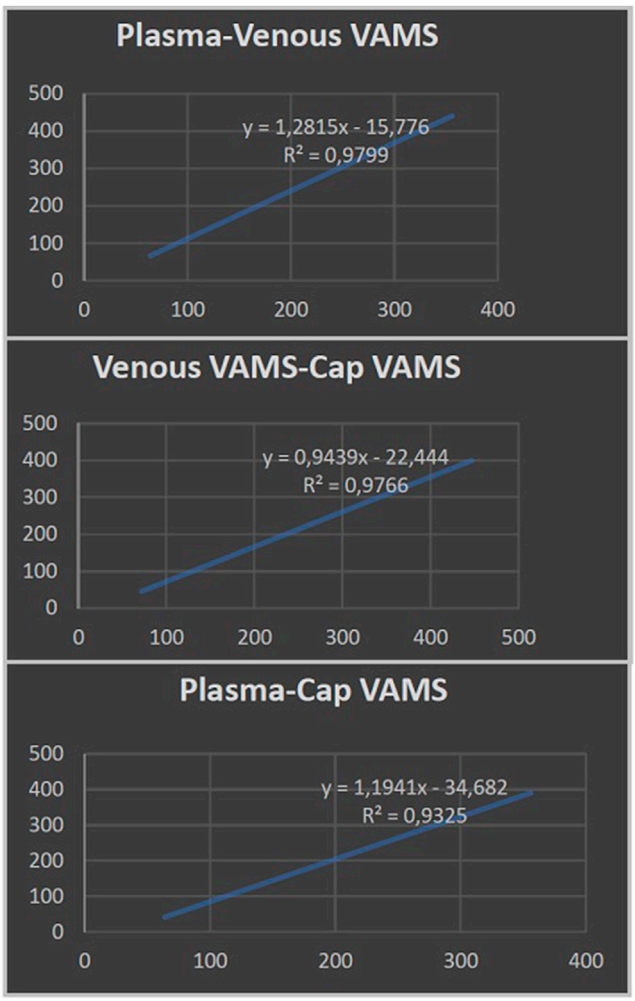

FIGURE 1 | Linear regression analysis showing the correlation between cannabidiol plasma levels from venipuncture and cannabidiol concentrations measured by venous and capillary volumetric absorptive microsampling (VAMS).

DISCUSSION

TDM is often indispensable in the follow-up of epilepsy patients for the need of dose adjustments to optimize the clinical outcome (Kok and Fillet, 2018; Patsalos et al., 2018). VAMS devices are porous hydrophilic tips that enable an accurate collection of small blood volumes (Denniff and Spooner, 2014) avoiding the volumetric HCT bias and erythrocyte volume fraction bias associated with the non-volumetric dried blood spots approach (De Kesel et al., 2014; Denniff and Spooner, 2014; Spooner et al., 2015). Moreover, a significant advantage of this less invasive and easily reproducible procedure is that it limits the discomfort caused to patients in obtaining venous samples. However, to date, the TDM of medical cannabis has few validated analytical methods on plasma (Grauwiler et al., 2007; Aizpurua-Olaizola et al., 2017; Lomonaco et al., 2018; Pigliasco et al., 2020).

We used a new microsampling method for the determination of CBD blood levels in patients with drug-resistant epilepsy using VAMS, which had previously proven useful for quantitative measurement of several venous and capillary blood drugs, including first and third-generation antiepileptic drugs (Velghe and Stove, 2018; D'Urso et al., 2019), antibiotics (Barco et al., 2017) and immunosuppressants (Koster et al., 2019).

Specifically, we aimed to evaluate the correspondence between the CBD levels detected in plasma and those measured using the VAMS technique, by pricking the patient's finger. CBD concentrations that were taken from capillary blood by VAMS were not statistically different from those of venous blood obtained in the laboratory from the same device. Also, this statistical comparison proved to be valid between the results collected from VAMS microsampling and the CBD plasma levels obtained by venipuncture.

Several factors may influence the pharmacokinetics of CBD-related products used (Lucas et al., 2018; Birnbaum et al., 2019). In particular, CBD is related to a high potential of drug-drug interactions due to the influence on the activity of several enzymes involved in the metabolism of antiseizure medications, including cytochromes CYP2C and CYP3A, isoenzymes of CYP450. The known increase in plasma levels of N-desmethylclobazam (N-CLB), an active metabolite of clobazam, due to inhibition of the catalytic activity of CYP2C19 by CBD, is responsible for the most common dose-dependent adverse event in the clinical practice (Lattanzi et al., 2020) In this study, we did not methodically collect the plasma N-CLB levels in our patients treated with clobazam. Moreover, our study was not designed to monitor high intra- and inter-individual pharmacokinetic variability, although the implementation of the patient cohort could provide additional investigation material.

CONCLUSION

VAMS device can be used as valuable support for patients with refractory epilepsy allowing control of CBD concentrations and dosage regulation, minimizing interindividual pharmacokinetic and pharmacodynamic problems, obtaining an effective personalized treatment and better control of therapeutic adherence. Our findings should be confirmed in further follow-up studies on larger series to identify a standardized match between the administered CBD dose and its detectable plasma concentration.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CER Liguria. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Medium-Dose Chronic Cannabidiol Treatment Reverses Object Recognition Memory Deficits of *APP$_{Swe}$/PS1ΔE9* Transgenic Female Mice

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Alzheimer's disease (AD) is a neurodegenerative disease that causes behavioral and cognitive impairments. The phytocannabinoid cannabidiol (CBD) has anti-inflammatory, antioxidant, and neuroprotective properties, and *in vitro* and limited *in vivo* evidence suggests that CBD possesses therapeutic-like properties for the treatment of AD. Cannabinoids are known to have dose-dependent effects and the therapeutic potential of medium-dose CBD for AD transgenic mice has not been assessed in great detail yet. 12-month-old control and *APP$_{Swe}$/PS1ΔE9* (*APPxPS1*) transgenic female mice were treated daily via intraperitoneal injection with 5 mg/kg bodyweight CBD (or vehicle) commencing three weeks prior to the assessment of behavioral domains including anxiety, exploration, locomotion, motor functions, cognition, and sensorimotor gating. *APPxPS1* mice exhibited a hyperlocomotive and anxiogenic-like phenotype and had wild type-like motor and spatial learning abilities, although AD transgenic mice took generally longer to complete the cheeseboard training (due to a lower locomotion speed). Furthermore spatial learning and reversal learning was delayed by one day in *APPxPS1* mice compared to control mice. All mice displayed intact spatial memory and retrieval memory, but *APPxPS1* mice showed reduced levels of perseverance in the cheeseboard probe trial. Importantly, vehicle-treated *APPxPS1* mice were characterized by object recognition deficits and delayed spatial learning, which were reversed by CBD treatment. Finally, impairments in sensorimotor gating of *APPxPS1* mice were not affected by CBD. In conclusion, medium-dose CBD appears to have therapeutic value for the treatment of particular behavioral impairments present in AD patients. Future research should consider the molecular mechanisms behind CBD's beneficial properties for AD transgenic mice.

Keywords: Alzheimer's disease, *APP$_{Swe}$/PS1ΔE9*, transgenic mouse model, cannabidiol, treatment, behavior

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INTRODUCTION

Alzheimer's disease (AD) is an insidious neurodegenerative disease that is caused by progressive damage to neuronal cells and results in irreversible cognitive and behavioral deficits including memory loss, spatial disorientation, and language impairments. AD is the most common form of dementia and is currently incurable and without effective preventative options and usually leads to death due to secondary diseases such as pneumonia (Burns et al., 1990; Brunnström and Englund,

2009). Often a verified diagnosis of AD can only be made postmortem, with the two main pathological hallmarks of AD being (1) the extracellular accumulation of amyloid-beta (Aβ) protein fragments around the neurons in the brain, forming Aβ plaques, and (2) the intracellular accumulation of hyperphosphorylated microtubule-associated protein tau (MAPT), forming neurofibrillary tangles (NFT). Cerebral atrophy, microglial activation, oxidative stress, and chronic inflammation of the brain are also seen postmortem (Chen and Mobley, 2019).

AD is most commonly categorized as either late-onset (> 65 years of age) sporadic AD or early-onset (< 65 years of age) familial AD. Sporadic AD is the most common form of AD and is sporadic in nature, with the most widely studied genetic risk factor for sporadic AD being the gene encoding apolipoprotein E (Liu et al., 2013; Mendiola-Precoma et al., 2016). Familial AD is estimated to represent less than 5% of all AD cases and results from the inheritance of an autosomal dominant mutation in the genes encoding amyloid precursor protein (APP), presenilin 1 (PS1), or presenilin 2 (PS2), the latter two being enzymes participating in the processing of APP. Mutations in *APP*, *PS1*, and *PS2* result in the aberrant cleavage of APP into Aβ peptides of 40 residues ($A\beta_{40}$) or of 42 residues ($A\beta_{42}$), which are thought to form toxic Aβ plaques responsible for causing neuronal cell death in AD (Hardy and Higgins, 1992).

Currently available treatments for AD include three acetylcholinesterase inhibitors (donepezil, rivastigmine, and galantamine) and one N-methyl-D-aspartate (NMDA) receptor antagonist (memantine). These treatments have numerous side effects and only provide symptomatic relief to patients in early disease stages without altering disease progression (Wong, 2016). A recent approach in the race for the treatment for AD involves targeting the endocannabinoid system, which is involved in numerous basic functions of the human body (Di Marzo et al., 2004; Benito et al., 2007), and testing constituents of the *cannabis sativa* plant (i.e., phytocannabinoids). Among the group of phytocannabinoids tested for therapeutic interventions, cannabidiol (CBD) is of particular interest. CBD is the main nontoxic (nonhigh producing) phytocannabinoid of *C. sativa* and possesses antioxidant, antiapoptotic, neuroprotective, immunosuppressive, and anti-inflammatory properties. Limited *in vitro* and *in vivo* evidence suggests that CBD may also reduce amyloid and tau pathologies and unlike other cannabinoids does not impair cognition (reviewed in Karl et al. (2017)). These properties suggest that CBD may be suitable for the treatment of neurodegenerative diseases including dementia.

Indeed, CBD has shown potential as a therapeutic for AD in preclinical studies. *In vitro* studies have shown that CBD dose-dependently inhibits tau hyperphosphorylation in Aβ-simulated PC12 cells (Esposito et al., 2006a). Furthermore, CBD can increase cell survival, reduce Aβ-induced lipid peroxidation and reactive oxygen species production (Iuvone et al., 2004), attenuate nitric oxide (Esposito et al., 2006b), and counteract the elevation of APP expression in transfected human neuroblastoma cells, thereby increasing cell survival (Scuderi et al., 2014). *In vivo*, CBD has been found to attenuate Aβ-evoked neuroinflammation in a pharmacological mouse model of AD (Esposito et al., 2007). In addition, 20 mg/kg CBD treatment has been shown to prevent an Aβ-induced learning deficit in the Morris Water Maze and to reduce the Aβ-induced increase in IL-6 (Martín-Moreno et al., 2011). Furthermore, a previous study from our lab reported that CBD at a dose of 20 mg/kg reversed social recognition and novel objection recognition deficits in 6-month-old $APP_{Swe}/PS1\Delta E9$ (*APPxPS1*) mice (a transgenic model for familial AD) when delivered chronically after the onset of disease-relevant symptoms (Cheng et al., 2014a). This dosage also prevented the development of a social recognition deficit in the *APPxPS1* model when delivered for 8 months prior to the onset of disease symptoms (Cheng et al., 2014c). More recently, 50 mg/kg CBD was found to restore impaired social recognition memory and reversal spatial learning and tended to reduce insoluble Aβ40 levels in the hippocampus of 12-month-old *APPxPS1* males (Watt et al., 2020). International colleagues also evaluated CBD-rich cannabis extract (at a dose of 0.75 mg/kg of CBD) which also improved object recognition memory of *APPxPS1* mice when chronically administered during the early symptomatic disease stage (Aso et al., 2015).

The *APPxPS1* mouse model exhibits fast-developing amyloid pathology (Borchelt et al., 1997; Jankowsky et al., 2004a; Jankowsky et al., 2004b), with Aβ plaques appearing as early as at 4–6 months of age and accumulating further with age (Jankowsky et al., 2004b; Savonenko et al., 2005; Garcia-Alloza et al., 2006; Ruan et al., 2009; Hamilton and Holscher, 2012). A sexual dimorphism profile for this model has also been identified, with female *APPxPS1* mice exhibiting higher pathological levels of phosphorylated tau, proinflammatory cytokines, astrocytosis, microgliosis, neuronal and synaptic degeneration (Jiao et al., 2016), and soluble Aβ40 and Aβ42 peptides (Wang et al., 2003) when compared to males. *APPxPS1* mice also exhibit a range of behavioral deficits relevant to the study of AD including spatial learning and memory impairments in various test paradigms (Savonenko et al., 2005; O'Leary and Brown, 2009; Zhang et al., 2011; Cheng et al., 2014b) and recognition memory impairments (NORT; Lalonde et al., 2004; Cheng et al., 2013; Cheng et al., 2014a; Cheng et al., 2014c) as well as task-dependent hyperlocomotion and anxiolytic-like phenotypes (Cheng et al., 2013; Cheng et al., 2014b). In line with brain pathology, male and female *APPxPS1* mice show differences in the nature of their behavioral impairments (Jardanhazi-Kurutz et al., 2010; Cheng et al., 2014a; Cheng et al., 2014b) so research strategies need to be developed sex-specifically. Unlike previous studies that combined male and female mice and assessed them together without consideration for the impact of gender (Lalonde et al., 2004; Reiserer et al., 2007), the current study decided to limit investigations to one gender only, and female mice were selected because of their more pronounced brain pathology.

It is important to note that CBD produces biphasic dose responses (Tzavara et al., 2003; Rey et al., 2012). It is therefore pivotal to investigate a range of dosages to determine the window of the therapeutic effectiveness of the drug. In addition, evaluating lower CBD doses than in our previous studies may have a positive impact on future financial burdens of dementia patients. Thus, the major aim of this explorative study was to determine if a chronic administration regime of a medium CBD

dose of 5 mg/kg bodyweight can reverse or ameliorate behavioral impairments of *APPxPS1* transgenic females at an advanced symptomatic disease stage.

MATERIALS AND METHODS

Animals

12-month-old female double transgenic $APP_{Swe}/PS1\Delta E9$ (*APPxPS1*) mice were used in this study. The *APPxPS1* mouse model of familial AD carries the chimeric mouse/human *APP* gene with Swedish mutation (Mo/HuAPP695swe/Swedish mutations K595N/M596L) and the mutant human *PS1* gene with exon nine deletion (*PS1/ΔE9*) and is generated on a mixed congenic C57BL/6JxC3H/HeJ background and maintained as a hemizygote (Borchelt et al., 1997; Jankowsky et al., 2004a; Jankowsky et al., 2004b). 12-month old female mice which were chosen as *APPxPS1* females show significantly higher levels of soluble $A\beta_{40}$ and $A\beta_{42}$ compared to male *APPxPS1* mice (Wang et al., 2003) and at 12 months of age, these females are considered to be in advanced stages of the symptomatic phase of AD (Aso et al., 2016). *APPxPS1* mice (n = 22) and their nontransgenic wild type-like littermates (WT: n = 28) were 361 ± 8 days old at the onset of the study, with a total of three cohorts of mice being used. Mice were bred at Australian BioResources (ABR: Moss Vale, NSW Australia) where they were group housed in individually ventilated cages (Type Mouse Version 1: Airlaw, Smithfield, Australia) under a 12/12 h light/dark cycle with a dawn/dusk simulation. Mice were transported to the Western Sydney University animal facility (School of Medicine, Campbelltown, Australia) once they had reached adulthood where littermates were group housed (two to three mice per cage) in filter top cages (1284L: Tecniplast, Rydalmere, Australia). Mice were provided with food (Rat & Mouse Pellets: Gordon's Specialty Stockfeeds Pty Ltd., NSW, Australia) and water *ab libitum* unless otherwise described. Corncob bedding (PuraCob Premium: Able Scientific, Perth, Australia), crinkle paper (Crink-l'Nest, The Andersons, Maumee, Ohio, United States), and tissue for nesting were used as enriching structures. Cages were changed fortnightly. Standard laboratory conditions were applied with a 12/12 h light/dark cycle (light phase beginning 0900 with white light at an illumination of 124 lux and dark phase beginning 2100 with a red light at an illumination of less than 2 lux). Temperature and relative humidity were automatically controlled between 20 and 22°C and 40 and 60%, respectively. All procedures were approved by the Western Sydney University Animal Care and Ethics Committee (#A12905) and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

Drug Preparation and Administration

Preparation of powdered cannabidiol (CAS: 13956-29-1; THC Pharma GmbH, Frankfurt/Main, Germany) dissolved to a concentration of 0.5 mg/ml in equal parts of Tween80 (Sigma-Aldrich Co., St Louis, United States) and 100% ethanol and diluted in 0.9% saline, to a ratio by volume of 1 : 1 : 18 ethanol: Tween80: saline, was used to prepare the CBD treatment solution. A similar solution without the addition of powdered cannabidiol (1 : 1 : 18 ethanol : Tween80 : saline) was used as the vehicle. At approximately 12 months of age, mice began treatment via daily intraperitoneal (i.p.) injection (10 ml/kg bodyweight, site alternated daily) of CBD or vehicle administered at a dose of 5 mg/kg body weight (WT-VEH n = 15; WT-CBD n = 13; *APPxPS1*-VEH n = 10; and *APPxPS1*-CBD n = 12). Treatment began 3 weeks prior to the start of the experiments and continued throughout the behavioral assessment. CBD or vehicle was administered in the afternoon to avoid acute effects of the injections modifying the behavioral performance of the mice tested in the morning, in line with our other studies (Cheng et al., 2014a; Watt et al., 2020). Bodyweight was monitored weekly.

TABLE 1 | Test biography. Test order and test age of wild type-like (WT) control and double transgenic $APP_{Swe}/PS1\Delta E9$ (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD). Ages (d) are presented as mean ± standard error of means (SEM). NORT: novel object recognition task. No significant differences between days of test.

	WT-VEH	*APPxPS1*-VEH	WT-CBD	*APPxPS1*-CBD
Start of CBD treatment	361 ± 2	357 ± 2	363 ± 2	360 ± 2
Light-dark test	382 ± 2	378 ± 2	384 ± 2	381 ± 2
Pole test	384 ± 2	380 ± 2	386 ± 2	383 ± 2
Accelerod	384 ± 2	380 ± 2	386 ± 2	383 ± 2
NORT	389 ± 2	385 ± 2	391 ± 2	388 ± 2
Cheeseboard	392 ± 2	388 ± 2	394 ± 2	391 ± 2
Prepulse inhibition	406 ± 2	404 ± 2	408 ± 2	405 ± 2

Behavioral Test Battery

Mice were tested in behavioral domains that have been found to be affected in dementia or AD-relevant mouse models. In line with previous studies conducted in our laboratory (Cheng et al., 2014a; Watt et al., 2020), all experiments were performed during the first 5 h of the light phase to reduce the effects of the circadian rhythm on mice performance (i.e., to avoid the less active period of the light phase (Grech et al., 2019)), and a 48 h intertest interval was applied to all testing to minimize the effect of repeated testing and to allow mice to rest between tests (with the exception of low-impact motor function tests, which were performed over three consecutive days). Mice were habituated to the test room for 30–60 min prior to testing. 80% ethanol was used to clean each apparatus between mice. For an overview of test order and test age, please see **Table 1**.

Light Dark

Anxiety-related behaviors can be assessed in the light-dark (LD) test. The LD apparatus (for details, see Karl et al. (2007), Cheng et al. (2014b)) consisted of two equally sized zones in an open-field chamber: a "light" zone (illumination > 200 lux) and a "dark" zone (illumination < 20 lux; dark box insert in the rear half of test arena). After a 60 min habituation to the test room, mice were placed into the dark zone and allowed to explore the entire apparatus for 10 min. The activity was recorded by MED Associates Activity Monitor software. Distance traveled was used as an indicator of locomotion. Exploration was shown by the frequency of *rearing* (vertical activity). Time spent and

percentage distance traveled in the light zone were calculated to identify anxiety-related behaviors.

Pole Test

Climbing behavior was assessed using the vertical pole test (Brooks and Dunnett, 2009). Mice were placed with snouts facing upwards on the end of a vertical pole (50 cm long by 1 cm diameter) and allowed to turn around and climb down the pole to a platform. This was repeated three times with a 30 min intertrial interval (ITI). The performance was measured by the average time taken to (1) turn around (latency to inversion) and (2) descend the pole once turned around (time to descend) and (3) total time taken to reach the platform (latency to platform; "cut-off" time of 60 s).

Accelerod

An accelerating rotarod paradigm was used to measure the motor coordination and balance of the test mice (Brooks and Dunnett, 2009). Training and testing were carried out as described previously (Kreilaus et al., 2019), but with two consecutive test days with two trials per day. The mean of the four trials was considered for analysis, as was the worst-performing trial. The performance was measured as the latency to fall from the cylinder ("cut-off" time of 300 s).

Novel Object Recognition Task

The innate preference of a mouse for novelty and its ability to distinguish a novel object from a familiar object (Dere et al., 2007) are utilized in this test to determine object recognition memory. The NORT was conducted as published previously (Kreilaus et al., 2019). The percentage of time spent *nosing* the novel object during the second "testing" trial was calculated as

$$\frac{\text{novel object nosing time}}{\text{novel} + \text{familiar object nosing time}} \times 100,$$

and it was used as an indication of object recognition memory. In line with previously published studies from our lab (Cheng et al., 2014a), mice were excluded if they did not show a minimum of 20 s of object exploration during both trials (one WT-VEH and one *APPxPS1*-CBD mouse were excluded).

Cheeseboard

Spatial memory was assessed through the cheeseboard (CB) paradigm. Details on the apparatus used can be found in previous studies from our lab (Cheng et al., 2013; Kreilaus et al., 2019; Watt et al., 2020). Briefly, mice were habituated over two days to the blank side of the board (i.e., 3 × 2 min trials per day, 20 min ITI). Next, mice were trained over five days to locate a well containing a food reward (i.e., 3 trials per day, 20 min ITI). The latency of the mice to find the baited well was recorded and if the mouse had not found the food reward within the maximum trial time of 2 min, it was gently guided to the well by the experimenter. To ensure motivation to find the food reward (i.e., sweetened condensed milk), mice were food restricted (access to food for 1–2 h following completion of daily testing) to a maximum of 85% of their free-feeding body weight throughout the entire testing period.

The average latency to find the reward and the mean speed and distance traveled during training were analyzed as a general indication of learning, while the first trial per day across training was analyzed to assess long-term reference memory (retention of ≥ 24 h), and the average of trials two and three each day across training was analyzed to assess intermediate-term memory (retention falling between short-term (2 min) and long-term (24 h) memory) (Taglialatela et al., 2009). Further, day-by-day learning in the CB, where the average latency for day 1 was compared to day 2, day 3, and so on, was performed to determine when the mice acquired the task.

A CB probe trial for spatial memory was performed on day 8, whereby mice were given 2 min to explore the board with no food reward present. The percentage of duration spent in the target zone (the zone containing the target well during training, i.e., 12.5% of the board) was analyzed using AnyMaze™ (Stoeting, Wood Dale, United States) tracking software, thereby analyzing target zone preference for total test time. As it has been observed that some mice do not leave the center zone immediately and therefore do not spend the entire 2 min of the probe trial exploring the board, a secondary calculation was carried out to ensure that the data presented was representative of the actual test time that mice spent exploring and was not skewed by an extended latency to leave the central start zone. This was calculated as

$$\frac{\text{time (s) in target zone}}{120\,s - \text{latency (s) to leave the centre zone}} \times 100.$$

The percentage of duration spent in the target zone for the first and second 30 s of the full 2 min trial was also analyzed to account for potential differences in behavioral flexibility rather than spatial memory, as a target zone preference in the first/second 30 s is indicative of intact retrieval memory or perseverative behavior, respectively, while decreased time in the target zone over the second 30 s is indicative of cognitive flexibility in adaptation to the lack of food reward (Grech et al., 2019). A reversal CB was also completed (4 days of training followed by a reversal probe trial, where the opposite well was baited). One *APPxPS1*-CBD mouse was excluded from probe analysis as it *froze* for 80 s (three times greater than any other mouse).

Prepulse Inhibition

The prepulse inhibition (PPI) test was used to assess the acoustic startle response (ASR) and sensorimotor gating (the occurrence by which a nonstartling prestimulus attenuates the startle response (Wang et al., 2012)). Mice were habituated to the apparatus (apparatus described in Cheng et al. (2014b)) for 10 min twice per day (1 h ITI) over two consecutive days prior to the test day. On day 3, mice were returned to the apparatus for the PPI test, which was carried out as previously described (Cheng et al., 2014b). A 120 dB startle pulse, prepulse intensities of 74, 82, and 86 dB, and interstimulus intervals of 32, 64, 128, and 256 ms were used in this test protocol. Percentage PPI (%PPI) was calculated as

$$\frac{\text{mean startle response (120 dB)} - \text{PPI response}}{\text{mean startle response (120 dB)}} \times 100.$$

%PPI was averaged across ISIs to produce a mean %PPI for each prepulse intensity.

Statistical Analysis

Analysis of behavioral data was performed using two-way ANOVA to determine the main effects of between-subject factors "genotype" and "treatment" and to test for "genotype" by "treatment" interactions. Three-way repeated measure (RM) ANOVA was also used to investigate repeated measure effects of the within-subject factors "time" (CB), "startle pulse intensity," "startle block," and "prepulse intensity" (all PPI). A "time" by "genotype" by "treatment" interaction was further investigated in CB by splitting data by "genotype" and then by both "genotype" and "treatment" and using mixed ANOVA and/or one-way RM ANOVA, respectively. To investigate day-by-day learning in CB, one-way RM ANOVA for "time" for day 1 versus respective day/s was performed. One-sample *t*-tests were also used for NORT and CB probe to determine whether a specific behavior was above chance levels (i.e., 50% for NORT–12.5% for CB). In line with Perneger (1998) and Rothman (1990), the data were not adjusted for multiple comparisons and were interpreted as such in Discussion. Significant differences were determined when $p < .05$. F-values and degrees of freedom are presented for ANOVA and significant effects of "genotype" are shown in figures and tables by "*" ($^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$), and significant effects of "treatment" are shown by "$^{\#}$" ($^{\#}p < .05$). Significant RM results are indicated by '^' ($\hat{}\,p < .05$, $\hat{}\hat{}\,p < .01$, and $\hat{}\hat{}\hat{}\,p < .001$). A "time" by "treatment" interaction is indicated by "†" ($^{\dagger}p < .05$). Significant *t*-test results are also shown by "+" ($^{+}p < .05$, $^{++}p < .01$, and $^{+++}p < .001$). Trends were reported when $.05 \leq p < .07$, and all other nonsignificant data were reported as "n.s." (i.e., $p \geq .07$) or with specific *p*-values. Data are shown as means ± standard error of means (SEM). All statistical analyses were conducted using IBM SPSS Statistics 25.0 for Mac.

RESULTS

Locomotion and Exploration

APPxPS1 mice displayed increased total distance traveled in the LD test (two-way ANOVA for "genotype": F (1,46) = 9.2 and $p = .004$) and this increase in locomotion was not affected by treatment (i.e., no "genotype" by "treatment" interaction: F (1,46) = .07 and $p = .8$, **Table 2**). Importantly, this hyperlocomotive phenotype of *APPxPS1* mice was also evident in the dark zone, which is least affected by anxiety behaviors (F (1,46) = 16.3 and $p < .001$, **Table 2**). No differences in exploration (i.e., *rearing* frequency) were detected between genotypes for the LD arena or any particular zone (all *p's* n.s., **Table 2**). Interestingly, mice treated with CBD exhibited increased *rearing* compared to vehicle-treated mice in the dark zone (F (1,46) = 5.5 and $p = .02$, **Table 2**) but no other LD area (all *p's* n.s.). However, follow-up analysis of exploration per minute spent in this zone did not detect a treatment effect (F (1,46) = 1.1 and $p = .3$, **Table 2**).

Anxiety

APPxPS1 transgenic mice were more anxious than controls during LD testing. In particular, *APPxPS1* mice spent significantly less time in the light zone of the LD test (F (1,46) = 4.6 and $p = .04$, **Table 2**) and they also tended to exhibit less locomotion in that zone (i.e., percentage distance traveled: F (1,46) = 3.6 and $p = .06$, **Table 2**). Chronic CBD had no effect on anxiety parameters or genotype differences detected (all *p's* n.s.).

Motor Function

In the pole test, no significant main effects of *APPxPS1* genotype or CBD treatment were found for the measures latency to inversion, time to descend, and latency to platform (all *p's* n.s., **Table 3**). Similarly, in the accelerod, no effects of "genotype" or "treatment" were evident for the average latency to fall from the accelerod (all *p*'s n.s., **Table 3**). However, *APPxPS1* mice fell from the accelerod significantly earlier than controls when comparing the worst performance of test mice across trials (F (1,46) = 7.1 and $p = .01$, **Table 3**) and this was not affected by CBD (no "genotype" by "treatment" interaction: F (1,46) = .3 and $p = .6$).

Cognition

Object Recognition Memory

In the NORT testing trial, all experimental groups except for vehicle-treated *APPxPS1* transgenic mice had a significant preference for the novel object, as indicated by one-sample *t*-tests for the percentage time spent *nosing* the novel object (WT-VEH: t (13) = 4.5 and $p = .001$; *APPxPS1*-VEH: t (9) =

TABLE 2 | Locomotion, exploration, and anxiety measures in the light-dark test. Data shown for wild type-like (WT) control and double transgenic $APP_{Swe}/PS1\Delta E9$ (*APPxPS1*) female mice treated chronically with either vehicle (VEH) or cannabidiol (CBD). Data are presented as mean ± SEM. Main "genotype" effects are presented as $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ or trend value given. Significant main effects of "treatment" are indicated by "$^{\#}$" ($^{\#}p < .05$).

	WT-VEH	*APPxPS1*-VEH	WT-CBD	*APPxPS1*-CBD
Total distance traveled (m)**	32.5 ± 1.6	40.0 ± 2.9	32.4 ± 1.5	38.7 ± 3.1
Dark zone distance traveled (m)***	17.0 ± 1.1	21.4 ± 1.8	17.1 ± .9	23.4 ± 1.5
Total *rearing* frequency (n)	120.1 ± 7.8	129.0 ± 15.1	128.7 ± 11.1	141.9 ± 12.7
Dark zone *rearing* frequency (n)$^{\#}$	51.3 ± 2.5	56.5 ± 6.7	60.5 ± 7.2	76.0 ± 7.6
Dark zone *rearing* frequency per minute in the zone (n/min)	12.2 ± 1.1	12.3 ± 1.7	13.4 ± 1.4	14.2 ± 1.6
Time spent in light zone (s)*	294.1 ± 17.8	277.6 ± 27.0	290.4 ± 8.4	230.9 ± 15.8
Distance traveled in light zone (%), $p = .06$	47.2 ± 2.4	46.1 ± 3.7	47.2 ± 1.6	38.6 ± 2.4

TABLE 3 | Motor functions in the pole test and accelerod. Data shown for wild type-like (WT) control and double transgenic *APP$_{Swe}$/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD). Data are presented as mean ± SEM. Significant "genotype" effects are indicated with "*" (*$p < .05$).

	WT-VEH	*APPxPS1*-VEH	WT-CBD	*APPxPS1*-CBD
Pole test, latency to inversion (s)	13.8 ± 2.9	14.0 ± 3.8	7.6 ± 1.2	10.7 ± 2.5
Pole test, time to descend (s)	17.8 ± 2.0	18.2 ± 2.7	15.0 ± 1.3	18.0 ± 2.2
Pole test, latency to platform (s)	31.6 ± 4.4	32.2 ± 5.2	22.7 ± 1.8	28.7 ± 3.9
Accelerod, average latency to fall (s)	202.9 ± 9.1	171.3 ± 11.7	193.9 ± 17.1	176.2 ± 17.8
Accelerod, latency to fall in worst performance (s)*	170.8 ± 9.3	120.5 ± 13.8	151.8 ± 17.6	119.0 ± 20.1

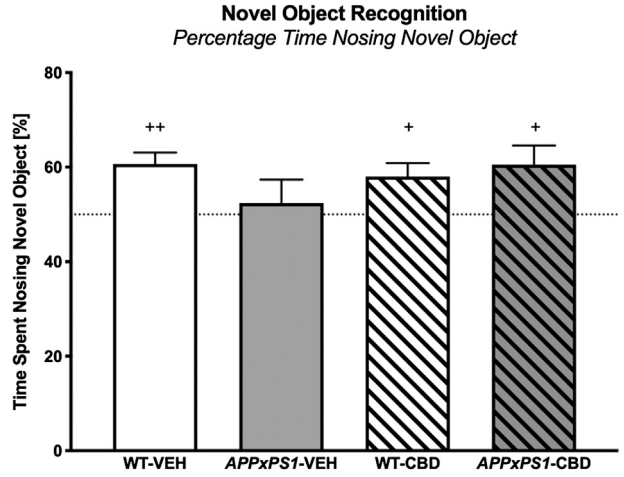

FIGURE 1 | Novel object recognition. The percentage of time spent *nosing* the novel object in the NORT. Data for wild type-like (WT) control and double transgenic *APP$_{Swe}$/PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant *t*-test results against chance levels (i.e., 50%) are indicated with "+" ($^{+}p < .05$ and $^{++}p < .01$).

.5 and $p = .6$; WT-CBD: t (12) = 2.8 and $p = .02$; *APPxPS1*-CBD: t (9) = 2.6 and $p = .03$, **Figure 1**). Comparing percentage time spent *nosing* the novel object across experimental groups using 2-way ANOVA did not reveal significant main effects or interaction thereof (all *p's* n.s.).

Cheeseboard - Spatial Learning and Memory

Task Acquisition

In the CB training trials, all mice demonstrated successful task acquisition as they learned the position of the baited well. This was indicated by a reduced latency to find the food reward over time and reduced distance traveled during training when averaged across the three trials per day (three-way RM ANOVA for "time": latency: F (4,180) = 88.6 and $p < .001$; distance: F (4,180) = 23.4 and $p < .001$) and successful learning was not affected by genotype or treatment (no interactions of "genotype" or "treatment" with "time"; all *p*'s n.s., **Figures 2A and B**). In line with this, intact learning was evident in all groups for both intermediate-term memory (i.e., averaged across trials two and three per day) (latency: F (4,180) = 59.3 and $p < .001$; distance: F (4,180) = 11.6 and $p < .001$, **Figures 2C and D**) and reference memory (i.e., trial one per day) (latency: F (4,180) = 41.6 and $p < .001$; distance: F (4,180) = 13.1 and $p < .001$, **Figures 2E and F**) (no interactions of "genotype" or "treatment" with "time"; all *p*'s n.s.). Interestingly, comparing the learning performance of each experimental group separately *day by day* revealed that all groups except the *APPxPS1*-VEH exhibited significant improvement in the latency to find food reward for the first time by day 2 (RM ANOVA for "time" for day 1 *versus* day 2: WT-VEH: F (1,14) = 39.9 and $p < .001$; WT-CBD: F (1,12) = 20.5 and $p = .001$; *APPxPS1*-CBD: F (1,10) = 7.5 and $p = .02$). The learning of the vehicle-treated *APPxPS1* group was delayed and only evident by day 3 (day 1 *versus* day 3, F (1,9) = 10.0 and $p = .01$) (**Supplementary Figure S1A**).

It should be noted that *APPxPS1* transgenic mice were generally slower on the board than their WT littermates when mean speed was averaged across daily trials, regardless of treatment (F (1,45) = 24.5 and $p < .001$; no interactions with "time" or "treatment," **Supplementary Figure S2A**). In line with this, *APPxPS1* mice took generally longer to find the reward than control mice, both averaged across all daily trials (latency to find a reward: F (1,45) = 6.8 and $p = .01$. **Figure 2A**) and averaged across trials two and three (F (1,45) = 11.1 and $p = .002$, **Figure 2C**) but no main effect of genotype was detected for reference memory (F (1,45) = 1.3 and $p = .3$, **Figure 2E**).

Reversal Task Acquisition

Successful reversal training was evident in all mice when averaged across three trials (latency F (3,135) = 47.2 and $p < .001$; distance: F (3,135) = 40.1 and $p < .001$, **Figures 3A and B**) and also when considering intermediate-term memory (latency: F (3,135) = 20.5 and $p < .001$; distance: F (3,135) = 18.4 and $p < .001$, **Figures 3C and D**) and reference memory (latency: F (3,135) = 38.9 and $p < .001$; distance: F (3,135) = 24.1 and $p < .001$, **Figures 3E and F**). No "time" by "genotype" or "time" by "treatment" interactions were detected for latencies (all *p's* n.s.). However, there were "time" by "genotype" by "treatment" interactions for distance traveled across three daily trials (F (3,135) = 4.6 and $p = .004$, **Figure 3B**) and when considering intermediate-term memory (F (3,135) = 2.9 and $p = .04$, **Figure 3D**). Split by genotype, mixed ANOVA revealed a significant "time" by "treatment" interaction for *APPxPS1* mice when considering the distance traveled for the average of the three trials (distance: F (3,57) = 3.5 and $p = .02$, **Figure 3B**) and a trend interaction when considering the intermediate-term memory (trend: F (3,57) = 2.6 and $p = .06$, **Figure 3D**). These interactions were not evident in WT mice (all *p*'s n.s.). However, split by "genotype" and "treatment," all experimental

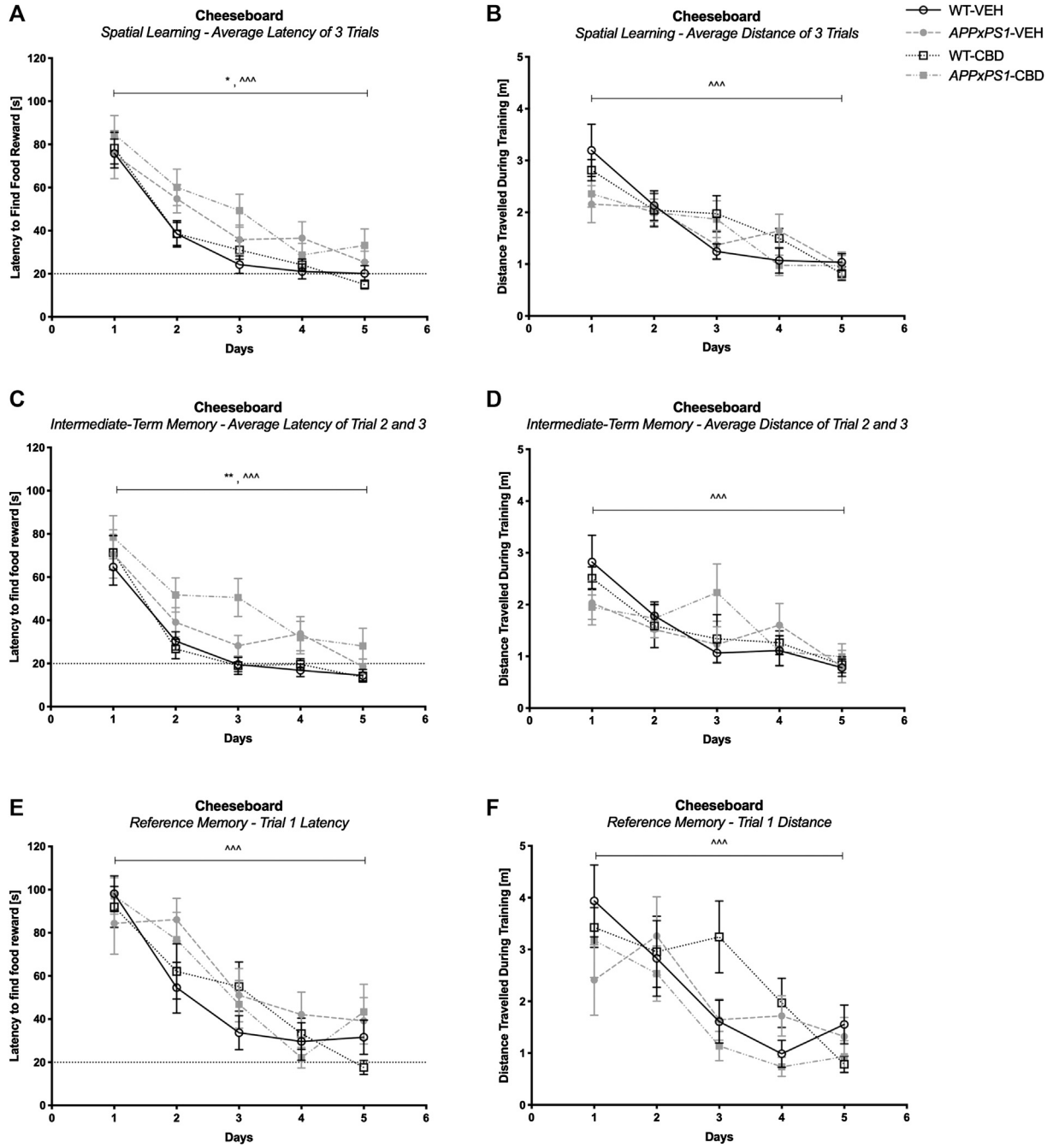

FIGURE 2 | Spatial learning in the cheeseboard (CB). **(A, C, and E)** Latency (s) to find the food reward and **(B, D, and F)** distance traveled (m) during CB training **(A and B)** averaged across all three trials, **(C and D)** for intermediate-term memory and **(E and F)** for reference memory. Data for wild type-like (WT) control and double transgenic *APP*$_{Swe}$/*PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means ± SEM. Significant "genotype" effects are indicated by "*" (*$p < .05$ and **$p < .01$) and successful learning is indicated by '^' (^^^$p < .001$).

groups displayed intact learning as indicated by significant RM effects of "time" for all groups (WT-VEH: F (3,42) = 23.8 and $p < .001$; *APPxPS1*-VEH: F (3,27) = 11.2 and $p < .001$; WT-CBD: F (3,36) = 12.7 and $p < .001$; *APPxPS1*-CBD: F (3,30) = 5.7 and $p = .003$). Looking at *day-by-day* learning, WT mice regardless of treatment condition displayed significantly improved latencies to find the food reward by day 2 (RM ANOVA for "time" for day 1 *versus* day 2; WT-VEH: F (1,14) = 30.9 and $p < .001$; WT-CBD: F (1,12) = 9.9 and $p = .008$), whereas *APPxPS1* mice of both treatments show improvement by day 3 (day 1 versus day 3; *APPxPS1*-VEH: F (1,9) = 16.9 and $p = .003$; *APPxPS1*-CBD: F (1,10) = 16.0 and $p = .003$) (**Supplementary Figure S1B**).

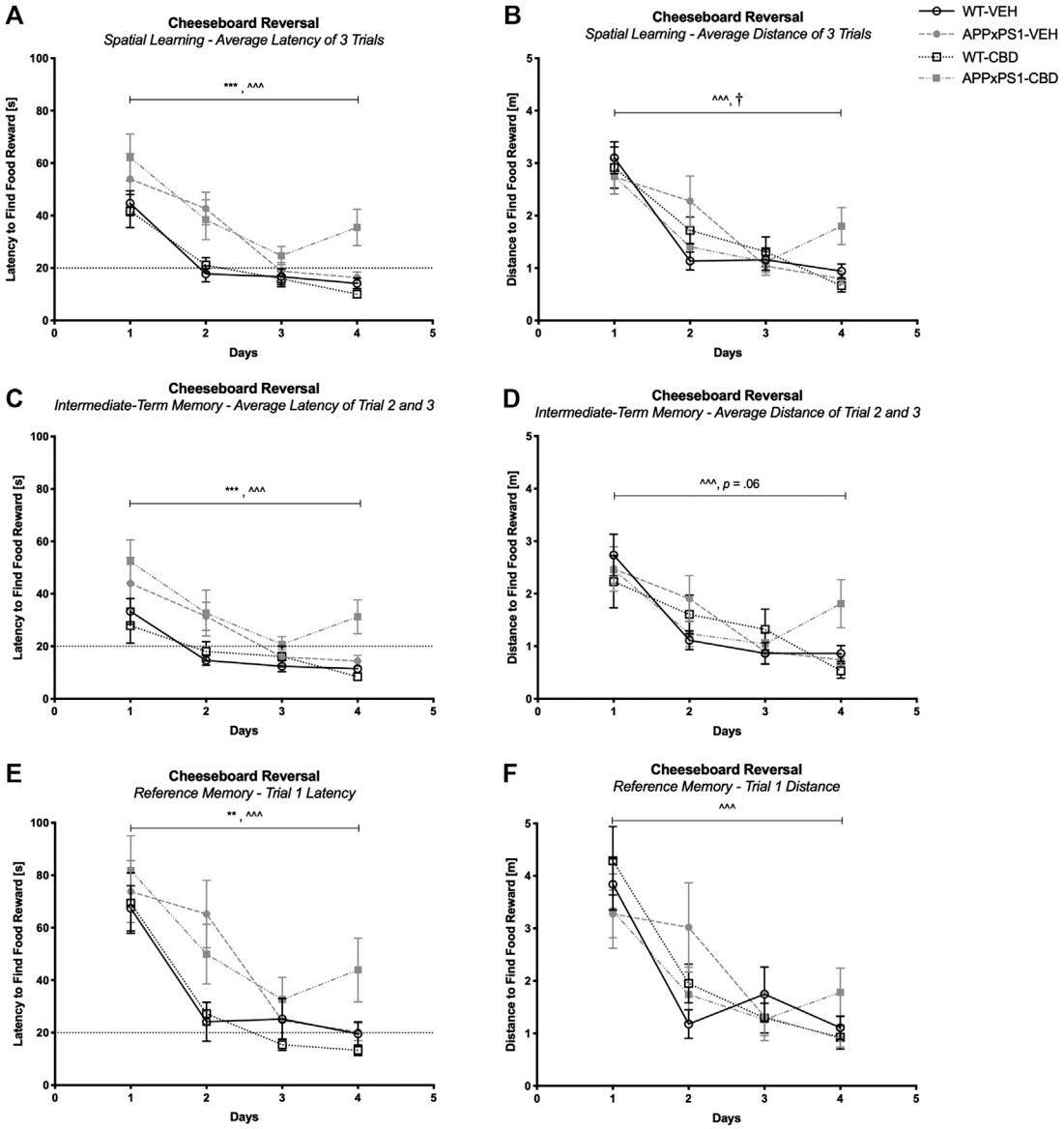

FIGURE 3 | Spatial learning in the reversal cheeseboard (reversal CB). **(A, C, and E)** Latency (s) to find the food reward and **(B, D, and F)** distance traveled (m) during reversal CB training **(A and B)** averaged across all three trials, **(C and D)** for intermediate-term memory and **(E and F)** for reference memory. Data for wild type-like (WT) control and double transgenic $APP_{Swe}/PS1\Delta E9$ (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means ± SEM. Significant "genotype" effects are indicated by "*" (**p < .01 and ***p < .001) and successful learning is indicated by '^' (^^p < .001). There was a "time" by "genotype" by "treatment" interaction for distance traveled across all three trials (p = .004) and for intermediate-term memory (p = .04). The "time" by "treatment" interactions for *APPxPS1* mice are indicated by "†" ($^{\dagger}p$ < .05) or the exact trend level has been indicated by "p = .06."

APPxPS1 mice were also slower on the board than their WT littermates during reversal training when averaged across the three trials per day (F (1,45) = 17.2 and p < .001, **Supplementary Figure S2B**). Again, *APPxPS1* mice took longer per day to find the reward when assessing latency across the average of the three trials (F (1,45) = 17.9 and p < .001, **Figure 3A**), across trials 2 and 3 (F (1,45) = 22.3 and p < .001, **Figure 3C**), and also for trial 1 only (F (1,45) = 7.0 and p = .007, **Figure 3E**). We also detected a "time" by "genotype" by "treatment" interaction (F (3,135) = 3.6 and p = .02, **Supplementary Figure S2B**). Split by genotype, a "time" by "treatment" interaction was evident in WT mice (F (3,78) = 2.8 and p = .048) with CBD-treated controls showing a more pronounced increase in average speed across days than the respective vehicle treatment group (p n.s. for *APPxPS1* mice).

Probe Trial

During the CB probe trial, all mice showed a preference for the target zone in the full 2 min test period (one-sample t-test: WT-VEH: t (14) = 4.4 and p = .001; *APPxPS1*-VEH: t (9) = 4.2 and p = .002; WT-CBD: t (12) = 5.5 and p < .001; *APPxPS1*-CBD: t

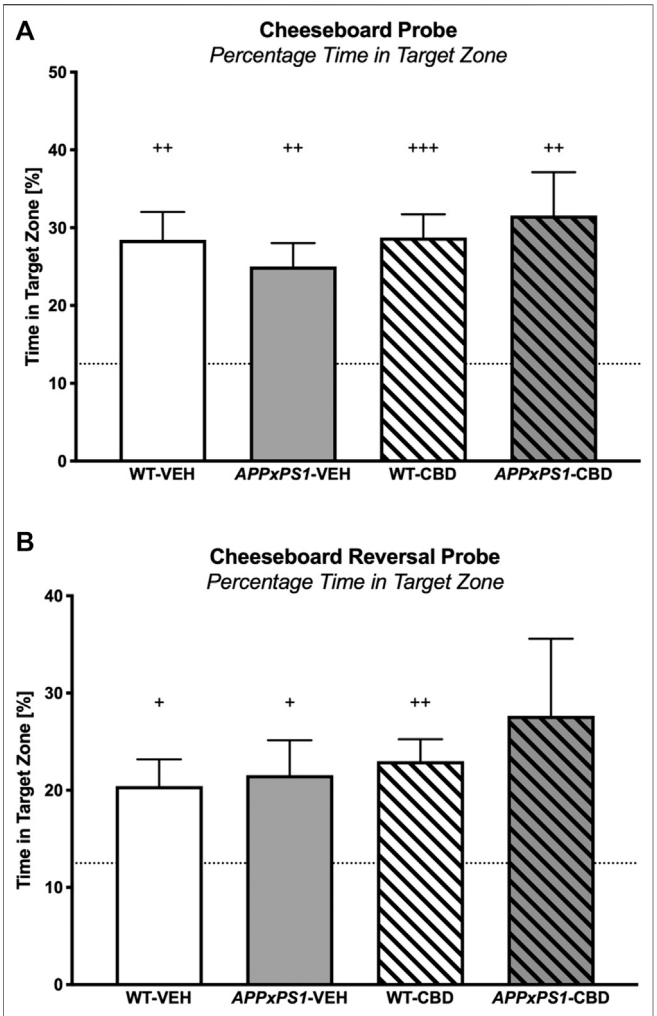

FIGURE 4 | Spatial memory in the cheeseboard (CB) probe and reversal cheeseboard (reversal CB) probe. **(A)** Percentage of time spent (%) in the target zone for the CB probe and **(B)** for the reversal CB probe. Data for wild type-like (WT) control and double transgenic $APP_{Swe}/PS1\Delta E9$ (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant *t*-test results against chance levels (i.e., 12.5%) are indicated by "+" ($^{+}p < .05$, $^{++}p < .01$, and $^{+++}p < .001$).

(9) = 3.4 and $p = .007$, **Figure 4A**). This was also evident when considering the target zone preference postleaving the start zone (WT-VEH: t (14) = 4.0 and $p = .001$; *APPxPS1*-VEH: t (9) = 4.1 and $p = .003$; WT-CBD: t (12) = 5.5 and $p < .001$; *APPxPS1*-CBD: t (9) = 3.3 and $p = .009$, **Supplementary Figure S3A**). Importantly, splitting up the full 2 min probe trial data into 30 s bins, all mice also demonstrated intact retrieval memory in the first bin (WT-VEH: t (14) = 4.8 and $p < .001$; *APPxPS1*-VEH: t (9) = 3.1 and $p = .01$; WT-CBD: t (12) = 5.3 and $p < .001$; *APPxPS1*-CBD: t (9) = 3.3 and $p = .009$, **Table 4**). However, when investigating perseverance in the second 30 s bin, WT mice persevered to find the food reward (WT-VEH: t (14) = 3.3 and $p = .005$; WT-CBD: t (12) = 3.3 and $p = .006$), whereas *APPxPS1* mice did not (*APPxPS1*-VEH: t (9) = 1.8 and $p = .1$; *APPxPS1*-CBD: t (9) = 1.2 and $p = .3$, **Table 4**). Comparing percentage time spent in target zone in the full 2 min test period using two-way ANOVA did not reveal significant main effects or interaction thereof (all *p's* n.s.).

Reversal Probe Trial

In the reversal probe trial, all experimental groups (except the *APPxPS1*-CBD group, which was affected by a statistical outlier) had a significant preference for the target zone (WT-VEH: t (14) = 2.9 and $p = .01$; *APPxPS1*-VEH: t (9) = 2.5 and $p = .03$; WT-CBD: t (12) = 4.6 and $p = .001$; *APPxPS1*-CBD: t (10) = 1.9 and $p = .09$, **Figure 4B**) and that preference was also evident when taking into consideration the latency of mice to leave the start zone (WT-VEH: t (14) = 2.9 and $p = .01$; *APPxPS1*-VEH: t (9) = 2.6 and $p = .03$; WT-CBD: t (12) = 4.7 and $p = .001$; *APPxPS1*-CBD: t (10) = 2.0 and $p = .08$, **Supplementary Figure S3B**). As before, all mice demonstrated intact retrieval memory (WT-VEH: t (14) = 2.8 and $p < .01$; *APPxPS1*-VEH: t (8) = 3.4 and $p = .01$; WT-CBD: t (12) = 4.3 and $p = .001$; *APPxPS1*-CBD: t (9) = 2.7 and $p = .03$) but *APPxPS1* transgenic mice did not persevere to find the food reward (WT-VEH: t (14) = 3.7 and $p = .002$; WT-CBD: t (12) = 2.8 and $p = .02$; *APPxPS1*-VEH: t (8) = 1.8 and $p = .1$; *APPxPS1*-CBD: t (9) = 1.7 and $p = .1$, **Table 4**). Comparing percentage time spent in target zone in the full 2 min test period using two-way ANOVAs did not reveal significant main effects or interaction thereof (all *p's* n.s.).

Sensorimotor Gating

Acoustic Startle Response. The ASR of all mice was similar as there were no main effects of "genotype" or "treatment" (all *p*'s n.s.). Importantly, all experimental groups responded to increasing startle pulse intensities with more pronounced startle responses (RM ANOVA for "startle intensity": F (2,90) = 411.2 and $p < .001$; no interactions with "genotype" or "treatment", all *p's* n.s., **Figure 5A**). In addition, all mice regardless of test condition displayed decreasing ASR across the three blocks of five 120 dB pulses each, confirming that all mice habituated to the 120 dB startle pulse (RM ANOVA for "startle block": F (2,90) = 25.4 and $p < .001$; no interactions with "genotype" or "treatment", all *p's* n.s., **Supplementary Figure S4**).

TABLE 4 | Retrieval memory and perseverance in the cheeseboard (CB) probe and reversal cheeseboard (reversal CB) probe trials. Data shown as the percentage of time spent in the target zone (%) in the first (indicative of retrieval memory) and second 30 s (indicative of perseverance) of each probe test for wild type-like (WT) control and double transgenic $APP_{Swe}/PS1\Delta E9$ (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD). Data are presented as mean ± SEM. Significant *t*-test results against chance levels (i.e., 12.5%) are shown by "+" ($^{+}p < .05$, $^{++}p < .01$, and $^{+++}p < .001$).

	WT-VEH	*APPxPS1*-VEH	WT-CBD	*APPxPS1*-CBD
CB probe, % time spent in target zone				
First 30 s bin	$27.5 \pm 3.1^{+++}$	$30.3 \pm 5.8^{+}$	$34.8 \pm 4.2^{+++}$	$34.2 \pm 6.5^{++}$
Second 30 s bin	$31.8 \pm 5.9^{++}$	28.9 ± 8.9	$22.6 \pm 3.0^{++}$	21.0 ± 7.1
Reversal CB probe, % time spent in target zone				
First 30 s bin	$29.5 \pm 6.0^{+}$	$27.1 \pm 4.3^{+}$	$30.2 \pm 4.1^{++}$	$34.0 \pm 8.0^{+}$
Second 30 s bin	$26.3 \pm 3.8^{+}$	19.1 ± 3.8	$21.6 \pm 3.2^{+}$	27.2 ± 8.7

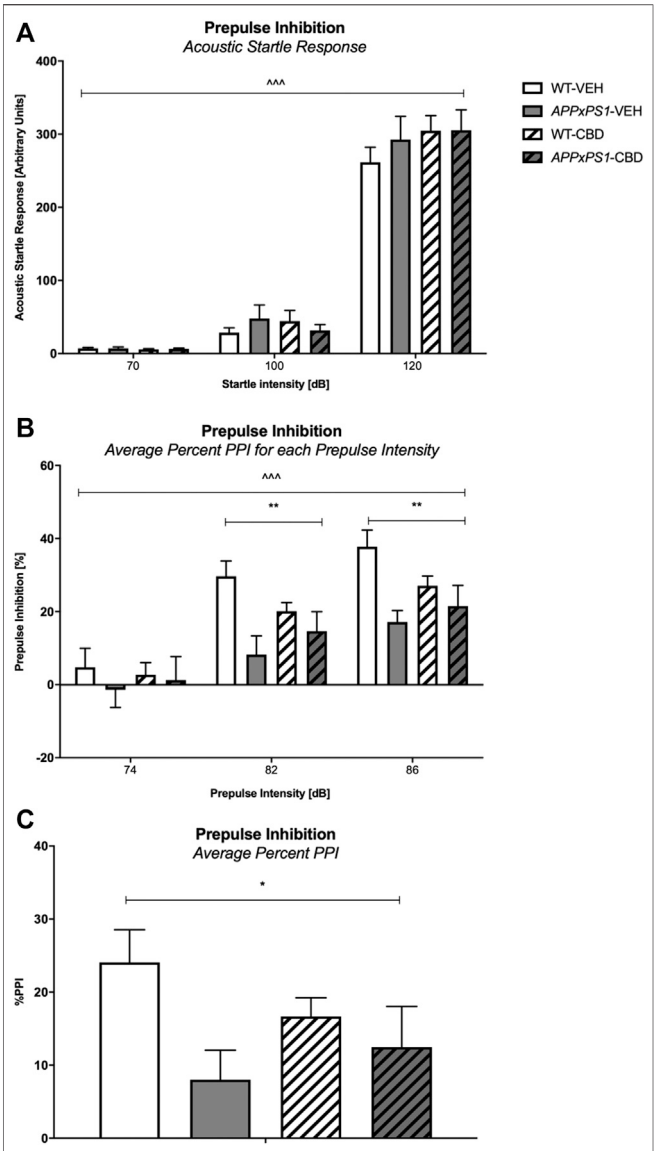

FIGURE 5 | Acoustic startle response (ASR) and sensorimotor gating (PPI). **(A)** ASR to increasing startle pulse intensity (70/100/120 dB), **(B)** percentage prepulse inhibition (%PPI) averaged over trials for increasing prepulse intensities (74/82/86 dB), and **(C)** %PPI averaged over prepulse intensity and interstimulus interval (ISI). Data for wild type-like (WT) control and double transgenic *APP*$_{Swe}$/*PS1ΔE9* (*APPxPS1*) female mice treated with either vehicle (VEH) or cannabidiol (CBD) are shown as means + SEM. Significant "genotype" effects are indicated with "*" (*$p < .05$ and **$p < .01$) and RM effects are indicated by '^' (^^^$p < .001$). There was a "genotype" by "prepulse intensity" interaction for average %PPI for increasing prepulse intensities ($p < .001$).

Prepulse Inhibition

Three-way RM ANOVA found that as prepulse intensities increased, the %PPI (averaged across ISI) of all mice became more robust as well ("prepulse intensity": F (2,90) = 166.7 and $p < .001$, **Figure 5B**). Importantly, a significant "genotype" by "prepulse intensity" interaction (F (2,90) = 8.3 and $p < .001$) was found. Data split by "prepulse intensity" revealed significant effects of "genotype" for %PPI at prepulse intensities of 82 dB (F (1,45) = 9.8 and $p = .003$) and 86 dB (F (1,45) = 9.3 and $p = .004$) but not 74 dB (F (1,45) = .6 and $p = .5$) with *APPxPS1* mice showing reduced prepulse inhibition compared to WT mice (**Figure 5B**). In line with this, we detected a genotype difference for the average %PPI (F (1,45) = 5.5 and $p = .02$, **Figure 5C**) with AD transgenic mice exhibiting lower % PPI. CBD treatment had no overall effect on sensorimotor gating and also did not change any genotype effect (i.e., no overall "treatment" effect and no "genotype" by "treatment" interactions for any prepulse intensity; all p's n.s.).

DISCUSSION

This study demonstrated that chronic administration of a medium dose of 5 mg/kg CBD reversed novel object recognition deficits in 12-month-old female double transgenic *APPxPS1* mice. CBD treatment did not affect the hyperlocomotive or anxiety-like phenotype of the *APPxPS1* mice, nor did CBD moderate the mild motor impairment shown by *APPxPS1* mice. *APPxPS1* mice, although being slower than WT mice, showed intact spatial learning and memory but exhibited impaired perseverance in the CB probe and reversal CB probe, which was not affected by chronic medium-dose CBD. Spatial learning and reversal learning was in fact delayed in *APPxPS1* mice by one day when considering performance across three daily trials compared to WT mice on a day-to-day basis Finally, the ASR of all mice was similar but *APPxPS1* transgenic mice showed a deficit in PPI.

The current study detected an object recognition deficit in 12-month-old *APPxPS1* female mice which is in line with our previous studies in male *APPxPS1* mice tested at the age of 5–6 months (Cheng et al., 2014a) and 12 months (Watt et al., 2020). Furthermore, object recognition impairments in female *APPxPS1* mice have been reported at 12 months of age by international colleagues when using a slightly different test protocol (Aso et al., 2016). Object recognition impairments correlate with the symptomatic stage of the disease, whereby AD patients often have difficulties recognizing faces and objects (Laatu et al., 2003). Importantly, chronic treatment with 5 mg/kg CBD was able to rescue this object recognition deficit. This finding expands on our earlier study reporting therapeutic effectiveness of 20 mg/kg CBD to restore object recognition memory in 5-6-month-old males (Cheng et al., 2014a) and has a similar effect to that seen by Aso et al. (2015) and Aso et al. (2016), whereby a botanical extract containing a combination of delta-9-tetrahydrocannabinol and CBD restored object recognition memory in a V-maze NORT paradigm in 6- and 12-month-old male *APPxPS1* mice, respectively. Interestingly, 50 mg/kg of purified CBD alone did not restore object recognition memory in 12-month-old males (Watt et al., 2020), and this outlines the importance to consider not only dose effects but also testing both male and female mice at early as well as later disease stages. Furthermore, these studies suggest that a combination of cannabinoids may be therapeutically more beneficial (in particular at later disease stages) than CBD alone treatment strategies. Interestingly, impairments in object recognition have been linked to glutamatergic dysfunction and inhibition

of the glutamate transporter 1 (Tian et al., 2019), and preclinical studies suggest that antagonism of the glutamate NMDA receptor via memantine can improve object recognition memory (Scholtzova et al., 2008). Importantly, CBD has previously been found to indirectly interact with the NMDA receptor via augmentation of the psychopathological effects of the NMDA receptor antagonist ketamine (Hallak et al., 2011). Thus, CBD may have reversed the object recognition deficits of *APPxPS1* mice in the current study through manipulations of the glutamatergic system. The potential involvement of the glutamate signaling pathway in CBD's therapeutic-like properties requires further study. The experimental outcomes suggest that lower doses of CBD may have more potential as a therapeutic in clinical settings and at later disease stages and adds further evidence to the biphasic nature of CBD.

The task-dependent hyperlocomotive phenotype of *APPxPS1* female mice confirms and expands our previous findings on task-specific hyperlocomotion in younger AD transgenic mice of both sexes (Cheng et al., 2013; Cheng et al., 2014b). A previous study suggested that this increase in locomotion may be related to increased anxiety or impaired habituation evident in this mouse model (Hooijmans et al., 2009); however, it is important to note that hyperlocomotion and anxiety phenotypes in the *APPxPS1* model appear to be task-specific and are not consistently reported in the literature (see, e.g., O'Leary et al. (2018)). CBD had no effect on the locomotion of WT mice nor on the hyperlocomotive phenotype of *APPxPS1* mice in line with other studies evaluating the effect of various CBD dosing on the locomotion of wild type-like mice (Moreira and Guimarães, 2005; Long et al., 2010; Todd and Arnold, 2016). CBD treatment increased the frequency of *rearing* of both WT and AD transgenic mice specifically in the dark zone, but when corrected for by time, it became clear that CBD had no effect on explorative behavior, confirming previous findings of our laboratory on the absence of CBD effects on *rearing* in male C57BL/6JArc mice (Long et al., 2010).

12-month-old *APPxPS1* female mice displayed an anxiogenic phenotype in the LD test, whereas an anxiolytic-like phenotype was evident in younger, 7-month-old AD females (Cheng et al., 2014b). It is important to note that the *APPxPS1* model of AD shows progressive age-related changes in behavior, cognition, and pathology (Arendash et al., 2001; Trinchese et al., 2004; Pugh et al., 2007; Lok et al., 2013) suggesting a potentially age-dependent change in anxiety behaviors in this mouse model although task and protocol sensitivity of this phenotype have also been raised as potential anxiety behavior-modulating factors in this model (as reviewed in O'Leary et al. (2018)). Importantly, the interpretation of the findings in the LD paradigm is affected by the observation that vehicle-treated WT mice did not show a strong aversion to the light zone. Chronic medium-dose CBD had no effect on anxiety parameters in the LD test, similar to our previous study on the effects of 20 mg/kg CBD in *APPxPS1* males (Cheng et al., 2014a) as well as male and female C57BL/6J mice (although in that study, CBD decreased EPM anxiety (Schleicher et al., 2019)). In this context, it is important to note that CBD has a biphasic dose-response in relation to anxiety effects (Rey et al., 2012; Zuardi et al., 2017). Furthermore, it has been suggested that the anxiolytic effects of CBD may only be evident after an external stressor has been applied, for example, following daily unpredictable stress (Campos et al., 2013).

Motor function impairment has recently been considered as an associated noncognitive symptom of AD (Buchman and Bennett, 2011). In our study, all mice performed equally well in the pole test and in the accelerod, when assessing motor functions across trials. However, *APPxPS1* mice fell off the accelerod sooner than WT mice on their worst-performing trial. Similarly, 6-month-old male and female *APPxPS1* mice tended to slip more often than WT mice in the balance beam test (Kuwabara et al., 2014). Other researches confirm that the motor phenotype of the *APPxPS1* mouse model is task-specific and likely affected by age also (Lalonde et al., 2004; Kemppainen et al., 2014; Kuwabara et al., 2014). Chronic CBD had no impact on motor performance. In line with this, CBD has previously been found to demonstrate few extrapyramidal side effects (Iffland and Grotenhermen, 2017) and not affect motor performance in male Swiss mice either (Ten Ham and De Jong, 1975).

Spatial disorientation is commonly seen in patients with AD (Lithfous et al., 2013). The current study found that the overall ability to acquire the CB and reversal CB task (i.e., the ability to learn the position of a food reward within the overall training period) was not affected in 12-month-old *APPxPS1* mice and that CBD did not affect spatial learning when considering intermediate-term and reference memory. Investigating CB learning in that detail has only recently been described (Kreilaus et al., 2019); thus, this is the first study to identify that the intermediate-term and long-term retention learning memory of 12-month-old *APPxPS1* female mice appear intact. Interestingly, spatial learning and reversal spatial learning of the *APPxPS1* mice were delayed by one day when considering performance across three daily trials compared to WT mice on a day-to-day basis. Importantly, CBD was able to restore this learning delay in the initial training period but did not restore the delay seen in the reversal CB training. It should be noted here that the average speed of *APPxPS1* mice was reduced and therefore transgenic mice took generally longer per day to find the food reward. Chronic CBD had no effect on the spatial learning of control mice in line with previous studies (Fagherazzi et al., 2012).

All mice showed a preference for the target zone indicating intact spatial memory (as well as reversal memory). The target zone preference of *APPxPS1*-CBD mice during reversal testing did not reach significance. However, this appears to be driven by one individual animal, which met the criterion as a statistical outlier but was not excluded from analysis as the mouse did not show any health issues when the test video was reviewed. More stressful tests for spatial memory, that is, the Morris Water Maze, detected memory deficits in 12-month-old and 18-month-old female *APPxPS1* mice (Savonenko et al., 2005; Zhang et al., 2011). Furthermore, 16-month-old *APPxPS1* mice exhibited impaired learning and memory in the Barnes maze (O'Leary and Brown, 2009) suggesting that stress levels may affect the cognitive performance of this AD transgenic mouse model. In addition, our previous work using CB testing detected general spatial memory deficits in 8-9-month-old *APPxPS1* female mice in the reversal CB probe when tested at baseline (Cheng et al., 2014b). Importantly, baseline studies cannot easily be compared

to cannabinoid treatment studies as all mice of the latter studies are exposed to daily injections and the necessary handling stress (Gouveia and Hurst, 2019), as well as the effects of the vehicle compound which can shift behavioral phenotypes (Long et al., 2013). Furthermore, the current study was carried out in a new test facility and by a female researcher; both factors have been found to impact on behavioral test outcomes (Lewejohann et al., 2006; Sorge et al., 2014). Finally, we detected intact retrieval memory across experimental conditions but also reduced the persistence of AD transgenic mice to find the food reward. The lack of preference for the target zone of these mice in the second 30 s bin could be discussed as heightened cognitive flexibility in adaptation to the lack of food reward (since the probe trial can be considered as an extinction trial (Grech et al., 2019)). Further analysis into the search patterns of mice during the CB and rCB could be conducted in future studies to determine any deficits in allocentric or egocentric navigational strategies that might explain the lack of perseverance in *APPxPS1* mice during probe trials.

The present study found that the sensorimotor gating of *APPxPS1* mice was reduced compared to control littermates, particularly at higher prepulse intensities, and was not accompanied by any changes in the baseline startle response or habituation thereof. These findings are in line with Wang et al. (2012) who found robust PPI deficits in female mice of a similar *APPxPS1* model as early as at 7 months of age but are different to the work by Cheng and coworkers (Cheng et al., 2014b), which found PPI deficits at the 128 ms ISI only in 10-11-month-old *APPxPS1* female mice (although *APPxPS1* mice in that study "generally" exhibited lower %PPI than WT mice). Chronic treatment with medium-dose CBD did not reverse deficits in PPI. Acute CBD has been found to either attenuate pharmacologically induced disruptions of PPI (but no effect in untreated control mice) (Long et al., 2006; Pedrazzi et al., 2015) or not affect such deficits (Gururajan et al., 2011) and one study utilizing chronic CBD treatment even caused a PPI deficit (Schleicher et al., 2019). Some of these discrepancies may be due to the fact that PPI test outcomes are heavily dependent on the protocol characteristics used in each study (Karl et al., 2011). For example, the experiments of this treatment study compared to the findings of the baseline study of Cheng et al. (2014b) were performed in different locations and utilized different PPI test enclosure habituation procedures (the Cheng protocol used three days of 5 min habituations). It has previously been shown that test location (Karl et al., 2011) and habituation procedures (Swerdlow et al., 2000) are important factors that can impact PPI test outcomes. Furthermore, CBD therapeutic effects on PPI impairments have previously been evaluated in genetic and pharmacological mouse models for schizophrenia, which are characterized by PPI-relevant pathological changes in, for example, the dopaminergic and glutamatergic pathways not necessarily evident in AD mouse models.

The study's outcome is affected by some limitations: the present study did not investigate male mice for reasons outlined earlier and thus requires follow-up experiments testing the effects of medium-dose CBD on behavioral deficits of *APPxPS1* males. Furthermore, the present study focused on the assessment of the behavioral effects of CBD in this model. Future investigations of AD-relevant neuropathological markers could help explain potential mechanisms regarding the behavioral effects of CBD seen in this study. In brief, *in vitro* studies have shown that CBD acts against Aβ-induced toxicity in various ways, including inhibition of tau hyperphosphorylation (Esposito et al., 2006a) which was associated with a reduction in the phosphorylated glycogen synthase kinase 3-β, the protein responsible for NFT formation in AD. In addition, CBD can increase cell survival, reduce Aβ-induced lipid peroxidation, reactive oxygen species production (Iuvone et al., 2004), and attenuate nitric oxide production via inhibition of phosphorylated p38 mitogen-activated protein kinase and transcription factor nuclear factor-κB (Esposito et al., 2006b). Finally, CBD can counteract the elevation of APP expression by inducing ubiquitination of APP through activation of peroxisome proliferator-activated receptor-γ (PPARγ; Scuderi et al., 2014). In pharmacological mouse models of AD, CBD prevented Aβ-induced spatial learning deficits, reduced the Aβ-induced increase in IL-6 (Martín-Moreno et al., 2011), and attenuated Aβ-evoked neuroinflammation (Esposito et al., 2007) and this appeared to be mediated via PPARγ as well (Esposito et al., 2011). Interestingly, PPARγ has been shown to be elevated in AD patients (de la Monte and Wands, 2006) although our recent research using a 50 mg/kg CBD dose did not find any genotype- or treatment-related changes in this receptor (Watt et al., 2020).

In conclusion, this study found that 12-month-old female *APPxPS1* transgenic mice were hyperlocomotive and showed cognitive impairments (i.e., object recognition memory and spatial learning) as well as PPI deficits. Importantly, chronic treatment with 5 mg/kg CBD reversed object recognition deficits in *APPxPS1* transgenic female mice suggesting a therapeutic-like effect in this established mouse model for AD. To conclude, this study suggests that CBD has therapeutic value for specific behavioral impairments present in AD. Importantly, to date, there is a lack of completed clinical trials on the therapeutic effects of CBD or CBD-rich cannabis extracts on AD symptoms. The current study assists in defining therapeutic dose regimes potentially effective in AD patients and lower-dose CBD treatment would reduce not only the therapy costs for patients but also potential side effects (most important for therapeutic cannabis compounds containing not only CBD but also other cannabinoids such as Δ^9-tetrahydrocannabinol, as would be the case for CBD-enriched cannabis extract therapies).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Ethics Committee of Western Sydney University.

AUTHOR CONTRIBUTIONS

TK conceptualized the study design and experimental protocol. MC carried out the drug administration and behavioral experiments following the training provided by GW and FK. MC collected the data and prepared the figures, and TK, MC, GW, and FK performed the statistical analysis. MC and TK drafted and revised the manuscript. All authors approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.587604/full#supplementary-material

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REVIEW
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Cannabidiol as a Therapeutic Target: Evidence of its Neuroprotective and Neuromodulatory Function in Parkinson's Disease

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The phytocannabinoids of *Cannabis sativa* L. have, since ancient times, been proposed as a pharmacological alternative for treating various central nervous system (CNS) disorders. Interestingly, cannabinoid receptors (CBRs) are highly expressed in the basal ganglia (BG) circuit of both animals and humans. The BG are subcortical structures that regulate the initiation, execution, and orientation of movement. CBRs regulate dopaminergic transmission in the *nigro-striatal* pathway and, thus, the BG circuit also. The functioning of the BG is affected in pathologies related to movement disorders, especially those occurring in Parkinson's disease (PD), which produces motor and non-motor symptoms that involving GABAergic, glutamatergic, and dopaminergic neural networks. To date, the most effective medication for PD is levodopa (L-DOPA); however, long-term levodopa treatment causes a type of long-term dyskinesias, L-DOPA-induced dyskinesias (LIDs). With neuromodulation offering a novel treatment strategy for PD patients, research has focused on the endocannabinoid system (ECS), as it participates in the physiological neuromodulation of the BG in order to control movement. CBRs have been shown to inhibit neurotransmitter release, while endocannabinoids (eCBs) play a key role in the synaptic regulation of the BG. In the past decade, cannabidiol (CBD), a non-psychotropic phytocannabinoid, has been shown to have compensatory effects both on the ECS and as a neuromodulator and neuroprotector in models such as 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and reserpine, as well as other PD models. Although the CBD-induced neuroprotection observed in animal models of PD has been attributed to the activation of the CB1 receptor, recent research conducted at a molecular level has proposed that CBD is capable of activating other receptors, such as CB2 and the TRPV-1

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Abbreviations: 2-AG 2-arachidonoyl-glycerol; 6-OHDA 6-hydroxydopamine; AEA, anandamide; BG, basal ganglia; CB1, cannabinoid receptor type-1; CB2, cannabinoid receptor type-2; CBD, cannabidiol; CBDA, cannabidiol acid; CBRs, cannabinoid receptors; CPu, caudate-putamen; eCBs, endocannabinoids; ECS, endocannabinoid system; FAAH, fatty acid amide hydrolase; GPe, globus pallidus external; GPi, globus pallidus internal; GPR55, G protein receptor-55; l-DOPA Levodopa; LID l-DOPA-induced dyskinesia; MPTP 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; PPAR-γ, Peroxisome proliferator-activated gamma; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; STN, subthalamic nucleus; THC Δ9-tetrahydrocannabinol; THCA Δ9-tetrahydrocannabinol acid; TRPV-1, transient receptor potential vanilloid-1.

receptor, both of which are expressed in the dopaminergic neurons of the *nigro-striatal* pathway. These findings open new lines of scientific inquiry into the effects of CBD at the level of neural communication. Cannabidiol activates the PPARγ, GPR55, GPR3, GPR6, GPR12, and GPR18 receptors, causing a variety of biochemical, molecular, and behavioral effects due to the broad range of receptors it activates in the CNS. Given the low number of pharmacological treatment alternatives for PD currently available, the search for molecules with the therapeutic potential to improve neuronal communication is crucial. Therefore, the investigation of CBD and the mechanisms involved in its function is required in order to ascertain whether receptor activation could be a treatment alternative for both PD and LID.

Keywords: cannabidiol (CBD), neuroprotective, neuromodulatory, L-DOPA-induced dyskinesia, parkinson's diasese

CANNABIDIOL: ORIGIN, PHARMACOKINETICS, AND PHARMACODYNAMICS

Taxonomically, *Cannabis sativa* L. pertains to the Cannabaceae family (Russo, 2007), which has recently been found to include the genera *Cannabis*, *Humulus*, and *Celtis*. *Cannabis sativa* has three varieties, *sativa*, *indica* and *ruderalis* (McPartland, 2018). Phytocannabinoids are the active compounds in *Cannabis sativa* L., the most abundant compound of which is Δ^9-tetrahydrocannabidiol (THC), which has psychoactive pharmacological effects, while cannabidiol (CBD), its second most abundant compound, has psychoactive/non-psychotropic pharmacological effects and is more medically promising than THC (Ibeas Bih et al., 2015) (**Figure 1**). THC and CBD are initially formed as carboxylic acids (e.g., Δ^9- THCA, CBDA) that are decarboxylated into neutral form, a process occurring naturally as the plant ages and when it is exposed to light or heat (Hanuš et al., 2016; Wang et al., 2016).

While preclinical and clinical studies have shown that THC induces anxiety and psychotic symptoms in healthy subjects, the consumption of CBD has been found to significantly reduce the effects of THC, with CBD shown to have an antagonistic effect against THC (Dalton et al., 1976; Zuardi et al., 2006). Interestingly, both compounds have been shown to affect inflammation, anxiety, emesis, and nausea; moreover, it has been proposed that they act as both neuroprotective agents and antioxidants (Pertwee 2004; Cascio and Pertwee, 2014). The strategic use of both compounds has been reported for pain relief in cancer and neuropathic pain relief in multiple sclerosis (Zajicek and Apostu, 2011; Fine and Rosenfeld, 2014; Dariš et al., 2019). Studies on CBD have shown that it participates in the regulation of the endocannabinoid system (ECS), the important characteristics of which will be summarized in this review.

The ECS is a complex lipid network consisting of cannabinoid receptors (CBRs), endogenous ligands, and the enzymes involved in endocannabinoid degradation and synthesis (**Figure 2**). Chemicals derived from fatty acid amides and diacylglycerols, endocannabinoids (eCBs) are synthesized endogenously in mammals and are produced on demand in response to increased intracellular calcium levels ($[Ca^{2+}]_i$) (Di Marzo et al., 1998; Mechoulam and Parker, 2013). The main eCBs are arachidonoylethanolamine, also known as anandamide (AEA), and 2-arachidonoyl-glycerol (2-AG) (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995).

The synthesis of AEA is produced by the hydrolysis of a phospholipid precursor, N-acyl-phosphatidylethanolamine (NAPE), which is carried out by the enzyme N-acyl-phosphatidylethanolamine-phospholipase D (NAPE-PLD). There is evidence that AEA is formed from N-acyl-lysophosphatidylethanolamine (NALPE) by the enzymes lysophospholipase D (lysoPLD), α/β-hydrolase 4 (ABH4), and phospholipase C (PLC). The other eCB, 2-AG, is synthetized via the activation of a PLC, thus producing 1,2-diacylglycerol (DAG), which, in turn, is converted into 2-AG by diacylglycerol lipase

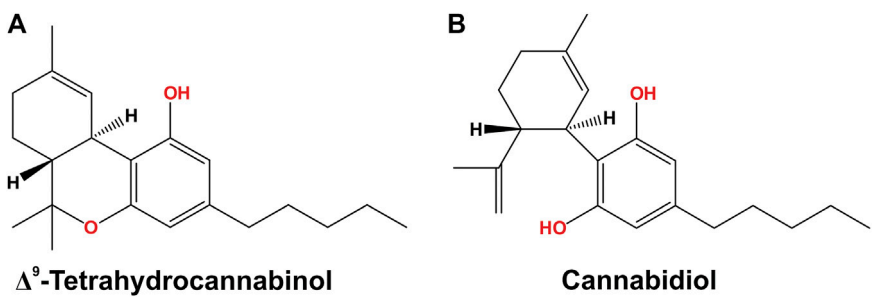

FIGURE 1 | Chemical structures, **(A)** THC and **(B)** CBD, the main phytocannabinoids extracted from the Cannabis plant THC, tetrahydrocannabinol; CBD, cannabidiol.

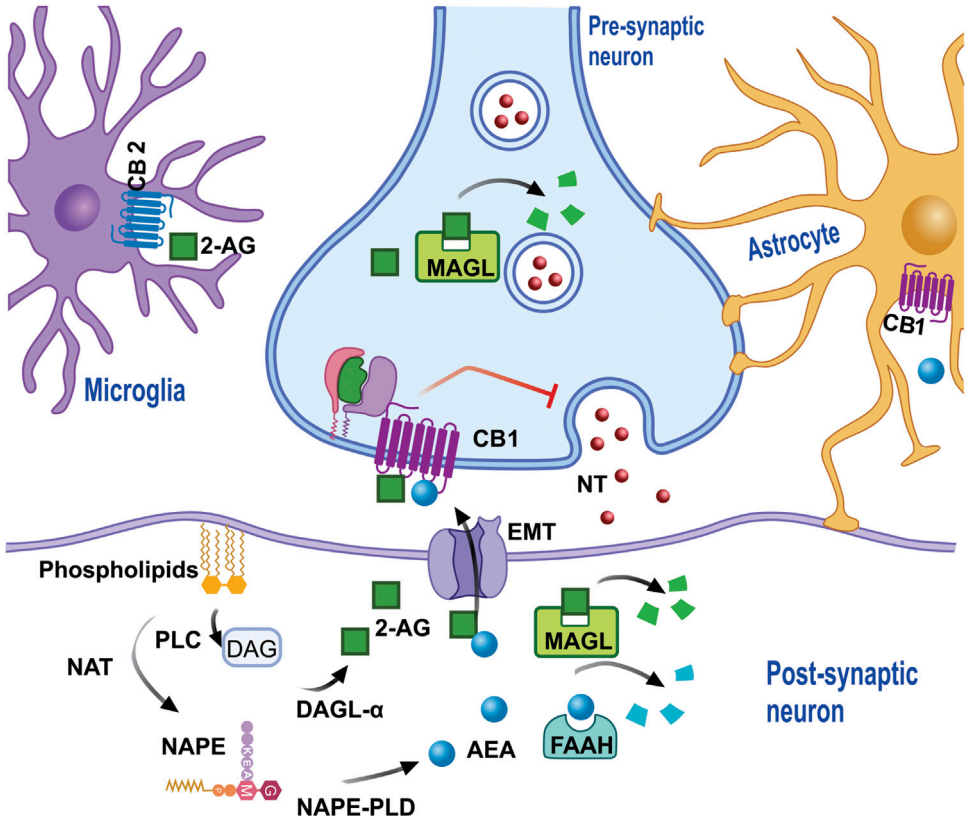

FIGURE 2 | eCB is synthesized from membrane phospholipids. NAT synthesizes the precursor NAPE, which subsequently, through the action of PLD, produces AEA in the cytoplasm of the post-synaptic neuron (or neuron spine). AEA leaves the cytoplasm and enters the synaptic space via diffusion and/or the action of EMT in order that, once it has onside, AEA activates the cannabinoid receptors which inhibit the release of NT. The degradation of AEA in EMT is regulated by FAAH, which produces metabolites such as AA and ETA. 2-AG requires the formation of the DAG precursor by PLC, which then through the action of diacylglycerol lipase α, and together with arachidonic acid generates 2-AG, which then leaves the synaptic space to activate cannabinoid receptors, which are also present in the microglia and/or astrocytes, and can be degraded by MAGL both in the pre and post-synapse, generating AA and Gro as metabolites. Abbreviations: eCB, endocannabinoids; NAT, N-acyl transferase; NAPE, N-acyl-phosphatidylethanolamine; PLD, phospholipase D; AEA, anandamide; NT, neurotransmitters; EMT, endocannabinoid membrane transporter; FAAH, fatty acid amide hydrolase; AA, arachidonic acid; ETA, ethanolamine; 2-AG, 2- Arachidonoylglycerol; DAG, diacylglycerol; PLC, phospholipase C; MAGL, monoacylglycerol lipase.

(DAGL), which can also be synthesized from sn-1-lysophospholipids, via the sequential action of phospholipase A1 (PLA1) and lysophospholipase C (Di Marzo et al., 2015). The main degradation enzymes of the eCBs are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL). FAAH is located in both the Soma and the post-synaptic neuronal dendrites and is associated with the membranes of cytoplasmic organelles that serve as a reservoir of $[Ca^{2+}]_i$, the mitochondria, and the smooth endoplasmic reticulum. DAGL and MAGL are located in the postsynaptic dendrites and the pre-synaptic neurons, respectively, whit the latter expressed when 2-AG, the main substrate, is metabolized. Both AEA and 2-AG are metabolized by FAAH, with other enzymes, such as the α/β-hydrolase families 6 and 12 (ABHD6 and ABHD12), also participating, although to a lesser extent (<10%) (Mechoulam and Parker, 2013).

Unlike the classical form of neurotransmitter release, eCBs are released from the post-synaptic neuron to then interact with its specific receptors in a retrograde manner (Di Marzo et al., 1998; Di Marzo et al., 2015). It has been proposed that the release of eCBs, but mainly AEA, occurs via a transporter called the *endocannabinoid membrane transporter*: (Yates and Barker, 2009; Fowler 2013). Once released into the synaptic space, these eCBs interact with their specific receptors (**Figure 2**). CBRs have been cloned, characterized, and classified into two subtypes, cannabinoid receptor type 1 (CB1) (Matsuda et al., 1990) and cannabinoid receptor type 2 (CB2) (Munro et al., 1993), which are proteins containing seven transmembrane domains coupled to inhibitory G proteins ($G\alpha_i$). At a molecular level, CB1-receptor activation inhibits the release of presynaptic neurotransmitters via the inhibition of the enzyme adenylyl cyclase (AC), the adenylate monophosphate circle/protein kinase A (cAMP/PKA) pathway, and the inhibition of the voltage-dependent Ca^{2+} channels (Mackie, 2006; Kano et al., 2009). This physiological mechanism ensures that the ECS plays a neuromodulatory role.

The pharmacokinetics of CBD is variable and depends on the route of administration (oral, intravenous, sublingual, topical,

inhalation, and transdermal), the type of product administered, concomitant food intake, and drug-drug interactions. Pharmaceutical forms with lipid excipients have been reported to improve CBD absorption (Zgair et al., 2016), with a study, conducted on subjects who had ingested food prior to administration via an erosol containing THC/CBD, finding a five-fold increase in the area under the curve (AUC) and a three-fold increase in the C*max*, as well as the prolongation of the T*max* (Stott et al., 2013). The use of sublingual drops at a dose of 20 mg obtained a T*max* of 2.17 h and a C*max* of 2.05 ng/ml (Guy and Flint, 2004). Administration via inhalation obtained a T*max* of 0.17 h and a C*max* of 28.2 ng/ml in occasional users, while a T*max* of 0.29 h and a C*max* of 76.3 ng/ml were obtained in frequent users, both via the administration 1.5 mg doses (Swortwood et al., 2017). Idiosyncratic differences mean that the mechanisms of administering cannabinoids are highly variable, with the oral administration of CBD shown to have a bioavailability of 13–19% (Mechoulam et al., 2002). Vaporization should be considered a promising route of administration, given that it can improve bioavailability without presenting a risk to the consumer, as long as it is used correctly (Varlet et al., 2016).

Cannabidiol is mainly excreted in feces, with part of the drug excreted unchanged (Perez-Reyes et al., 1976), while approximately 100 CBD metabolites are estimated to be excreted via the kidneys (Huestis, 2007). It is mainly metabolized via both oxidation and hydroxylation at various sites in the molecule, generating a complex degradation process. Other metabolites are formed via the *ß*-oxidation and biotransformation of the pentyl side chain and hydroxylations at C-6 and C-7 (Harvey and Mechoulam, 1990), while the highest concentration metabolites are 7-COOH-CBD and 6- OH-CBD, which are excreted intact or as glucuronide acid conjugates (Devinsky et al., 2018).

The metabolite 7-OH-CBD has been reported to both inhibit FAAH (IC50 = 34 μM) and decrease the metabolism of AEA in a basophil culture (IC50 = 50 μM) (Bisogno et al., 2001). Moreover, both 7-OH-CBD and 7-COOH-CBD have been reported to have anti-inflammatory effects and inhibit the production of nitric oxide (NO), reactive oxygen species (ROS), and tumor necrosis factor-alpha (TNF-α) (Mechoulam et al., 2010).

While the inhibition of the CYP1A1, 1A2, 2C9, and 1B1 isoforms has been reported, the inhibition of the CYP2C19 and 3A4 isoforms is more potent (Bornheim and Grillo, 1998; Zendulka et al., 2016; Arellano et al., 2017). This inhibition can have a synergistic effect in the presence of barbiturates (Paton and Pertwee 1972). Although CBD can interrupt the hydrolysis of THC by Cytochrome P450, no pharmacokinetic changes are reported in either compound. CBD metabolites, such as 7-OH or 7-COOH-CBD, have anti-inflammatory effects and inhibit the formation of NO, ROS, TNF-α, IL-1ß, NF-κβ, and IL-6 (Watzl et al., 1991; Kozela et al., 2010; Mechoulam et al., 2010). In addition, CBD reduces the production of prostaglandins (Costa et al., 2004) and nitric oxide synthases (NOS) (Esposito et al., 2006), indicating a route for anti-inflammatory or antinociceptive effects, while one effect of 6-oxo-CBD (a CBD metabolite) is anticonvulsant activity (Carlini et al., 1975). As CBD does not affect the metabolism of 2-AG (Rimmerman et al., 2011), it does not influence the action of the CB1 and CB2 receptors. It should be noted that the effects of eCB and phytocannabinoids, especially CBD, depend on the expression and anatomical location of CBRs in the brain.

While the mechanism of action by which CBD exerts its therapeutic effects remains, to date, unclear, the interactions between CBD and various molecular targets can be divided into interactions that are either dependent on or independent of the ECS. The ECS-dependent effects of CBD occur via the CB1, CB2, and TRPV-1 receptors, as does its interaction with the FAAH enzyme. As CBD is a lipophilic structure, it is able to cross the blood-brain barrier (BBB) and thus modulate specific zones of the CNS (Calapai et al., 2020). While CBD was thought to have a low affinity for CBRs and able to activate them at high concentrations (>10 μM) (Howlett and Fleming, 1984), at low concentrations, it has been reported to act on the allosteric site of the CB1 receptor (Laprairie at al., 2015), which may maximize the binding of the orthosteric probe. At higher concentrations, meanwhile, this action may reduce the binding of the orthosteric probe, producing a bell-shaped curve (Tham et al., 2019). Cannabidiol acts as an inverse agonist on the CB1 and CB2 receptors, as demonstrated by the efficacy of its antagonist properties against the agonistic effects induced by CP55940 on the CB1 and CB2 receptors in [35S]GTPγS binding assays undertaken on membrane preparations (Thomas et al., 2007). The K_B values obtained for CBD as an antagonist of CP55940-induced [35S]GTPγS binding were 79 and 65 nM for CB1 and CB2, respectively, while the Ki values for the displacement of [3H]CP55940 were 4.9 and 4.2 μM for CB1 and CB2, respectively (Thomas et al., 2007). These findings have been supported by recent reports showing that CBD does not necessarily have to be present at the orthosteric site to act as an inverse agonist, meaning that it could induce a non-competitive negative allosteric modulation of the CB1 receptor (Laprairie et al., 2015). In addition, at 100 nM, CBD was found to be a negative allosteric modulator of CB2, with a Ki value of 4.2 μM observed for the displacement of WIN55212.2 (Martínez Pinilla et al., 2017). These findings demonstrate that cannabidiol is a high-potency antagonist of CBR agonists in the brain and has a negative allosteric modulatory effect (**Figure 3**).

It has also been reported that CBD is capable of inhibiting some of THC's effects (Zuardi et al., 1982) by acting as a negative allosteric modulator on both CB1 and CB2 (Laprairie et al., 2015; Martí nez-Pinilla et al., 2017). Furthermore, computational models have identified an allosteric site on the CB1 receptor which is able to bind with CBD and, thereby, promote conformational changes to the receptor in either its active or inactive state. These findings may explain the negative allosteric modulatory effects of CBD on the CB1 receptor, with the possible participation of other molecular targets (namely independent interactions in the ECS) that, together, contribute to the effects observed in both *in vitro* and *in vivo* experimental studies (Chung et al., 2019). The mechanism by which CBD functions may be explained as a biased agonism, namely that it selects "which signaling pathways become activated upon binding to the receptor". In 2018, it was reported that CBD, applied at 100 nM concentrations, produces a biased agonism effect

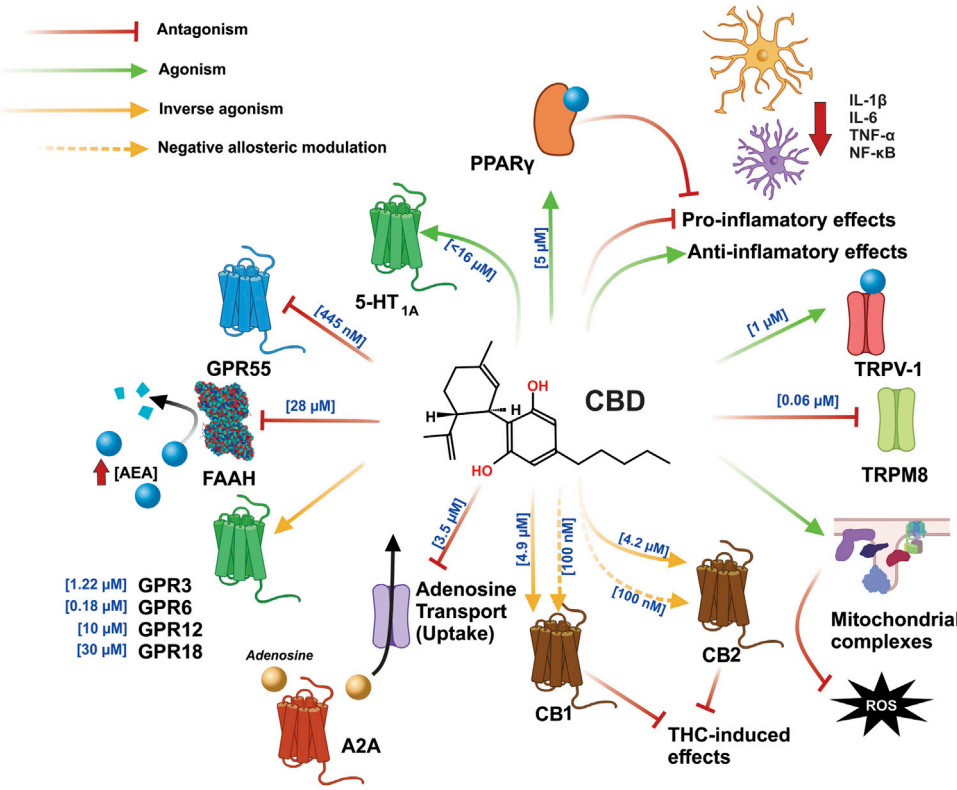

FIGURE 3 | CBD exerts an agonist-like effect on the PPARγ, TRPV1, CB1, and CB2 receptors, by inhibiting the enzyme that degrades AEA and FAAH, leading to increased AEA concentration and greater interaction with said receptors. In addition, CBD inhibits GPR55 and TRPM8 and exerts an effect as an inverse antagonist on the GPR3, GPR6, GPR12, CB1, and CB2 receptors; moreover, in CB1 and CB2, it can function as a negative allosteric modulator, which is involved in blocking the effects of THC. The anti-inflammatory effects of CBD function by directly decreasing the synthesis of pro-inflammatory cytokines and increasing the synthesis of anti-inflammatory cytokines. CBD also reduces inflammation by stimulation PPARγ. Part of its antioxidant effects are achieved via the increased activity of mitochondrial complexes I, II, II-III, and IV. Abbreviations: CBD, cannabidiol; PPARγ, peroxisome proliferator activated receptor γ; TRPV1, transient potential receptor V1; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; AEA, anandamide; FAAH, fatty acid amide hydrolase; GPR55, G protein coupled receptor 55; TRPM8, transient potential receptor M8; GPR3, G protein coupled receptor 3; GPR6, G protein coupled receptor 6; GPR12, G protein coupled receptor 12; THC, tetrahydrocannabinol.

against the effects of THC, by increasing cAMP levels and decreasing ERK ½ activity, thus countering the effects induced by the THC. These data may explain the controversial pharmacology of CBD, namely whether or not it interacts with cannabinoid receptors and results from the binding of the two receptors, CB1 or CB2, to allosteric sites (Navarro et al., 2018). In the ECS, CBD can exert an effect as an indirect agonist of the CB1 receptor by inhibiting both the FAAH enzyme and the AEA transporter (Bisogno et al., 2001; Ligresti et al., 2006; De Petrocellis et al., 2011), which leads to an increase in AEA levels and, consequently, in the activation of the CB1 receptor (Howlett et al., 2010), although it can also interact with CB2, TRPV1, and PPARɣ (Pertwee and Ross, 2002; Ross, 2003; Bouaboula et al., 2005). The TRPV-1 receptor is a molecular target for the pharmacological effects of CBD (Bisogno et al., 2001), which are highly potent (producing an EC50 of 1 μM) (De Petrocellis et al., 2011) and, via TRPV-1, increase Ca2+ levels (producing an EC50 of 0.7 μM) (Ligresti et al., 2006). Moreover, CBD, including its precursor, binds at [5 μM - >11.6 μM] and activates PPARγ at [10 –20 μM], while cannabidiolic acid (CBDA) binds at [7.6 μM] and activates PPARγ and is more effective than CBD in activating PPARγ at concentrations of [10 –25 μM] (O'Sullivan et al., 2009; Nadal et al., 2017) (**Figure 3**).

Notable among the independent mechanisms exerted by CBD in the ECS is the agonist binding of the G protein coupled receptors (GPCR) GPR3, GPR6, GPR12, and GPR18, which are considered orphan receptors (Morales and Reggio, 2017). However, lysophosphatidylinositol is considered an endogenous receptor for GPR55 (Alhouayek et al., 2018). CBD exhibits a decrease in concentration-dependent ß-arrestin two recruitment to both GPR3 and GPR6, but with greater potency for the latter (EC50 values of 1.22 and 0.18 μM, respectively) (Laun and Song, 2017). Furthermore, CBD significantly decreases the cAMP accumulation stimulated by GPR12, in a concentration-dependent manner, corresponding to an approximate EC50 of 10 μM (Brown et al., 2017). Therefore, these findings show the inverse agonist effect of the GPR3, GPR6, and GPR12 receptors. CBD is reported to present a low level of efficacy as an agonist in the recruitment, via GPR18, of ß-arrestin at a concentration of

30 μM (Console-Bram et al., 2014). However, CBD acts as an antagonist of the effects, induced by N-arachidonoyl glycine (NAGly) and THC, on the migration and morphology of microglia (McHugh et al., 2014). It is likely that the functionality of CBD as an agonist and antagonist depends on the expression of the GPR18 receptor and that CBD may also act as a biased agonist in this GPCR (Morales et al., 2020). It should be noted that, based on the inverse agonist effect that CBD has been shown to have on the GPR55 receptor, novel therapeutic strategies are proposed for treating neurodegenerative diseases via the probable mechanism of this phytocannabinoid (Ryberg et al., 2007; Kaplan et al., 2017). The GPR55 receptor is coupled to the Gα12/13 and Gαq proteins, while its activation promotes the release of Ca^{2+} stores from the endoplasmic reticulum and, in turn, the activation of MAPKs (Alhouayek et al., 2018). On the other hand, antagonism inhibits Ca^{2+} currents and causes neuronal inhibition at a presynaptic level. CBD is likely to promote both neuronal repolarization via GPR55, at a presynaptic level, and neuromodulation (Morano et al., 2020). Interestingly, as GPR55 is expressed in a similar way to the CB1 receptors, they are also expressed in the BG circuit (Celorrio et al., 2017).

In addition to the foregoing findings, evidence reported on the mechanism via which CBD affects the CNS has shown that CBD activates the serotonin receptor 5-HT1A (Russo et al., 2005) and the adenosine A2A receptors (Mecha et al., 2013). CBD behaves as an antagonist with TRPM8 (De Petrocellis et al., 2011), while, acting alone, it has also been found to stimulate the activity of mitochondrial complexes (Valvassori et al., 2013), in addition to, directly and indirectly, stopping the pro-inflammatory process and promoting the anti-inflammatory process, via PPARγ (Esposito et al., 2011; Malfait et al., 2000) (**Figure 3**). It should be noted that the CB1 receptor, TRPV-1, GPR55, and the A2A receptor are all abundantly expressed in the BG, which are main structures that participate in the control of movement (Fernández-Ruiz et al., 2010a; Hickey and Stacy, 2012; Chaves-Kirsten et al., 2013; Celorrio et al., 2017).

THE BASAL GANGLIA IN PARKINSON'S DISEASE AND THE NEUROMODULATORY ROLE OF CANNABIDIOL

The BG are subcortical nuclei that constitute a parallel and partially closed circuit and are brain structures essential for promoting the initiation and execution of voluntary movements (Lanciego et al., 2012; Klaus et al., 2019). The following nuclei make up the motor circuit of the BG: the caudate-putamen (CPu), which is known as the striatum in rodents and is the main entry nucleus of the BG; the internal and external *globus pallidus* (GPi and GPe, respectively); the subthalamic nucleus (STN); and, the *substantia nigra pars reticulata* and *substantia nigra pars compacta* (SNpr and SNpc, respectively). The main exit nuclei of the BG are the GPi and SNpr (Albin et al., 1989; Lanciego et al., 2012). The neural information that enters the circuit mainly arrives from the sensorimotor cortex and then circulates through the BG and the thalamus, finally returning to the cerebral cortex. (Alexander and Crutcher, 1990; Haber and Calzavara, 2009). This loop plays an important role in guiding motor behavior; however, the release of dopamine from SNpc neurons in the entry nucleus of the BG is necessary for the motor circuit to properly function (Gerfen and Surmeier, 2011). Various movement disorders emerge from neurochemical dysfunction in the CPu, with dopamine deficiency and the loss of dopaminergic neurons comprising the main characteristics of PD (**Figure 4**).

Parkinson's disease is a chronic and progressive neurodegenerative condition that manifests in people with characteristic clinical symptoms, such as tremor at rest, bradykinesia, muscle stiffness, and postural instability (Clarke, 2007). Parkinson's disease is the second most common neurodegenerative disease in the world, manifesting in people over 60 years of age (Mhyre et al., 2012), with a global prevalence of 5–35 cases per 100,000 people (Savica et al., 2017). Since the discovery of the decrease in dopamine in the striatum of patients with PD in the 1960s (Hornykiewicz, 1962), the treatment of choice for the disease has been the administration of L-DOPA (Carlsson et al., 1957; Birkmayer and Hornykiewicz, 1961; Bogetofte et al., 2020). However, the chronic administration of L-DOPA in parkinsonian patients shows choreatetotic-type motor complications, called L-DOPA-induced dyskinesia (LID). (Tran et al., 2018). As it has been proposed that this movement disorder, is more disabling than the disease itself, pharmacological proposals for reducing LIDs, such as the use of CBD, could improve quality of life for the parkinsonian patient.

With neurochemical and therapeutic findings showing that dopamine is a key regulatory neurotransmitter in the motor circuit of the BG, the activation of the dopaminergic system is generally associated with increased movement, while its inhibition is associated with hypokinesis (Fernández-Ruiz et al., 2010a). Dopaminergic deficit in the striatum is associated with morphological changes across all BG, a decrease in the number of dendritic spines of the medium spiny neurons (MSNs) in the striatum, and alterations in the neuronal connectivity of the striatopallidal pathway (an indirect pathway) and the *striatum-nigral* pathway (a direct pathway) (Galvan, et al., 2015).

Due to the fact that the CB1 receptor is found at the presynaptic level, its activation promotes neuromodulatory action via retrograde eCB signaling, mainly in the synapses located in those brain structures that regulate the motor process, namely the corticostriatal pathway and the direct and indirect pathways of the BG (Covey et al., 2017). This action is significant for the functioning of the excitatory and glutamatergic neurons which carry neuronal information through the cortex to the CPu, while the neurons that carry information from the CPu to the output nuclei are inhibitory and GABAergic in nature (DeLong, 1990). The neurotransmitter dopamine is closely related to the action performed by cannabinoids (Fernández-Ruiz et al., 2010a; Covey et al., 2017). The CB1 receptor has been considered to be the main receptor involved in controlling the synaptic activity of the dopaminergic neurons of the *nigro-striatal* pathway, although these neurons do not express CB1 as well as other subpopulations of dopaminergic neurons, such as the

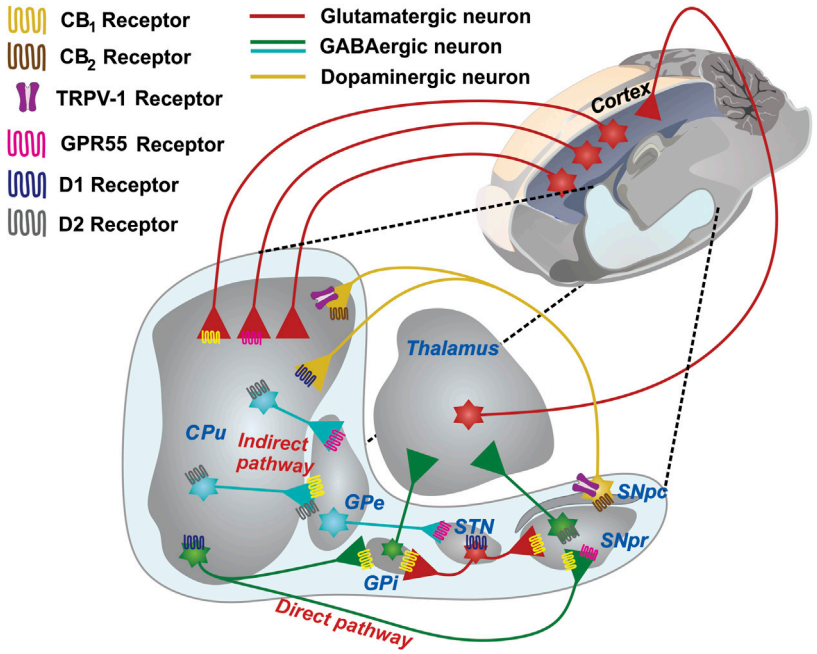

FIGURE 4 | The basal ganglia network and its wide expression of receptors in presynaptic neurons. A schematic representing the GABAergic and glutamatergic connections in a sagittal section of the rat brain is shown. The CPu, the main input nucleus of the circuit, receives cortical projections of glutamatergic neurons. The expression of D1 receptors in the striatum forms the direct pathway of the BG circuit, which is projected toward the GPi and SNpr. The expression of the D2 receptors forms the indirect pathway, which is projected toward the GPe and subsequently toward the STN, which then sends projections to the GPi and SNpr. The projections that emerge from the exit nuclei direct the thalamus and return the processed information to the cerebral cortex. The expression of CB1 receptors mostly occurs in both GABAergic and glutamatergic neurons. GPR55 is present in the GPe, CPu, STN, and SNpc. CB2 and TRPV-1 are the sole in terminals of the SNpc. Abbreviations: CPu, Striatum or caudate-putamen; GPi, Internal *globus pallidus*; SNpr, *Substantia nigra pars reticulata*; SNpc, *Substantia nigra pars compacta*; GPe, External *globus pallidus*; STN, subthalamic nucleus.

mesostriatal pathway and the cortico-limbic system (García et al., 2016). However, the expression of the CB2 receptor has recently been reported in the dopaminergic neurons of the ventral tegmental area, an important neuronal area that modulates reward (Liu et al., 2017). In human brains, the expression of CB2 in the *nigro-striatal* pathway (García et al., 2015) has been demonstrated and has even been shown to be associated with pathological conditions (Cassano et al., 2017). The specific deletion of CB2 from the dopaminergic neurons of DAT-Cnr2 conditional knockout (cKO) mice has shown that CB2 may play an important role in modulating psychomotor behaviors, anxiety, and depression, as well as the rewarding effects of alcohol and cocaine. Furthermore, human genome-wide association studies have shown that the Cnr2 gene is associated with PD and substance abuse disorders (Liu et al., 2017). Therefore, the regulation that the CB2 receptor may exert on dopaminergic neurons and that which the CB1 receptor may exert on the GABAergic neurons of the striatum, GPi, and SNpr could be crucial for neuroprotective and neuromodulatory cannabinoid therapy using CBD (**Figure 4**).

The effects observed when the CB1 receptor is activated or blocked in the BG circuit are caused by its action on other neuronal populations, such as the GABAergic (da Silva et al., 2015), glutamatergic (Ren et al., 2009), or opiodergic populations (Prud'homme et al., 2015), which comprise neurons interconnected with the dopaminergic neurons (Stampanoni Bassi et al., 2017). However, it should be noted that anandamide (AEA), N-arachidonoyl-dopamine (NADA), and the synthetic compound AM404 interact and activate the TRPV1 receptor, which is expressed in the *nigro-striatal* pathway, thus enabling the direct activation of eCB in the dopaminergic system (Mezey et al., 2000; Cristino et al., 2006) (**Figure 4**). The CB1 receptors are capable of forming heterodimers with the D2 receptors in striatal projection neurons, enabling both systems to interact directly in postsynapse (Blume et al., 2013). Therefore, the range of receptors found in the striatum may be involved in the modulation, via eCB and cannabinoids such as CBD, that is proposed in the present study.

A study conducted on intact rat striatal synaptosomes identified various modulatory mechanisms that cannabinoids may execute on the reuptake of dopamine, glutamate, and adenosine (Pandolfo et al., 2011). Specifically, CBD has shown a low capacity for inhibiting dopamine reuptake (IC50 = 16.5 μM), a finding similar to that reported by Poddar and Dewey 1980, who found that, in striatum synaptosomes, high concentrations of CBD were needed to produce an inhibitory effect on dopamine recapture. This finding is important for understanding the modulatory role of CBD in PD, as 90% of dopaminergic neuronal death occurs in PD (Cheng et al., 2010),

meaning that CBD treatment in the latter stages of the disease is likely to be ineffective. Furthermore, CBD had potent inhibitory effects on adenosine reuptake (IC50 = 3.5 μM) (Pandolfo et al., 2011), which may explain its neuromodulatory activity via the expression of the A2A receptors in the BG circuit (Schiffmann et al., 2007). The A2A receptor has been shown to be widely expressed in the striatopallidal pathway, in presynaptic and postsynaptic GABAergic neurons (Rosin et al., 2003; Diao et al., 2017). Moreover, as the function of the A2A receptor is to inhibit the release of GABA, it will promote movement in an animal PD model (Schwarzschild et al., 2006). However, it is likely that interactions with various neurotransmitter receptors can activate, in addition to neuronal modulation, neuronal signaling pathways that promote neuronal survival. To address the probable neuroprotective effect of CBD, it is necessary to identify the proposed pharmacological approaches that harness the medicinal properties of phytocannabinoids as an adjuvant in PD treatment.

CANNABIDIOL AS AN ADJUVANT IN PARKINSON'S DISEASE TREATMENT

Various studies have suggested that both genetic (5–10%) and idiopathic factors may contribute to the neurodegeneration that occurs in *p*D. However, the etiology of this disease, namely the underlying cause of dopaminergic neuronal death, is unknown (Kalinderi et al., 2016; Deng et al., 2018). Several studies have linked the idiopathic factors in PD to both aging and environmental factors (heavy metals, pesticides, head trauma, and viral infections) (Ascherio and Schwarzschild, 2016; Pang et al., 2019). Two processes, oxidative stress and neuroinflammation, are closely related to both the genetic and idiopathic factors observed in PD (Hald and Lotharius, 2005). There is evidence that the dopaminergic neurons of the SNpc are vulnerable to oxidative damage, as they present low levels of antioxidant enzymes, such as glutathione peroxidase, and high levels of pro-oxidants, such as free iron and neuromelanin (González-Hernández et al., 2010). The oxidative characteristics of the SNpc promote increased ROS levels, induce the inhibition of the mitochondrial electron transport chain, increase glutamate levels, stimulate NMDA receptors, and, finally, induce the processes of excitotoxicity and neuronal death (Hernandez-Baltazar et al., 2019). Indeed, one of the aims of neuroprotective therapeutic strategies for PD is to reduce the cytotoxic effects of oxidative stress, namely lipid peroxidation, protein nitration, and DNA oxidation, a point reviewed in the next section.

In addition to the role played by the neuronal population, the participation of glial cells (astrocytes and microglia) is essential for the development of PD (Hernandez-Baltazar et al., 2019; Domingues et al., 2020). Glial cells are associated with neuroinflammation and the neurodegenerative process, with the former characterized by reactive microglia and the presence of astrocytes alongside neurons with dopaminergic injury (Bachiller et al., 2018). Microglia are considered the resident innate immune cells and are, therefore, capable of robust chemotaxis, phagocytosis, and cytokine production and release (Domingues et al., 2020). It should be noted that recent studies have phenotypically categorized microglia cells into M1 (pro-inflammatory) and M2 (anti-inflammatory) states (Tang and Le, 2016) (**Figure 5**). In the M2 state, microglia improve neuronal survival by releasing glial cell line-derived neurotrophic factor (GDNF) (Ding et al., 2004) and are involved in the upregulation of tissue repair and gene regeneration (Le et al., 2016). In contrast, in the M1 state, microglia promote the neurodegeneration of the nigrostriatal pathway in *p*D. Microglia produce and increase ROS levels in a pro-inflammatory state, thus producing IL-1β, IL-6, TNFα, chemokines, $NO^{\bullet}$, and $O_2^{\bullet-}$ (Liu and Hong, 2003; Subramaniam and Federoff, 2017). This release of pro-inflammatory cytokines activates signaling pathways in order to promote microglia recruitment and, thus, dopaminergic cell death (Hernandez-Baltazar et al., 2019).

Astrocytes, the class of glial cells that are most present in the mammalian CNS, are metabolic, supportive of neuronal structure, and release neurotrophic factors. Furthermore, they maintain the integrity of the BBB and modulate neuronal transmission and excitability (Domingues et al., 2020). Following dopaminergic neuronal injury, mature astrocytes proliferate and promote neuronal regeneration via brain-derived neurotrophic factor (BDNF) and cerebral dopamine neurotrophic factor (CDNF) (Hernandez-Baltazar et al., 2019). Astrocytes detect cellular insult signals and trigger an immune response through the secretion of cytokines and chemokines. However, an imbalance in the secretion of pro-inflammatory/anti-inflammatory substances contributes to neuroinflammation and chronic neurodegeneration (Colombo and Farina, 2016; Liddelow et al., 2017). For this reason, one of the novel and opportune treatment strategies for PD is to modulate the neuroinflammation occurring during the progression of the disease.

Interestingly, while both the CB1 and CB2 receptors are expressed in astrocytes, the CB2 receptor is overexpressed under neuroinflammatory conditions in both the microglia and astrocytes (Benito et al., 2005; Cassano et al., 2017). Although the main receptor involved in the modulation of reactive glia is the CB2 receptor, a neuroprotective effect of the CB1 receptor that directly involves the glia cannot be ruled out (Chung et al., 2011). The modulating effects of astrocytic activity with brain injury is mediated by cannabinoids via the CB2 receptor, or the CB1 and CB2 receptors combined (Fernández-Ruiz et al., 2010a; Stella, 2010) (**Figure 5**). These effects promote a trophic role or provide anti-inflammatory mediators that can rescue damaged neurons (IL-10, TGF-β) and promote the reduction of chemokine levels by astrocytes such as fractalkine, an effect that would be predominantly mediated by the activation of CB2 receptors (Smith et al., 2000; Molina-Holgado et al., 2003). Particularly when activated, microglia are affected by the activation of CB2 receptors in the CNS, while the CB2 receptors also play a role in the proliferation and migration of these cells at injury sites (Walter et al., 2003; Carrier et al., 2004). The activation of CB2 receptors in microglia dampens the generation of

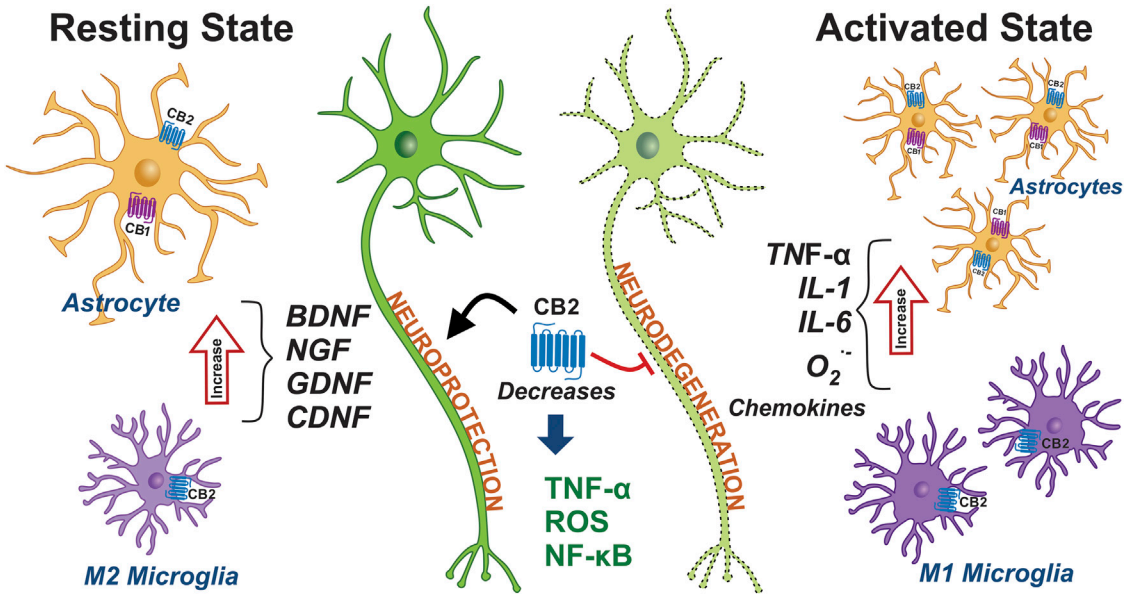

FIGURE 5 | Expression of cannabinoid receptors in glial cells and their role in PD. The phenotypes of microglia and astrocytes are schematically represented under non-inflammatory conditions, namely in a resting state. The microglia and astrocytes are shown in pro-inflammatory conditions and in an active state. The activation of microglia and astrocytes promotes and triggers the neuroinflammation that contributes to *PD*. The activation of the CB1 and CB2 receptors may decrease the inflammation seen in *PD*. Abbreviations: CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; GDNF, glial cell line-derived neurotrophic factor; CDNF, cerebral dopamine neurotrophic factor; ROS, reactive oxygen species; NF-κB, nuclear factor kappa B; IL-1, interleukin-1; IL-6, interleukin-1; $O_2^{\cdot-}$, Superoxide anion; TNF-α, tumor necrosis factor alpha.

neurotoxic factors such as TNF-α (Stella, 2010) and the transcription factor NF-κB, which regulates pro-inflammatory responses (Oh et al., 2010). Therefore, the expression of the CB2 receptor in both reactive microglia and astrocytes suggests that it could be a target for promoting neuroprotection (Fernández-Ruiz et al., 2015). It is likely that CBD is able to regulate the CB1 and CB2 receptors in both the glial cells and the BG circuit, via non-canonical mechanisms.

Currently, orthodox L-DOPA therapy reduces the symptoms of PD; however, there are no therapies that can prevent or rescue neurons from death (Choonara et al., 2009; Schapira et al., 2014). In these circumstances, L-DOPA is no longer metabolized by dopaminergic neurons, due to the degeneration of between 50 and 70% of nigral DA neurons (Carta and Bezard, 2011). Serotonergic neurons possess the enzymatic machinery for synthesizing dopamine via L-DOPA, promoting vesicular storage, and expressing the vesicular monoamine transporter (VMAT). However, serotonergic neurons lack a regulatory mechanism for dopamine release and regulation, a function which is carried out by the presynaptic D2 receptor and, therefore, induces the excessive release of dopamine into the CPu (Arai et al., 1995). As the disease progresses and dopaminergic neuronal death increases, the efficiency of L-DOPA decreases and patients experience the abnormal involuntary movements known as LIDs (Putterman et al., 2007; Francardo et al., 2011). For this reason, it is necessary to develop new non-dopaminergic drugs capable of reducing or attenuating motor symptoms without inducing dyskinesias, with the use of cannabinoids an interesting therapeutic approach to PD, one which has emerged alongside a new class of drugs. Cannabidiol has no psychoactive effects and has shown encouraging results in preclinical and clinical trials conducted on different neurodegenerative diseases. It is also a multi-target drug, as, in addition to acting on the ECS, it can act on the serotonin, adenosine, dopamine, and opioid receptors (Russo et al., 2005; Carrier et al., 2006; Kathmann et al., 2006; Thomas et al., 2007; Pandolfo et al., 2011; Linge et al., 2016; Sonego et al., 2018). As most of the aforementioned CBD-activated receptors are coupled to an inhibitory G protein, they are capable of acting as neuromodulators, given that they regulate the release of other neurotransmitters.

Clinical trials evaluating the effects of cannabinoids on PD show conflicting results. Nabilone (a non-selective CB1 receptor and CB2 receptor agonist) decreases L-DOPA-induced dyskinesias. It has been suggested that the lateral *globus pallidus* (GPl) exbibits hyperactive behavior in the dyskinetic process and that the stimulation of CBRs improves GABAergic transmission by reducing GABA reuptake in the GPl (Sieradzan et al., 2001). The oral administration of a cannabis extract in PD patients was well tolerated but did not produce an anti-parkinsonian effect (Carroll et al., 2004), while the administration of cannabis has been shown to have a beneficial effect on tremor and stiffness, a minor effect on bradykinesia, and a tendency to improve posture, all of which are motor symptoms of *p*D. Cannabis been found to have a positive impact on non-motor symptoms, such as sleep and pain

(Lotan et al., 2014), with the latter finding potentially attributable, in part, to nighttime pain relief and, in part, to the drug's calming and soporific effects. Some studies have found cannabis-induced improvements in sleep quality rather than motor symptoms (Finseth et al., 2015). While a single smoked dose of marijuana has not been found to decrease tremor in PD patients, its sedative or anxiolytic effect benefits some patients when anxiety is a significant trigger (Frankel et al., 1990). The different results obtained by these studies are related to variations in the amount of plant extract administered and the different routes of administration, where, for example, oral administration produces lower plasma concentrations than inhalation.

A double-blind, placebo-controlled, cross-sectional study evaluated the severity and duration of LIDs and the effect on these symptoms of the administration of a cannabis extract (Cannador®), comprising THC/CBD, to eighteen patients, in doses of up to 0.5 mg/kg/day. The results obtained did not show any significant difference, although some patients did report reduced tremor and improved sleep quality compared to those who had received the placebo, as well as an improved dementia score via the Mini-Mental State Examination (MMSE) (Carroll et al., 2004). Some of the first clinical studies in this area were driven by testimonies, such as the anecdotal account of a patient with severe Parkinson's tremor who had been resistant to different types of medication until experiencing dramatic relief from smoking marijuana. This prompted a study to be conducted on five idiopathic PD patients, who were evaluated via the Webster scale and revealed improvements in tremor after the administration of smoked marijuana (1 g ≈ 29 mg of THC), L-DOPA, and apomorphine. These authors found no evidence to show that smoking cannabis reduces tremor or other parkinsonian symptoms (Frankel et al., 1990). Another evaluation of smoked cannabis was undertaken in an open study, in which, of the 22 patients who received ultimately ineffective treatments to relieve pain and tremor for ten months, seven experienced motor fluctuations. Moreover, an improvement in motor symptoms was obtained, with a greater benefit observed in tremor and bradykinesia, while, in addition to improved posture, improvements in non-motor symptoms and good tolerability were also found (Lotan et al., 2014).

Recent studies have highlighted the synergistic effect among the components of cannabis (Samarut et al., 2019), especially those with the highest concentrations, namely THC and CBD (Jin et al., 2020). This effect may generate limitations, as it is difficult to determine the mechanism by which results were obtained, although cannabis also has advantages over other treatments due to its benefits in terms of various therapeutic objectives, mainly achieved via the use of CBD (Morales et al., 2017; Peres et al., 2018). As the improvement of parkinsonian symptoms was observed to be related to the use of cannabis for more than two months (Venderová et al., 2004), the time period in which the treatment is applied is an important factor. More studies are required in order to evaluate the effect of cannabinoids on LIDs, considering the duration, the dosing, and the use of routes of administration that do not cause secondary damage (Varlet et al., 2016; Millar et al., 2018).

CANNABIDIOL AS A DRUG WITH PROBABLE NEUROPROTECTIVE PROPERTIES

The first reports of clinical research on the treatment of PD with CBD were followed by the studies carried out by Snider and Consroe (1985) and Consroe et al. (1986). They showed that, in two patients with dystonia and coexisting Parkinsonian characteristics, oral CBD treatment at doses higher than 300 mg/day exacerbated hypokinesia and tremor at rest, and had a positive effect on dystonic movements (Snider and Consroe, 1985). Consroe showed CBD to be effective in treating LIDs in PD patients, a finding that was perhaps the first to show the beneficial effect of CBD on LIDs (Consroe et al., 1986); however, given that he did not report the beneficial effects of CBD on PD, it is likely that interest in the potential of CBD as a treatment for this condition dwindled. It was not until the 2000s that CBD regained relevance in PD research, as a result of a study conducted on healthy recreational users of both marijuana (plant) and resin (hashish). The study analyzed subjects' hair samples for both THC and CBD levels (as determined by chromatography/mass spectrometry), finding an increase in the levels of the markers of the cerebral metabolism of N- Acetylaspartate (NAA)/Total Creatinine (tCr) in the putamen/*globus pallidus*, as determined by magnetic resonance spectroscopic imaging (MRSI). A positive correlation between NAA/tCr and CBD was also observed in the striatopallidal pathway (Hermann et al., 2007), a finding which may reflect a possible improvement in the neuronal and axonal integrity of the indirect BG pathway due to the effects of CBD. In addition, this finding led to the proposal of CBD as a therapeutic target during the initiation of PD, given that GPe is the nucleus with the highest expression of the CB1 receptor and is the nucleus with the highest level of GABAergic activity, thus promoting hypokinesia.

Studies on the administration of CBD have been undertaken on patients with non-motor PD symptoms (**Table 1**). In 2009, Zuardi carried out a study on six patients presenting both psychosis and the motor symptoms of *p*D. The four-week treatment regime began with a 150 mg dose, which, depending on the clinical response, was increased by 150 mg each week. All evaluations were performed via tests and clinical evaluation scales for anxiety and cognition, with CBD observed to decrease PD psychosis, while no difference was observed in motor processes (Zuardi et al., 2009). The same author conducted two parallel studies evaluating PD motor disorders and REM sleep behavior disorder (RBD). The effect of the CBD treatment on RBD was evaluated in a group of four patients with symptoms characteristic of PD and the sleep disorders caused by the disease. The CBD dose was 75 mg/day and 300 mg/day per patient, with both treatments lasting six weeks. The polysomnograph evaluation conducted revealed that the CBD attenuated RBD (Chagas et al., 2014a).

In order to explore the role of CBD in the motor symptoms of PD, a study was conducted with 21 PD patients who had recorded a score of one to three on the Hoehn and Yahr scale, using a CBD dose of 75 mg/day or 300 mg/day for three weeks. While an increase in general well-being and functioning in daily tasks was

TABLE 1 | Clinical research reports on the effect of CBD on Patients of PD.

Patient characteristics	Symptoms	CBD dosage and temporary treatment	Medical evaluations and study techniques	Main findings	Author (reference)
13 male recreational cannabis users (six users consumed marijuana, three hashish and four marijuana and hashish). Mean age 22 years, approximately	All participants were medication free. They had not brain disorders and other diseases	6 years of using marijuana or hashish	Chromatography/mass spectrometry (GC/MS) to hair analysis of cannabinoids (THC and CBD) ^{1}H magnetic resonance spectroscopic imaging (MRSI) markers of brain metabolism: NAA, cho and tCr	↑ positive correlation of NNA/tCr and CBD in the putamen/*globus pallidus*	Hermann et al. (2007)
Six patients (four men and two women). Mean age 58 years, approximately	Patients had psychosis for 3 months and motor sympotms of PD	150 mg CBD (p.o.), and increasing 150 mg every week depending on the clinical response. Four weeks of treatment	Bech's version of the brief psychiatric rating scale (BPRS) structured interview guide with test–retest reliability of the BPRS Parkinson psychosis questionnaire (PPQ) unified Parkinson's disease rating scale (UPDRS) clinical global impression – Improvement scale (CGI–I) Mini-Mental State Examination (MMSE) frontal assessment battery (FAB)	↓ psychotic symptoms in PD CBD did not worsen the motor function CBD did not induce any decrease in cognitive function	Zuardi et al. (2009)
Four male PD patients with RBD. Mean age 63 years, approximately	Alterations during sleep characterized by swearing, talking, yelling, pushing, kicking, punching and gesturing and motor sympotms of PD	75 mg/day CBD (p.o.) in three patients 300 mg/day CBD (p.o.) in one patient duration of treatment, six weeks	Polysomnography (PSG) periodic limb movement index (PLMI)	↓ frequency of RBD-related events	Chagas et al. (2014a)
21 (15 male and 6 female) PD patients. Mean age 65 years, aproximately	Motor symptoms of idiopathic PD, score between 1 and 3 in the hoehn and yahr scale	75 mg/day or 300 mg/day CBD (p.o.) duration of treatment, six weeks	UPDRS to assess PD symptoms Parkinson'|'s disease questionnaire – 39 (PDQ-39) plasma levels of BDNF (ELISA) proton magnetic resonance scans (MRS)	↑ functioning and well-being of PD patients (NC) motor score evaluated with the UPDRS (NC) BDNF plasma levels (NC) NAA/Cr for MRS	Chagas et al. (2014b)
24 (male and female) idiophatic PD patients	Motor symptoms of idiopathic PD, and anxiety an absence of marked cognitive alterations	300 mg CBD (p.o.) with interval between the first and the second experiment was 15 days (two administration)	UPDRS to assess PD symptoms hoehn and yahr scale schwab and england scale simulated public speaking test (SPST)-VAMS; SPST; SSPS systemic blood pressure and heart rate tapping test accelerometer (tremors measured)	↓ SPST-induced anxiety ↓ tremor amplitude in patients with PD	De Faria et al., 2020

BDNF, brain-derived neurotrophic factor; CBD, cannabidiol; Cho, choline; ELISA, enzyme-linked immunosorbent assay; NAA, N-acetylaspartate; RBD, REM sleep behavior disorder; SPST, simulated public speaking test; SSPS, self-statements during public speaking scale; tCr, total Creatine; THC, tetrahydrocannabinol; VAMS, visual analog mood scales; (↑) increase; (↓) decrease; NC, no changes.

observed, alterations were not observed in the motor score evaluated via the Unified Parkinson's disease Rating Scale (UPDRS) or the plasma levels of BDNF and NAA/Cr, as measured via proton magnetic resonance scans (MRI) (Chagas et al., 2014b). The results for effects of CBD on PD in clinical research are likely not as encouraging, suggesting that increased clinical research efforts are required in this area.

Hampson et al. first reported the neuroprotective effects of CBD in 1998, using the primary cultures of cortical neurons exposed to toxic concentrations of the neurotransmitter glutamate [250 μM] for 10 min, finding that CBD prevented both glutamatergic neurotoxicity, with an EC50 of 2–4 μM, and cell death induced by oxidative stress. In addition, they observed that the antioxidant effect of CBD was more powerful than *a*-tocopherol and ascorbate in equimolar concentrations, finding that neuroprotection was not inhibited by CBR antagonism, thus indicating the independent therapeutic effects of CB1 and CB2 (Hampson et al., 1998). The CBR-independent antioxidant effect of CBD has also been demonstrated in B-lymphobalstoid and fibroblast cell cultures serum-starved for survival (Chen and Buck, 2000). The ability of CBD to attenuate the neurotoxicity induced by two oxidative insults was also observed, finding stress and mitochondrial dysfunction in cultured granular neurons at 2.5 μM concentrations due to the effect of both CBR-independent and 5-HT-1A mechanisms (Echeverry et al., 2020). As the antioxidant effect of CBD was observed to play an important role in neuroprotection, it was proposed as a possible therapeutic agent in the treatment of highly oxidative neurodegenerative disorders, such as PD.

TABLE 2 | Preclinical research reports of effect CBD on various *in vivo* models of PD.

Animal species studied	Lesion model	Lesion time	CBD dosage	Treatment time	Brain nuclei studied	Biochemical markers	Proteins/mRNA evaluated	Evaluated animal behavior	Author (reference)
Sprague–Dawley rat	6-OHDA [8 μg/2 μl] in MFB	2 weeks	CBD (3 mg/kg i.p.)	Two weeks, CBD administration 16 h post 6-OHDA	Striatum SNpc	↑ dopamine (NC) DOPAC. (NC) TH activity	(NC) mRNA SP (ISH) (NC) mRNA PENK (ISH) (NC) mRNA TH (ISH)	Not evaluated	Lastres-Becker et al. (2005)
Sprague–Dawley rat	6-OHDA [8 μg/2 μl] in MFB	2 weeks	CBD (3 mg/kg i.p.)	1 week (1W), CBD administration 7 days' post 6-OHDA 2 weeks (2W), CBD administration 16 h post 6-OHDA	Striatum SNpc	↑ dopamine 2W (NC) dopamine 1W	↑ mRNA SOD Cu/Zn 2W (ISH) (NC) mRNA SOD Cu/Zn 1W (ISH)	Not evaluated	García-Arencibia et al. (2007)
Sprague–Dawley rat	6-OHDA [200 μg/ 5 μl] i.c.v injection	2 weeks	CBD enriched botanical extract (equivalent to 3 mg·kg-1 of pure CBD i.p.)	2 weeks, CBD administration 16 h post 6-OHDA	SNpc	Not evaluated	↑ TH (IHC) ↓ OX-42 (IHC)	Not evaluated	García et al. (2011)
Wistar rat	Reserpine (dosage 1 mg/kg s.c.) 2 days administration	6 days	CBD (0.5 and 5 mg/kg i.p.)	7 days, administration one day before reserpine	Not evaluated	Not evaluated	Not evaluated	(NC) locomotor activity ↓ time bar test catalepsy ↓ vacuous chewing movements plus-maze discriminative avoidance task (attenuate the reserpine-induced memory deficit)	Peres et al. (2016)
C57BL/6Mice	MPTP (dosage 20 mg/kg i.p.)	5 weeks	CBD (5 mg/kg i.p.)	5 weeks. Joint administration with MPTP	Striatum SNpc	Not evaluated	(NC) TH (IHC) (NC) Iba-1 (IHC) (NC) TH (IHC)	(NC) time to descended in pole test ↓ latency to fell in rotarod (CBD in animals control)	Celorrio et al. (2017)
	Haloperidol (dosage 1 mg/kg i.p.)	180 min induced catalepsy	CBD (5 mg/kg and 20 mg/kg i.p.)	60 min, administration 2 h after injection of haloperidol	Not evaluated	Not evaluated	Not evaluated	(NC) time in the bar test catalepsy	
C57BL/6Mice	6-OHDA [5 μg/2 μl] dorsolateral striatum injection	21 and 38 days	CBD (10 mg/kg i.p.)	CBD chronic administration, 14 days; 24 days post 6-OHDA	Not evaluated	Not evaluated	Not evaluated	↑ latency in tail flick test	Crivelaro do Nascimento et al. (2020)
			CBD (10 mg/kg i.p.)	Acute administration, a single injection at 21 days' post 6-OHDA.				↑ latency in tail flick test ↑ latency hot plate test ↑ latency to mechanical stimulus in von frey test ↑ latency to cold stimulus in acetone drop test	
			CBD (30 mg/kg i.p.)					↓ latency to mechanical stimulus in von frey test ↓ latency to cold stimulus in acetone drop test	
			CBD (100 mg/kg i.p.)					↑ latency to mechanical stimulus in von frey test ↑ latency to cold stimulus in acetone drop test	

6-OHDA, 6-hydroxydopamine; CBD, cannabidiol; DOPAC, dihydroxyphenylacetic; i.c.v, Intracerebroventricular; Iba-1, ionized calcium-binding adaptor protein-1; IHC, immunohistochemistry; ISH, in situ *hybridization; MFB, medial forebrain bundle; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; OX-42, oxycocin-42; PENK, proenkephalin; SNpc, substantia nigra pars compacta; SOD Cu/Zn, Copper Zinc - superoxide dismutase; SP, substance p; TH, tyrosine hydroxylase; (↑) increase; (↓) decrease; NC, no changes.*

The animal PD models most frequently used in preclinical research are administered neurotoxic agents such as 6-OHDA (mostly used in rats and mice) and MPTP (mostly used in monkeys and mice) (Duty and Jenner, 2011; Blesa et al., 2012). One of the goals of using an animal PD model is to study the drugs that prevent dopaminergic neuronal death. Attempts have been made to delay, or even arrest, dopaminergic degeneration with different chemicals, such as synthetic antioxidants (Moosmann and Behl, 2002), N-Methyl-D-aspartate receptor antagonists (NMDA) (Alexi et al., 2000; Olivares et al., 2012), Ca^{2+} channel blockers (Rodnitzky, 1999; Kang et al., 2012), and anti-inflammatory substances (McGeer et al., 2001; Martinez and Peplow, 2018). However, these pharmacological strategies, which aim to treat the main predisposing factors for PD, are yet to yield satisfactory neuroprotection results.

The proposal of phytocannabinoids as a potential promoter of dopaminergic neuroprotection in animal PD models began with the work of Lastres-Becker et al. (2005) (**Table 2**), who administered THC and CBD every 24 h for 2 weeks at a dose of 3 mg/kg i.p., one day after injury with 6-OHDA in the medial bundle of the forebrain. They showed that THC and CBD play a neuroprotective role by decreasing dopaminergic neuronal death, although it should be note that CBD increased dopamine concentrations in the striatum. While a modification of the expression of the CB1 and CB2 receptors was not found in the 6-OHDA model, decreased TRPV1 receptor expression, a receptor expressed in the *nigro-striatal* pathway, was observed (Mezey et al., 2000).

Evidence of the neuroprotective effect of CBD may be found in a hemiparkinsonian model 6-OHDA injury. However, neurorestorative effects were not evident when CBD was administered one week after injury with 6-OHDA (García-Arencibia et al., 2007). The joint administration of intraperitoneal CBD and intrastriatal 6-OHDA showed increased levels in the mRNA of the enzyme superoxide dismutase Cu/Zn (SOD Cu/Zn) (García-Arencibia et al., 2007), which suggests that CBD affects the expression of antioxidant enzymes, an effect which may, thus, decrease ROS levels in the striatum (Indo et al., 2015). In addition to the probable antioxidant role of CBD, an anti-inflammatory role was demonstrated when a marijuana extract enriched with CBD was administered to rats injured with 6-OHDA i.c.v. The dose was adjusted to an approximate concentration of 3 mg/kg and administered intraperitoneally. It was found that the decrease observed in the levels of OX-42, a marker of reactive microglia, may have induced an increase in the dopaminergic phenotype, as observed via TH immunohistochemistry conducted (García et al., 2011).

Other models of PD enable the evaluation of the effect of neuroprotective drugs. The neurotoxin reserpine decreases dopamine concentrations, leaving dopaminergic synaptic vesicles without a neurotransmitter, thus producing reversible parkinsonism. In an animal PD model, two 1 mg/kg reserpine doses were administered for six days along whit a CBD 0.5 and 5 mg for seven days, resulting in a decrease in catalepsy behavior, a decrease in vacuous chewing movements, and an attenuation of reserpine-induced memory deficit (Peres et al., 2016). Another study found neither neuroprotective effect of CBD in 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models in mice, nor beneficial effects on motor behavior or haloperidol-induced catalepsy (Celorrio et al., 2017).

Crivelaro do Nascimento et al. (2020) observed the neuroprotective effect of CBD in parkinsonian models in order to evaluate non-motor behaviors. After administrating 6-OHDA in the dorsolateral striatum for 21 days, they induced nosciceptive behaviors via the tail flick, hot plate, von frey, and acetone drop test, cannabidiol caused antinosciceptive effects at intraperioteneally administered doses of 10 and 1,000 mg/kg (Crivelaro do Nascimento et al., 2020). This antinosciceptive effect is likely to be more pronounced on interaction between CBD and the TRPV-1, CB2, and GPR55 receptors due to their analgesic and anti-inflammatory properties (Bisogno et al., 2001; Fernández-Ruiz et al., 2010b).

In vitro PD models enable the determination of the effect of neuroprotective drugs on cell viability, the modulatory role of the drug, and the signaling pathway that they active, namely the molecule of interest in the present study (**Table 3**). The most commonly studied cell culture has been the SH-SY5Y neuroblastoma culture, with one study finding that when the neurotoxic MPP^+ was administered at concentrations of 1 and 7 mM, phenotype and cell viability decreased. However, when CBD was administered at 10 μM, cell viability increased (Gugliandolo et al., 2020), although no differences were observed on the administration of either a 1 and 2.5 μM CBD concentration (Carroll et al., 2012) and in 6-OHDA models (Schönhofen et al., 2015). In the MPP^+ model at concentrations of 100 μM, the protective effect of 1 μM CBD concentrations was notable in PC12 pheocromocytoma cells, while increased cell differentiation and an increase in the NGF markers, synaptophysin, synapsin-1, and the GAP-43 protein, proteins that promote neural proliferation, were also observed (Santos et al., 2015). The mTOR pathway and the MAPK pathway, signaling pathways that promote cell survival and decrease the level of cell death markers such as Caspase-3 and Bax, are the signaling pathways that are activated by CBD (Gugliandolo et al., 2020).

THE ROLE OF CANNABIDIOL IN LEVODOPA-INDUCED DYSKINESIA

The word *dyskinesia* is derived from the Greek word *dis*, meaning difficult or abnormal, and *kinesis*, meaning movement, and is used to indicate abnormal involuntary movements (AIMs) (Rascol et al., 2010). Initially, the administration of L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor of DA, produces significant motor symptom improvements in PD patients, reducing tremor, muscle stiffness, difficulty initiating gait, and bradykinesia (slow movements) (Malek et al., 2019). The beneficial effects of L-DOPA last for approximately five years from the start of treatment, considered a "honeymoon period" between L-DOPA and PD, although they can sometimes last longer. After chronic L-DOPA administration, most patients develop motor fluctuations (*on-off*) or dyskinesias, due to increases and decreases in L-DOPA plasma levels (Cotzias et al., 1969).

TABLE 3 | Research reports of effect CBD on various *in vitro* models of PD.

Cell culture	Cell toxicity induced	Temporary cell toxicity	CBD treatment	Biochemical markers	Proteins evaluated	Author (Reference)
SH-SY5Y	MPP^+ [7mM]	48 h	CBD [0.01, 0.1 y 1 μM] Joint administration with MPP^+	(NC) LDH release	Not evaluated	Carroll et al. (2012)
PC12	MPP^+ [100 μM and 1 mM]	24 h	CBD [1, 5 y 10 μM] Joint administration with MPP^+	↑ Cell viability MTT assay		Santos et al. (2015)
		72 h	CBD [1 μM]	↑ Differentiation cellular	↑ NGF (ELISA) ↑ Synaptophysin (WB) ↑ Synapsin I (WB) ↑ GAP-43 (WB)	
		72, 96, 120, 144, 168 h		↑ Differentiation cellular whitout MPP^+		
SH-SY5Y	Without toxin	Without toxin	CBD [1 μM] 72 h	(NC) Differentiation cellular	Not evaluated	
SH-SY5Y	6-OHDA [6.25 μM]	24 h	CBD [2.5 μM]	(NC) Cell viability MTT assay	Not evaluated	Schönhofen et al. (2015)
SH-SY5Y	MPP^+ [1 mM]	24 h	CBD [10 μM]	↑ Cell viability MTT assay		Gugliandolo et al. (2020)
		48 h			↓ Caspase-3 (WB) ↓ Bax (WB) ↓ PARP-1 (WB) ↑ TH (WB) ↑ pERK ½/ERK2 (WB) ↑ pAKT/AKT (WB) ↑ pmTOR/mTOR (WB) (NC) Beclin-1 (WB) ↓ LC3-II/LC3-I (WB)	

6-OHDA, 6-hydroxydopamine; Bax, BCL2 associated X; CBD, cannabidiol; ELISA, enzyme-linked immunosorbent assay; GAP-43, growth associated protein 43; LC3, microtubule-associated protein 1A/1B-light chain 3; LDH, lactate dehydrogenase; MPP^+, 1-methyl-4-phenylpyridinium; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; NGF, nerve growth factor; pAKT, protein kinase B phosphorylated; PARP-1, poly (ADP-ribose) polymerase-1; pERK, extracellular-signal-regulated kinase phosphorylated; pmTOR, mammalian target of rapamycin phosphorylated; TH, tyrosine hydroxylase; WB, western blot; (↑) increase; (↓) decrease; NC no changes.

Normally starting on the side contralateral to the hemisphere most affected by PD and occurring first in the lower extremities, LIDs are either choreiform or dystonic (Mones et al., 1971; Thanvi et al., 2007; Calabresi and Standaert, 2019). Factors such as the age of PD onset and its severity, gender, and the L-DOPA dose administered are factors related to the onset and intensity of LIDs (Eusebi et al., 2018; Zhou et al., 2019), which commonly affects the extremities, head, neck, and trunk, and are characterized by rapid and irregular movements (Luquin et al., 1992; Thanvi et al., 2007; Rascol et al., 2010).

L-DOPA-induced dyskinesias are classified in terms of the plasma levels of the drug administered and the appearance of certain symptoms post-administration. The symptoms that appear when L-DOPA concentrations are at their highest circulation, known as peak-dose dyskinesias, are characterized by stereotyped, ballistic, or choreiform movements (Luquin et al., 1992; Rascol et al., 2010) are usually the most common, and have the greatest impact on quality of life (Luquin et al., 1992; Pahwa et al., 2019). The dyskinesias that begin to occur when L-DOPA reaches its half-life have been termed diphasic and mainly cause rapidly alternating stereotyped movements in the legs (Luquin et al., 1992), as well as ballistic kicking or dystonia (Rascol et al., 2010; Pandey and Srivanitchapoom, 2017). Given that it occurs between the *on* and *off* phases, this period presents mixed dyskinesias, which occur at a low incidence and are difficult to treat (Luquin et al., 1992; Thanvi et al., 2007; Vijayakumar and Jankovic, 2016). When L-DOPA falls to a low level of circulation, the movements caused are known as *off* dyskinesias, in which patients usually suffer from a dystonic posture, especially in the morning (Marconi et al., 1994), and problematic lower limb sensations (Thanvi et al., 2007; Rascol et al., 2010).

Few studies have obtained evidence of the antidyskinetic activity of CBD, whit its pleiotropic effects (Devinsky et al., 2014) depending on the concentration administered (Jones et al., 2010). As determining the molecular mechanism that is involved in LIDs (see below) is a complex process, the preclinical and clinical evidence depends on the experimental design used to evaluate CBD as a potential treatment for reducing both PD symptoms and the adverse effects of L-DOPA administration. A study was conducted on tardive dyskinesias, which are related to the *nigro-striatal* pathway, which, itself, is affected by the administration of reserpine (1 mg/kg), a drug which has been shown to reduce glutamate consumption (Burger et al., 2005). Said research obtained favorable results in behavioral evaluations via the administration of CBD (0.5 and 5 mg/kg), finding improved memory and reductions in oral dyskinesia and the cataleptic effect (Peres et al., 2016), which describes the subject's inability to correct an imposed posture (Pertwee, 1972), without modifying locomotor activity and anxiety in the model (Peres et al., 2016).

As previously mentioned, given that L-DOPA must be metabolized in the serotonergic neurons, the participation of these neurons in LIDs is an important factor to consider. It has been shown that the pharmacological silencing of serotonergic neurons can be accomplished via the agonists of serotonergic auto-receptors. Therefore, several studies show a decrease in LIDs as induced by selective agonists of the 5-HT1 receptors in animal models of PD (Bibbiani et al., 2001; Muñoz et al., 2008). As CBD has been shown to interact with the 5-HT1A receptor, this phytocannabinoid is able to modulate serotonergic neurotransmission (Magen et al., 2010; Zanelati et al., 2010; Espejo-Porras et al., 2013). *In vitro* studies have demonstrated that, at concentrations higher than 10 mM, CBD is able to activate 5-HT1A; however, at concentrations of 100 nM, it is

also able to improve the agonist capacity of the 5-HT1A receptor in rat brainstem membranes (Rock et al., 2012). Given this effect of CBD on serotonergic receptors, it has been proposed as an anxiolytic (Gomes et al., 2011) and antidepressant agent (Zanelati et al., 2010). Moreover, it has been shown, at high doses (>10 mg/kg), to modify motor behavior, wherein 20 mg/kg doses administered during motor behavior were not antagonized by rimonabant, a CB1 antagonist, but were antagonized by a selective 5-HT1A receptor antagonist (WAY100,635; 0.5 mg/kg) (Espejo-Porras et al., 2013). These data demonstrate that CBD has significant effects on both motor behavior and the 5-HT1A receptor. While it is likely that CBD exerts effects on serotonergic receptors during LIDs, the ability of CBD to activate various mechanisms that exert a synergistic effect on PD and LIDs should not be excluded.

The antidyskinetic effect of CB1 activation, by means of AEA or WIN55212–2, is exhibited only when co-administered with a TRPV1 receptor antagonist (Morgese et al., 2007; Martinez et al., 2012; Martinez et al., 2015; Dos Santos-Pereira et al., 2016), namely capsazepine (CPZ) or N-arachidonoyl serotonin (AA-5-HT) (Dos Santos-Pereira et al., 2016). In addition, AA-5-HT inhibits the FAAH (Gobira et al., 2017) mechanism that increases concentrations of AEA, an ECB with a greater affinity with the CB1 receptor (Lam et al., 2005). However, the use of the TRPV1 antagonist (CPZ) is necessary in order to demonstrate the antidyskinetic effects of the inhibition of FAAH. Promising results were obtained in research conducted on the 6-OHDA model in mice with a dorsolateral striatum lesion. After L-DOPA treatment for 21 days (50 mg/kg/day, ip), CBD and capsazepine (CPZ, 1 and 5 mg/kg, ip, respectively), an antagonist of TRPV1, were administered during the last days of an AIMs evaluation, showing a decrease in AIMs with the administration of CBD + CPZ (Dos Santos-Pereira et al., 2016). These results were similar to those obtained with the use of N-araquidonoil serotonin (AA-5-HT), an eCB antagonist of FAAH and TRPV1 (Gobira et al., 2017). While these effects were previously evaluated using synthetic cannabinoids, with similar results obtained, it should be noted that the administration of WIN55212–2 (0.5 and 1 mg/kg ip) was shown to have antidyskinetic effects. Although the study's authors used WIN55212–2 as a selective agonist for CB1 (Martinez et al., 2012), it is known to have both a higher affinity for CB2 and the ability to inhibit TRPV1 (Morgese et al., 2007).

The mechanism by which AIMs are reduced by CBD + CPZ suggests that improving endocannabinoid tone and blocking TRPV1 receptors exerts a compensatory effect on aberrant endocannabinoid transmission, which occurs in the striatum of dyskinetic rats in 6-OHDA models after L-DOPA treatment (Wang et al., 2018). The increase in AEA, which preferentially activates CB1 over TRPV1 (McPartland et al., 2007; Lam et al., 2005), is the result of the inhibition of FAAH by CBD, which can also activate TRPV1 (Peres et al., 2018) (**Figure 6**). While stimulating CB1 alone reduces the hyperactivity of the cAMP/PKA pathway, which has been implicated in the development of dyskinesias (Aubert et al., 2005; Martinez et al., 2012), the coactivation of CB1 and TRPV1 generates the opposite effect (Lam et al., 2005), with their participation in the induction of LTP also observed (Cui et al., 2018). This explains why CBD or FAAH antagonist (URB597) treatments do not reduce AIMs; however, when they are co-administered with CPZ, they exert an antidyskinetic effect on all types of AIMs (Morgese et al., 2007; Martinez et al., 2015; Dos-Santos-Pereira et al., 2016).

An examination of the above-described mechanisms reveals evidence concurring with the results obtained via the subchronic administration of CBD + CPZ, which reduces the severity of LIDs and reduces the levels of biochemical markers such as pERK, pAcH3, NF-κβ, and COX-2 (Dos-Santos-Pereira et al., 2016), which increase with chronic L-DOPA treatment (Santini et al., 2007; Bastide et al., 2014). Both pERK and pAcH3 are generated by D1-receptor sensitization (Park et al., 2014), which maintains

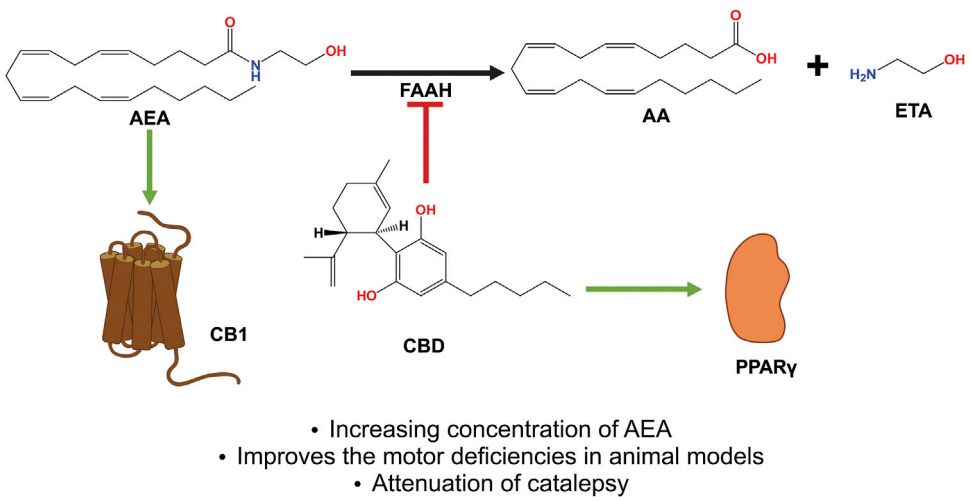

FIGURE 6 | CBD acts as an indirect agonist of the CB1 receptor by inhibiting the enzyme that degrades AEA and FAAH, thus increasing the concentration of said eCB, which has a greater affinity to activating CB1, thus exerting neuroprotective and antiparkinsonian effects. In addition, CBD activates the PPARγ receptors, which have been shown to be involved in neuroinflammatory processes. Abbreviations: CBD, cannabidiol; CB1, cannabinoid receptor type 1; AEA, anandamide; FAAH, fatty acid amide hydrolase; eCB, endocannabinoids; PPARγ, peroxisome proliferator activated receptor γ.

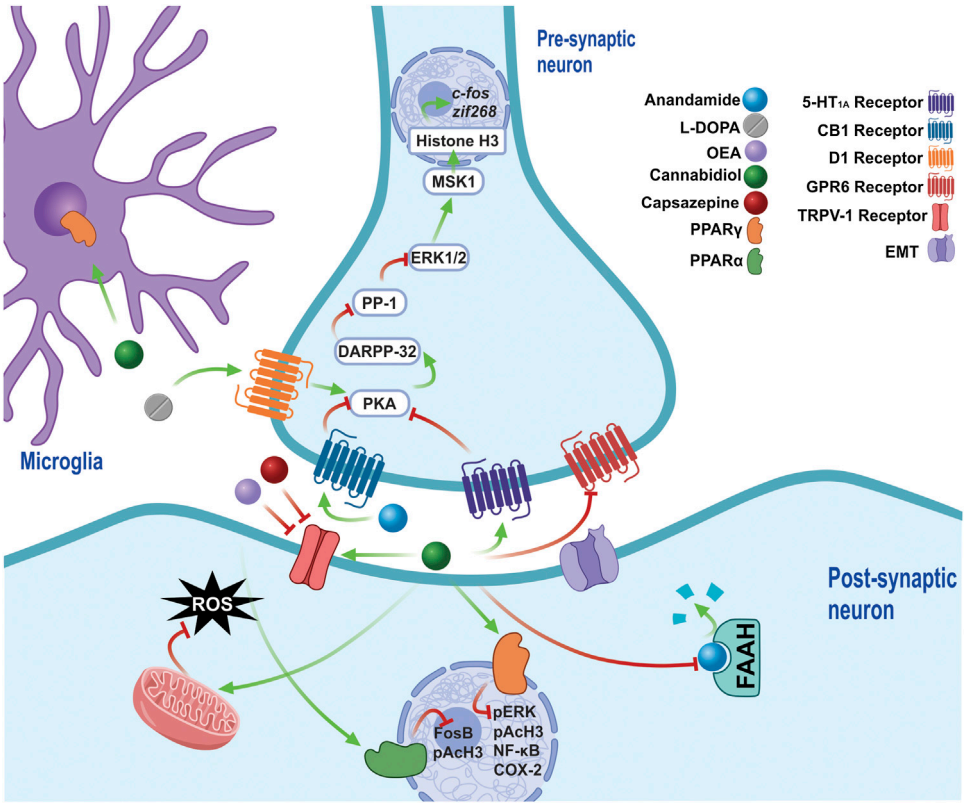

FIGURE 7 | The chronic administration of L-DOPA leads to a sensitization of D1 receptors, which maintain the over-activation of PKA in LIDs. PKA regulates the pathway that activates DARPP-32, which inhibits the modification by PP-1, of ERK1/2 signaling, which acts on nuclear targets, such as MSK1, and, along with histone H3, regulates the expression of early genes such as c-fos and zif268. CBD exerts antidyskinetic effects by increasing AEA concentration by inhibiting of FAAH, thus stimulating the CB1 receptors, which decrease PKA activity. The CB1 requiere the co-administration of a TRPV1 inhibitor (CPZ), because they stimulate TRPV1 via AEA and CBD, both of which generate opposite effects to the activation of CB1. Furthermore, increased OEA is generated via the inhibition of FAAH, an endocannabinoid able to block TRPV1 and stimulate PPARσ receptors, reducing biochemical markers such as FosB and pAcH3. In addition, CBD activate the 5-HT1A receptor, a receptor that had previously only been implicated in the anticataleptic effect of CBD. By activating PPARγ receptors, CBD reduces the levels of molecular markers involved in LIDs, such as pERK, pAcH3, NF-Kβ and COX-2, while it also generates an anti-inflammatory effect by stimulating said receptors, which are present in the glia. Furthermore, CBD is able to reduce oxidative damage, decreasing the production of ROS by increasing the activity of mitochondrial complexes. The inverse agonism that CBD exerts on GPR6 could form part of its antidyskinetic mechanisms. Abbreviations: L-DOPA, L-3,4-Dihydroxyphenylalanine; D1, Dopamine receptor 1; PKA, cAMP-dependent protein kinase; LID, L-DOPA-induced dyskinesias; DARPP-32–32 KDa Phosphoprotein regulated by cAMP and dopamine; PP-1, phosphoprotein 1; ERK, extracellular signal-regulated kinase; MSK-1, mitogen and stress regulated protein kinase; CBD, cannabidiol; AEA, anandamide; FAAH, fatty acid amide hydrolase; TRPV1, transient potential receptor V1; CPZ, cpazazepine; OEA, oleoylethanolamide; PPARα, peroxisome proliferator activated receptor α; pAcH3, Histone 3 phosphoacetylation; 5HT1A, Serotonin receptor 1A; PPARγ, peroxisome proliferator activated receptor γ; NF-Kβ, nuclear factor Kβ; COX-2, cyclooxygenase 2.

an overactivated signaling pathway and which, in turn, results in an increase in the level of these markers (Aubert et al., 2005). NF-κβ and COX-2 are characteristic of a neuroinflammatory process, with their levels increasing due to the depletion of DA and the neurotoxicity caused by L-DOPA (Bortolanza et al., 2015; Pisanu et al., 2018), while a reduction in their levels causes the activation of PPARγ (Randy and Guoying, 2007) and occurs due to CBD's own anti-inflammatory effects (Peres et al., 2018).

It has been shown that the administration of the eCB oleoylethanolamide (OEA) generates antidyskinetic effects (González-Aparicio and Moratalla, 2014), results which show that its mechanisms block TRPV1 and stimulate PPARσ (Almási et al., 2008; Thabuis et al., 2008). Moreover, a decrease in the levels of markers such as FosB and pAcH3 has also been observed (González-Aparicio and Moratalla, 2014). In addition, an increase in the concentration of OEA has been reported with the administration of URB597 in an EP model injured with MPTP (Celorrio et al., 2016), as it shares the same degradation pathway as AEA (**Figure 7**) (Thabuis et al., 2008), with the same study also observing a motor deficit improvement (Celorrio et al., 2016).

Other molecular targets of CBD, such as the GPR6 orphan receptor, which is mainly expressed in the *striato-pallidal* neurons of the striatum, have been studied in recent years (Lobo et al., 2007). One study found an AIMs reduction in knockout mice after GPR6 ablation, as well as a cAMP reduction and an increase in both DA levels and the phosphorylation of DARPP-32 in the striatum. This suggests that blocking GPR6 exerts an antidyskinetic effect (Oeckl et al., 2014), with research showing that CBD acts as an inverse agonist on GPR6 (Laun and Song, 2017), an effect forming part of its therapeutic mechanisms.

Clinical evaluations of CBD have found improvements in terms of certain symptoms, depending on the doses administered (Jones et al., 2010). In an open preliminary pilot study, which evaluated dystonia under weekly CBD dose escalations (100 mg/week), one of the patients, who was received 1,000 mg/day L-DOPA doses, presented a 50% improvement (Consroe et al., 1986). Given that it is a type of LID (Marconi et al., 1994), the use of CBD, at appropriate doses, is suggested to reduce the severity of dystonia. An increase in hypokinesia has been reported with the administration of high CBD doses (300–400 mg/day); however, there is currently no complete understanding of its pharmacokinetics, although significant improvements in symptoms have been observed in a dosage range of 1–50 mg/kg/day in different pathologies (Millar et al., 2019). Furthermore, no changes were found when quantifying brain-derived neurotrophic factor (BDNF), the level of which decreases in PD, while a greater susceptibility to LID was found in patients with a polymorphism in the gene encoding BNDF (Howells et al., 2000; Foltynie et al., 2009). Both the increase in BDNF (Sales et al., 2019) and the improvement in LID symptoms that can be caused by CBD require further study in order to be applied in clinical practice. Moreover, it must also be established whether results improve with the use of other phytocannabinoids that exert a synergistic effect (Samarut et al., 2019).

CONCLUSION

The bibliographic evidence shown in the present review suggests the clinical utility of CBD for treating both LIDs and the motor symptoms of PD, as well as the neuromodulatory, neuroprotective and antidyskinetic effects of CBD in animal models and *p*D. Furthermore, the evidence shown on the pharmacological mechanisms and molecular interactions of CBD with various receptors may explain the wide range of therapeutic utility in various neurological disorders.

Despite the promising results for CBD pharmacology, unknowns remain about dosages and mechanisms of action. However, the essential role of CBD as an antioxidant and anti-inflammatory is affirmed, as these processes are important in the pathogenesis of *p*D. The neuromodulatory mechanism of CBD in the BG circuit remains to be studied in greater depth, in order to establish this phytocannabinoid's physiological role and function as a coadjuvant in PD.

AUTHOR CONTRIBUTIONS

FP, AM-A, AP-M, and IL performed the bibliography searches and participated in the manuscript writing, all authors reviewed and approved the final version of the manuscript. FP and AM-A made all the figures in this review.

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frontiers in Pharmacology

REVIEW
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Cannabidiol for the Treatment of Neonatal Hypoxic-Ischemic Brain Injury

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Each year, more than two million babies die or evolve to permanent invalidating sequelae worldwide because of Hypoxic-Ischemic Brain Injury (HIBI). There is no current treatment for that condition except for therapeutic hypothermia, which benefits only a select group of newborns. Preclinical studies offer solid evidence of the neuroprotective effects of Cannabidiol (CBD) when administered after diffuse or focal HI insults to newborn pigs and rodents. Such effects are observable in the short and long term as demonstrated by functional, neuroimaging, histologic and biochemical studies, and are related to the modulation of excitotoxicity, inflammation and oxidative stress—the major components of HIBI pathophysiology. CBD protects neuronal and glial cells, with a remarkable effect on preserving normal myelinogenesis. From a translational point of view CBD is a valuable tool for HIBI management since it is safe and effective. It is administered by the parenteral route *a posteriori* with a broad therapeutic time window. Those findings consolidate CBD as a promising treatment for neonatal HIBI, which is to be demonstrated in clinical trials currently in progress.

Keywords: cannabidiol, hypoxia-ischemia, neuroprotection, brain, newborn

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HYPOXIC-ISCHEMIC BRAIN INJURY IN NEWBORNS

Diffuse or focal acute hypoxic-ischemic brain injury (HIBI) is a prevalent condition affecting 1 to 9 out of 1000 live newborns (Martínez-Orgado, 2014). As far as focal HIBI is concerned, incidence in the neonatal period is in fact as high as in adulthood (Kratzer et al., 2014). In global terms nearly 2 million babies die or remain with long-lasting detrimental consequences, including motor and cognitive deficits each year (Martínez-Orgado, 2014; Parikh and Juul, 2018). Thus, neonatal HIBI is the main known cause of Cerebral Palsy, a devastating non-progressive degenerative disorder that compromises the lives of children and families and represents a tremendous socio-economic burden on society (Nelson, 2008). Those figures have not changed substantially in the last few years because clinical management of neonatal HIBI is challenging. On the one hand, the complex pathophysiology of HIBI implies that only therapies encompassing multiple mechanisms can be really effective (Juul and Ferriero, 2014; Linsell et al., 2016; Parikh and Juul, 2018). On the other hand, early treatment is hardly achievable. In the case of diffuse HIBI although the neurologic condition derived from this situation, known as neonatal hypoxic-ischemic encephalopathy (NHIE) has a well-characterized clinical picture, determining the exact moment when the HI insult took place before delivery is very difficult (Gonzalez and Ferriero, 2008). In the case of focal HIBI, Perinatal Arterial Ischemic Stroke (PAIS), the clinical picture is so subtle that less than 25% of cases are diagnosed immediately (Armstrong-Wells and Ferriero, 2014). Therefore, neuroprotective therapies aiming to reduce HIBI

should be effective when administered *a posteriori* to the HI event and show a broad therapeutic time window (TTW) (Gonzalez and Ferriero, 2008). Finally, treatments to be administered to newborns must not only be free from serious side effects in the short-term but free from detrimental effects on development as well (Juul and Ferriero, 2014; Parikh and Juul, 2018).

Pathophysiology of Hypoxic-Ischemic Brain Injury in Newborns

Immature brain is particularly susceptible to HIBI mainly because of high metabolic rate, immature cerebral blood flow autoregulation mechanisms, paucity of anti-oxidant defenses, high density and sensitivity of receptors to excitatory amino acids such as glutamate and high sensitivity to inflammation (Martínez-Orgado et al., 2007; Johnston et al., 2011; Rainaldi and Perlman, 2016). Energy failure during HI causes dysfunction of ionic pumps leading to changes in membrane potential, deregulation of ion homeostasis and glutamate excitotoxicity, which triggers increased intracellular calcium levels that activates different enzymes involved in neuronal cell death including caspases, lipases, endonucleases and nitric oxide production (Martínez-Orgado et al., 2007; Johnston et al., 2011; Rainaldi and Perlman, 2016). During post-ischemic reperfusion, inflammation and exacerbated oxidative stress increase and spread neuron and glial cell damage (Martínez-Orgado et al., 2007; Johnston et al., 2011; Rainaldi and Perlman, 2016). In the immature brain, the initial HI insult is followed by a latent period lasting 6–24 h, during which excitotoxicity, inflammation and oxidative stress act to induce the development of cell events which lead to a secondary deterioration because of delayed energy failure (Gonzalez and Ferriero, 2008; Johnston et al., 2011; Parikh and Juul, 2018). This latent phase offers a window of opportunity for treatment with neuroprotectants. Although apoptotic and necrotic cell death are a continuum in immature brain after HI, apoptosis plays a particularly relevant role in neonatal HIBI pathophysiology (Rocha-Ferreira and Hristova, 2016). In addition, immature brain is characterized by a greater impact of autophagy-related cell death after HI insults than adult brain (Descloux et al., 2015).

Glial cells play a key role in HIBI pathophysiology. Immature oligodendroglial cells are extremely vulnerable to inflammatory, excitotoxicity and oxidative damage (Back et al., 2002), which results in extensive myelination disturbance leading to long-lasting motor, sensorial and cognitive disabilities (Nelson, 2008; Volpe, 2010). Astrocytes are essential to support the neurons that survive acute HI damage, attenuate oxidative stress and glutamate excitotoxicity, release neurotrophic factors and preserve the integrity of the blood-brain barrier thereby limiting brain invasion by inflammatory cells during reperfusion (Takuma et al., 2004; Barreto et al., 2011). Increased population of astrocytes, known as astrogliosis, is a well-known long-term marker of HIBI due to the formation of a glial scar in areas of infarct (Colangelo et al., 2014). However, shortly after the HI insult activation and reduction of astrocyte population corresponds to the severity of HIBI since such a response is related to excitotoxic damage of astrocytes which implies blunting of its homeostatic role (Takuma et al., 2004; Barreto et al., 2011; Mallard et al., 2014). HI insults lead to a greater increase of microglia and more robust expression of pro-inflammatory cytokines in activated microglia -the M1 phenotype-in immature than in mature brain (Ferrazzano et al., 2013), although the protective effects of the anti-inflammatory M2 microglial phenotype is also particularly relevant in immature brain (Mallard et al., 2014). The increasing importance paid to the interrelated, complex and time-dependent roles that astrocytes and microglial cells play in neonatal HIBI makes glial cell protection from HI injury a critical component of neuroprotective strategies (Mallard et al., 2014).

Treatment

Therapeutic hypothermia (TH) became the standard of care to treat newborns with NHIE after revealing an improved outcome, which reduces death and/or severe disability in the long term (Natarajan et al., 2016; Parikh and Juul, 2018). The total tissue water (TTW) for TH is 6 h, although TH initiated between 6 h and 24 h after birth may have some beneficial effects (Laptook et al., 2017). However, TH efficacy has been revealed mostly in mild NHIE, in babies over 35 weeks of gestational age at birth and when provided in selected centers in developed countries (Natarajan et al., 2016; Parikh and Juul, 2018). In addition to those limitations, an unacceptable 40%–50% of NHIE patients eligible to receive TH still show no benefits from the treatment (Parikh and Juul, 2018). Thus, synergistic or complementary therapies to hypothermia are warranted. Different strategies have been tested in the experimental setting such as melatonin, erythropoietin, xenon or stem cells (Parikh and Juul, 2018) but they have not demonstrated clinical benefits so far.

There is no specific treatment for PAIS (Armstrong-Wells and Ferriero, 2014). In preclinical studies melatonin or erythropoietin are effective on reducing brain damage, whereas hypothermia offers just mild and contradictory results (Harbert et al., 2011; Villapol et al., 2011; Gonzalez et al., 2013).

CANNABIDIOL FOR NEUROPROTECTION IN NEONATAL HYPOXIC-ISCHEMIC BRAIN INJURY

CBD has a complex poly-pharmacologic profile and regulates the activity of different signaling proteins and receptors acting on various molecular targets (Izzo et al., 2009; Campos et al., 2017). In different experimental paradigms CBD shows robust anti-oxidant and anti-inflammatory activity, inhibits calcium flux across membranes, inhibits endocannabinoid uptake and enzymatic hydrolysis, reduces glutamate release, stabilizes the mitochondrial membrane, regulates different receptor types including serotonin $5HT_{1A}$ and PPARγ receptors, augments the extracellular concentration of adenosine and prevents NF-κB activation (Pertwee, 2004; Mechoulam et al., 2007). All those effects (**Figure 1**) account for a potential neuroprotective efficacy and prompted the study of CBD as a neuroprotectant for neonatal HIBI.

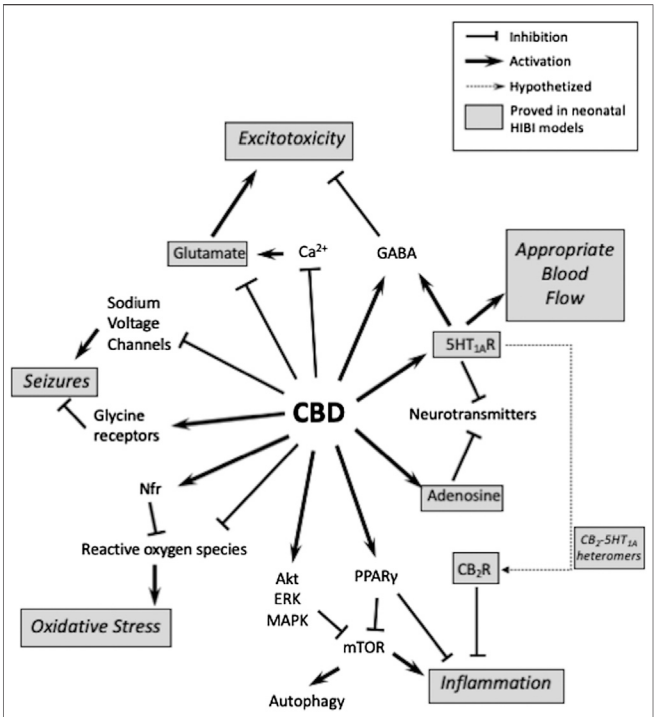

Figure 1 | Schematic representation of different molecular targets of cannabidiol (CBD) and their involvement on the mechanisms of hypoxic-ischemic brain injury (HIBI). Dot-lined boxes indicate which ones have been studied in preclinical models of neonatal HIBI.

Europrotective Efficacy of Cannabidiol in Experimental Models of Hypoxic-Ischemic Brain Injury

Incubation of newborn mice forebrain slices *in vitro* exposed to oxygen-glucose deprivation (OGD) with CBD 100 μM reduces LDH release and caspase 9 expression, which indicates reduced necrotic and apoptotic cell death (Castillo et al., 2010).

CBD has been tested *in vivo* in different experimental paradigms of diffuse neonatal HIBI. In the model most widely used for that purpose, known as the Rice-Vannucci model and consisting of unilateral carotid artery clamp plus exposure to 8%–10% O_2 in seven-to-ten-day-old (P7-P10) rodents, post-insult administration of CBD 1 mg/kg i.p. reduces the volume of damage in rats (Pazos et al., 2012) and mice (Mohammed et al., 2017). Those neuroprotective effects are sustained in the long term and associated with remarkable neurofunctional benefits; with HI rats treated with CBD after the HI insult showing normal motor and cognitive performance when they become adults (Pazos et al., 2012). In newborn piglets studied for 6 h–72 h after being exposed to moderate HI insult by temporary bilateral carotid artery occlusion and exposure to 10% O_2, post-insult administration of CBD 0.1–1 mg/kg i.v. restores brain electrical activity and reduces seizure burden as assessed by continuous EEG monitoring. This also preserves regional cerebral blood flow as assessed by near-infrared spectroscopy (NIRS), prevents the increase of lactate/N-acylaspartate (Lac/NAA) ratio thought to be the most predictive early biomarker of a poor outcome to infant HI and a surrogate endpoint used to assess neuroprotective strategies (Pazos et al., 2013); as assessed by proton magnetic resonance spectroscopy (H + -MRS) and restoring motor and behavioral performance (Alvarez et al., 2008; Lafuente et al., 2011; Pazos et al., 2013; Arruza et al., 2017). However, when piglets were exposed to very severe HI insults CBD administration does not lend neuroprotection (Garberg et al., 2016; Barata et al., 2019). In this case TH administered to the piglets was also ineffective (Garberg et al., 2016; Barata et al., 2019).

Noteworthy, CBD neuroprotection remains unaffected in spite of delaying administration of CBD up to 18 h after the HI insult in newborn mice, a TTW broader than that of TH and other neuroprotective substances (Mohammed et al., 2017).

Regarding focal neonatal HIBI, CBD efficacy was studied using a model of Middle Cerebral Artery Occlusion (MCAO) adapted to newborn rats. In this model of PAIS post-insult administration of CBD 3 mg/kg reduces the volume of infarct and restores motor and cognitive performance in the long term (Ceprián et al., 2017).

Effects of Cannabidiol on Neuron and Glia Damage

Since glioprotection is now considered as important as neuroprotection to reduce HIBI (Mallard et al., 2014; Parikh and Juul, 2018) it is important to remark that CBD has demonstrated protection of both neuronal and glial cells in models of HIBI.

Post-HI administration of CBD to piglets reduces neuronal death, as reflected by the prevention of HI-induced increase on cerebrospinal fluid concentration of neurospecific enolase (Lafuente et al., 2011) in association with the prevention of necrotic and apoptotic neuronal death, as observed in parietotemporal cortex 6 h and 72 h after the end of the HI insult by Nissl and TUNEL staining and caspase 3 concentration measurement by Western blot analysis (Alvarez et al., 2008; Lafuente et al., 2011; Lafuente et al., 2016; Pazos et al., 2013; Arruza et al., 2017; Barata et al., 2019). In newborn rodents CBD reduction of diffuse HI-induced neuronal death in the cortex is significant but less dramatic, although the effect is still observable when animals become adults (Pazos et al., 2012) and includes the modulation of apoptotic death (Mohammed et al., 2017). In the PAIS model in newborn rats CBD fully prevents stroke-induced reduction of the neuronal population and increased density of TUNEL+ neurons in the cortex (Ceprián et al., 2017).

Treatment of HI piglets with CBD results in the prevention of HI-induced astrocyte activation and population reduction as assessed by immunohistochemistry GFAP labelling in the cortex as well as cerebrospinal fluid S100ß protein concentration measurement (Lafuente et al., 2011; Pazos et al., 2013). In addition, CBD administration to HI newborn rats reduces the severity of long-term astrogliosis (Mohammed et al., 2017). In focal HIBI, in addition to the reduction of long-term astrogliosis early post-stroke protection of astrocyte integrity by CBD is demonstrated by the prevention of ischemic-induced reduction of myoinositol/creatine ratio, a marker of

cytolytic astrocyte dysfunction, as assessed by H^+-MRS (Ceprián et al., 2017).

In HI piglets, 6 h after diffuse HI insult CBD does not reduce the number of microglial cells in the cortex but modifies the proportion of M1 and M2 phenotypes, which reduces the presence of the pro-inflammatory M1 phenotype (Barata et al., 2019). Since microglial activation leads to increased microglial proliferation, the fact that in newborn rats exposed to diffuse HI insult CBD prevents HI-induced proliferation of microglial cells in the cortex as assessed seven days post-insult is not surprising (Mohammed et al., 2017). After focal HIBI in newborn rats, CBD reduction of microglial activation and subsequent proliferation is even more remarkable 30 days post-stroke, which indicates that modulation of microglial activation by CBD is sustained in the long term (Ceprián et al., 2017).

CBD administration to newborn rats after diffuse HIBI preserves normal myelination. Thus, in HI rats studied 30 days after the insult CBD prevents HI-induced reduction in the number of mature oligodendrocytes and myelin basic protein signal as assessed by immunohistochemistry in the cortex and White Matter, as well as HI-induced reduction of axonal density and myelin sheath thickness as assessed by Electronic Microscopy in the same areas (Ceprián et al., 2019). This reasonably accounts for the fact that CBD treatment is more effective in restoring neurobehavioral function than in reducing the volume of brain damage (Pazos et al., 2012). Post-insult administration of a treatment implies that a substantial amount of brain tissue is irreversibly damaged by HI at the time the treatment is administered. However, preserving normal myelination in the perilesional surviving tissue is the basis for the brain to develop compensatory mechanisms to eventually attain normal function. There are no reports of the effects of CBD treatment on myelination after stroke in newborn animals.

Mechanisms of Action of Cannabidiol

CBD acts on the main factors leading to cell death in HIBI: excitotoxicity, oxidative stress and inflammation.

In vitro, incubation of newborn mice forebrain slices exposed to OGD with CBD dramatically reduces the increase in glutamate release observed in the first 30 min after the insult (Castillo et al., 2010). *In vivo*, CBD treatment fully prevents HI-induced increase of glutamate/N-acylaspartate (Glu/NAA) ratio in the brain -which is proportional to the severity of encephalopathy in human newborns (Pazos et al., 2013)- as assessed in piglets 6 h after a moderate HI insult by H^+-MRS studies (Pazos et al., 2013; Lafuente et al., 2016). This effect is still observable in newborn rats seven days after the HI insult (Pazos et al., 2012) but not in piglets three days after a very severe HI insult (Barata et al., 2019).

In *in vivo* studies in newborn piglets CBD prevents HI-induced increase in brain concentration of MDA (Lafuente et al., 2011) as well as HI-induced consumption of reduced glutathione 6 h after the HI insult (Pazos et al., 2013). This effect is still observable seven days after the HI insult in newborn rat brain (Pazos et al., 2012). In newborn piglets CBD prevents HI-induced increase of protein carbonylation, a specific marker of increased oxidative stress involved in HIBI pathophysiology (Pazos et al., 2013; Lafuente et al., 2016; Arruza et al., 2017). However, to determine the real efficacy of CBD against HI-induced oxidative stress in HIBI, further research is warranted about the effects of CBD on brain neuroprostane or neurofurane concentration as reliable biomarkers of neuronal lipid peroxidation after HI (Garberg et al., 2016).

Given the modulatory effect of CBD on microglial activation CBD was also expected to moderate the release of pro-inflammatory cytokines. *In vitro,* incubation of newborn mice forebrain slices exposed to OGD with CBD prevents post-insult increase of IL-1a and TNFa concentration as well as COX-2 expression (Castillo et al., 2010). *In vivo*, CBD administration after moderate HI insult in newborn piglets prevents the increased brain concentration of those cytokines as studied by Western blot or microarrays six (Pazos et al., 2013; Lafuente et al., 2016; Arruza et al., 2017) or 72 h (Lafuente et al., 2011) after the insult. CBD prevention of HI-induced increased brain TNFa concentration can still be observed seven days after the HI insult in newborn rats (Pazos et al., 2012). By contrast, CBD administration to newborn piglets after a very severe HI insult does not reduce HI-induced increase in brain TNFa concentration (Barata et al., 2019). It is known that PPARγ activation plays a key role in the anti-inflammatory effects of CBD (Esposito et al., 2011) but this has not been studied in models of neonatal HIBI. This point is of interest since PPARγ activation is involved in CBD inhibition of mTOR, the main activator of autophagy which plays a relevant role in neonatal HIBI pathophysiology (Galluzzi et al., 2016).

Modulation of those mechanisms is likely key to CBD neuroprotection, since prevention of cell loss by CBD in immature rat brain after HI insults is not associated with increased expression of neuroproliferative factors such as BDNF or GDNF (Ceprián et al., 2019).

CBD is a $5HT_{1A}$ receptor agonist and inhibits 5HT re-uptake (Russo et al., 2005). In newborn piglets exposed to moderate HI insult, administration of a $5HT_{1A}$ receptor antagonist together with CBD eliminates all the beneficial effects of CBD (Pazos et al., 2013), which supports the key role of $5HT_{1A}$ activation in the neuroprotective effects of CBD in immature brain. CBD is traditionally known to not act through CB_1 or CB_2 receptor activation (Pertwee, 2004; Mechoulam et al., 2007). Accordingly, blockade of CB_1 receptors does not modify CBD effects in mice forebrain slices exposed to OGD (Castillo et al., 2010). The role of CB_2 receptors on CBD neuroprotection in immature brain, however, is more controversial. Coincubation with CB_2 antagonists reversed all the neuroprotective effects of CBD in mice forebrain slices exposed to OGD (Castillo et al., 2010). Similarly, administration of CB_2 antagonists together with CBD to newborn piglets after HI insult attenuated all the neuroprotective effects of CBD (Pazos et al., 2013). Since it has been repeatedly demonstrated that CBD does not act as an activator of CB_2 receptors, those effects could be accounted for by an indirect cross-activation of CB_2 receptors in CB_2-$5HT_{1A}$ heteromers. CB_2-$5HT_{1A}$ heteromers are present and functioning in rat brain. Their density is higher in immature than in mature brain and increased after HI insults particularly in immature brains (Franco et al., 2019). *In vitro*, antagonism of adenosine A_{2A}

receptors blocked the neuroprotective effects of CBD in newborn mice forebrain slices exposed to OGD, suggesting that A_{2A} receptors are involved in CBD neuroprotection in immature brain, with a particular relevance on the anti-apoptotic effects of CBD (Castillo et al., 2010). The involvement of other receptors known to be a target for CBD, such as PPARγ, GPR55 or TRPV1 (Pertwee, 2004), has not yet been explored in models of neonatal HIBI.

CBD inhibits endocannabinoid uptake and enzymatic hydrolysis *in vitro* (Pertwee, 2004; Mechoulam et al., 2007). This appears to be of marginal importance at least in the early moments after HI insults in newborn brain since in fact CBD administration prevents HI-induced increase in endocannabinoid concentration observed in piglet brain 6 h after insult (Pazos et al., 2013).

Cannabidiol and Therapeutic Hypothermia

Since TH is the standard of care for asphyxiated newborns, studying how CBD can substitute or collaborate with TH is mandatory.

In newborn piglets exposed to diffuse HI insult, CBD and TH show a similar neuroprotective profile. When the HI insult is moderate, either CBD or TH similarly prevent HI-induced increase in neuronal loss, Lac/NAA ratio, caspase 3 expression, excitoxicity, inflammation and oxidative stress as assessed 6 h after the insult (Lafuente et al., 2016). When the HI insult is severe, neither 48 h-long TH nor CBD 1 mg/kg/d for three days are able to prevent HI-induced increased apoptosis, Lac/NAA ratio, excitotoxicity and cytokine concentration (Garberg et al., 2016; Barata et al., 2019). Noteworthy, even under those circumstances CBD exerts a modulatory effect on microglial activation, an effect not observable in piglets treated with TH (Barata et al., 2019).

When administered in combination after a moderate HI insult in newborn piglets, CBD and TH reveal additive effects, with the combination of both therapies leading to better neuroprotective effects than CBD or TH alone (Lafuente et al., 2016). However, combining CBD with TH in piglets exposed to severe HI insult led to controversial results depending on the experimental model. In piglets exposed to severe anoxia and systemic hypotension the combination of CBD and TH does not result in additive affects (Garberg et al., 2016). By contrast, in piglets exposed to severe hypoxia and brain ischemia combining CBD and TH has synergistic effects, because if CBD or TH alone are not protective, the combination of both therapies effectively reduces apoptotic death, Lac/NAA increase, inflammation and excitotoxicity as assessed in brain cortex three days after the HI insult (Barata et al., 2019).

Pharmacologic Aspects of Cannabidiol in Neonatal Hypoxic-Ischemic Brain Injury

Although CBD is a lipid substance, a formulation of CBD in saline, ethanol and a solvent as solutol or cromophor is suitable for parenteral administration (Alvarez et al., 2008; Lafuente et al., 2011; Pazos et al., 2012; Pazos et al., 2013; Lafuente et al., 2016; Ceprián et al., 2017; Barata et al., 2019; Ceprián et al., 2019). PK studies in newborn piglets receiving 1 mg/kg i.v and using that formulation indicate that plasma CBD concentration peaks by 15 min after the end of the infusion and attains 200–300 ng/ml. It is nearly undetectable 12 h post infusion, with $t_{1/2}$ approximately 2 h (Barata et al., 2019). CBD administered in this way to HI piglets attains a brain concentration of about 60 ng/g 6 h post-infusion (Pazos et al., 2013; Barata et al., 2019), which is equivalent to 200 nM. In newborn rats receiving CBD at the same dose using the same formulation i.p. brain concentration peaked 3 h post administration reaching about 30 ng/g. It was still detectable 36 h post administration (Pazos et al., 2012). Studies in piglets of CBD and TH revealed that hypothermia did not modify plasma or brain concentration of CBD or its metabolites, with the exception of a mild increase in the very low plasma levels of 6-OH-CBD in piglets receiving CBD and TH (Barata et al., 2019).

CBD administration to HI newborn piglets is not only free from significant side effects but is associated with hemodynamic and respiratory benefits. In HI piglets, CBD prevents HI-induced myocardial troponin increase and hypotension (Alvarez et al., 2008; Pazos et al., 2013), the latter effect even more remarkable in HI piglets receiving TH (Barata et al., 2019). CBD treatment prevents the ventilatory deterioration observed in newborn piglets in the hours following the HI insult (Alvarez et al., 2008; Pazos et al., 2013; Arruza et al., 2017), an effect attributed to the prevention of distant inflammatory lung damage induced by HIBI (Arruza et al., 2017). In newborn rats CBD administration has no effects on growth, brain volume or neurobehavioral performance as assessed when rats become adults (Pazos et al., 2012).

CONCLUSION

Preclinical studies offer solid evidence of the neuroprotective efficacy of CBD to limit HIBI in newborns. CBD prevents the functional deficits appearing after neonatal HIBI, which reduces the extent of brain damage and protects myelinogenesis. From a translational point of view CBD is a valuable tool for HIBI management since it is safe and effective and administered by the parenteral route *a posteriori*, with a broad therapeutic time window. CBD shows a similar neuroprotective profile as TH, which augments its efficacy when administered in combination. The fact that combining CBD and TH affords neuroprotection in severe cases of HIBI after they were ineffective when administered separately offers a promising opportunity to give some hope to the high number of asphyxiated babies showing no benefits with TH. Accordingly, a clinical trial testing CBD in asphyxiated infants is now underway (GWEP1560, EudraCT 2016-000936-17).

Although it is clear that CBD protects neuronal and glial cells by modulating key factors leading to HIBI such as excitotoxicity, oxidative stress and inflammation, further information is needed about the ultimate mechanisms of CBD neuroprotection in neonatal HIBI and how they can be determined by developmental status. At least in newborn piglets CBD could act as a substitute for TH. When combined with TH, CBD could lend neuroprotection in some cases in which TH alone is ineffective.

AUTHOR CONTRIBUTIONS

MV and AP participated in many of the experiments described in the text and participated in the writing and correction of the manuscript. JM-O was the Principal Investigator of all the experiments described in the text and wrote the manuscript.

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The Impact of Cannabidiol on Human Brain Function: A Systematic Review

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Background: Accumulating evidence suggests that the non-intoxicating cannabinoid compound cannabidiol (CBD) may have antipsychotic and anxiolytic properties, and thus may be a promising new agent in the treatment of psychotic and anxiety disorders. However, the neurobiological substrates underlying the potential therapeutic effects of CBD are still unclear. The aim of this systematic review is to provide a detailed and up-to-date systematic literature overview of neuroimaging studies that investigated the acute impact of CBD on human brain function.

Methods: Papers published until May 2020 were included from PubMed following a comprehensive search strategy and pre-determined set of criteria for article selection. We included studies that examined the effects of CBD on brain function of healthy volunteers and individuals diagnosed with a psychiatric disorder, comprising both the effects of CBD alone as well as in direct comparison to those induced by Δ9-tetrahydrocannabinol (THC), the main psychoactive component of *Cannabis*.

Results: One-ninety four studies were identified, of which 17 met inclusion criteria. All studies investigated the acute effects of CBD on brain function during resting state or in the context of cognitive tasks. In healthy volunteers, acute CBD enhanced fronto-striatal resting state connectivity, both compared to placebo and THC. Furthermore, CBD modulated brain activity and had opposite effects when compared to THC following task-specific patterns during various cognitive paradigms, such as emotional processing (fronto-temporal), verbal memory (fronto-striatal), response inhibition (fronto-limbic-striatal), and auditory/visual processing (temporo-occipital). In individuals at clinical high risk for psychosis and patients with established psychosis, acute CBD showed intermediate brain activity compared to placebo and healthy controls during cognitive task performance. CBD modulated resting limbic activity in subjects with anxiety and metabolite levels in patients with autism spectrum disorders.

Conclusion: Neuroimaging studies have shown that acute CBD induces significant alterations in brain activity and connectivity patterns during resting state and

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Abbreviations: AIS, intoxication scale; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; CBD, cannabidiol; CHR, clinical high risk; fMRI, functional magnetic resonance imaging; GABA, γ-aminobutyric acid; GPR18, G protein-coupled receptor 18; GPR55, G protein-coupled receptor 55; Glx, glutamate and glutamine; H-MRS, proton magnetic resonance spectroscopy; $5HT_{1A}$, serotonin type 1A receptor; PANNS, positive and negative syndrome scale; PPARgamma, peroxisome proliferator-activated receptor gamma; PRISMA, preferred reporting items for systematic reviews and meta-analyses; SPECT, single photon emission computed tomography; STAI, state-trait anxiety inventory; ^{99m}Tc-ECD, ^{99m}Tc-ethyl cysteinate dimer; THC, tetrahydrocannabinol; TRPV1, transient receptor potential vanilloid 1; VAMS, visual analog mood scales.

performance of cognitive tasks in both healthy volunteers and patients with a psychiatric disorder. This included modulation of functional networks relevant for psychiatric disorders, possibly reflecting CBD's therapeutic effects. Future studies should consider replication of findings and enlarge the inclusion of psychiatric patients, combining longer-term CBD treatment with neuroimaging assessments.

Keywords: cannabidiol, delta9-tetrahydrocannabinol, *Cannabis* (marijuana), neuroimaging, functional MRI

INTRODUCTION

Recently, there has been a growing interest in cannabidiol (CBD) as a therapeutic substance, due to its putative antipsychotic, anxiolytic and anti-craving effects (Iseger and Bossong, 2015; Rohleder et al., 2016; Batalla et al., 2019). CBD is one of the more than 100 cannabinoids that can be derived from the cannabis plant and is, unlike the main psychoactive compound delta-9-tetrahydrocannabinol (THC), devoid of intoxicating effects (Freeman et al., 2019). Since most conventional treatments in psychiatry, such as antipsychotics and antidepressants, are associated with limited response rates and adverse events that often limit tolerability and adherence (Blessing et al., 2015; Samara et al., 2019), there is an urgent need for developing novel pharmaceutical treatments (Leucht et al., 2013; Blessing et al., 2015; Lally and MacCabe, 2015). In this regard, CBD has been proposed as novel therapeutic compound in several psychiatric disorders, such as psychosis (Iseger and Bossong, 2015; Batalla et al., 2019), anxiety disorders (Blessing et al., 2015), substance use disorders (Chye et al., 2019; Freeman et al., 2020) and autism spectrum disorders (Poleg et al., 2019; Fusar-Poli et al., 2020).

CBD effects are most likely related to the endocannabinoid system (Rohleder et al., 2016), although its precise mechanism of action is not yet fully understood. Animal studies have shown that CBD has no significant affinity with the cannabinoid receptors CB_1 and CB_2 (Bisogno et al., 2001; Jones et al., 2010), but may act as an antagonist of both in presence of CB_1 agonists (Thomas et al., 2007). It has been hypothesized that the antagonistic effects of CBD might be through negative allosteric modulation of the CB_1 receptor (Laprairie et al., 2015; Rohleder et al., 2016). Other suggested molecular targets include different types of receptors, such as serotonin type 1A ($5HT_{1A}$), peroxisome proliferator-activated receptor gamma (PPARgamma), vanilloid receptor 1 (TRPV1), GPR55, and GPR18 (Pertwee, 2008; Gururajan and Malone, 2016). In addition, CBD has been shown to increase plasma levels of the endogenous cannabinoid anandamide, which was related to its antipsychotic effects (Leweke et al., 2012). Hence, CBD may exert a protective effect on disturbances of the endocannabinoid system, as observed in several psychiatric disorders (Leweke et al., 2007; Morgan et al., 2013; Minichino et al., 2019).

Neuroimaging techniques provide a highly useful insight into the human neural processes involved in the behavioral effects of cannabinoids. An increasing number of neuroimaging studies have been performed to examine the human neural mechanisms underlying the effects of CBD. Although some of these studies have been included in excellent reviews that describe the impact of cannabis on human brain function in a broader context (Martín-Santos et al., 2010; Bhattacharyya et al., 2012a; Batalla et al., 2014; Weinstein et al., 2016), the aim of the current review is to provide a systematic and up-to-date overview of neuroimaging studies that investigated the effects of CBD on human brain function. This includes studies that examined the impact of CBD on brain function of healthy volunteers, comprising both the acute effects of CBD alone as well as in direct comparison to those induced by THC, and studies that investigated the neural substrates of acute CBD effects in patients with a psychiatric disorder.

METHODOLOGY

Search Strategy

This review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Moher et al., 2009). PubMed was searched for neuroimaging studies investigating the impact of CBD on human brain function published until May 2020. See for the exact Pubmed search syntax the **Supplementary Methods**. References were screened for additional relevant studies.

Data Inclusion

Titles and abstracts were screened blind for eligibility by two authors (AB and JB). Discrepancies were discussed with a third author (MB). Inclusion criteria were: 1) use of neuroimaging techniques, and 2) administration of CBD to human subjects. Reviews and case reports were excluded.

Data Extraction

Data extraction included: study information (e.g., title, authors, study design); sample characteristics (mean age, sex, handedness); cannabinoid dose and administration route; time interval between administration and imaging; imaging modality; cognitive task performed during imaging; and degree of sample overlap.

RESULTS

The search strategy yielded 194 studies, of which 15 studies met inclusion criteria. Two studies were found by additional references, resulting in a total of 17 included studies (**Figure 1**). In total, the current review comprised 115 healthy

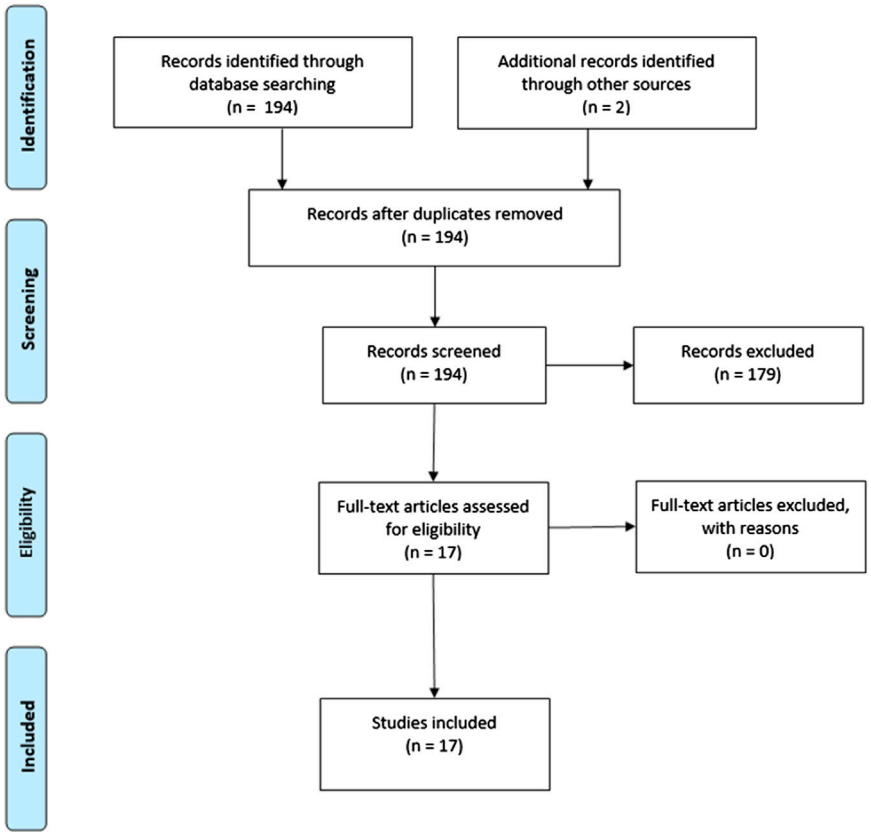

FIGURE 1 | PRISMA flow diagram.

subjects, 33 individuals at clinical high risk (CHR) for psychosis, 13 patients with a psychotic disorder, 10 patients with anxiety disorder and 17 patients with an autism spectrum disorder.

In healthy subjects, 12 studies reported the acute effects of CBD compared to placebo (Crippa et al., 2004; Borgwardt et al., 2008; Bhattacharyya et al., 2009; Fusar-Poli et al., 2009; Fusar-Poli et al., 2010) or compared to THC (Bhattacharyya et al., 2010; Winton-Brown et al., 2011; Bhattacharyya et al., 2012b; Bhattacharyya et al., 2015; Freeman et al., 2018; Grimm et al., 2018; Wall et al., 2019). In individuals with a psychiatric disorder, five studies assessed the acute effects of CBD compared to placebo (Crippa et al., 2011; Bhattacharyya et al., 2018; Pretzsch et al., 2019; Wilson et al., 2019; O'Neill et al., 2020).

A cluster of eight studies performed different cognitive tasks (i.e., go-no go, verbal learning, emotional processing, visual and auditory processing) using the same sample of healthy subjects (Borgwardt et al., 2008; Bhattacharyya et al., 2009; Fusar-Poli et al., 2009; Bhattacharyya et al., 2010; Fusar-Poli et al., 2010; Winton-Brown et al., 2011; Bhattacharyya et al., 2012b; Bhattacharyya et al., 2015). In addition, the studies of Freeman et al. (2018) and Wall et al. (2019) used an overlapping sample of healthy participants, and those of Bhattacharyya et al. (2018) and Wilson et al. (2019) a similar cohort of CHR individuals. See **Tables 1–3** for study characteristics and results of studies included in the current systematic review.

Acute Effects of CBD on Brain Function of Healthy Volunteers

Nine double-blind placebo-controlled studies investigated the acute effects of CBD on brain function of healthy volunteers. One of these studies used Single Photon Emission Computed Tomography (SPECT) measuring regional cerebral blood flow, whereas eight studies applied functional Magnetic Resonance Imaging (fMRI), either at rest or during the performance of a cognitive task (**Table 1**).

Resting State

Two studies investigated the acute effects of CBD during resting state (Crippa et al., 2004; Grimm et al., 2018). Crippa et al. (2004) measured cerebral blood flow using ^{99m}Tc-ethyl cysteinate dimer (^{99m}Tc-ECD) SPECT imaging in 10 healthy male volunteers using a cross-over design (Crippa et al., 2004). Administration of an oral dose of 400 mg CBD enhanced blood flow compared to placebo in an area consisting of the left parahippocampal and fusiform gyrus. Conversely, CBD attenuated blood flow in the left posterior cingulate cortex and in a cluster comprising the left amygdala, hippocampus, uncus and hypothalamus (Crippa et al., 2004). In another resting state study using fMRI to measure connectivity, 16 healthy male volunteers were given placebo, 10 mg oral THC and 600 mg oral CBD using a cross-over study design (Grimm et al.,

TABLE 1 | The acute effects of CBD on brain function of healthy volunteers.

First author	Imaging modality	Condition	Image analysis	Study design	HC	M/F	Mean age (SD)	*Cannabis* use	Dose	Route	Imaging findings
Grimm et al. (2018)	3T fMRI	Resting state	Connectivity	DB, PC, Ra, BS	16	NR	NR	NR	600 mg CBD	Oral	↑ Connectivity R putamen with R middle frontal gyrus, BL superior frontal gyrus/paracingulate gyrus, R frontal pole
Bhattacharyya et al. (2015)	1.5T fMRI	Go-no go salience	Connectivity	DB, PC, PR, WS	15	15/0	26.7 (5.7)	<15 times lifetime. Not in the last month	600 mg CBD	Oral	↓ R inferior frontal gyrus with R insula; L anterior lobe of cerebellum; L lingual gyrus; L thalamus; L dorsal striatum with L caudate nucleus body; L inferior frontal gyrus; L dorsal striatum with L anterior cingulate; L medial frontal gyrus; L posterior hippocampus with L parahippocampus; L posterior hippocampus with R parahippocampus; L posterior cingulate; L caudate tail
Bhattacharyya et al. (2012b)	1.5T fMRI	Go-no go salience	Whole brain	DB, PC, PR, WS	15	15/0	26.7 (5.7)	<15 times lifetime. Not in the last month	600 mg CBD	Oral	↓L medial prefrontal cortex
Winton-Brown et al. (2011)	1.5T fMRI	Visual and auditory processing	Whole brain	DB, PC, PR, WS	14	14/0	26.7 (5.7)	<15 times lifetime. Not in the last month	600 mg CBD	Oral	Auditory: BL temporal cortex, BL insula, BL parahippocampal gyri, BL hippocampi; L superior temporal gyrus, L insula, L posterior middle temporal gyrus, L supramarginal gyrus. Visual: R (inferior, middle) occipital lobe, R lingual gyrus, R cerebellum, R cuneus
Fusar-Poli et al. (2010)	1.5T fMRI	Fearful faces	Connectivity	DB, PC, PR, WS	15	15/0	26.7 (5.7)	<15 times lifetime. Not in the last month	600 mg CBD	Oral	Disruption of anterior cingulate cortex–amygdala effective connectivity
Fusar-Poli et al. (2009)	1.5T fMRI	Fearful faces	Whole brain	DB, PC, PR, WS	15	15/0	26.7 (5.7)	<15 times lifetime. Not in the last month	600 mg CBD	Oral	Neutral faces: NS: Intermediate fearful faces: ↓ BL posterior lobe cerebellum. Intensely fearful faces: ↓ L medial temporal region (amygdala and anterior parahippocampal gyrus), anterior and posterior cingulate gyri, R posterior lobe cerebellum
Bhattacharyya et al. (2009)	1.5T fMRI	Verbal learning task	Whole brain	DB, PC, PR, WS	15	15/0	26.7 (5.7)	<15 times lifetime. Not in the last month	600 mg CBD	Oral	NS
Borgwardt et al. (2008)	1.5T fMRI	Go-no go response inhibition	Whole brain	DB, PC, PR, WS	15	15/0	26.7 (5.7)	<15 times lifetime. Not in the last month	600 mg CBD	Oral	↓ L posterior insula, L superior temporal gyrus, L transverse temporal gyrus
Crippa et al. (2004)	99mTc-ECD SPECT rCBF	Resting state	Whole brain	DB, Ra, PC, WS	10	10/0	29.8 (5.1)	<5 times lifetime. Not in the last year	400 mg CBD	Oral	↑ L mediotemporal cortex (parahippocampus, fusiform gyrus). ↓ L. amygdala/hippocampus/hypothalamus, L posterior cingulate cortex

BL, bilaterally; BS, between-subject; CBD, cannabidiol; DB, double-blinded; HC, healthy controls; L, left; M/F, male/female; NB, non-blinded; NC, non controlled; NR, not reported; NS, nonsignificant results; PC, placebo controlled; PR, pseudorandomized; R, right; Ra, randomized; THC, tetrahydrocannabinol; WS, within subject. Grey areas: overlapping samples of subjects.

TABLE 2 | The acute effects of CBD vs. THC on brain function of healthy volunteers.

First author	Imaging modality	Condition	Image analysis	Study design	HC	M/F	Mean age (SD)	*Cannabis* use	Dose	Route	Imaging findings	Clinical correlations
Wall et al. (2019)	fMRI	Resting state	ROI	DB, PC, R, WS	17	8/9	26,2 (7.1)	<3 times/week and >4 times last year	8 mg THC (Cann-CBD). 8 mg THC +10 mg CBD (Cann + CBD)	Inhalation	Cann + CBD vs. placebo, and Cann-CBD vs. placebo: ↓ mean connectivity in default mode network (defined as positive connectivity with the posterior cingulate cortex). Cann-CBD vs. Cann + CBD: ↓ mean connectivity in salience (defined as positive connectivity with anterior insula)	Cann-CBD: Disruptions in posterior cingulate cortex function in default mode network with subjective feelings of stoned, high, feel drug effect, dry mouth. Frontal pole region in salience network was negatively correlated with feelings of paranoia
Freeman et al. (2018)	fMRI	Auditory processing	ROI	DB, PC, R, WS	16	8/8	26.3 (7.4)	<3 times/week and >4 times last year	8 mg THC (Cann-CBD). 8 mg THC +10 mg CBD (Cann + CBD)	Inhalation	Cann-CBD vs. placebo: ↓ BL auditory cortex, R hippocampus, R ventral striatum, R amygdala. Cann-CBD vs. placebo, and Cann + CBD vs. placebo: ↑ connectivity R ventral striatum with BL auditory cortex (Cann + CBD greater effects)	Cann-CBD and Cann + CBD: ↑ R ventral striatum correlated with pleasure ratings and response to music
Grimm et al. (2018)	3T fMRI	Resting state	ROI	DB, PC, Ra, BS	16	NR	NR	NR	600 mg CBD. 10 mg THC	Oral	CBD > THC: R putamen with frontal pole and paracingulate gyrus	NS
Bhattacharyya et al. (2015)	1.5 T fMRI	Go-no go salience	ROI. Whole brain	DB, PC, PR, WS	15	15/0	26.7 (5.7)	<15 times lifetime. Not in the last month	600 mg CBD. 10 mg THC	Oral	THC > placebo > CBD: R inferior frontal with R parahippocampal gyrus. L posterior hippocampus with L superior-, middle- and inferior frontal gyri, anterior cingulate/medial prefrontal cortex, L precentral gyrus. THC < placebo < CBD: L dorsal striatum with L ventral striatum and with L inferior frontal gyrus. L posterior hippocampus with parahippocampal gyrus	THC: ↓ connectivity between striatum and inferior frontal gyrus with ↑ response latency during standard condition relative to oddball condition
Bhattacharyya et al. (2012b)	1.5 T fMRI	Go-no go salience	Whole brain	DB, PC, PR, WS	15	15/0	26.7 (5.7)	<15 times lifetime. Not in the last month	600 mg CBD: 10 mg THC	Oral	THC > placebo > CBD: R superior, R middle, R inferior and R orbitofrontal gyri. THC < placebo < CBD: L caudate, L putamen, L parahippocampal gyrus, L thalamus, L lingual gyus	THC: ↓ BL caudate head with ↑ severity of psychotic symptoms. ↓ BL caudate head with ↑ response latency to standard stimuli. ↑ R prefrontal cortex with ↑ response latency to standard stimuli
Winton-Brown et al. (2011)	1.5 T fMRI	Visual and auditory processing	Whole brain	DB, PC, PR, WS	14	14/0	26.7 (5.7)	<15 times lifetime. Not in the last month	600 mg CBD. 10 mg THC	Oral	Auditory processing: THC < CBD: R superior and middle temporal gyrus (R side homolog to Wernicke). Visual processing: THC > CBD: L lingual and middle occipital gyriTHC < CBD: BL occipital regions	NS

(Continued on following page)

TABLE 2 | (*Continued*) The acute effects of CBD vs. THC on brain function of healthy volunteers.

First author	Imaging modality	Condition	Image analysis	Study design	HC	M/F	Mean age (SD)	*Cannabis* use	Dose	Route	Imaging findings	Clinical correlations
Bhattacharyya et al. (2010)	1.5 T fMRI	Verbal learning, go-no go, visual and auditory processing, fearful faces	Whole brain	DB, PC, PR, WS	15	15/0	26.7 (5.7)	<15 times lifetime. Not in the last month	600 mg CBD. 10 mg THC	Oral	Verbal learning (recall):CBD > THC: Striatum, anterior cingulate/medial prefrontal, lateral prefrontal. Go-no go: CBD > THC: BL parahippocampus, L insula, caudate. Visual processing: CBD > THC: BL occipital cortex. Auditory processing: CBD > THC: BL lateral temporal cortex: Fearful faces: CBD < THC: L amygdala, fusiform, lingual gyri, lateral prefrontal cortex, cerebellum	THC: ↓ striatum with ↑ severity of psychotic symptoms. ↑ L amygdala with anxiety (STAI). ↑ skin conductance response. CBD: ↓ L amygdala with a trend toward less anxiety (VAMS). ↓ skin conductance response

BL, bilaterally; BS, between-subject; CBD, cannabidiol; DB, double-blinded; HC, healthy controls; L, left; M/F, male/female; NB, non-blinded; NC, non controlled; NR, not reported; NS, nonsignificant results; PC, placebo controlled; PR, pseudorandomized; R, right; Ra, randomized; THC, tetrahydrocannabinol; WS, within subject. Grey areas: overlapping samples of subjects.

2018). The striatum was set as a seed region and a whole brain analysis was performed. CBD increased connectivity between the right putamen and three clusters, situated mainly in the right prefrontal cortex (Grimm et al., 2018).

Cognitive Tasks

Seven studies performed different cognitive tasks (i.e., go-no go, verbal learning, emotional processing, visual and auditory processing) using the same sample of 15 healthy volunteers (Borgwardt et al., 2008; Bhattacharyya et al., 2009; Fusar-Poli et al., 2009; Fusar-Poli et al., 2010; Winton-Brown et al., 2011; Bhattacharyya et al., 2012b; Bhattacharyya et al., 2015). The authors explored the acute effects of 600 mg CBD, 10 mg THC and placebo on brain activity measured by fMRI using a double-blind cross-over design. During the sessions, ratings of anxiety (STAI), intoxication (AIS), psychotic symptoms (PANNS) and subjective feelings (VAMS) were obtained. The acute effects of CBD in direct comparison to those induced by THC administration are described in the next section.

A go-no go task was used to investigate brain activity during response inhibition and detection of salient stimuli (Borgwardt et al., 2008; Bhattacharyya et al., 2012b). Under conditions of response inhibition, CBD attenuated brain activity compared to placebo in the left posterior insula, left superior temporal gyrus and left transverse temporal gyrus (Borgwardt et al., 2008). During the presentation of a salient relative to a non-salient stimuli CBD attenuated activity in the left medial prefrontal cortex (Bhattacharyya et al., 2012b). In the same salient relative to non-salient stimuli comparison, Bhattacharyya et al. (2015) conducted connectivity analyses with the inferior frontal gyrus, dorsal striatum and posterior hippocampus set as seed regions. Compared to placebo, a CBD decreased connectivity was found between the following areas: the right inferior frontal gyrus and right insula, left cerebellum, left lingual gyrus and left thalamus; the left dorsal striatum and left anterior cingulate and left medial frontal gyrus; the left posterior hippocampus and right parahippocampus, left posterior cingulate gyrus and caudate tail. Conversely, CBD increased connectivity between the following areas: the left dorsal striatum and left body of the caudate nucleus and left inferior frontal gyrus; left posterior hippocampus and left parahippocampus (Bhattacharyya et al., 2015).

The verbal learning task consisted of an encoding block, where participants had to evaluate whether pairs of words fitted well together, and a recall block, during which participants matched the presented with the previously associated word (Bhattacharyya et al., 2009). CBD modulated activation during encoding conditions in the insula, midtemporal gyrus, lingual gyrus, precuneus and precentral gyrus. During recall, CBD modulated activation in the hippocampus. However, none of these findings were statistically significant (Bhattacharyya et al., 2009).

The emotional processing task consisted of a series of faces, including neutral, intermediate and extremely fearful faces (Fusar-Poli et al., 2009). Relative to placebo, administration of CBD did not alter brain activity during the presentation of neutral faces. During the presentation of intermediately fearful faces, CBD attenuated activity bilaterally in the posterior lobe of the

TABLE 3 | The acute effects of CBD on brain function of patients with a psychiatric disorder.

First author	Imaging modality	Condition	Image analysis	Study design	HC	Patients	M/F	Mean age (SD)	*Cannabis* use	Dose	Route	Imaging findings	Clinical correlations
O'neill et al. (2020)	3T fMRI	Verbal learning	ROI	DB, R, PC, WS	19	13 with psychotic disorders, all treated with antipsychotics except for one subject	Pt: 10/5. HC: 11/8	Pt: 27.7 (4.6). HC: 23.9 (4.2)	HC:<10 times lifetime	600 mg CBD	Oral	Encoding: Placebo > control: R inferior frontal gyrus, L inferior and middle frontal gyrus: Placebo < control: L middle frontal gyrus. Placebo > CBD > control: BL inferior frontal gyrus, L middle frontal gyrus. Placebo < CBD < control: L middle frontal gyrus. Recall: Placebo > control: R parahippocampus, R middle and inferior frontal gyri, placebo < control: L parahippocampal gyrus. Placebo > CBD > control: R middle -, R frontal gyrus, R parahippocampal gyrus. Placebo < CBD < control: L parahippocampal gyrus. Connectivity (recall): Placebo > control: Hippocampus with the right caudate head and left caudate body. CBD showed intermediate connectivity relative to placebo and healthy controls between hippocampus and R caudate head, L caudate body, L putamen	↓ activation in inferior frontal gyrus with increase in PANSS score
Wilson et al. ((2019)	3T fMRI	Monetary incentive delay	ROI. Whole brain	DB, PC, R, BS	19	33 CHR, antipsychotic naïve	HC: 11/8. CHR: 17/16	HC: 23.9 (4.2). CHR (CBD) 22.7 (5.1). CHR (placebo) 24.1 (4.5)	HC: NR, CHR (CBD): Current users: 43.8%. CHR (placebo) current users: 41.2%	600 mg CBD	Oral	CHR (placebo) > HC: BL frontal operculae; L insula, parietal operculum; L superior frontal gyri, L inferior frontal gyrus, frontal operculae; L superior temporal gyrus. CHR (placebo) > CHR (CBD) > HC: L insula, parietal operculum; L frontal operculum; L superior frontal gyrus	CHR (placebo): Negative correlation between b-values and mean reaction time difference between salience and neutral conditions. Positive correlation between activation in L insula/parietal opercula and CAARMS positive subscale. HC: Negative correlation b value in L insula/parietal opercula with mean reaction time for salience condition
Pretzsch et al. (2019)	MRS	Resting state	ROI	DB, PC, PR, WS	17	17 with ASD, unmedicated except for two subjects (methylphenidate and sertraline)	34/0	Pt: 31.3 (9.9). HC: 28.5 (6.6)	NR	600 mg CBD	Oral	HC and ASD: ↑ Glx basal ganglia. ↓ Glx dorsomedialprefrontal cortex. HC: ↑ GABA + basal ganglia and dorsomedialprefrontal cortex. ASD: ↓ GABA + basal ganglia and dorsomedialprefrontal cortex	

(Continued on following page)

TABLE 3 | (*Continued*) The acute effects of CBD on brain function of patients with a psychiatric disorder.

First author	Imaging modality	Condition	Image analysis	Study design	HC	Patients	M/F	Mean age (SD)	*Cannabis* use	Dose	Route	Imaging findings	Clinical correlations
Bhattacharyya et al. (2018)	3T fMRI	Verbal learning	Whole brain	DB, PC, R, BS	19	33 CHR, antipsychotic naïve	HC: 11/8. CHR (CBD): 10/6. CHR (placebo): 7/10	CHR (CBD): 22.4 (5.0). CHR (placebo): 25.4 (5.2). HC: 23.9 (4.1)	CHR: Most more than once a week. HC: <10 times lifetime	600 mg CBD	Oral	Encoding: CHR (placebo) > HC: R middle frontal gyrus, inferior frontal gyrus, insula; L insula/claustrum, inferior frontal gyrus, putamen; R precentral gyrus, postcentral gyrus, inferior parietal lobule; L cerebellum, lingual gyrus. CHR (placebo) < HC: R subcallosal gyrus, caudate head; L anterior cingulate; R caudate tail, posterior cingulate cortex; R precuneus, cuneus. CHR (placebo) > CHR (CBD) > HC: R inferior frontal, middle frontal gyri, insula; L insula, putamen; 3 clusters in precentral gyri; R fusiform gyrus, cerebellum; L cerebellum, fusiform gyrus. CHR (placebo) < CHR (CBD) < HC: L caudate head, putamen, anterior cingulate cortex; R subcallosal gyrus, caudate head; R caudate tail, posterior cingulate cortex; precuneus, R cuneus, fusiform gyrus. Recall: CHR (placebo) > HC: R inferior frontal, middle frontal, precentral gyri, insula; R cuneus, fusiform, lingual gyri, posterior cingulate gyri; L cerebellum, middle occipital, fusiform gyri. CHR (placebo) < HC: Parahippocampal gyrus, midbrain, cerebellum, thalamus; superior temporal, middle temporal gyri; superior transverse temporal gyri; middle frontal gyrus. CHR (placebo) > CHR (CBD) > HC: R inferior frontal gyrus, middle frontal gyrus, insula; R precuneus, cuneus, lingual, middle occipital, fusiform gyri, cerebellum; L cerebellum, fusiform, lingual, inferior occipital gyri. CHR (placebo) < CHR (CBD) < HC: L parahippocampal gyrus, midbrain, cerebellum; L thalamus; L transverse temporal gyrus, superior temporal gyrus; L precentral, cingulate gyri, caudate body	
Crippa et al. (2011)	99mTc-ECD SPECT rCBF	Resting state	Whole brain	DB, PC, R, WS		10 with social anxiety disorder, unmedicated	10/0	Pt: 27.7 (4.6). HC: 23.9 (4.2)	<5 times lifetime. Not in the last year	400 mg CBD	Oral	↓ L parahippocampal gyrus/hippocampus. ↑ R posterior cingulate gyrus	NS

ASD, autism spectrum disorders; BL, bilaterally; BS, between-subject; CBD, cannabidiol; CHR, clinical high risk of psychosis; DB, double-blinded; HC, healthy controls; L, left; M/F, male/female; NB, non-blinded; NC, non controlled; NR, not reported; NS, nonsignificant results; PC, placebo controlled; PR, pseudorandomized; Pt, patients; R, right; Ra, randomized; THC, tetrahydrocannabinol; WS, within subject. Grey areas: overlapping samples of subjects.

cerebellum. During the processing of intensely fearful faces, CBD attenuated activity in the left medial temporal region (amygdala and anterior parahippocampal gyrus), the anterior and posterior cingulate gyri and the right posterior lobe of the cerebellum (Fusar-Poli et al., 2009). In addition, CBD decreased the number of skin conductance response fluctuations, a physiological measure of emotional response. Moreover, this decrease in skin conductance response covaried with the attenuation of activity in both the left amygdala and the anterior cingulate (Fusar-Poli et al., 2009). Based on these results Fusar-Poli et al. (2010) investigated the connectivity between these two regions in the same sample. Compared to placebo, administration of CBD disrupted connectivity between the left anterior cingulate cortex and the left amygdala while viewing fearful faces (Fusar-Poli et al., 2010).

While listening to neutral words, brain activity was increased during CBD relative to placebo in the bilateral temporal cortex, insula, parahippocampal gyrus and hippocampus (Winton-Brown et al., 2011). Conversely, CBD attenuated activity in the left superior temporal gyrus, insula, posterior middle temporal gyrus and supramarginal gyrus. During visual stimulation, CBD increased activity in the right occipital lobe, lingual gyrus, cerebellum and cuneus (Winton-Brown et al., 2011).

In summary, CBD enhanced fronto-striatal connectivity and decreased limbic activity during resting state, and modulated brain activity showing task-specific patterns during different cognitive paradigms. For example, CBD increased activation relative to placebo in the parahippocampus during auditory processing, and reduced activation in this region during the processing of fearful faces. In addition, CBD decreased connectivity between fronto-limbic regions (i.e., anterior cingulate cortex and amygdala) during the processing of fearful faces and enhanced fronto-limbic-striatal connectivity (i.e., inferior frontal gyrus, dorsal striatum and posterior hippocampus) during salience processing.

Acute Effects of CBD vs. THC on Brain Function of Healthy Volunteers

Seven fMRI studies investigated the acute effects of CBD in direct comparison to those induced by THC, during resting state or a cognitive task. Some studies analysed regions in the brain where CBD and THC showed opposite activity relative to placebo, whereas others directly compared both substances (**Table 2**).

Resting State

Grimm et al. (2018) conducted a resting state connectivity analysis on 16 healthy volunteers, where the striatum and frontal regions were set as regions of interest. While CBD enhanced frontal-striatal connectivity, THC did not alter this connectivity significantly, possibly due to low THC plasma concentrations during scanning. Direct comparison between the two substances showed that CBD increased connectivity relative to THC between the right putamen and frontal pole and paracingulate gyrus (Grimm et al., 2018).

In a double-blind, pseudo-randomized, within-subject study, Wall et al. (2019) investigated the effects on the resting-state functional connectivity of two strains of inhaled cannabis, containing THC (8 mg) without or with CBD (10 mg), and placebo in 17 occasional cannabis users. Connectivity analyses were performed to investigate the default mode network (defined as positive connectivity with the posterior cingulate cortex), executive control network (defined as negative connectivity with the posterior cingulate cortex) and salience (defined as positive connectivity with the anterior insula). Both strains of cannabis showed a significant reduction in mean connectivity in the default mode network relative to placebo. In the salience network, cannabis containing both THC and CBD caused a significant increase in connectivity compared to cannabis without CBD, but both strains did not differ significantly from placebo. No significant effects were found within the executive control network (Wall et al., 2019). Significant correlations between the subjective measures of feeling the drug's effect and brain effects were only found after cannabis without CBD was administered. These correlations involved the posterior cingulate cortex region and the frontal pole region (Wall et al., 2019).

Cognitive Tasks

Freeman et al. (2018) investigated the acute effects of inhaled cannabis with and without CBD, while they listened to classical music and scrambled sound, using the same sample of occasional cannabis users as described by Wall et al. (2019). Both types of cannabis increased ratings of wanting to listen to music and enhanced sound perception. Inhalation of cannabis without CBD relative to placebo resulted in a dampened response to music bilaterally in the auditory cortex, right hippocampus, right ventral striatum and right amygdala. Cannabis with CBD did not significantly modulate activity relative to placebo or cannabis without CBD. Across all sessions, activation in the right ventral striatum was correlated with pleasure ratings and response to music. Moreover, this region showed an increased functional connectivity with the bilateral auditory cortex during music relative to scrambled sound. Cannabis with CBD had a greater impact on the functional connectivity between these two regions relative to cannabis without CBD (Freeman et al., 2018).

The other series of four studies performed different cognitive tasks (i.e., go-no go, verbal learning, emotional processing, visual and auditory processing) in a double-blind cross-over design of 600 mg CBD, 10 mg THC and placebo, using the same sample of 15 healthy volunteers as described by Borgwardt et al. (2008).

During an emotional processing task, CBD and THC had opposite effects relative to placebo in the left amygdala, fusiform, and lingual gyri, the lateral prefrontal cortex and the cerebellum (Bhattacharyya et al., 2010). The increased activity in the left amygdala following THC administration covaried with the level of anxiety assessed by the STAI, while the attenuated activity after CBD in the amygdala correlated to its anxiolytic effect measured by the VAMS. Opposite effects on skin conductance response fluctuations were also found following the administration of THC compared to CBD (Bhattacharyya et al., 2010).

During the recall phase of a verbal memory task, CBD enhanced and THC reduced brain activity in the striatum (Bhattacharyya et al., 2010). The reduction in the striatum activity after THC administration correlated with the severity of psychotic symptoms. Furthermore, during the recall phase opposite effects were found in a cluster consisting of the anterior

cingulate and medial prefrontal cortex and in the lateral prefrontal cortex (Bhattacharyya et al., 2010).

During response inhibition, CBD increased and THC reduced activity in the left insula, left caudate and bilateral parahippocampal gyrus (Bhattacharyya et al., 2010). During a go-no go task, CBD attenuated and THC increased activity in the right superior, middle, inferior and orbifrontal gyri compared to placebo (Bhattacharyya et al., 2012b). Conversely, in left caudate, putamen, parahippocampal gyrus, thalamus and lingual gyrus, activation was attenuated by THC but augmented by CBD (Bhattacharyya et al., 2012b). Bhattacharyya et al. (2015) conducted a connectivity analyses on the same data with the inferior frontal gyrus, dorsal striatum and posterior hippocampus set as seed regions. CBD and THC modulated functional connectivity between these seeds and clusters in the rest of the brain in opposite direction (Bhattacharyya et al., 2015).

During processing of speech, CBD and THC showed opposite effects relative to placebo in the bilateral temporal cortex, whereas opposite effects were found in the bilateral occipital cortex while viewing a visual checkerboard (Bhattacharyya et al., 2010). A direct comparison of CBD and THC effects revealed significantly reduced activity after THC in the right superior and middle temporal gyrus during processing of speech. During visual processing, THC increased activity relative to CBD in the bilateral lingual and middle occipital gyrus, but reduced activity in several other occipital regions. Mixed effects were reported in the cerebellum (Winton-Brown et al., 2011).

In summary, CBD and THC showed dissonant effects during resting state and during several cognitive tasks. During resting state, CBD enhanced connectivity between fronto-striatal regions compared to THC, and cannabis with both THC and CBD increased connectivity within the salience network compared to cannabis without CBD. THC and CBD showed task-specific opposite effects during emotional processing (fronto-temporal), verbal memory (fronto-striatal), response inhibition (fronto-limbic-striatal), and auditory/visual processing (temporo-occipital).

The Acute Effects of CBD on Brain Function of Patients With a Psychiatric Disorder

Five neuroimaging studies reported the acute effects of CBD on brain function in patients with a psychiatric disorder. Three of these studies used fMRI: two in a similar cohort of individuals at clinical high for psychosis and one in a group of patients with established psychosis. One study used SPECT to investigate cerebral blood flow in patients with social anxiety disorder and one study examined metabolite concentrations using proton Magnetic Resonance Spectroscopy (^{1}H-MRS) in patients with autism spectrum disorder (**Table 3**).

Bhattacharyya et al. (2018) conducted an fMRI double-blind randomized trial on 33 medication-naïve CHR subjects and 19 healthy controls, using the same verbal learning task as described in previous studies (Bhattacharyya et al., 2009; Bhattacharyya et al., 2010). Patients were administered 600 mg CBD or placebo, while healthy controls were not given any drug. During encoding conditions, the group of patients who received placebo (indicative of the at-risk state) showed altered brain activity compared to the healthy control group in clusters involving the frontal gyrus, the insula, claustrum, dorsal striatum, pre- and postcentral gyrus, parietal gyrus, cerebellum, lingual gyrus, subcallosal gyrus, cingulate cortex, precuneus and cuneus. During recall conditions, the group of patients who received placebo showed altered brain activity relative to the healthy control group in clusters comprising the frontal gyrus, insula, cuneus, fusiform, lingual gyrus, posterior cingulate, cerebellum, occipital gyrus, fusiform gyrus, parahippocampal gyrus, midbrain, cerebellum, thalamus and temporal gyrus. A linear comparison across the three groups (patients receiving CBD, patients receiving placebo, and control subjects receiving no drug) revealed several clusters in which CBD showed intermediate activation compared to the placebo and healthy control group. For instance, during encoding, the CBD group showed intermediate activation in clusters encompassing the frontal gyrus, insula, striatum, precentral gyrus, cerebellum, fusiform gyrus, cingulate cortex, subcallosal gyrus and occipital gyrus. During recall, the CBD group showed intermediate activation (relative to the placebo and control group) in clusters comprising the frontal gyrus, insula, striatum, precentral gyrus, cerebellum, fusiform gyrus, cingulate cortex, occipital gyrus, parahippocampal gyrus, midbrain, thalamus and temporal gyrus (Bhattacharyya et al., 2018).

Wilson et al. (2019) conducted a monetary incentive delay task in the same 32 CHR medication-naïve subjects and 19 healthy controls reported by Bhattacharyya et al. (2018). This tasked was used to investigate motivational salience conditions by comparing brain activation during reward and loss relative to neutral anticipation. The group of patients who received placebo showed greater brain activity compared to the healthy control group in clusters encompassing the frontal opercula, insula, parietal operculum, frontal gyri, and temporal gyri. A linear comparison between the three groups revealed intermediate activation in the CBD group (compared to the placebo and control group) in three clusters: the left insula and parietal operculum, left frontal operculum, and left superior frontal gyrus (Wilson et al., 2019).

One fMRI study explored the effects of CBD on patients with established psychosis (O'Neill et al., 2020), where 15 patients on antipsychotic treatment were given 600 mg CBD or placebo in a double-blind, randomized, within-subject design. In this study, 19 healthy participants were scanned but were not given any drugs. During the scanning procedure all participants performed a verbal learning task, the same used in previously described studies (Bhattacharyya et al., 2009; Bhattacharyya et al., 2010; Bhattacharyya et al., 2018). The medial temporal lobe, prefrontal cortex and striatum/*pallidum* were selected as regions of interest and activation patterns as well as a connectivity analysis were performed (O'Neill et al., 2020). Patients after CBD administration showed a trend level towards a greater decrease in median total PANSS score compared to those receiving placebo. Healthy controls scored better on both encoding and recall of the task compared to patients (after CBD or placebo). Patients under placebo showed increased activation compared to controls in the right inferior frontal gyrus and left inferior and middle frontal gyrus during encoding, while having both increasing and attenuating effects in two different clusters in

the left middle frontal gyrus. A linear comparison between the three groups showed that patients under CBD treatment had intermediate activation in several clusters located bilaterally in the inferior frontal gyrus, and the left middle frontal gyrus. The two clusters in the right inferior frontal gyrus were similar to clusters found in the placebo vs. control analysis (O'Neill et al., 2020). During recall, patients under placebo showed increased activation relative to healthy controls in the right middle and inferior frontal gyri and right hippocampus, but decreased activation in the left hippocampus. Similar clusters were found in all of these areas such that CBD had intermediate activation relative to the placebo and control group. Patients under placebo condition displayed increased connectivity between the hippocampus and the right caudate head and left caudate body during recall conditions. CBD had intermediate functional connectivity relative to the other two groups in connections between the hippocampus and right caudate head, left caudate body and left putamen (O'Neill et al., 2020).

Crippa et al. (2011) investigated the acute effect of an oral dose of 400 mg CBD in 10 medication-naïve patients with social anxiety disorder, while using $_{99}$mTc-ECD SPECT imaging to measure cerebral blood flow in a within-subject design. Compared to placebo, CBD decreased subjective anxiety and blood flow in a cluster consisting of the left parahippocampal gyrus and hippocampus, but enhanced blood flow in the right posterior cingulate gyrus (Crippa et al., 2011).

Pretsch et al. (2019) investigated the acute effects of 600 mg CBD on 17 patients with autism spectrum disorder and 17 healthy controls. Magnetic resonance spectroscopy was used to measure glutamate and glutamine (Glx) and inhibitory γ-aminobutyric acid and macromolecules (GABA+) levels in two voxels placed in the basal ganglia and dorsomedial prefrontal cortex. Both groups received placebo and CBD. The effect of CBD on Glx levels showed the same pattern in both patients and controls: CBD increased Glx levels relative to baseline in the basal ganglia and decreased Glx levels in the prefrontal cortex. However, the effects of CBD on GABA + levels showed an opposite pattern between groups: GABA + levels in both the basal ganglia and prefrontal cortex increased in the control group after CBD administration but decreased in the patients with autism (Pretzsch et al., 2019).

In summary, acute brain effects after CBD administration were different in patients with a psychiatric disorder compared to healthy controls. In subjects at CHR for psychosis, CBD administration showed intermediate activity in brain areas involved in memory and reward processing compared to placebo and healthy controls. An intermediate activity was also reported in patients with psychosis after CBD administration during a memory task. CBD also modified limbic activity in subjects with social anxiety, and showed similar (glutamate) and opposite (GABA) patterns of metabolite levels in patients with autism compared to healthy controls.

DISCUSSION

The current review provides a systematic literature overview of studies that investigated the acute effects of CBD on the human brain of healthy volunteers and individuals diagnosed with a psychiatric disorder. Overall, studies in healthy subjects showed that CBD modulated brain activity and had opposite effects when compared to THC in resting state and during several cognitive paradigms (i.e., salience, emotional, memory, response inhibition, auditory/visual processing), following task-specific activation patterns. Acute CBD administration also modulated brain activity in patients with psychiatric disorders by 1) showing intermediate activity compared to placebo and healthy controls in individuals at CHR and with established psychosis, 2) engaging with resting limbic activity in subjects with anxiety disorders, and 3) exhibiting similar (glutamate) and opposite (GABA) metabolite levels in patients with autism compared to healthy controls.

The acute administration of CBD in healthy volunteers modulated networks relevant for psychiatric disorders during resting state and several cognitive tasks, such as fronto-striatal and fronto-limbic circuitry. Fronto-striatal connectivity was enhanced after CBD administration during resting state (Grimm et al., 2018) and activity increased during salience processing (Bhattacharyya et al., 2015). Interestingly, lower functional connectivity in fronto-striatal circuitry has been reported in psychosis, and has been associated with more severe positive symptoms (Fornito et al., 2013). In addition, CBD decreased fronto-limbic activity during resting state (Crippa et al., 2004) and emotional processing (Fusar-Poli et al., 2010). Functional fMRI studies have shown activation of limbic areas in anxiety disorders (e.g., during panic attacks or panic anticipation) (Pfleiderer et al., 2007; Dresler et al., 2013). Based on a mechanistic account of these networks, these findings suggest that CBD might prove useful as treatment by restoring imbalanced networks in these and probably other neurological (Nenert et al., 2020) and psychiatric conditions, such as substance use disorders (Freeman et al., 2020). Regarding the last, converging preclinical and clinical evidence have shown promising effects of CBD on reducing craving, negative affect and motivation for substance use (Chye et al., 2019; Hurd et al., 2019; Freeman et al., 2020; Spanagel, 2020), phenomena associated with fronto-striatal and limbic network disbalances (Koob and Volkow, 2016; Volkow and Boyle, 2018).

Along these lines, CBD also showed opposite effects compared to THC during resting state and several cognitive paradigms in healthy volunteers. It is known that THC has pro-psychotic and anxiogenic properties, particularly evident with high potency cannabis strains (rich in THC) and at high doses (Campeny et al., 2020; Van der Steur et al., 2020). Opposite neurophysiological effects were reported on prefrontal, striatal and limbic areas, which are relevant neural substrates of psychosis and anxiety, and during several cognitive processes, such as salience, verbal memory, response inhibition, emotional processing and auditory/visual processing. Importantly, striatum activity correlated with severity of psychotic symptoms after THC (Bhattacharyya et al., 2010; Bhattacharyya et al., 2012b), and divergent amygdala activity correlated with severity of anxiety after CBD and THC (Bhattacharyya et al., 2010). These opposite brain effects may therefore underlie the neural basis for the antipsychotic and anxiolytic properties of CBD, and suggest that CBD might be

able to counterbalance THC induced effects (Colizzi and Bhattacharyya, 2017). However, CBD concentrations needed to offset the effects of THC in healthy individuals are still unclear, as CBD might also have different effects when administered at different doses (Solowij et al., 2019).

Acute CBD administration also affected brain networks of subjects diagnosed with a psychiatric disorder. In individuals at CHR for psychosis, CBD showed intermediate activity compared to patients receiving placebo and healthy subjects in regions involved in reward and salience processing (Bhattacharyya et al., 2018; Wilson et al., 2019). A similar intermediate activity was reported in subjects with established psychosis during a memory task (O'Neill et al., 2020). These findings are consistent with the enhanced activity observed in fronto-striatal regions in healthy subjects after CBD (Bhattacharyya et al., 2015; Grimm et al., 2018). Altogether, these findings suggest that CBD could contribute to normalise disbalanced fronto-striatal activity in patients at CHR or with established psychosis. In addition, Crippa and colleagues showed that CBD reduced cerebral blood flow in (para) limbic areas (i.e., hippocampus, parahippocampal and inferior temporal gyrus) in subjects with social anxiety (Crippa et al., 2011). This is congruent with decreased fronto-limbic activity in healthy individuals reported after CBD (Crippa et al., 2004; Fusar-Poli et al., 2010), and suggest that the anxiolytic effects of CBD may be related to the capacity of this compound to modify brain activity in (para) limbic areas (Crippa et al., 2011). Finally, a spectroscopy study in autism spectrum disorder and healthy controls showed similar glutamate (i.e., increased in basal ganglia, and decreased in prefrontal cortex in both groups) and opposite GABA (i.e., decreased levels in patients and increased in controls in both basal ganglia and prefrontal cortex) effects after CBD administration (Pretzsch et al., 2019). This study adds to preclinical evidence that CBD may modulate the activity of other neurotransmitters, even after a single dose (Crippa et al., 2018). This has implications for the homeostasis of other neurotransmitter systems, such as glutamate, GABA and dopamine. However, the underlying molecular mechanisms explaining the relationship between CBD and other neurotransmitters needs further study.

One of these molecular mechanisms may involve the ability of CBD to directly inhibit the reuptake of anandamide. This endocannabinoid has shown anti-inflammatory activity (Pisanti et al., 2017), and its increase after CBD has been related to antipsychotic effects (Leweke et al., 2012; Rohleder et al., 2016). Because endocannabinoids act as retrograde messengers, it has been hypothesized that increased endocannabinoid concentrations after CBD may attenuate presynaptic release of GABA and glutamate, as well as stabilise dopamine neurotransmission (Gururajan and Malone, 2016). In addition, most of the reported effects after CBD administration occurred in brain areas rich in CB_1 receptors (Burns et al., 2007). Chronic cannabis use is associated with reductions in endogenous cannabinoids and down-regulation of CB_1 receptors (Hirvonen et al., 2012; Morgan et al., 2013), while CBD antagonistic effects could be related to modulation of cannabinoid receptors by binding to a distinct allosteric site (Laprairie et al., 2015). Given that CBD may attenuate THC effects, it has also been speculated that CBD may be able to prevent down-regulation of CB_1 receptors on the long-term, and thus decrease the risk of developing psychosis and/or substance use disorders (Wall et al., 2019). Other possible mechanisms of action of CBD involve its agonist activity towards $5HT_{1A}$ receptors (Soares and Campos, 2017), partial agonist activity on dopamine D2 receptors (Seeman, 2016), and the activation of vanilloid receptor 1, a non-selective calcium channel, facilitating glutamate pre-synaptic release (Campos et al., 2012).

This review must be read with a series of limitations taken into account. First, included papers often employed different methodologies (e.g., imaging method, route of administration, applied doses), although we used strict inclusion and exclusion criteria for article selection to avoid excessive heterogeneity between studies. For example, whereas most studies administered CBD and THC as individual cannabinoid compounds in separate sessions (Borgwardt et al., 2008; Bhattacharyya et al., 2012b; Grimm et al., 2018), some studies examined the impact of CBD on brain function by comparing effects of cannabis containing THC only to cannabis with both THC and CBD (Freeman et al., 2018; Wall et al., 2019). Regarding differences in applied cognitive paradigms, it is important to note that the described effects on brain activity might depend on the nature of the task used or stimuli presented, as different tasks might provoke distinct brain activity patterns. For instance, whereas memory paradigms may heavily rely on recruitment of temporal and prefrontal areas (Bhattacharyya et al., 2009; Bhattacharyya et al., 2018; O'Neill et al., 2020), emotional and salience processing mainly involve limbic activation (Fusar-Poli et al., 2009; Bhattacharyya et al., 2012b). One final methodological aspect that should be taken into account is that the clinical and brain effects of CBD might be different depending on age and illness progression (Di Marzo et al., 2015; Batalla et al., 2019; Colizzi et al., 2020), or be influenced by the concomitant use of medication (e.g., antipsychotics) or drugs of abuse. However, the within-subject design of the studies where concomitant use of medication or cannabis was allowed probably mitigated these confounding effects (**Table 3**). Second, most of the included studies used overlapping samples of mainly male healthy subjects or patients with psychiatric disorders to explore the effects of CBD. Although the studies reviewed herein offer a consistent picture indicating that CBD has modulatory effects over neural networks relevant for psychosis, anxiety and addiction, this highlights the need for replication of findings in independent and larger cohorts also including female subjects.

Suggestions can be made for future research on the impact of CBD on brain function. First, because all studies included in the current review examined the acute effects of CBD administration, future research should focus on longer-term CBD treatment of patients with a psychiatric disorder in combination with neuroimaging assessments, in order to elucidate neural substrates underlying the therapeutic effects of CBD. In this respect, two excellent examples of studies nearing completion are 1) 3 weeks CBD treatment of individuals at CHR for psychosis (Institute of Psychiatry, King's College London), and 2) 4-week add-on CBD treatment of early-onset patients with a psychotic disorder (University Medical Center Utrecht, Netherlands), both in combination with baseline and follow-up functional MRI and ^{1}H-MRS techniques. Second, because the clinical response to

CBD has been shown to differ between patients (Batalla et al., 2019), future studies could also apply neuroimaging techniques to contribute to identification of those patients that may particularly benefit from CBD treatment.

In conclusion, neuroimaging studies have shown that CBD modulates brain activity and connectivity in neural systems relevant for psychosis and anxiety, possibly reflecting CBD's therapeutic effects. Future studies should consider replication of findings and enlarge the inclusion of psychiatric patients, combining longer-term CBD treatment with neuroimaging assessments.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AB and JB performed the systematic search and drafted the manuscript, and MB screened potentially eligible publications and critically revised the manuscript for important intellectual content. AB, JB, AP, and MB provided critical revisions for important intellectual content and significantly contributed to the manuscript. All authors have read and agreed to the published version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.618184/full#supplementary-material.

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REVIEW
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Is Cannabidiol During Neurodevelopment a Promising Therapy for Schizophrenia and Autism Spectrum Disorders?

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Schizophrenia and autism spectrum disorders (ASD) are psychiatric neurodevelopmental disorders that cause high levels of functional disabilities. Also, the currently available therapies for these disorders are limited. Therefore, the search for treatments that could be beneficial for the altered course of the neurodevelopment associated with these disorders is paramount. Preclinical and clinical evidence points to cannabidiol (CBD) as a promising strategy. In this review, we discuss clinical and preclinical studies on schizophrenia and ASD investigating the behavioral, molecular, and functional effects of chronic treatment with CBD (and with cannabidivarin for ASD) during neurodevelopment. In summary, the results point to CBD's beneficial potential for the progression of these disorders supporting further investigations to strengthen its use.

Keywords: cannabidiol, Cannabidivarin, schizophrenia, Autism, neurodevelopmental disorders, Prodrome, Prevention, animal models

INTRODUCTION

Brain development is a critical period for an individual's life; many physiological changes occur during this period, such as neurogenesis and neuronal migration, axonal growth and dendritic maturation, the establishment of nerve cell networks, the formation of new synapses, the proliferation of glial cells, and the myelination (Andersen, 2003). The events and experiences during neurodevelopment will affect the individual's behavioral phenotype and his/her future mental health. It is well established that disturbances occurring throughout critical periods of brain development can disrupt normal brain maturation leading to long-lasting pathological alterations. This highlights the impact of environmental insults on neurodevelopmental psychopathologies such as autism spectrum disorder (ASD) and schizophrenia (Ikonomidou et al., 1999; Kaindl and Ikonomidou, 2007; Dawson et al., 2014; Nicolini and Fahnestock, 2018; Lord et al., 2020). In schizophrenia, a substantial amount of evidence suggests that these disturbances occur during neurodevelopment and are brought about by a combination of genetic and environmental risk factors (Harrison and Weinberger, 2005; Owen et al., 2016; Seshadri et al., 2018). Early periods of brain development are also critical for the establishment of ASD. Even though genetic and epigenetic factors are significant risk factors, environmental events such as gestational

and/or perinatal complications could increase the risk of ASD development (Lord et al., 2020). Although the association between neurodevelopmental injuries and neuropsychiatric disorders is not restricted to ASD and schizophrenia, these two disorders share considerable clinical and neurobiological features, ranging from risk factors (e.g., maternal immune activation) to symptoms (such as social disabilities and cognitive deficits) (Boulanger-Bertolus et al., 2018; Barlati et al., 2020). ASD symptoms are frequently observed in patients with schizophrenia and vice versa, with the severity of ASD symptoms being a possible predictor of the severity of schizophrenia symptoms (Barlati et al., 2020).

Furthermore, they also share some pathophysiological mechanisms such as neuroinflammation (Bjorklund et al., 2016; Cattane et al., 2018; Araujo et al., 2019), reduction in thalamus volume, amygdala and thalamus dysfunctions when processing social stimuli (Barlati et al., 2020), as well as glutamatergic, GABAergic (Cattane et al., 2018), and endocannabinoid (ECB) system dysfunctions (Zamberletti et al., 2017; Zador et al., 2019; Borgan et al., 2020; Pietropaolo et al., 2020). The ECB system is widely expressed in the central nervous system, playing roles in synaptic plasticity regulation through retrograde signaling. In a strict sense, it is composed of the cannabinoid receptors type 1 (CB_1, which is widely expressed in the nervous system) and type 2 (CB_2, mainly expressed in immune cells), their endocannabinoid signaling molecules (e.g., anandamide (AEA); and 2-arachidonoylglycerol (2-AG)), and their metabolic enzymes (NAPE-PLD, DAGL, FAAH, and MAGL) (Schonhofen et al., 2018).

In this context, the *Cannabis sativa* second-most abundant compound, cannabidiol (CBD), emerges as a potential treatment for these neurodevelopmental psychiatric disorders. CBD is an ECB system modulator that also presents several other mechanisms of action [for detailed information, see Peres et al. (2018b); Schonhofen et al. (2018)]. CBD exerts its effects on both developing and mature brains through several mechanisms, such as modulating the ECB system (either directly via cannabinoid receptors or indirectly by regulating endocannabinoid levels), being an agonist of the vanilloid receptor $TRPV_1$, facilitating serotoninergic transmission through $5\text{-}HT_{1A}$ receptors, and interacting with the peroxisome proliferator-activated receptor γ (PPARγ) acting on G-protein-coupled receptor (such as GPR55, GPR3, GPR6, and GPR12) and anti-inflammatory and antioxidant actions.

In this review, we will discuss behavioral and molecular aspects of both clinical and preclinical studies investigating the effects of CBD during neurodevelopment as a potential therapy for ASD and schizophrenia.

General Aspects of Schizophrenia

Schizophrenia is a psychiatric neurodevelopmental disorder with a lifetime prevalence of just under 1% (Kahn et al., 2015), with the burden of the disease increasing globally (Charlson et al., 2018). It stands out as one of the most debilitating psychiatric disorders because it impairs brain functioning in multiple ways, triggering the expression of positive symptoms (psychosis, characterized by hallucinations, delusions, and disorganized speech), negative symptoms (social dysfunction, avolition, among others), and cognitive symptoms. Negative and cognitive symptoms are more enduring and can precede the first psychotic episode by years, characterizing the prodromal phase (Marenco and Weinberger, 2000; Munro et al., 2002; Schenkel and Silverstein, 2004; Schenkel et al., 2005; Insel, 2010; Larson et al., 2010; Dawson et al., 2014; Millan et al., 2016). More recently, it has been argued that pharmacological interventions during the prodromal phase could delay or even prevent the full-blown manifestation of schizophrenia and preclinical data support this hypothesis (Piras et al., 2014; Gomes et al., 2016; Sommer et al., 2016; Hashimoto, 2019). The establishment of preventive strategies for schizophrenia is essential since the currently available treatment with antipsychotics is most effective for positive symptoms, but ineffective in preventing or slowing schizophrenia progression, besides inducing some serious side effects. On the other hand, there are a significant number of adolescents and young adults presenting reduced social abilities, attenuated psychotic symptoms, and progressive decline in functioning—the so-called individuals at "ultra-high risk" for psychosis—who will not convert to the full-blown manifestation of psychosis (Sommer et al., 2016; Ding et al., 2019). Therefore, potential preventive pharmacological approaches should be beneficial in ameliorating the neurodevelopmental changes associated with schizophrenia. At the same time, they must be safe enough for the approximately 60–70% of at-risk individuals that will not convert to the disorder (Gee and Cannon, 2011; Mokhtari and Rajarethinam, 2013; Piras et al., 2014).

The full comprehension of the mechanisms that underlie schizophrenia progression from the prodromal phase (or earlier) until establishing a psychotic acute state is far from complete. However, at least a portion of these mechanisms have already been elucidated. Impaired functional integration between brain subsystems (e.g., between the hippocampus and the prefrontal cortex (PFC)) and dysfunctions in the organization of brain networks has been suggested to be responsible for the neurocognitive deficits observed in schizophrenia (Peled et al., 2001; Kim et al., 2003; Kim et al., 2005; Meyer-Lindenberg et al., 2005; Benetti et al., 2009; Lee et al., 2012; Dawson et al., 2014; Oh et al., 2017). Neuroinflammation and oxidative stress are also implicated in neurodevelopmental alterations associated with this disorder (Buckley, 2019; Lin and Lane, 2019). Impairments in neurotransmission functions are also described, such as the compromised dopaminergic system in the mesocortical, mesolimbic, and nigrostriatal pathways (Guillin et al., 2007; McCutcheon et al., 2019), the glutamatergic hypofunction in the PFC (Bondi et al., 2012; Snyder and Gao, 2020), and GABAergic, serotoninergic, and ECB system dysfunctions (Eggers, 2013; Schmidt and Mirnics, 2015; Fakhoury, 2017; Cattane et al., 2018; Zador et al., 2019).

Some clinical and preclinical evidence suggests the antipsychotic property of CBD (Zuardi et al., 2012; Saito et al., 2013; Rohleder et al., 2016; Schoevers et al., 2020). Furthermore, CBD does not promote the side effects commonly induced by the traditional antipsychotic drugs (Briles et al., 2012; Leweke et al., 2012; Gomes et al., 2013; Dos-Santos-Pereira et al., 2016; Park

et al., 2018). In contrast, the effects that preventive treatments with CBD might have on behavioral and molecular aspects of schizophrenia neuroprogression are still being debated and will be reviewed here.

General Aspects of Autism Spectrum Disorder

Autism spectrum disorder (ASD) is the fastest-growing neurodevelopmental disorder worldwide, affecting about 1% of the global population and presenting a prevalence four times higher in boys than in girls (Bonnet-Brilhault, 2017; Maenner et al., 2020). According to the DSM-V, ASD core symptoms include impairments in social communication and interaction, restricted or repetitive behaviors, and sensory abnormalities, usually associated with cognitive deficits, intellectual disability, and language delay (American Psychiatric Association, 2013). Also, at least one comorbidity such as epilepsy, gastrointestinal and sleep disorders, and mental health conditions (anxiety, depression, attention-deficit/hyperactivity disorder, and obsessive-compulsive disorder) are present in more than 95% of the patients. At least four comorbidities are associated with ASD in 70% of the cases (Soke et al., 2018). The presence of comorbidities causes a delay in diagnosis, which occurs on average at 4 years old or later (Miodovnik et al., 2015). On the other hand, clinical evidence suggests that the probability of treatment success and the improvement in children's outcomes increase when interventions occur at very-early ages (2 years old or earlier) (Dawson et al., 2010; Anderson et al., 2014; MacDonald et al., 2014; Rogers et al., 2014; Estes et al., 2015; Pierce et al., 2019).

While improvements in ASD diagnosis have been achieved and cannot be disregarded, early-age diagnostic stability is still not optimal (due to the overlap of clinical symptoms between ASD and other disorders). For this reason, the US Preventive Services Task Force has not yet endorsed early universal screening for ASD (Siu et al., 2016). In contrast, ASD patients still need alternative treatment strategies since current available pharmacological therapies are scarce. Aripiprazole and risperidone (the only FDA-approved drugs for ASD) present limited efficacy besides inducing some side effects such as sedation, increased sleep duration, and weight gain (Tural Hesapcioglu et al., 2020). Therefore, promising therapies should be effective in treating ASD symptoms. Simultaneously, they must be safe enough for both ASD patients and the individuals who will eventually lose their ASD status in a final diagnosis.

The complexity of the pathophysiological mechanisms of ASD is still far from having been fully elucidated. However, knowledge of this topic has advanced considerably, shedding light on important aspects of the disorder. Monogenic mutations with a high risk for the development of ASD partially explain some autistic traits (Shemesh et al., 2016), but a high load of common low-risk variants is also associated with the development of the disorder (Chahrour et al., 2016; Griesi-Oliveira and Sertie, 2017). Moreover, ASD-distinctive genetic architecture produces highly heterogeneous behavioral phenotypes which produces unique symptoms for each patient (Griesi-Oliveira and Sertie, 2017; Lombardo et al., 2019), including some approaches that have classified ASD into subgroups according to the patients' phenotype (Jacob et al., 2019; Tillmann et al., 2020), while others attempt to classify ASD according to the different patients' genetic variants (Jeste and Geschwind, 2014). Alterations related to pleiotropic genes associated with ASD can be seen at distinct neurodevelopmental stages (Mitra et al., 2016; Courchesne et al., 2019). During the first and second trimesters of pregnancy, the autistic brain has a high rate of proliferation in the frontal and temporal cortex when compared to neurotypical brains (Courchesne et al., 2007; Courchesne et al., 2011). This leads to irregularities in migration as well as in maturation and differentiation of neurons that result in neural connectivity abnormalities, synaptogenesis damage, and brain overgrowth (Yenkoyan et al., 2017; Courchesne et al., 2019). Local hyperconnections are established in the cortex due to these changes, preventing the functioning of global long-distance connections between brain regions (Courchesne et al., 2007). These cortical changes are accompanied by disruptions in the excitation/inhibitory balance that can cause neuroinflammation and cell death by excitotoxicity (Fang et al., 2014; Courchesne et al., 2019). Other encephalic regions are also disrupted in ASD, including the thalamus and hypothalamus, the amygdala, the striatum, and the hippocampus (Ferhat et al., 2017; Barlati et al., 2020).

At a molecular level, several neurotransmission systems, such as the glutamatergic and the GABAergic (Cattane et al., 2018), are altered in ASD. Similarly, the ECB system (that plays an important role in the modulation of several signaling systems) has also been implicated in the pathophysiology of ASD and has become a target for the development of pharmacological therapies (Wei et al., 2016; Zamberletti et al., 2017; Pietropaolo et al., 2020). Preclinical evidence suggests that its modulation impacts socioemotional reactivity (Servadio et al., 2016; Wei et al., 2016; Folkes et al., 2020), stereotyped behaviors (Servadio et al., 2016; Melancia et al., 2018), learning and memory (Griebel et al., 2015; Qin et al., 2015; Melancia et al., 2018), susceptibility to seizures (Kaplan et al., 2017; Patra et al., 2019; Patra et al., 2020), and regulation of circadian rhythm (Atkinson et al., 2010; Vaughn et al., 2010). All of them are directly or indirectly related to ASD (for detailed review, see Zamberletti et al., 2017).

REVIEWED STUDIES ON SCHIZOPHRENIA

The terms "cannabidiol" and "schizophrenia" were paired with "neurodevelopment," "development," or "preventive" for the search of clinical and preclinical studies in the PubMed database. The inclusion criteria were a) describing the use of CBD-containing products and medications and b) the treatments occurring chronically and during neurodevelopment (from early ages up to late adolescence/beginning of adulthood). Our search yielded only ten results, all on preclinical studies (**Table 1**). The low number of studies highlights that even though schizophrenia has been recognized for over two decades as a

neurodevelopmental disorder (Insel, 2010; Kahn et al., 2015) and that CBD has shown potential antipsychotic properties (Zuardi et al., 2006; Zuardi et al., 2012; Iseger and Bossong, 2015), its use as a potential preventive strategy for at-risk individuals is still poorly explored (Lambert et al., 2016). Four of the studies used a peripubertal/adolescence CBD treatment without continuing it throughout adulthood (Peres et al., 2016a; Peres et al., 2018a; Stark et al., 2019; Stark et al., 2020). In the other six, CBD administration started at late adolescence and extended throughout adulthood (Gomes et al., 2014; Gomes et al., 2015; Osborne et al., 2017; Osborne et al., 2019a; Osborne et al., 2019b; Jimenez Naranjo et al., 2019). Considering the long-term effects of CBD as a preventive strategy, it should be noted that, in four studies (Osborne et al., 2017; Osborne et al., 2019a; Osborne et al., 2019b; Jimenez Naranjo et al., 2019), the chronic preventive effect of CBD could be confounded with a subacute effect (or even an acute effect). In the other two studies, CBD administration occurred concomitantly with the pharmacological induction of the schizophrenic-like phenotype (Gomes et al., 2014; Gomes et al., 2015).

Long-Lasting Effects of Cannabidiol Administration as a Preventive Strategy

This section will discuss the long-lasting impact of CBD treatment during earlier periods of development (peripubertal/adolescence) on schizophrenia-like phenotypes in adulthood. Three different schizophrenia animal models were used in these studies: maternal immune activation (MIA) through polyinosinic:polycytidylic acid (poly I:C) administration during the gestational period (Meyer and Feldon, 2012; Haddad et al., 2020), the late gestational antimitotic administration of methylazoxymethanol acetate (MAM) (Lodge and Grace, 2009; Sonnenschein and Grace, 2020), and the spontaneous development of schizophrenia-like behaviors in the Spontaneously Hypertensive Rat (SHR) strain (Calzavara et al., 2009; Calzavara et al., 2011a; Calzavara et al., 2011b; Levin et al., 2011). Chronic administration of CBD during periadolescence presented several benefits regarding the emergence of a schizophrenic-like phenotype in all studies (Peres et al., 2016a; Peres et al., 2018a; Stark et al., 2019; Stark et al., 2020). First, CBD-treated animals showed neither prepulse inhibition of startle deficits (PPI) in the SHR strain model nor spontaneous hyperlocomotion in both the SHR strain and poly I:C models (Peres et al., 2016a; Peres et al., 2018a), behavioral alterations that mimic sensorimotor gating deficits and positive-like symptoms, respectively (van den Buuse, 2010; Almeida et al., 2014; Peres et al., 2016b). Also, cognitive improvements after chronic treatment with CBD were reported for deficits both in the contextual fear conditioning paradigm (CFC, a long-term associative memory task) in the SHR strain model (Peres et al., 2018a) and in the novel object recognition task (NOR, an explicit short-term memory) in the gestational MAM model (Stark et al., 2019). These findings show that the CBD benefits for behaviors that mimic cognitive symptoms are not restricted to a single behavioral phenotype, as they encompass aversive and nonemotional related behaviors, as well as short- and long-term memories. Regarding CBD effects on social interaction impairments, a series of behaviors that mimics the negative symptoms (Almeida et al., 2014; Miyamoto and Nitta, 2014; Wilson and Koenig, 2014), the findings are not consistent. Stark and colleagues (2019) observed improvement in MAM offspring's social behaviors after CBD treatment, while Peres and colleagues (2018a) did not observe any improvement in the SHR strain's poor social performance, suggesting that CBD effects on social behaviors can be model-dependent. In parallel, another possible explanation is that CBD effects on social behaviors present a dose-dependent profile since a low range of dosage (0.5, 1, 5, and 10 mg/kg/day) (Peres et al., 2018a; Stark et al., 2019) did not improve social behavior deficits, while a higher dosage (30 mg/kg/day) did (Stark et al., 2019). These results suggest long-lasting beneficial effects of CBD for behaviors that mimic different symptoms of schizophrenia when the treatment occurs during the peripubertal/adolescence period. Clinical and preclinical evidence has already reported that treatment with CBD reduced psychotic symptoms of schizophrenia (Zuardi et al., 2006; Zuardi et al., 2012; Peres et al., 2016b). These studies expand the beneficial effects of CBD, suggesting that it could also be considered as a preventive strategy for at-risk individuals.

Considering the safety requirements of a novel long-term treatment for individuals at risk that will not convert to schizophrenia, potential side effects of prolonged early treatment with CBD were also investigated in these studies. Regarding the positive-, negative-, and cognitive-like behaviors assessed, the authors reported that CBD treatment did not induce any impairment on control animals. In addition, Peres et al. (2018a) observed that chronic CBD treatment did not cause other behavioral alterations (such as catalepsy and oral dyskinesia) or metabolic dysfunctions (such as altered body weight gain, serum levels of glucose, and triglycerides) in both Wistar and SHR strains. Importantly, the absence of behavioral and metabolic dysfunctions following prolonged CBD treatment was observed both immediately and one month after CBD discontinuation. These findings present high translational relevance because CBD showed significant improvements for core schizophrenic-like behaviors without inducing side effects commonly associated with antipsychotic drugs (Muench and Hamer, 2010; Briles et al., 2012; Park et al., 2018). On the other hand, undesired effects of prolonged treatment with CBD have been reported in patients of a wide age range (as reviewed in Schonhofen et al., 2018) and also in mice during peripubertal/adolescence periods (Carvalho et al., 2018a; Carvalho et al., 2018b; Carvalho et al., 2020), highlighting the importance of studies evaluating specifically the potential side effects of chronic treatment with CBD.

Neurochemical alterations following chronic CBD administration were also reported. Stark et al. (2019) investigated the ECB system in the gestational MAM model. They observed increased CB_1 expression in the PFC as a result of reduced *CNR1* promoter DNA methylation and consequent increase in CB_1 mRNA expression. These changes were reversed by early chronic treatment with 30 mg/kg/day CBD. The content of the ECB molecules, AEA and 2-AG, and

ECB-related molecules, N-palmitoylethanolamide (PEA) and N-oleoylethanolamide (OEA), were also assessed in the PFC. They observed that chronic treatment with CBD increased AEA only in the control offspring and affected 2-AG levels distinctly in control and MAM offspring and that these findings did not directly explain the behavioral alterations. Regarding the dopaminergic neurotransmission, Stark et al. (2020) observed an increased D_2 mRNA content in PFC of MAM offspring that was not affected by chronic treatment with CBD. Intriguingly, alterations in D_2 mRNA content did not reflect changes either in D_2 protein expression or in DNA methylation of D_2 gene regulatory regions that were not affected by the MAM insult or CBD treatment. They also found that D_3 mRNA content was increased in PFC, hippocampus, and NAc of the MAM offspring, while treatment with CBD reduced it in all three regions without altering it in control offspring. In fact, D_3 mRNA content was almost absent in PFC and NAc of the MAM offspring treated with CBD. However, similar to D_2 results, D_3 mRNA content alterations did not reflect DNA methylation changes of D_3 gene regulatory regions while D_3 protein expression was not evaluated. An absence of effect of CBD on the dopaminergic system was reported by Peres and colleagues (2018a): the early long-term treatment did not change the increased dopamine levels in PFC of the SHR strain at 90th postnatal day with CBD (lower doses than the 30 mg/kg/day used in the study by Stark et al., 2020). Additionally, Stark et al. (2020)—using molecular modeling approaches—proposed that CBD may act as a weak partial agonist of D_3 receptors once it can favorably bind to dopamine D_3 rather than to dopamine D_2 receptors. This finding is in accordance with a previous study that computationally predicted the D_3 receptor as a potential target for CBD (Bian et al., 2019). Nevertheless, D_2 receptors cannot be disregarded as a potential target for CBD, since CBD has also been proposed to act as a partial agonist of these receptors, similarly to the antipsychotic aripiprazole (Seeman, 2016).

Besides the CBD effects on ECB and dopaminergic systems discussed above, chronic CBD treatment effects on the serotoninergic system and the brain-derived neurotrophic factor (BDNF) were also reported for the SHR strain model (Peres et al., 2018a). The authors found that the SHR strain presents reduced levels of serotonin in PFC at the 61st but not at the 90th postnatal day and that chronic CBD treatment was not able to recover it. On the other hand, increased levels of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) were observed in the PFC of both Wistar- and SHR-treated animals one month after CBD discontinuation. In the same direction, the 5-HIAA/serotonin ratio was also increased one day after CBD administration ceased, although a more pronounced effect was observed in the SHR strain. Regarding BDNF levels, no CBD effects were reported. These data suggest that chronic treatment with CBD during peripubertal/adolescence periods increases serotonin turnover in the PFC and supports the role of the serotoninergic system in the CBD effects on the brain (Russo et al., 2005; Linge et al., 2016).

Finally, neuroanatomical and functional alterations were also evaluated (Stark et al., 2020). An elevated regional cerebral blood flow (CBF) in the circle of Willis and a regional CBF reduction in the hippocampus were observed in the MAM offspring, following other clinical and preclinical studies showing altered CBF in schizophrenia (Goozee et al., 2014; Drazanova et al., 2018; Drazanova et al., 2019). Chronic treatment with CBD reversed the changes in the circle of Willis but not in the hippocampus (Stark et al., 2020). Moreover, CBD reduced regional CBF in the somatosensory cortex of MAM offspring but not of control offspring. No alterations were observed in relation to PFC and NAc. In parallel, the enlargement of lateral ventricles—a structural alteration commonly observed in both patients and animal models of schizophrenia (Le Pen et al., 2006; Kempton et al., 2010)—in the MAM offspring was not prevented by the long-term treatment with CBD (Stark et al., 2020). Interestingly, although the authors have not discussed the possible relationship between the CBF and the anatomical changes, the enlargement of lateral ventricles could be a consequence of the reduced hippocampal blood flow resulting in a reduction of the hippocampal volume, as observed by Stark et al. (2020) and by others that also used the gestational MAM model to investigate this issue (Le Pen et al., 2006). Even though this topic needs to be further explored, it seems that neither chronic treatment with CBD nor chronic treatment with an antipsychotic drug (haloperidol) can reverse these neuroanatomical and functional alterations (Stark et al., 2020).

Despite the limited number of studies investigating the effects of CBD treatments during an early prodromal-like phase of schizophrenia (so far, only three studies investigated its impact on animals' behavior), the results pointing out the benefits for its use are quite robust and promising. Nevertheless, it remains unclear whether CBD administration is hindering the emergence of schizophrenia-like behaviors or reversing the early signs already present in a prodromal phase. Some aspects of schizophrenia-like behaviors in those animal models were previously described and speculations can be inferred from them. In the SHR strain, social impairments and CFC deficits have already emerged during puberty/adolescence, while spontaneous hyperlocomotion and PPI deficits appear only during adulthood (Niigaki et al., 2019). Similar results about the early emergence of social impairments and the late emergence of hyperlocomotion were observed in other animal models, including the gestational MAM model (Sams-Dodd et al., 1997; Le Pen et al., 2006). Also, the early emergence of cognitive deficits (Su et al., 2014; Latusz et al., 2017) and the late emergence of PPI deficits were also reported in other animal models, including the MIA (through poly I:C administration) and the gestational MAM models (Le Pen et al., 2006; Ozawa et al., 2006; Uehara et al., 2010; Latusz et al., 2017; Takahashi et al., 2019). These preclinical results are in agreement with the course of schizophrenia: the early appearance of negative- and cognitive-like symptoms (i.e., a prodromal phase) followed by a later emergence of sensorimotor gating deficits and positive-like symptoms (Marenco and Weinberger, 2000; Larson et al., 2010; Millan et al., 2016). Based on the above-discussed reports, even though there are some conflicting results about the timing in which the emergence of the behavioral alterations occurs (Le Pen et al., 2006; Takahashi et al., 2019), it can be speculated that early chronic treatment with CBD during

peripubertal/adolescence may be able to recover the already established behavioral deficits and/or prevent the emergence of the late abnormalities observed in schizophrenic-like models. Notably, CBD effects last more than a month after the treatment was discontinued, suggesting that prolonged treatment with CBD during a "prodromal phase" induced long-lasting brain changes that altered the course of the pathophysiological mechanisms underlying schizophrenia, delaying the progression of the disorder.

Effects of Prolonged Cannabidiol Administration During Later Periods of Development on the Schizophrenia-Like Phenotype

This section will discuss CBD treatment's impact during later periods of development (end of adolescence/early adulthood) on the schizophrenia-like phenotype in adulthood. Two different schizophrenia animal models were used: the already mentioned MIA through poly I:C administration during the gestational period (Osborne et al., 2017; Osborne et al., 2019a; Osborne et al., 2019b; Jimenez Naranjo et al., 2019) and a late adolescence/early adulthood transient NMDA receptor antagonism model (Li et al., 2011; Uttl et al., 2018; Ma et al., 2020) through daily MK-801 administration during 28 days (Gomes et al., 2014; Gomes et al., 2015). Similar to the above-discussed data, prolonged administration of CBD during late adolescence/early adulthood also presented several benefits regarding the manifestation of a schizophrenia-like phenotype in all the studies. Osborne and colleagues (2017, 2019a) showed that MIA through poly I:C administration in the dams induced social impairments and cognitive deficits in male and female offspring. Interestingly, working memory deficits in the "rewarded T-maze test" at early adulthood were sex-dependent, being observed only in male offspring. Short-term explicit memory impairment in the NOR task was observed in both male and female offspring, suggesting that different cognitive processes are affected in distinct ways in this model. Regardless of the sex, prolonged treatment with CDB (10 mg/kg twice a day, i.e., 20 mg/kg/day) from PND56 to PND80 attenuated all the behavioral impairments evaluated. In contrast, control females treated with CBD presented a reduction in social interaction that was not observed in male ones. Although this result indicates a putative sex-specific side effect of CBD in healthy individuals, this study's experimental design does not allow identifying if this alteration is a consequence of chronic or acute CBD administration. Moreover, from the ten studies included in this review (**Table 1**), only one of them evaluated behavioral alterations in females, challenging the discussion of a possible sex-dependent effect of CBD.

Effects of prolonged treatment with CBD on social performance and short-term explicit memory impairments were also evaluated in the transient NMDA receptor antagonism model through chronic MK-801 administration at late adolescence/early adulthood (Gomes et al., 2015). The authors found that treating the animals for 23 days (starting on the sixth day after the first MK-801 administration) with 60 mg/kg/day CBD, but not 30 mg/kg/day, attenuated negative- and cognitive-like symptoms (in the social interaction test and the NOR, respectively). They also found that neither the late chronic MK-801 administration nor the late prolonged treatment with CBD induced changes in locomotor behaviors (in the OF task) and anxiety-like behaviors (in the EPM task), which are in accordance with some other reports (Li et al., 2011; Schiavon et al., 2016; Uttl et al., 2018) but not with others (ElBatsh et al., 2012; Uttl et al., 2018) that investigated their effects in similar age periods. Although further investigation is needed, the use of distinct species/strains and protocols to investigate CBD or MK-801 effects in these studies can account for the different outcomes (Viola and Loss, 2014; Uttl et al., 2018). In another study, Gomes and colleagues (2014) investigated the effects of the same prolonged treatment with CBD on sensorimotor gating deficits induced by the same protocol (chronic MK-801 administration at late adolescence). Their results suggest that prolonged treatment with 60 mg/kg/day CBD produced only a slight attenuation of PPI impairments.

These studies follow the data discussed in the previous topic, giving further support for the beneficial effects of CBD even when its administration occurs during late periods of neurodevelopment. Nevertheless, it should be noted that some of the results are conflicting (e.g., the effects of prolonged treatment with CBD on anxiety-like behaviors) and that the data are scarce (so far, only four studies investigated the effects of prolonged treatment with CBD during late adolescence/early adulthood on schizophrenia-like behaviors).

Side effects of prolonged treatment with CBD were poorly explored in the above-mentioned studies. Osborne and colleagues' (2017, 2019a) findings suggest a sex-dependent effect of poly I:C treatment on body weight and water intake but not on food intake. Poly I:C female offspring seem to be heavier and consume more water at adulthood than the control female offspring. No differences in these variables were observed in male subjects. Regarding the transient NMDA receptor antagonism model, no conclusions can be drawn about the influence of sex on these variables, since only males were used in these studies (Gomes et al., 2014; Gomes et al., 2015). Similar to the poly I:C model, MK-801 male subjects did not present differences in body weight when compared to control subjects. Regardless of the sex and the schizophrenia-like model, prolonged treatment with CBD did not induce any alteration in these variables. Therefore, besides the above-discussed decreased social interaction observed in females, no other adverse effects of prolonged treatment with CBD during late development periods were reported in these studies. However, some studies observed the emergence of adverse effects after repeated CBD administration in similar age periods, such as increased anxiety-like behaviors (ElBatsh et al., 2012) and decreased neurogenesis (Schiavon et al., 2016), highlighting the fact that further confirmatory studies are needed.

Molecular and functional alterations in the brain following prolonged treatment with CBD were also reported. Regarding the ECB system, Osborne and colleagues (2019a, 2019b) observed that in the poly I:C model CB_1 binding density was affected in a

sex-dependent way. While CB_1 binding density was decreased in the PFC of poly I:C male offspring, it was not altered in female ones. The prolonged treatment with CBD reversed the changes in male offspring (Osborne et al., 2019b). In addition, it decreased CB_1 binding density in the control female offspring (Osborne et al., 2019a).

Regarding FAAH expression, it was not affected either in the poly I:C and control offspring, independently of the sex and of the treatment with CBD (Osborne et al., 2019a; Osborne et al., 2019b). In contrast to the decreased CB_1 binding density found in the above-mentioned study, the previously discussed study by Stark et al. (2019) found an increased CB_1 expression in MAM male offspring. Moreover, early chronic treatment with CBD (in a different dose and developmental period) in MAM male offspring reversed this change by reducing CB_1 expression to control levels (Stark et al., 2019) while in the study by Osborne et al. (2019b) the late prolonged treatment with CBD in poly I:C male offspring normalized CB_1 binding density by increasing it to control levels. Together, these results suggest that the CB_1 receptor is affected distinctly in the different models and by the different protocols of CBD administration.

Sex-dependent results were also found for the glutamatergic system, in which the poly I:C model decreased NMDA receptor binding density in the PFC of female offspring (Osborne et al., 2019a), but not of male ones (Osborne et al., 2019b). Interestingly, expression of the obligatory GluN1 subunit was unaffected in either the poly I:C and control offspring, independently of the region analyzed (PFC or hippocampus), and the sex and the treatment with CBD (Osborne et al., 2019a; Osborne et al., 2019b), suggesting that gestational poly I:C injection is affecting the functionality of the glutamatergic system (glutamate synthesis, release, or reuptake, for instance, or even the composition of NMDA receptor) without necessarily interfering in the amount of NMDA receptor expressed. Prolonged treatment with CDB (10 mg/kg twice a day; i.e., 20 mg/kg/day) from PND56 to PND80 effectively reverted the decreased NMDA receptor binding density in the poly I:C female offspring. In contrast, in control female offspring, it decreased NMDA receptor binding density in the PFC similarly to gestational injection of poly I:C (Osborne et al., 2019a). These data are not in accordance with Gomes and colleagues' study (2014) that showed no alteration in *GRIN1* mRNA expression in the PFC and striatum of male mice subjected to chronic MK-801 administration (daily injections for 28 days) at late adolescence/early adulthood but did show a decrease in the hippocampus. This change was slightly attenuated when prolonged treatment with 60 mg/kg/day CBD occurred concomitantly (for 23 days) with MK-801 administrations.

Regarding the GABAergic system, Osborne et al. (2019a); Osborne et al. (2019b) reported that prolonged treatment with CBD increased parvalbumin (PV) expression in the hippocampus (but not in the PFC) regardless of the gestational manipulation or the sex of the offspring, while gestational poly I:C injection did not induce any alteration *per se*. On the other hand, Gomes et al. reported a decreased number of PV-positive cells in the PFC (but not in the striatum or the hippocampus) of male mice subjected to chronic injections of MK-801 during late adolescence/early adulthood (Gomes et al., 2014). This alteration was slightly attenuated when CBD was concomitantly administrated. It is important to note that these results are not necessarily conflicting, because the expression of PV can be altered without affecting the number of PV-positive cells and vice versa. Sex-dependent effects were reported for GAD_{67} expression (Osborne et al., 2019a; Osborne et al., 2019b). Gestational poly I:C injection decreased GAD_{67} expression in the hippocampus of male offspring but not female ones. Prolonged treatment with CBD increased hippocampal expression of GAD_{67} regardless of the sex or gestational manipulation, bringing it back to control levels in male offspring while increasing it above control levels in female ones. No alterations were observed regarding $GABA_A$ receptor binding density (Osborne et al., 2019a; Osborne et al., 2019b).

The effects of prolonged treatment with CBD on the cholinergic system were also investigated. Jimenez Naranjo et al. (2019) results showed that gestational poly I:C administration reduced muscarinic M1/M4 receptors binding density in the PFC and hippocampus of male offspring, while the prolonged treatment with CDB (10 mg/kg twice a day; i.e., 20 mg/kg/day) from PND56 to PND80 slightly attenuated this alteration in the poly I:C male offspring. On the other hand, this treatment with CBD reduced muscarinic M1/M4 receptors binding density in the control male offspring at similar levels of the poly I:C ones. There was no evidence of M1/M4 receptors binding density alterations induced by either the gestational poly I:C administration or the postnatal treatment with CBD in female offspring. The authors also reported that gestational poly I:C administration reduced hippocampal choline acetyltransferase (ChAT) expression of male offspring, but not female ones, while acetylcholinesterase (AChE) protein expression was not altered in either sex. Prolonged treatment with CBD did not affect these proteins in both male and female offspring.

To investigate putative functional effects of prolonged treatment with CBD on chronic administration of MK-801 at the late adolescence/early adulthood model, Gomes et al. (2014) also evaluated the FosB/ΔFosB expression (an indication of sustained neuronal activation) (Nestler et al., 1999). The authors reported an increased number of FosB/ΔFosB-positive cells in PFC and NAc (but not in dorsal striatum and hippocampus) after chronic MK-801 injection. Concomitant administration of CBD was able to revert this increase in the PFC but failed to alter it in the NAc. On the other hand, CBD treatment did not change the number of FosB/ΔFosB-positive cells in control animals.

Finally, only one study investigated the effects of prolonged treatment with CBD on neuroinflammation. Gomes and colleagues (2015) reported astrogliosis in the PFC of chronic MK-801-treated animals in late adolescence/early adulthood. Microglial reactivity was also observed in both the PFC and the hippocampus of these animals. Concomitant administration of CBD for 23 days attenuated the astrogliosis induced by MK-801 in the PFC. Furthermore, prolonged CBD treatment was also capable of reverting microglial reactivity in both the PFC and hippocampus of these animals. Prolonged treatment with CBD did not induce any glial changes in control animals. These results

confirm the already described anti-inflammatory effects of CBD (Burstein, 2015).

Although the results of the studies employing the poly I:C model are interesting (Osborne et al., 2017; Osborne et al., 2019a; Osborne et al., 2019b; Jimenez Naranjo et al., 2019), yielding sex-dependent differences in the schizophrenia-like phenotype, which are in accordance with the course of the disorder in humans (Abel et al., 2010; Ochoa et al., 2012; Barajas et al., 2015), these studies performed behavioral and neurochemical evaluations while the treatment with CBD was still ongoing. CBD's long-term effects can only be speculated as we cannot distinguish them from its acute effect. In parallel, the studies employing a blockade of NMDA receptors at late adolescence/early adulthood (Gomes et al., 2014; Gomes et al., 2015) performed the CBD treatment concomitantly to the MK-801 administration (starting on the sixth day after the beginning of MK-801 injections). Recently, a study from the same group (Rodrigues da Silva et al., 2020) showed that MK-801 administrations twice a day (in the dose range of up to 2 mg/kg/day) for seven consecutive days were not enough to induce schizophrenia-like behavioral alterations (measured eight days after the last MK-801 injection, i.e., on the 15th day of the experiment). In contrast, MK-801 injections twice a day (0.5 mg/kg, i.e., 1 mg/kg/day) for fourteen consecutive days induced social impairments and cognitive deficits (in the social interaction test and in the NOR, respectively, which were measured at both one and eight days after the last MK-801 injection, i.e., on the 15th and 22nd days of the experiment). Thus, in the two studies by Gomes et al. (2014), Gomes et al. (2015), CBD's effects on the development and progression of the behavioral and neurochemical changes cannot be distinguished from the action of CBD directly interfering with MK-801 mechanisms of action. On the other hand, it should be noted that the subacute treatment with CBD was effective in reversing the NMDA receptor antagonism-induced behavioral changes even after MK-801 injections were suspended (Rodrigues da Silva et al., 2020).

Clinical evaluations of the effects that a long-term CBD treatment might have on the course of the neurodevelopmental pathophysiological mechanisms associated with the emergence of schizophrenia are still lacking. Notwithstanding, beneficial effects of acute or subacute treatments with CBD for individuals at clinical high risk for psychosis (CHR, at late adolescence/early adulthood) have been recently described. Functional magnetic resonance imaging studies have shown that individuals at clinical high risk for psychosis (CHR, aging from 18 to 35 years) present altered activation of some brain regions—such as the striatum and the medial temporal cortex—during cognitive and emotional processing. Although the direction of changes in these regions may vary according to the task, the administration of a single dose of CBD (600 mg) promotes a normalization of the dysfunction observed (Bhattacharyya et al., 2018; Davies et al., 2020). In addition, the insular dysfunction presented by CHR subjects during motivational salience processing is also attenuated by this same single dose of CBD (Wilson et al., 2019). Adding to the beneficial effects of CBD on abnormal brain activities, another study of the same group reported that a seven-day treatment with CBD (600 mg/kg) partially attenuated abnormal cortisol levels and anxiety and stress perception induced by social stress in CHR individuals (Appiah-Kusi et al., 2020).

REVIEWED STUDIES ON AUTISM SPECTRUM DISORDER

Here, we reviewed the impact that treatment with CBD during neurodevelopment has on behavioral and molecular aspects of ASD. Firstly, the term "cannabidiol" was paired with "autism" or "autism spectrum disorder" for the search of clinical and preclinical studies in the PubMed database. Additional searches were carried out in the reference list of the studies found in the first search. Since no preclinical studies were found, we expanded the search using the term "cannabidivarin" (CBDV, a propyl analog of CBD) as an alternative phytocannabinoid molecule for CBD. The final inclusion criteria were a) describing the use of products and medications containing CBD or CBDV in the treatment of ASD and b) the treatments occurring chronically and during the neurodevelopment (from early ages up to late adolescence/beginning of adulthood). Only five studies were included: four clinical trials using cannabis oil extract and one preclinical study using CBDV (**Table 2**). Case reports were not included.

Clinical Evidence of Early Treatment with Products Containing CBD for ASD

The subjects in the clinical trials were ASD patients in distinct developmental stages (age range of 4–22 years) being a majority of boys. In all four studies, CBD was delivered as CBD-enriched cannabis extract oil containing both CBD and THC (and probably other cannabinoid molecules) administered orally. In three of them, the CBD/THC ratio was 20:1 (Barchel et al., 2018; Aran et al., 2019a; Bar-Lev Schleider et al., 2019), while in one study, it was 75:1 (Fleury-Teixeira et al., 2019). The treatments with CBD/THC oil presented elevated retention rates, achieving more than 80% retention after six months of treatment (Bar-Lev Schleider et al., 2019; Fleury-Teixeira et al., 2019), around 77% after nine months of treatment (Fleury-Teixeira et al., 2019) and 73% retention with a mean treatment duration of around 11 months (Aran et al., 2019a). On the other hand, in one study, the median retention rate was around two months (i.e., 50% of patients discontinued 1–2 months after starting treatment), ranging from one up to ~19 months (Barchel et al., 2018). One can argue that lower retention rates in this study were due to the higher CBD dose used (16 mg/kg/day) when compared to lower doses in others with better retention rates (mean daily dose below 5 mg/kg; maximum dose of 10 mg/kg/day or less) (Aran et al., 2019a; Fleury-Teixeira et al., 2019). Since CBD dosage variation was broad in these studies, plus the fact that CBD-containing oil also contained other cannabinoids, an accurate conclusion about retention rates is difficult to be made. Notwithstanding, evidence regarding elevated adherence and

retention rate for low doses of CBD in ASD patients is quite robust.

Around the reasons for discontinuation of CBD treatment, the most common were treatment ineffectiveness/low efficacy, the appearance of side effects, and a combination of both. Among the side effects reported, the most frequent were sleep disturbances, restlessness, sleepiness, irritability, and also loss or increase of appetite. It is essential to highlight that concomitant to CBD treatment, most patients were also receiving at least one of the following medications: typical or atypical antipsychotics, benzodiazepines or other anticonvulsants, selective serotonin reuptake inhibitors (SSRIs) or other antidepressants, stimulants, melatonin, etc. One can speculate that the adverse events observed throughout CBD treatment could be partially due to the synergic actions of other medications with CBD treatment. In fact, drug-drug interactions between CBD and lithium were reported in a 13-year-old boy with ASD and Lennox-Gastaut syndrome who presented lithium toxicity after a few weeks of treatment with 10 mg/kg/day CBD (Singh et al., 2020). In addition, since all the clinical trials reviewed here delivered CBD through oil extract containing THC and other compounds, the so-called "entourage effect" (i.e., a cannabinoid-cannabinoid interaction) cannot be ignored as a putative adverse effect cause (Cogan, 2020; Koltai and Namdar, 2020).

Even though some of the patients experienced adverse effects throughout treatment with CBD, improvements in ASD- and comorbidity-related symptoms were reported in all four studies. Immediate improvements in the patients' behavior were observed, such as a decrease in anxiety, sleep problems, hyperactivity, rage attacks, and self-injury. Progress in the patients' autonomy, increased motor, and cognitive performances as well as communication and social interaction improvements were also reported. The expected anticonvulsant effect of CBD (Mullard, 2018; Silvestro et al., 2019; Alves et al., 2020; Aran and Cayam-Rand, 2020; Lazarini-Lopes et al., 2020) was confirmed in two studies in which seizures were at least partially or even completely controlled (Bar-Lev Schleider et al., 2019; Fleury-Teixeira et al., 2019). In accordance with these studies, a recent case report about a 15-year-old boy with ASD who was treated with CBD-enriched cannabis extract oil (CBD/THC ratio of 20:1; 4 mg CBD and 0.2 mg THC twice a day) reported that CBD-based treatment aided in the control of ASD-related behavioral symptoms, core social communication abilities, anxiety, sleep difficulties, and body weight (Ponton et al., 2020). Notably, this study also reported that no side effects of the CBD-based treatment were observed. In addition to the direct impact that CBD treatment had on patients' behavior, parents and caregivers' indirect benefits were also reported. A decrease in patients' disruptive behavior was observed and, consequently, improvements of 29% in the Home Situations Questionnaire-Autism Spectrum Disorder (HSQ-ASD) and of 33% in the Autism Parenting Stress Index (APSI) were reported (Aran et al., 2019a), indicating an increased quality of life for the whole family. A second indirect outcome regarded the concomitant use of other medications. Although few patients received more medications or higher doses after treatment with CBD, the proportion of patients who could reduce the dosage or even discontinue other medications was significantly higher (Aran et al., 2019a; Bar-Lev Schleider et al., 2019; Fleury-Teixeira et al., 2019).

The clinical evidence observed here suggests that early treatment with CBD might be a promising therapy for ASD. It yields important direct and indirect benefits (such as positive effects on multiple autistic symptoms and reduction in concomitant use of other medications). It also shows good tolerability without causing the typical side effects found in medicated ASD patients (in most cases, only mild and/or transient side effects were reported). However, it is essential to highlight the fact that methodological limitations were reported in all four studies. The two main self-reported limitations were due to 1) the unavailability of an objective assessment tool for symptom changes (the results were based on subjective reports of the patients' parents or caregivers); 2) the nature of the studies: the lack of control groups could bias the outcomes, resulting in potentially significant placebo effects. Therefore, it is crucial that CBD's efficacy in treating ASD symptoms is confirmed through randomized, double-blind placebo-controlled multicenter trials. Fortunately, a clinical study (investigating both CBD and other phytocannabinoids) is currently being carried out (NCT03900923; NCT03849456; NCT03202303), although its results are not available yet. Additional studies must be conducted to better understand if CBD treatment benefits are indeed due to CBD effects *per se* or due to the entourage effect of cannabinoid molecules present in the cannabis oil extracts used in these studies.

Preclinical Evidence of Early Treatment With Cannabinoids in ASD Models

Environmental manipulations during gestational periods have been used to induce an ASD-like phenotype in animals (Narita et al., 2002; Miyazaki et al., 2005; Schneider and Przewlocki, 2005; Narita et al., 2010; Malkova et al., 2012; Xuan and Hampson, 2014). These models focus on inducing at least some of the core ASD-like behaviors and/or neuroanatomical alterations in offspring. In rats, Zamberletti and colleagues (2019b) used the valproic acid (VPA) administration in the dams when they were in the 12th gestational day to induce an ASD-like phenotype. Their offspring were then treated with CBDV to investigate its effects on behavioral and molecular aspects related to ASD. As in VPA-exposed humans (Ornoy, 2009; Christensen et al., 2013; Veroniki et al., 2017; Macfarlane and Greenhalgh, 2018), VPA administration in pregnant rodents induced behavioral alterations in the offspring, including decreased social interaction, increased repetitive and stereotyped behaviors, hyperlocomotion, and impaired short-term recognition memory. In agreement with others (Schneider and Przewlocki, 2005; Servadio et al., 2016; Bronzuoli et al., 2018; Melancia et al., 2018), these behavioral alterations were observed in both the pubescent and early adulthood periods. The CBDV was administered in the offspring of VPA-treated dams (and in control ones) using two different therapeutic strategies. The first one was called the "symptomatic" approach in which

TABLE 1 | Preclinical results: effects of CBD administration during neurodevelopment on behavioral and molecular evaluations on animal models of schizophrenia.

Species/ strain/sex	Model of schizophrenia-like phenotype	Dose and schedule of CBD injections	Measurements	Key behavioral effects	Key molecular effects	Comments	References
Chronic treatment with CBD during peripubertal/adolescence periods							
Rats/ SHR/M	Spontaneous SCZ-like phenotype in the SHR strain	0.5, 1, or 5 mg/kg/ day (i.p.) from PND30 to PND60	*Behavioral assessment*: catalepsy assessment was performed throughout the period of treatment with CDB, OF, SI, PPI, and CFC, starting on PND90; oral dyskinesia on PND62 and at the end of the other behavioral tasks. *Molecular assessment*: glycemia and serum levels of triglycerides on PND61; quantification of monoamines and their metabolites and the levels of BDNF on PND61 or PND90	0.5 mg/kg CBD prevented the emergence of SHRs' hyperlocomotor activity and deficits in PPI and CFC	In both strains, 0.5 mg/kg CBD increased the 5-HIAA/serotonin ratio in the PFC on PND61; CBD increased the levels of 5-HIAA in the PFC on PND90	CBD did not induce catalepsy or oral dyskinesia; CBD did not induce metabolic side effects	Peres et al. (2018a)
Rats/SD/M	Single MAM administration (22 mg/kg; i.p) on pregnant dams (GD17); SCZ-like phenotype evaluated in their offspring	10 or 30 mg/kg/ day (i.p.) from PND19 to PND39	*Behavioral assessment:* OF, NOR (short-term memory), and SI tasks starting on PND100. *Molecular assessment*: quantification of AEA, 2-AG, PEA, and OEA in the PFC, Hp, and NAc after the last behavioral task; DNA methylation of CNR1 gene promotor and CB1 mRNA and protein expression in the PFC, Hp, and NAc after the last behavioral task	30 mg/kg CBD prevented MAM-induced behavioral alterations in both SI and NOR tasks	30 mg/kg CBD prevented MAM-induced changes in CNR1 promoter DNA methylation, in CB1 mRNA and protein expression in PFC	CBD prevented MAM-induced schizophrenia's negative- and cognitive-like symptoms in adulthood, without affecting control offspring	Stark et al. (2019)
Rats/SD/M	Single MAM administration (22 mg/kg; i.p) on pregnant dams (GD17); SCZ-like phenotype evaluated in their offspring	30 mg/kg/day (i.p.) from PND19 to PND39	MRI scanning, RT-qPCR, DNA methylation, and molecular modeling of D2 and D3 receptors in complex with CBD and HAL on PND90		30 mg/kg CBD prevented MAM-induced increase in encephalic regional blood flow at the level of the circle of Willis	Computational modeling suggested that CBD could bind preferentially to dopamine D3 receptor than to dopamine D2 receptor	Stark et al. (2020)
Mice/ C57Bl/ 6J/M	Single poly I:C administration (10 mg/kg; i.v.) on pregnant dams (GD9); SCZ-like phenotype evaluated in their offspring	1 mg/kg/day (i.p) from PND30 to PND60	SI and locomotor activity (measured during SI) on PND90	1 mg/kg CBD prevented poly I:C-induced hyperlocomotion		CBD did not alter body weight gain throughout all the experiments	Peres et al. (2016a)
Rats/SD/M	Single poly I:C administration (4 mg/kg; i.v.) on pregnant dams (GD15); SCZ-like phenotype evaluated in their offspring	10 mg/kg/twice a day (i.p., i.e., 20 mg/kg/day) from PND56 to PND80	NOR (short-term memory), T-maze reward alternation, and SI tasks starting on PND72 and finishing on PND79	10 mg/kg CBD prevented poly I:C-induced deficits in NOR, working memory, and social interaction performance		CBD did not affect total body weight gain, food, and water intake in all experimental groups	Osborne et al. (2017)
Rats/SD/F	Single poly I:C administration (4 mg/kg; i.v.) on pregnant dams (GD15); SCZ-like phenotype evaluated in their offspring	10 mg/kg/twice a day (i.p., i.e., 20 mg/kg/day) from PND56 to PND80	*Behavioral assessment:* NOR (short-term memory), T-maze reward alternation, and SI tasks starting after two weeks of treatment with CBD or vehicle and with a 24 h period interval between tasks. *Molecular assessment:* receptor autoradiography for CB1R, NMDAR, and $GABA_AR$ binding density assessment in the PFC and Hp measured approximately 10–12 h after the last treatment; FAAH, GluN1, GAD_{67}, and PV protein expression in the PFC and Hp measured approximately 10–12 h after the last treatment	10 mg/kg CBD prevented poly I:C-induced deficits in NOR, working memory, and social interaction performance	Poly I:C offspring presented reduced NMDAR binding density in the PFC, while treatment with 10 mg/kg CBD prevented it	CBD increased PV and GAD_{67} expression in Hp, regardless of the gestational manipulation. In control offspring, CBD reduced social interaction, besides NMDAR and CB1R binding density in the PFC	Osborne et al. (2019a)
Rats/SD/M	Single poly I:C administration (4 mg/kg; i.v.) on pregnant dams (GD15); SCZ-like phenotype evaluated in their offspring	10 mg/kg/twice a day (i.p., i.e., 20 mg/kg/day) from PND56 to PND80	Receptor autoradiography for CB1R, NMDAR, and $GABA_AR$ binding density assessment in the PFC and Hp on PND80; FAAH, GluN1, GAD_{67}, and PV protein expression in the PFC and Hp on PND80		Poly I:C offspring presented reduced CB1R binding density in the PFC, while treatment with 10 mg/kg CBD prevented it; poly I:C offspring presented reduced GAD_{67} expression in the Hp, while treatment with 10 mg/kg CBD prevented it	CBD increased GAD_{67} expression in Hp of control offspring; CBD increased PV expression in Hp, regardless of the gestational manipulation	Osborne et al. (2019b)

(Continued on following page)

TABLE 1 | (*Continued*) Preclinical results: effects of CBD administration during neurodevelopment on behavioral and molecular evaluations on animal models of schizophrenia.

Species/ strain/sex	Model of schizophrenia-like phenotype	Dose and schedule of CBD injections	Measurements	Key behavioral effects	Key molecular effects	Comments	References
Rats/SD/M and F	Single poly I:C administration (4 mg/kg; i.v.) on pregnant dams (GD15); SCZ-like phenotype evaluated in their offspring	10 mg/kg/twice a day (i.p., i.e., 20 mg/kg/day) from PND56 to PND80	Receptor autoradiography for M1/M4R binding density assessment in the PFC and Hp on PND80; ChAT and AChE protein expression in the PFC and Hp on PND80		In male offspring, 10 mg/kg CBD treatment attenuated poly I:C-induced changes in M1/M4R binding density in both PFC and Hp (CA1/CA2 and CA3 subregions). In male offspring, 10 mg/kg CBD prevented the poly I:C-induced changes in hippocampal ChAT expression	Neither treatment with poly I:C nor CBD affected the measurements in the female offspring	Jimenez Naranjo et al. (2019)
Mice/ C57Bl/ 6J/M	Daily injections of MK-801 (1 mg/kg; i.p.) for 28 days, starting when animals were 6 weeks old (P1)	15, 30, or 60 mg/kg/day (i.p.) from P6 to P28	*Behavioral assessment:* PPI test on P29. *Molecular assessment:* immediately after PPI, immunohistochemical detection of FosB/ ΔFosB and PV and RT-qPCR for GRIN1 gene	30 and 60 mg/kg CBD partially attenuated MK-801-induced impairment in PPI	MK-801 increased FosB/ΔFosB-positive cells in PFC and NAc, while treatment with 60 mg/kg CBD reversed it only in PFC; MK-801 decreased PV-positive cells in PFC, while treatment with 60 mg/kg CBD slightly attenuated it; MK-801 decreased PV-positive cells in PFC and GRIN1 mRNA expression in Hp, while treatment with 60 mg/kg CBD slightly attenuated them	Single CBD injection on P28 did not affect PPI impairments induced by MK-801 injections	Gomes et al. (2014)
Mice/ C57Bl/ 6J/M	Daily injections of MK-801 (1 mg/kg; i.p.) for 28 days (P1–P28), starting when animals were 6 weeks old (P1)	30 or 60 mg/kg (i.p.) from P6 to P28 (i.e., for 23 days)	*Behavioral assessment:* SI and EPM on P29 and NOR (short-term memory) and OF on P30. *Molecular assessment:* immunohistochemical detection of NeuN, GFAP, and Iba1 on P31	CBD (60 mg/kg) attenuated MK-801-induced impairment in SI and NOR	MK-801 increased GFAP-positive cells in PFC, while treatment with CBD (60 mg/kg) slightly attenuated it; MK-801 increased the Iba1-positive cells with a reactive phenotype in PFC and Hp, while treatment with CBD (60 mg/kg) reversed microglial reactivity in all regions		Gomes et al. (2015)

2-Arachidonoylglycerol (2-AG); 5-hydroxyindoleacetic acid (5-HIAA); acetylcholinesterase (AChE); anandamide (AEA); brain-derived neurotrophic factor (BDNF); cannabidiol (CBD); contextual fear conditioning task (CFC); choline acetyltransferase (ChAT); elevated plus maze (EPM); female (F); glutamate decarboxylase 67 kDa isoform (GAD_{67}); gestational day (GD); haloperidol (HAL); hippocampus (Hp); high-performance liquid chromatography (HPLC); male (M); methylazoxymethanol acetate (MAM); magnetic resonance imaging (MRI); nucleus accumbens (NAc); novel object recognition task (NOR); N-oleoylethanolamide (OEA); open field behavioral task (OF); N-palmitoylethanolamide (PEA); prefrontal cortex (PFC); offspring's postnatal day (PND); prepulse inhibition of startle (PPI); parvalbumin (PV); social interaction task (SI); schizophrenia (SCZ); Sprague-Dawley (SD); Spontaneously Hypertensive Rats (SHR).

TABLE 2 | Clinical and preclinical results: effects of CBD administration during neurodevelopment on behavioral and molecular evaluations in both animal models and patients of autism spectrum disorders.

Sex/age	Study design	Dose and schedule of CBD administration	Measurements	Main results	Comments	References
Clinical studies						
N = 60 (83% M)/ 5–18 years old (mean 11.8 ± 3.5)	Retrospective study; children with ASD and refractory disruptive behaviors investigated after 7–13 months of treatment	CBD/THC ratio of 20:1 oil (SL), 2–3 times a day with doses up-titrated over 2–4 weeks (starting CBD dose was 1 mg/kg/day; maximal CBD dose was 10 mg/kg/day). The mean total daily dose was 3.8 ± 2.6 mg/kg/day CBD and 0.29 ± 0.22 mg/kg/day THC for children who received three daily doses (*n* = 44) and 1.8 ± 1.6 mg/kg/day CBD and 0.22 ± 0.14 mg/kg/day THC for children who received two daily doses (*n* = 16)	CGIC; HSQ-ASD; APSI; retention rates; modified Liverpool adverse events profile	All had severe behavioral problems based on CGI-S (scores of 6 or 7); 29 patients with insufficient response used cannabis strains with lower CBD: THC ratios (6:1; maximal CBD dose was 5 mg/kg/day); retention rate of 73% (mean treatment duration: 10.9 ± 2.3 months); improvement in CGIC: 61% in behavioral outbreaks, 47% for communication, and 39% for anxiety; improvement in stress and disruptive behavior: HSQ 29% and APSI 33%; adverse events included sleep disturbances 14%, irritability 9%, and loss of appetite 9%. Following the cannabis treatment, 33% received fewer medications or lower dosage, 24% stopped taking medications, and 8% received more medications or higher dose	Uncontrolled retrospective study of a subgroup of children with severe and refractory behavioral problems. Participants used various cannabis strains from different growers and a broad range of CBD and THC dose. The number of participants was not large enough to evaluate the impact on different ASD subgroups	Aran et al. (2019a)
N = 18 (72% M)/ 6–17 years old (mean 10.9 ± 3.06)	Observational study; cohort of 18 patients undergoing 6–9 months treatment with compassionate use of standardized CBD-enriched *Cannabis sativa* extract	CBD:THC ratio of 75:1 CBDRx® (Colorado, USA), twice a day with an average CBD dose of 4.6 mg/kg/day and an average THC dose of 0.06 mg/kg/day. Starting CBD dose was ~2.90 mg/kg/day (minimum: 2.30 and maximum: 3.60 mg/kg/day). Dosage adjustment occurred over 150 days. At the end of the study, the minimal CBD dose was 3.75 and the maximum was 6.45 mg/kg/day	Parents perceived percentage change on ADHD; BD; MD; AD; CSID; CD; sleep disorders; seizures. Clinical assessments: side effects and changes, maintenance, reduction, or withdrawal of neuropsychiatric drugs that were already in use	Retention rate in 6 months was 83% and in 9 months was 77%. Parents perceived percentage change: 47% had improvements equal to or above 30% in four or more symptoms categories, 13% presented improvements equal to or above 30% in two symptom categories, and 33% presented improvements equal to or above 30% in one symptom category. At least 60% of patients showed improvements of 20% or more in ADHD, MD, CSID, BD, sleep disorders, and seizures. Patients who presented BD: eight (53.3%) had improvements equal to or above 20% in this symptom category. AD, only four (26.7%) had improvements equal to or above 20%. ADHD, sleep disorders, and seizures, with more than 80% of patients presenting improvements equal to or above 30%. Five epileptic patients, with seizure reduction of 50% in three cases and 100% in the other two cases	Lack of control groups; small cohort size; potentially significant placebo effects due to caregivers bias. This treatment made it possible to achieve a decrease in the dosage or to discontinue other neuropsychiatric medications in eight out of 10 patients that were receiving OM	Fleury-Teixeira et al. (2019)
N = 53 (85% M)/ 4–22 years old (mean 11)	Prospective study; ASD children treated with CBD-oil over 30–588 days (~1–19 months) had safety and comorbid symptoms assessed biweekly	CBD:THC ratio of 20:1 oil prepared by "Tikum Olam" at a concentration of 30%. Daily dose, maximal daily dose, and median interquartile range for CBD were 16 mg/kg, 600 mg, and 90 mg (45–143), respectively. Daily dose, maximal daily dose, and median interquartile range for THC were 0.8 mg/kg, 40 mg, and 7 mg (4–11)	According to parent's reports, the emerging adverse effects, medications in use, and ASD comorbidities, hyperactivity symptoms, sleep problems, self-injury, and anxiety, were evaluated. An overall change was defined based on the summation of all parent's reports. The change in each comorbid symptom in the study cohort was compared to published data using conventional treatment	Retention rate: 50% patients discontinued the treatment with 66 days. Overall improvement (*n* = 51) was reported in 74.5%, did not change in 21.6%, and worsened in 3.9%. Self-injury and rage attacks (n = 34) improved in 67.6% and worsened in 8.8%. Hyperactivity symptoms (n = 38) improved in 68.4%, did not change in 28.9%, and worsened in 2.6%. Sleep problems (*n* = 21) improved in 71.4% and worsened in 4.7%. Anxiety (*n* = 17) improved in 47.1% and worsened in 23.5%. Adverse effects were somnolence (*n* = 12) and decreased appetite (*n* = 6)	CBD shows noninferiority when compared to conventional treatments in the overall improvement of hyperactivity, self-injury, sleep problems, and anxiety symptoms	Barchel et al. (2018)

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TABLE 2 | (*Continued*) Clinical and preclinical results: effects of CBD administration during neurodevelopment on behavioral and molecular evaluations in both animal models and patients of autism spectrum disorders.

Sex/age	Study design	Dose and schedule of CBD administration	Measurements	Main results	Comments	References
N = 188 (81% M)/ 5–18 years old (mean 12.9 ± 7)	Prospective study; children with ASD treated with medical cannabis (30% CBD and 1.5% THC) between 2015 and 2017	Cannabis oil with CBD:THC ratio of 20:1. The dosage ranged from 1 drop (0.05 ml) three times a day to 20 drops three times a day, for 6 months. Each drop (0.05 ml) contained 45% olive oil, 30% CBD (15 mg), and 1.5% THC (0.75 mg). The average dose was 79.5 ± 61.5 mg CBD and 4.0 ± 3.0 mg THC; patients with insomnia received an additional average dose of THC (3%) 5.0 ± 4.5 mg	Patient's parents were interviewed and filled a medical questionnaire about demographics, comorbidities, habits, concomitant medications, measurements of quality of life, and a detailed symptom checklist. The evolution of patients was assessed after 1 and 6 months of treatment and intensity of symptoms, side effects, and quality of life were assessed. The global assessment approach and Likert scale were used to assess efficacy and quality of life, respectively	Quality of life (before the treatment): 31.3% of patients reported good quality of life, 3.3% reported good sleep, 0% reported good concentration, 42% reported positive mood, and 26.4% reported no difficulty in abilities to dress and shower independently. After one month, 179 patients (94.6%) continued treatment and 119 patients (66.4%) responded to the questionnaire. 48.7% reported a significant improvement, 31.1% reported a moderate improvement, 14.3% reported nonimprovement, and 5.9% reported side effects. After six months, 155 patients (86.6%) continued treatment and 93 patients (60%) responded to the questionnaire. 30.1% reported a significant improvement, 53.7% reported moderate improvement, 6.4% reported slight improvement, and 8.6% reported no change in their condition. 66.8% of patients reported good quality of life, 24.7% reported good sleep, 14% reported good concentration, 63.5% reported positive mood, and 42.9% reported no difficulty in abilities to dress and shower independently. 67 reported use of chronic medications, 8.9% reported an increase in their drug consumption, in 56.7%, drug consumption remained the same, and 34.3% reported a decrease. 23 patients discontinued the treatment and 17 (73.9%) responded to questionnaire for the treatment discontinuation: 70.6% reported no therapeutic effect and 29.4% reported side effects. Seven patients (41.2%) who discontinued the treatment had reported intentions to return to the treatment	The most prevalent side effect reported at six months was restlessness, appearing in less than 6.6% of patients. The compliance with the treatment was high and less than 5% have stopped the treatment due to the side effects. Absence of control group, therefore no causality between cannabis therapy and improvement in patient's well-being can be established. Self-selection bias due to parents seeking cannabis therapy for their children. High compliance (above 80%) with the treatment provides good evidence of the patients and parents' satisfaction with the treatment	Bar-Lev schleider et al. (2019)

Species/ Strain/Sex	Study design/animal model	Dose and schedule of CBDV injections	Measurements	Main results	Comments	References
Preclinical study						
Rats/SD/M/	Single valproic acid administration (500 mg/kg; i.p.) on pregnant dams (GD 12.5) → ASD-like phenotype evaluated in their offspring	Daily injections of CBDV (0.2, 2, 20, or 100 mg/kg; i.p) from PND34 to PND58 (symptomatic protocol); daily injections of CBDV (2 or 20 mg/kg; i.p.) from PND19 to PND32 (preventive protocol)	*Behavioral assessment*: symptomatic treatment: three-chamber test on PND56, NOR (short-term memory) on PND57, and activity cage on PND58; preventive treatment: the same tests were performed on PND30, PND31, and PND32, respectively. *Molecular assessment*: 24 h after the last behavioral test in symptomatic protocol: expression of several proteins in PFC and Hp; immunohistochemical detection of Iba1 in dorsal Hp	Key behavioral effects: CBDV symptomatic treatment recovered social impairments, social novelty preference deficits, NOR deficits, repetitive behaviors, and hyperlocomotion; CBDV preventive treatment improved sociability and social novelty deficits, NOR impairments, and hyperlocomotion, without affecting stereotypies. Key molecular effects: prenatal VPA exposure increased CB1 receptor, FAAH, and MAGL levels, enhanced GFAP, CD11b, and TNFα levels, and triggered microglia activation restricted to the Hp. All these alterations were restored after CBDV treatment	CBDV increased CB2 receptor expression in Hp regardless of the gestational manipulation; both CBDV administration and prenatal VPA exposure decreased DAGLα expression in PFC	Zamberletti et al. (2019b)

AD, autonomy deficits; ADHD, attention-deficit/hyperactivity disorder; APSI, autism parenting Stress Index; ASD, autism spectrum disorder; BD, behavioral disorders; CBDV, cannabidivarin; CD, cognitive deficits; CGIC, caregiver global impression of change; CGI-I, clinical global impression of improvement; CSID, communication and social interaction deficits; F, female; GD, gestational day; Hp, hippocampus; HSQ-ASD, home situations questionnaire-autism spectrum disorder; M, male; MD, motor deficits; NOR, novel object recognition task; PFC, prefrontal cortex; PND, offspring's postnatal day; SD, sprague-dawley; SL, sublingual; WB, western blotting.

several doses of CBDV (0.2, 2, 20, or 100 mg/kg/day) were tested: they were chronically administered throughout puberty (from PND34 to PND58) and the evaluations occurred at early adulthood (from PND56 to PND58). At this schedule, CBDV was efficient in reverting (or at least attenuating) all the VPA-induced behavioral abnormalities evaluated. The dose of 20 mg/kg/day was the most efficient one. The second CBDV therapeutic strategy was called "preventive": CBDV (2 or 20 mg/kg/day) was chronically administered during an earlier period of neurodevelopment that encompassed a preweaning period plus the prepubertal period (from PND19 to PND32), and the evaluations occurred at puberty (from PND30 to PND32). Also, in this treatment schedule, the CBDV dose of 20 mg/kg/day was the most efficient. It reverted (or at least attenuated) the VPA-induced behavioral abnormalities evaluated, except for repetitive and stereotyped behaviors (measured through self-grooming).

Similar beneficial effects of chronic CBDV administration were observed in studies using genetic syndrome models, in which autistic behaviors are among the symptoms. Zamberletti et al. (2019a) found that chronic CBDV administration (at 20 mg/kg/day and others) in Mecp2 knockout mice (a Rett syndrome-like animal model) rescued the impaired short-term recognition memory which was evaluated during adolescence and early adulthood. In addition to CBDV benefits, chronic CBD administration (100 mg/kg twice daily, i.e., 200 mg/kg/day from the neonatal period up to early adulthood) rescued several autistic-like behaviors (anxiety- and depression-like behavior, poor social interaction, and increased rearing behavior, as well as reference memory and working memory) in *Scn1a*$^{+/-}$ mice, a Dravet syndrome-like animal model (Patra et al., 2020). Importantly, CBD did not induce any adverse effects on motor function, giving further support for the benefits and safety of using these cannabinoids in treating ASD.

As already discussed, the ECB system is altered in ASD patients and this might be directly related to the behavioral and morphological alterations observed in these individuals. This observation is also true for the animal models (for more information, see Zamberletti et al., 2017). Zamberletti and colleagues (2019b) found that CB_1 and CB_2 receptors' expression was increased in the hippocampus of VPA-treated animals. In addition, they observed that the expression of the two enzymes responsible for AEA and 2-AG degradation (FAAH and MAGL, respectively) was also increased in these animals while the expression of the enzymes responsible for the synthesis of these molecules (NAPE-PLD and DAGL-a, respectively) was not altered in the hippocampus. The CBDV symptomatic schedule treatment (i.e., chronic administration of CBDV from PND34 to PND58) rescued all of them except the increased CB_2 receptor expression. The authors hypothesized that AEA and 2-AG concentrations are decreased in VPA animals (due to the increased expression of FAAH and MAGL) which agrees with other clinical and preclinical studies (Servadio et al., 2016; Karhson et al., 2018; Melancia et al., 2018; Wang et al., 2018; Aran et al., 2019b). They also suggest that the beneficial effects of CBDV could be related to the restoration of the ECB system abnormalities in the hippocampus. Contrary to the increase in ECB catabolic enzymes in the hippocampus, the DAGL-a expression was reduced in the PFC of VPA animals which agrees with the reduced 2-AG (but not AEA) hypothesis. However, the DAGL-a expression in PFC also decreased in response to CBDV treatment, which disagrees with the ECB system restoration hypothesis. A similar effect of CBDV was observed in cell culture experiments (De Petrocellis et al., 2011). In addition, reduced DAGL-a expression (related to decreased 2-AG levels) in response to chronic CBDV administration was also observed in the Rett syndrome model (Zamberletti et al., 2019a). In this case, administration of CBDV (at behaviorally effective doses) in the Mecp2 knockout mice increased the levels of AEA and oleylethanolamide (OEA, a monounsaturated analog of AEA that does not bind to cannabinoid receptors) while it reversed the increase in both CB_1 and CB_2 receptors. Interestingly, CBDV restored neurotrophic factor levels in Mecp2 knockout mice, which were related to a normalization of their common downstream AKT/mTOR signaling pathway and ribosomal protein six phosphorylation (Zamberletti et al., 2019a); both of them were expected to be impaired in ASD (Tai et al., 2020).

Substantial evidence suggests that immunological dysfunction plays a crucial role in the pathophysiology of ASD and that therapies able to control or reduce neuroinflammation could ameliorate ASD symptoms (Gottfried et al., 2015; Kern et al., 2015; Bjorklund et al., 2016; Bertolino et al., 2017; Bronzuoli et al., 2018). In the study by Zamberletti and colleagues (2019b), VPA injection during the gestational period induced hippocampal inflammation in the offspring, marked by enhanced levels of GFAP, CD11b, TNFα, and also microglial reactivity. The symptomatic schedule for chronic CBDV administration rescued both the hippocampal inflammation and autistic-like behavioral symptoms induced by gestational VPA injection, giving further support for this hypothesis. The anti-inflammatory actions of synthetic cannabinoids and phytocannabinoids have been extensively reported (Burstein, 2015; Schonhofen et al., 2018), especially for CBD and its derivative molecules. Some findings also support an anti-inflammatory property of CBDV (Tubaro et al., 2010; De Petrocellis et al., 2011; Amada et al., 2013; Pagano et al., 2019). On the other hand, chronic administration of this molecule induced an increase in GFAP expression in both control and VPA animals' PFC (Zamberletti et al., 2019b), reinforcing the necessity for further investigation about this topic.

CONCLUSION

Schizophrenia and ASD are psychiatric neurodevelopmental disorders that cause high levels of suffering, ranging from social isolation and cognitive deficits to severe debilitations and functional disabilities. The currently available treatments for these disorders are limited, stressing the importance of developing novel efficient and safe therapeutic strategies. The use of cannabinoids (as CBD and CBDV) during neurodevelopment (while the full-blown disorder symptoms are still in progress) has been investigated as a promising novel treatment for schizophrenia and ASD. However, the use

of cannabinoid therapy demands particular caution since it must be safe both for the patients and for the individuals without a formal full-blown diagnosis. The clinical and preclinical evidence discussed in this review point out the beneficial potential that the treatment with CBD-based products (and/or CBDV for ASD) presents. Furthermore, the use of these cannabinoids was shown to be safe in both humans and animal models. Nevertheless, further clinical and preclinical studies should be carried out to provide more robust evidence for the use of CBD- (or CBDV) based products as an early preventive treatment for schizophrenia and ASD.

Even though the studies discussed here presented promising translational results, the number of studies investigating CBD (and/or CBDV) administration during neurodevelopment as a treatment for schizophrenia or ASD is still scarce. For schizophrenia, results from clinical studies investigating the effects of long-term treatment are not available yet. In addition, only ten preclinical studies investigating this issue have been published until now, limiting the complete translation of the data to clinical settings. The use of CBD for the treatment of ASD has been observed in four clinical trials, all of them using erratic CBD-enriched cannabis extract oils with other phytocannabinoid molecules (such as THC). In relation to preclinical trials, none using CBD during the neurodevelopment were performed and only one study using CBDV could be found. Another essential aspect that deserves attention is the ongoing lack of studies using female subjects, limiting the conclusions about the putative sexual dimorphism reported in the studies reviewed here. This issue is not restricted to preclinical investigations of psychiatric disorders, drawing attention to the fact that researchers should carefully plan their future studies to contemplate female subjects. Finally, the studies discussed in this review present an exploratory research approach. Therefore, their suggestive findings need to be further investigated through confirmatory research specifically designed to test the effect sizes identified in these studies as presenting biological relevance (Festing and Altman, 2002; Duan, 2013). Finally, further clinical long-term, placebo-controlled trials using pharmaceutical grade cannabinoids, involving different doses and neurodevelopmental treatment periods, would be timely to elucidate these compounds' potential in predicting better outcomes.

AUTHOR CONTRIBUTIONS

CL and VA were responsible for the conceptualization and design of the review. LT, GR, LM, and CL were responsible for reviewing the literature and acquiring the review data. CL, LT, FP, JC, AZ, JH, and VA were responsible for writing and revising the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Glial Cells and Their Contribution to the Mechanisms of Action of Cannabidiol in Neuropsychiatric Disorders

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Cannabidiol (CBD) is a phytocannabinoid with a broad-range of therapeutic potential in several conditions, including neurological (epilepsy, neurodegenerative diseases, traumatic and ischemic brain injuries) and psychiatric disorders (schizophrenia, addiction, major depressive disorder, and anxiety). The pharmacological mechanisms responsible for these effects are still unclear, and more than 60 potential molecular targets have been described. Regarding neuropsychiatric disorders, most studies investigating these mechanisms have focused on neuronal cells. However, glial cells (astrocytes, oligodendrocytes, microglia) also play a crucial role in keeping the homeostasis of the central nervous system. Changes in glial functions have been associated with neuropathological conditions, including those for which CBD is proposed to be useful. Mostly *in vitro* studies have indicated that CBD modulate the activation of proinflammatory pathways, energy metabolism, calcium homeostasis, and the proliferative rate of glial cells. Likewise, some of the molecular targets proposed for CBD actions are f expressed in glial cells, including pharmacological receptors such as CB1, CB2, PPAR-γ, and 5-HT1A. In the present review, we discuss the currently available evidence suggesting that part of the CBD effects are mediated by interference with glial cell function. We also propose additional studies that need to be performed to unveil the contribution of glial cells to CBD effects in neuropsychiatric disorders.

Keywords: cannabidiol, pharmacology, neuropsychiatric disorders, glial cells, neurons

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INTRODUCTION

During the first decades of neuroscience and psychopharmacology research, glial cells and cannabidiol (CBD) did not play a major role in modifying brain functions. Currently, however, both CBD and glial cells, initially thought as secondary components, are recognized as major players in the central nervous system (CNS) physiology and *Cannabis sativa* pharmacology, respectively.

CBD was isolated in 1940 by Adams and Hunt (1940) and had its chemical structure described 23 years later by Mechoulam and Shvo (1963). In the early 1970s, CBD has been shown not to mimic the effects of Cannabis sp., and some believed CBD was an innocuous compound (Mechoulam et al., 1970). Most of the initial studies on CBD's actions aimed to investigate how it could interact and antagonize delta-9 tetrahydrocannabinol (THC) effects (Karniol et al., 1974; Hine et al., 1975; Brady

and Balster, 1980; Zuardi et al., 1982). However, several groups worldwide have dedicated their efforts to characterizing CBD's pharmacological properties and therapeutic applications, especially since the 1990s (Guimarães et al., 1990, 1994; Zuardi et al., 1993; Hampson et al., 1998; Moreira and Guimarães, 2005; Campos and Guimarães, 2008). These efforts produced evidence for CBD's potential actions against different disorders and have sustained the foundation for current public health policies around the globe that approved CBD-based medicines to treat conditions such as glaucoma, epilepsy, and cancer-associated pain (Campos et al., 2016).

Glial cells were named for their supposedly sole function of "gluing" the CNS matrix for sustaining the neuronal environment (Andriezen, 1893; Taylor, 1897). Nowadays, this vision has been expanded to include far more complex actions of glial cells on several vital aspects of the CNS homeostasis's maintenance (Allen and Barres, 2009; Valles et al., 2019; Verkhratsky et al., 2019; Salas et al., 2020).

Several pharmacological receptors used as drug targets to treat neurological and psychiatric conditions are expressed in glial cells. In the present review, we will address the pharmacological effects caused by CBD in these conditions and discuss how interaction with glial cell function could help to explain them.

THE GREAT POTENTIAL OF CBD AS AN ALTERNATIVE TO TREAT NEUROPSYCHIATRIC DISORDERS

The wide range of its therapeutic potential, together with, until now, good safety profile (Campos et al., 2016), has made CBD special among the almost 150 phytocannabinoids that have been already described (Hanuš et al., 2016). CBD is potentially useful in several of the main disorders that affect the CNS, including epilepsy, schizophrenia, autism, addiction, traumatic and ischemic brain injury, multiple sclerosis (MS), and anxiety, depressive, post-traumatic stress, obsessive–compulsive, and neurodegenerative disorders (Campos et al., 2016). In this section, we will briefly discuss the main studies that have investigated the effects of CBD in the context of these disorders.

Anticonvulsant Properties

The term epilepsy refers to a disorder of brain function characterized by a periodic and unpredictable occurrence of seizures due to hyper excitability and hyper synchrony of neurons (McNamara, 1994; Austin and Dunn, 2002; Dichter, 2009; Jacobs et al., 2009; Devinsky et al., 2013; Ali, 2018).

Izquierdo et al. (1973) and Carlini et al. (1973) were the first to report a potential therapeutic application for CBD in epilepsy by describing its action on diminishing seizures in rats. In the animal model of epileptic seizures induced by acute pilocarpine administration, CBD has reduced the percentage of rats experiencing severe scores of seizures (Jones et al., 2012; Patra et al., 2019). Additionally, other authors also demonstrate that intracerebroventricular injection of CBD during the significantly diminished seizure scores during the chronic phase (Hosseinzadeh et al., 2016). In another rodent model based on the administration of the GABA inhibitor, pentylenetetrazole (PTZ), CBD reduced seizure severity and lethality (Consroe et al., 1982; Jones et al., 2010; Patra et al., 2019; Lima et al., 2020). Moreover, Mao et al. (2015) demonstrated that CBD not only decreases the daily average grade of epileptic seizures, but also promoted reduction of neuronal loss due in the hippocampus (Mao et al., 2015).

Seizures can also occur after intoxication by the abuse of certain drugs, such as cocaine (Köppel et al., 1996; Gobira et al., 2015; Schifano et al., 2019). CBD is able to increase the latency and reduced the duration of cocaine-induced tonic seizures (Gobira et al., 2015; Vilela et al., 2015), as effect no mediate by CB_1 or CB_2 (Gobira et al., 2015). Conversely, in PTZ model, it is hypothesized that CB_1 and CB_2 receptors, primarily located in neurons are involved in the anti-seizure effects of CBD (Vilela et al., 2017).

The anti-seizure effects of CBD have been observed in several clinical studies (Porter and Jacobson, 2013; Lattanzi et al., 2018; Thiele et al., 2018; Silvestro et al., 2019). In infantile refractory epilepsy such as Lennox-Gastaut and Dravet syndrome, randomized controlled trials evaluated the efficacy of CBD oil as an adjuvant agent and the CBD addition significantly reduced the frequency of seizures compared to placebo (Devinsky et al., 2017; Devinsky et al., 2018).

Autism Spectrum Disorders

Autism spectrum disorders (ASDs) are a group of disabilities characterized by repetitive patterns behaviors and diminished social interaction that starts during childhood (Lai et al., 2014; Baxter et al., 2015; Goel et al., 2018). Currently pharmacological treatment of one part of the symptoms of ASDs includes antidepressants, anxiolytics and atypical antipsychotics (Stachnik and Gabay, 2010; Wink et al., 2010; Hurwitz et al., 2012; Goel et al., 2018). Recently, some studies have suggested CBD as a therapeutic strategy for the treatment of ASDs (Földy et al., 2013; Barchel et al., 2019; Poleg et al., 2019).

In this regard, low doses of CBD increased time of social exploration with the stranger mice in the Three-Chamber Test, and reduced autistic-type social deficits in genetic mouse model of Dravet syndrome (Kaplan et al., 2017). Besides ASDs, other psychiatric comorbidities as hyperactivity, Attention Deficit Hyperactivity Disorder and self-mutilation have been reported in the Dravet syndrome (Sturm et al., 2004; Murray, 2010; Berkvens et al., 2015).

The pharmacotherapy of behavioral changes in children with ASDs commonly involves the use of psychostimulants such as methylphenidate, however, the consensus has been that psychostimulants promote minimal clinical improvement for this population and many case reports have suggested a high rate of significant adverse effects (Handen et al., 2000). In a recent study conducted on children with ASDs, CBD treatment improved hyperactivity in over 68.4% of children. Comparing the overall improvement in hyperactivity symptoms in children treated with CBD to that of children treated with methylphenidate treatment as reported by literature, non-inferiority of CBD was observed. However, in this study, the main adverse effects induced by CBD were somnolence and

change in appetite that occurred in a transient way and resolved spontaneously. Still, no symptoms of toxicity were reported (Barchel et al., 2019). These initial findings point the promising therapeutic effects of CBD for ASD's. However, the exact action mechanism remains largely unknown.

Anxiolytic Properties

Anxiety disorders are highly prevalent psychiatric conditions commonly associated with a diminished sense of well-being and elevated rates of incapacity (Kroenke et al., 2007; Mata et al., 2015; Sjöberg et al., 2017). The treatment of these disorders is based on the use of benzodiazepines and antidepressants (serotonin reuptake inhibitors (SSRIs), serotonin–norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressant and partial 5-HT1A receptor agonists) as well as non-pharmacological treatments, such as psychotherapy and physical activity (Phillips, 2017; Marwood et al., 2018; Ribeiro et al., 2018; Kandola et al., 2019). Unfortunately, the late onset of therapeutic effects and important adverse reactions reduces adherence and success of the pharmacotherapy (Blessing et al., 2015; Pruckner and Holthoff-Detto, 2017; Artigas et al., 2018; Davies et al., 2019).

Several studies have investigated CBD as a possible tool for treating these disorders (Stern et al., 2012; Campos et al., 2013b; Shbiro et al., 2019). The first studies conducted in animals showed contradictory results. Low doses of CBD induced anxiolytic-like behaviors, while in high doses promoted an anxiogenic-like action (Zuardi and Karniol, 1983; Guimarães et al., 1990; Onaivi et al., 1990).

Using the elevated plus maze (EPM), a classic model for screening anti-anxiety drugs in rodents, Guimaraes et al. demonstrated that a single systemic administration of CBD promoted anxiolytic-like behavior in rats (Guimarães et al., 1990, 1994). The anxiolytic effect of CBD has been reported in other animal models such as the Vogel conflict test (VCT) (Moreira et al., 2006), open-field and in the light-dark test (Long et al., 2010). Chronic administration of CBD (14 days or 21 days) also produces anxiolytic-like effect in rodents previously exposed to chronic stress (Campos et al., 2013b).

In order to evaluate the possible neurobiology of the anxiolytic effects of CBD, several studies infused CBD into brain areas governing panic and anxiety (Fogaça et al., 2014; Lee et al., 2017). CBD injected into the dorsolateral periaqueductal gray (dlPAG) produced anxiolytic-like effects in the EPM and VCT. This effect was blocked by antagonism of 5HT1A receptors, but not by CB_1 receptors antagonism (Campos and Guimarães, 2009). The same mechanism was also responsible for the anti-panic effect of CBD in animals submitted to the electrical stimulation model of the dorsal PAG or elevated T maze (Soares et al., 2010). Corroborating these findings, in another brain region that modulates anxiety behavior, the prefrontal cortex, CBD also promotes the anxiolytic and anti-stress effects (Fogaça et al., 2014). Anxiolytic-like effect probably occurs by altering prefrontal-subcortical connectivity through amygdala and cingulate cortex and, a reduction in the activity of para-hippocampal gyrus, hippocampus and inferior temporal gyrus (Fusar-Poli et al., 2010; Crippa et al., 2011).

Zuardi et al. (1993) conducted a study in which the effect of CBD (300 mg) was compared with placebo, diazepam (10 mg; benzodiazepine) and ipsapirone (5 mg; 5-HT1A partial agonist compound) in healthy volunteers submitted to a simulated public test (SPS). The anxiety promoted by SPS was mitigated by ipsapirone and CBD, without triggering significant adverse reactions, while the anxiolytic effects induced by diazepam were accompanied by sedation (Zuardi et al., 1993).

Antidepressant-Like Effects

CBD also modulate depressant-like behaviors in rodents. Using the forced-swimming test (Porsolt et al., 1977; Cryan et al., 2002), it was observed that the administration of CBD induced an antidepressant-like effect (El-Alfy et al., 2010; Zanelati et al., 2010; Shbiro et al., 2019). The same results were found in other animal models of depression, such as tail suspension and olfactory bulbectomy (El-Alfy et al., 2010; Linge et al., 2016). Repeated administration of CBD (30 mg/kg) also induces antidepressant-like effects in swiss mice (Schiavon et al., 2016). Single doses of CBD can also induce long-term antidepressant effects, a ketamine-simile effect (Linge et al., 2016; Sales et al., 2019).

Recent studies have shown that the antidepressant effect promoted by the systemic administration of CBD in mice submitted to the forced swimming test is associated with increased expression of synaptophysin, PSD95 (synaptic plasticity marker) and BDNF levels in medial prefrontal cortex (PFC) (Sales et al., 2019). The similar effects were described in mice submitted previously to chronic mild stress mouse model (Xu et al., 2019). Indeed, preceding research shows that CBD injection into the ventral medial PFC also induces antidepressant like behavior (Sartim et al., 2016).

Stress-Related Disorders

Stress-related disorders are psychiatric conditions that could appear after the exposure and one or several stressful situations. It includes obsessive compulsive disorder (OCD) (Thomas et al., 2009; Seibell and Hollander, 2014) and posttraumatic-stress disorders (PTSD) (Rauch et al., 2006). SSRIs are the first-line drugs for the treatment of OCD and PTSD, which suggests that 5-HT-mediated neurotransmission is involved in their pathophysiology (Zohar et al., 2000).

Casarotto et al. (2010) demonstrated that CBD (single or repeated doses) decrease defensive responses in the marble burying test. Another study using the metachlorophenylpyperazine (mCPP), a nonselective 5-HT1A/D and 5-HT2C receptors agonist (Kennett et al., 1989) showed that CBD pre-treatment reduced the number of buried marbles (Casarotto et al., 2010; Umathe et al., 2011; Nardo et al., 2014).

Regarding the putative effects of CBD on PTSD, a number of good studies are available in the literature. Using the fear conditioning paradigm, several groups showed that the administration of CBD in rodents reduced the expression of fear, interrupting the reconsolidation of memory and facilitating the process of extinction (Stern et al., 2012; Berardi et al., 2016; Jurkus et al., 2016; Song et al., 2016; Bitencourt and Takahashi, 2018). CBD also promoted contextual fear conditioning

extinction when infused into the infra-limbic region of medial prefrontal cortex (Do Monte et al., 2013). In spontaneously hypertensive rats (SHR), the treatment with CBD mitigate acquisition of contextual fear memory (Levin et al., 2012).

Another animal model used to study some aspects of PTSD is based on prey *vs.* predator paradigm. The exposure of rats to the predator (cat) triggers a long-lasting anxiogenic behavior, symptoms found in patients with PTSD. Campos et al. (2013a) demonstrated that repeated administration of CBD prevents long-lasting anxiogenic effects promoted by a single predatory exposure followed by an upregulation of 5-HT1A mRNA in hippocampus and prefrontal cortex (Campos et al., 2012a). Similar effects CBD-induced were observed also when mice (prey) were exposed to a constricting snake (predator) (Uribe-Marĩo et al., 2012).

In humans, a case-report suggested the putative effects of CBD in PTSD (Shannon and Opila-Lehman, 2016). Recently, Elms et al. (2019) conducted a retrospective review of medical records of 11 adult psychiatric patients diagnosed with PTSD who consented to CBD treatment as a complement to their routine of psychiatric treatment (drugs + psychotherapy). CBD administration for 8 weeks decreased the severity of PTSD symptoms in 91%. Neuroimaging studies have shown that the CBD administration promoted a change in the activity of amygdala, thalamus, the anterior cingulate gyrus, ventromedial prefrontal cortex (vmPFC), important structures in modulating behavior in patients with diagnosis of PTSD (Lanius et al., 2003; Milad et al., 2007; Passie et al., 2012).

Drug Addiction

Addiction is a chronic and recurrent psychiatric disorder characterized by complex behavioral and neurobiological features that promote the compulsive and non-controlled use of a particular drug, such as cocaine, alcohol and opioids (Camí and Farré, 2003; Volkow and Li, 2005; Viudez-Martínez et al., 2018). It constitutes a public health problem in several countries (Lhermitte et al., 2012; Modesto-Lowe et al., 2017) with few effective treatments available.

In this scenario, CBD has been investigated as a possible therapeutic strategy for the treatment of drug addiction (Hay et al., 2018; Luján et al., 2018). In the self-administration model (Sanchis-Segura and Spanagel, 2006; Panlilio and Goldberg, 2007) CBD attenuated the self-administration of methamphetamine, but not heroin, in rats (Ren et al., 2009; Hay et al., 2018). Mahmud et al. (2017) also noted that acute administration of CBD did not alter cocaine self-administration or cue-induced relapse to cocaine seeking. However, in a 7-days treatment regimen, CBD attenuated cue-induced reinstatement of cocaine self-administration in rats (Gonzalez-Cuevas et al., 2018). In the conditioned place preference (CPP) test (Tzschentke, 2007), CBD potentiated the extinction of both cocaine and amphetamine use (Parker et al., 2004; Luján et al., 2018).

Regarding ethanol, CBD promoted significant reduction of ethanol consumption following by decreased neuronal tyrosine hydroxylase gene expression in the ventral tegmental area and reduced neuronal GPR55 signaling in the nucleus accumbens (NAc) (Viudez-Martínez et al., 2018).

In humans, a double-blind placebo randomized clinical suggested that CBD treatment (during one week) reduced the total number of cigarettes smoked (Morgan et al., 2013). In addition, 10-week treatment with CBD improved psychological and cognitive symptomatology observed in an open-label clinical trial realized in 20 ongoing cannabis users (Solowij et al., 2018). In individuals in abstinence of heroin acute administration of CBD, in contrast to placebo, significantly reduced the crack and anxiety induced by the presentation of protruding drug signs compared to neutral signs (Hurd et al., 2019). These data reinforce the results obtained in animal studies, however, the mechanisms involved in these actions need to be clarified.

Antipsychotic Properties

Schizophrenia is a complex disorder characterized by the presence of psychotic symptoms, such as delusions and hallucinations, and by a core of negative symptoms, social isolation and anhedonia, affecting about 1% of world's population (Egan and Weinberger, 1997; Freedman, 2003; Nucifora et al., 2019).

Zuardi et al. (1991) were pioneers in CBD research with potential antipsychotic properties. In this study, using apomorphine-induced stereotypy in rats, CBD, similar to the antipsychotic haloperidol, decreased the stereotyped behavior (related to positive symptoms of schizophrenia) in a dose-related manner. Moreover, contrary to haloperidol, CBD did not induce catalepsy, even at high doses (Zuardi et al., 1991).

Supporting this idea, CBD reduced the hyperlocomotion induced by the administration of D-amphetamine, an indirect dopaminergic agonist (Moreira and Guimarães, 2005). CBD also attenuated the hyperlocomotion observed after the administration of NMDA-antagonist, ketamine in the open field test (Moreira and Guimarães, 2005; Gururajan et al., 2012).

In the pre-pulse inhibition (PPI) of the startle response test, acute treatment with CBD ameliorates startle reflex deficits in rats (Long et al., 2006; Zuardi et al., 2012). Recently, Pedrazzi et al. showed that the pre-treatment with CBD (systemic or intra-NAc) attenuated the disruptive effects of amphetamine in mice submitted to the PPI ((Pedrazzi et al., 2015).

CBD is also effective in chronic models of schizophrenia in rodents. CBD can reduce psychotic-like effects induces by the chronic treatment with NMDA receptor antagonists, such as MK801 (during 28 days), by restoring the performance of mice in the social interaction test (related to negative symptoms) and new object recognition test (NOR-evaluates memory) (Gomes et al., 2015; Rodrigues da Silva et al., 2020). CBD treatment for 6 days rescued cognitive deficits induced by ketamine in rats submitted to NOR by reducing the transcriptional changes induced by ketamine in prefrontal cortex (Kozela et al., 2020).

In humans, a randomized, double-blinded study showed that CBD treatment produced clinical improvement of some symptoms of schizophrenia that is accompanied by a significant increase in serum levels of anandamide (AEA), resulted from the inhibition of the fatty-acid amide hydrolase

(FAAH), enzyme that metabolizes this endocannabinoid (Leweke et al., 2012). Additionality, in another study, schizophrenic patients received CBD or placebo along with their pre-existing antipsychotic medication for 6 weeks and it was observed that CBD reduced the negative symptoms of schizophrenia as well as improved the patients' cognitive performances (McGuire et al., 2018).

Neurodegenerative Diseases

Neurodegenerative diseases are severe and debilitating conditions produced by the progressive degeneration and death of neurons in the brain triggered by several factors such as inflammatory processes, reactive-oxygen species (ROS), cytotoxicity, mitochondrial and protein dysfunction (Lassmann et al., 2001; Tiraboschi et al., 2004; Almeida-Santos et al., 2017). CBD has antioxidant, anti-inflammatory, anti-apoptotic and neuroprotective properties that was demonstrated by several *in vitro* and *in vivo* studies using models of ischemia, cerebral malaria, Alzheimer's Disease (AD), Huntington's Disease (HD), MS and Parkinson's Disease (PD) (Martín-Moreno et al., 2011; Fernández-Ruiz et al., 2013; Mori et al., 2017; da Silva et al., 2018).

CBD reduced tau protein hyperphosphorylation (Casarejos et al., 2013; Aso et al., 2016) and the production of interleukins and nitric oxide in the brain (Iuvone et al., 2009; Walther and Halpern, 2010; Aso et al., 2016). In *in vitro* models of AD and MS, CBD pretreatment reduced ROS accumulation, mitochondrial dysfunction, lipid peroxidation, caspase-3 levels and DNA fragmentation (Iuvone et al., 2004; Vallée et al., 2017).

CBD also promotes neuroprotective action in an animal model of PD, presumably because of their antioxidant properties (García-Arencibia et al., 2007). In PD's animal model produced after the unilateral injection of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle, the administration of CBD immediately after the injury, recovered the dopamine depletion in nigrostriatal neurons, but did not revert the consequences of the dopaminergic neurodegeneration when the treatment started 1 week after the injury (Noor et al., 2002; García-Arencibia et al., 2007).

An open pilot study conducted in PD patients showed that CBD, when associated with medications used in the clinic to treat PD, reduced psychotic symptoms without influencing the cognitive and motor signs of the disease (Zuardi et al., 2009). In a subsequent clinical trial, Chagas and colleagues suggested that CBD may improve motor symptoms, sleep disturbances and, the quality of life in patients with PD (Chagas et al., 2014).

The neuroprotective effects of CBD have also been described in MS. In a mouse model of MS, CBD administration mitigated experimental autoimmune encephalomyelitis (EAE) by increasing anti-inflammatory and reducing pro-inflammatory cytokines (Elliott et al., 2018). In patients with MS, oromucous spray composed of Δ^9-THC/CBD (Sativex®) promoted a reduction of spasticity without serious adverse effects (Collin et al., 2010; Notcutt et al., 2012). The mechanism of action of Sativex in humans is not well elucidated, however in animal models of EAE, the treatment with Sativex-like combination of Δ^9-THC and cannabidiol attenuated the progression of EAE through the activation of CB_1 receptors (Hilliard et al., 2012; Moreno-Martet et al., 2015). Currently, Sativex® is approved in some countries for the treatment of MS-related spasticity and neuropathic pain (Fraguas-Sánchez and Torres-Suárez, 2018).

Similar to PD, HD is characterized by changes in behavior and motor disorders (Niccolini, 2014). In HD animal model, the administration of CBD completely reversed 3-nitro propionic acid (3NP) reductions in mRNA levels for SOD-2. However, a trial conducted in patients with HD, Sativex® did not significatively improve motor, cognitive or psychiatric impairment related to HD (López-Sendón Moreno et al., 2016).

CBD can also exert neuroprotective effects in animal models of brain ischemia. In brain slices of newborn rats submitted to oxygen and glucose deprivation CBD reduced acute (LDH efflux to the incubation medium) and apoptotic (caspase-9 concentration in tissue) (Castillo et al., 2010). CBD prevented the increase of excitotoxicity, oxidative stress and inflammation in hypoxic-ischemic (HI) brain injury model in newborn pigs. Mori et al. (2017) demonstrated that short-term treatment with CBD results in global functional recovery in ischemic mice. The main mechanisms of neuroprotection are mediated by the reduction of oxidative stress and anti-inflammatory action induced by CBD treatment.

THE WIDESPREAD FUNCTIONS OF GLIAL CELLS IN THE BRAIN

The first records of the use of the term glia (from the ancient Greek: glue) in the field of neuroscience are from 1850 (as a reference for their former attributed function: put the SNC together). Rudolf Virchow proposed the term neuroglia to describe the "substance ... which lies between the proper nervous parts, holds them together and gives the whole its form in a greater or lesser degree." The term was also generally used to emphasize the systematic identification of glial cells associated with pathological changes, such as glial tumors, encephalitis, and myelitis (Virchow, 1856; Fan and Agid, 2018). Later, several neuroanatomists and neurophysiologists have characterized different cell types as part of neuroglia. These groups of cells were divided into two categories according to their embryonic origin: macroglia (of ectodermal origin: astrocytes, oligodendrocytes, and polydendrocytes) and microglia (originated from the yok Salk's).

Astrocytes (from the Greek *Astron*: star, while *kytos*: hollow vessel) were the first to be identified by Michael Von Lenhossék in 1891 (Von Lenhossék, 1895). The most numerous cells in the CNS, astrocytes play a crucial role in its metabolic support, maintenance of ionic and osmotic homeostasis, regulation of neurotransmitter levels in the synaptic cleft, control of the communication between the brain and the periphery, support for synaptic signaling and mediation of neurovascular coupling. They also actively participate in the formation, maintenance, and proper signaling of the synapses (Khakh and Sofroniew, 2015; Kozela et al., 2017).

Astrocytes are classified in subtypes based on their morphological and functional properties: protoplasmic, fibrous, interlaminar, and varicose projection. The last two subtypes listed

are found in primates' brains, but not in rodents (Tabata, 2015). Protoplasmic astrocytes are widely distributed in the gray matter associated with neuronal synaptic terminals to comprise the tripartite synapses (Kozela et al., 2017; Tabata, 2015). On the other hand, fibrous astrocytes are primarily located in the white matter and express higher levels of Glial fibrillary acidic protein (GFAP), a protein described by Ramon y Cajal in the early 1900s as a marker of astrocytes. Nevertheless, expression of GFAP varies depending on the brain region and, in healthy states, some astrocytes do not express GFAP (Khakh and Sofroniew, 2015). Other molecules used as astrocytic markers are GLAST, GLT-1, connexin 30, S100β, glutamine synthetase, aquaporin four, and aldehyde dehydrogenase one family, member L1 (Yang et al., 2011; Khakh and Sofroniew, 2015).

Oligodendrocytes were described for the first time by Ford Robertson in 1899 and called mesoglia. Later, Cajal and his student Pio del Río-Hortega designated these cells as oligodendroglia (the interfascicular glia) (Ramóny Cajal, 1920). This glial cell is responsible for myelin production, providing energy-efficiency to neurons and maintaining axonal integrity through trophic and metabolic support (Michalski and Kothary, 2015; Simons and Nave, 2016).

Among the glial cells, microglia had the most intriguing discovery. In 1841, Gluge described phagocytic cells in the damaged brain for the first time. He called these cells "inflammatory corpuscles." Microglia was later described and named by other neuroanatomists: foam cells (Virchow, 1856), road cells (Nissl, 1899), granuloadipose cells (Achúcarro, 1909) and scavenger cells (Merzbacher, 1910). Although called by many names, they were always described as phagocytic cells in damaged or inflamed brain tissue (Rezaie et al., 2014). Finally, the term microglia was minted by the fantastic work of Pio Del Río-Hortega in 1919 to refer to these cells that are the resident macrophages of the CNS and promote early host defense against infections or injuries (Tremblay et al., 2015).

In the 1980s, the fourth known type of glial cells, distinct from mature oligodendrocytes, astrocytes, and microglia, the polydendrocytes (also called NG2 cells, oligodendrocyte precursor cells or synaptocytes), was described. These cells express the chondroitin sulfate proteoglycan NG2 and are present in both the gray and the white matter (Dawson et al., 2003; Hughes et al., 2013). Different from other glial cells, polydendrocytes are considered bipotential cells that putative generate both oligodendrocytes and protoplasmic astrocytes (Nishiyama et al., 2009). In several animal models of demyelination, NG2 cells are shown to rapidly replace oligodendrocytes (Gensert and Goldman, 1997; Abílio and Reynolds, 1999; Tanaka et al., 2004), suggesting that they play a role in remyelination and brain homeostasis. Nonetheless, their function goes further than the generation of new oligodendrocytes in the brain. NG2 cells are in close proximity to neurons and may be an integral component of synaptic connections (Butt et al., 2005).

After more than 150 years of research, the heterogeneous population of glial cells is much more than structures that fill the empty spaces between neurons. They play essential roles for the maintenance of critical aspects of brain homeostasis, including: (1) energy metabolism; (2) ion homeostasis; (3) network and cellular homeostasis; (4) neurotransmitter clearance; (5) organ homeostasis and osmotic control; and (6) immune response (for review see, Jäkel and Dimou, 2017).

GLIAL CELLS AND THEIR RESPONSE TO CNS DAMAGE

Reactive gliosis is observed under various neurological conditions such as infection, ischemia, trauma, and neurodegeneration, and psychiatric disorders. The activation of these glial cells usually involves hypertrophy and proliferation, changes in the patterns of gene expression, and release of chemokines, cytokines, and neurotrophic factors. Once released, these factors can either induce neuroprotection or produce damage in the neural tissue (Lee and Chung, 2019).

Activation of microglia is a common hallmark of a diverse range of neurodegenerative diseases, including AD (Esposito et al., 2007, 2011), MS, PD, HD (Sapp et al., 2001), and is considered to be responsible for the ongoing inflammatory condition occurring in neurodegenerative diseases. Of note, the activation of glial cells during insults or neuropsychiatric conditions are not only a secondary response to damage, but could also play a pathophysiological role in its development (Jonsson et al., 2013; Hong et al., 2016; Sekar et al., 2016).

For instance, reactive astrocytes and microglia are found to be important for protein misfolding removal in neurodegenerative diseases. Activated glial cells can be found around Aβ plaques and play beneficial or harmful roles in disease progression (Nagele et al., 2003; Olabarria et al., 2010; Simpson et al., 2010). A similar panorama is found in PD brains, were reactive astrocytes and microglial cells can be found close to *a*-synuclein inclusions. Astrocytes can internalize Aβ and *a*-synuclein *in vitro* (Wakabayashi et al., 2000; Wyss-Coray et al., 2003; Pihlaja et al., 2008; Lee et al., 2010; Braidy et al., 2013). Like astrocytes, microglial cells are located around Aβ plaques and *a*-synuclein inclusions in both human and mouse brains (Perlmutter et al., 1990; Bolmont et al., 2008; Grathwohl et al., 2009).

Oxidative stress and pro-inflammatory mechanisms are actively involved in misfolding protein aggregation. On the other hand, misfolded proteins can also lead to excessive oxidative stress and inflammation leading to neurotoxicity and neurodegeneration. Glial cells not only can produce ROS and proinflammatory signals, but also suffer the consequences of this "unfriendly" neurodegenerative environment (Singh et al., 2019). In addition, glial cells are involved in maintaining the inflammatory state during epilepsy by the release of inflammatory cytokines (Ravizza et al., 2008; Devinsky et al., 2013; Do Val-da Silva et al., 2017).

In the case of psychiatric disorders, the "new" Neuro-immune hypothesis states that circulating levels of cytokines and immune cells are found increased in patients with mood disorders, schizophrenia, and post-traumatic stress disorder. However, glial reductions are described by independent laboratories in different brain areas. For instance, mixed data is found in this regard in the anterior cingulate cortex, prefrontal cortex, and

orbitofrontal cortex of patients with mood disorders (Stockmeier and Rajkowska, 2004; Wilczyńska et al., 2018). Similarly, in schizophrenia, some studies demonstrate increased microglia activation. In contrast, others failed to replicate earlier studies and found no differences between patients and healthy controls (Trépanier et al., 2016). Therefore, although the role of gliosis in neurodegenerative diseases is established, in the case of psychiatric disorders, the panorama is not so clear. However, it is important to remember that besides their vital functions in the homeostatic and pathological brain, glial cells express several pharmacological receptors that are used as primary targets by drugs, including CBD, to produce their therapeutic actions, as we will discuss in the next topics.

CBD EFFECTS THAT NOT INCLUDE GLIAL CELLS IN ITS MECHANISM OF ACTION (YET)

Why it has been so difficult to figure out the mechanism of action of CBD? In every storyline, to understand the entire plot, it is essential to know the characters. Are all potential characters being considered to construct CBD's storyline? Most studies aimed at investigating these mechanisms do so with the assumption that they are located in neurons. Even when putative brain sites of CBD action are identified, their precise cellular location is unknown (Khan et al., 2020). Khan et al. (2020) showed that CBD exerted a CB_1-dependent panicolytic-like effect after the pharmacological excitation of the ventromedial hypothalamus in rats. Neuronal excitatory activity has been shown to cause an accumulation of lactate. Brain regions such as the hypothalamus respond to pH changes caused by the activity-dependent increase in lactate (Maddock et al., 2013). Lactate is generated via glycolytic metabolism, mainly in astrocytes, and then transferred to neurons as an energy source (Riske et al., 2017). Recently, it has been shown that mitochondrial CB_1 receptors present in astrocytes dampen lactate production (Jimenez-Blasco et al., 2020). Therefore, activation of astrocytic CB_1 could diminish activity-dependent lactate increase and interfere in neural activity and in resulting behavioral responses.

Other CBD effects that depend on cannabinoid CB_1 and CB_2 receptors, including the disruption of fear memories, alterations in reward-related responses, and neuroprotection (Castillo et al., 2010; Pazos et al., 2013; Stern et al., 2017; Bi et al., 2020; Galaj et al., 2020; Raymundi et al., 2020), could also involve glial cells. CB_1 receptors, apart from being expressed in astrocytes, are also found in microglia, oligodendrocytes, and NG2 cells (Molina-Holgado et al., 2002; Cabral, 2005; Stella, 2010). The cellular location of CB_1 receptors in the CNS is relevant for determining its signaling route. While in neurons, CB_1 receptors activate G_i and decrease neurotransmitter release from pre-synaptic terminals, in astrocytes these receptors increase intracellular calcium concentrations and ultimately lead to a potentiation of synaptic transmission (Navarrete and Araque, 2010). CB_2 receptors have also been found in astrocytes, oligodendrocytes, and microglia. There is a discussion, however, whether in these cells, CB_2 receptors are expressed in physiological conditions or only induced in pathological conditions (Molina-Holgado et al., 2002; Stella, 2010; Cassano et al., 2017; Tanaka et al., 2020).

Microglia show remarkable plasticity and can adopt a spectrum of polarized states in response to microenvironmental cues. Interestingly, cannabinoid receptors expressed in these cells vary depending on their activation profile. In intact, healthy brain tissue, microglial cells behave predominantly in a resting state, a condition where immunostaining assays often do not find CB_1 positive cells (Stella, 2010). Regarding CB_2 receptors, studies performed in rodent or human samples in general reports the lack of CB_2 receptors or their presence in levels too low to be quantified (Munro et al., 1993; Galiègue et al., 1995; Schatz et al., 1997; McCoy et al., 1999). On the other hand, as shown in mice models and patients, during specific neuroinflammatory conditions CB_2 receptor is upregulated in activated microglia. This effect has been associated with responses to microenvironment changes such as the presence of pathogens, cytokines, and other molecules (Maresz et al., 2005; Palazuelos et al., 2009). The regulatory mechanisms that drive the expression of specific microglia phenotype and whether CBD exert its anti-inflammatory effects by modulating these mechanisms remains to be understood. Mecha et al. (2015) reported that AEA and 2-arachidonoylglycerol are independently regulated in microglia by specific anti-inflammatory cues, suggesting that endocannabinoid signaling plays crucial role in regulating microglia phenotype during neuroinflammatory and neurodegenerative conditions.

The enhancement of endocannabinoid signaling, specially AEA, has been proposed as one of the actions of CBD in the CNS (Watanabe et al., 1996; Bisogno et al., 2001; Campos et al., 2013a). Neurons are not the only cells to populate the CNS that produce and release endocannabinoids. Microglial cells and astrocytes also express the AEA-synthesizing enzyme NAPE-PLD (Kallendrusch et al., 2012), and it was shown that *in vitro* astrocytes can produce AEA (Walter et al., 2002). Gabrielli et al. (2015) showed that microglial cells produce AEA and release the endocannabinoid to the perivascular space in association with macrovesicles or exosomes, modulating the activity of inhibitory neurons.

FAAH and fatty-acid binding proteins (FABPs), which participate in the transport and hydrolysis of AEA, respectively, are expressed in microglia, astrocytes, oligodendrocytes, and NG2-positive cells (Egertová et al., 2003; Kallendrusch et al., 2012; Graves, 2013; Sharifi et al., 2013; Young et al., 2013; Duffy et al., 2017; Gerstner et al., 2017; Foerster et al., 2020). FABP5 and FABP7 have been shown to regulate the proliferation of NG2-positive cells and their differentiation to oligodendrocytes (Sharifi et al., 2013). Brains astrocytes expressing FABP7 are largely concentrated at the hippocampal neurogenic niche, often in close proximity to proliferating precursor cells located in subgranular zone of the dentate gyrus (DG) (Boneva et al., 2011; Young et al., 2013). CBD has important effects in this neurogenic niche, and local enhancement of AEA levels is one of the mechanisms associated with its pro-neurogenic actions (Campos et al., 2013a; Fogaça et al., 2018).

Glial cells are important for the process of adult hippocampal neurogenesis. In the neurogenic niches, apoptosis in an important mechanism. Microglial cells located in subgranular zone of the

DG are sensors of cell death and rapidly eliminate cell debris through phagocytosis, an essential step of the neurogenic process (Sierra et al., 2010; Diaz-Aparicio et al., 2020). In turn, astrocytes control the proliferation, survival, and differentiation of progenitor cells (Song et al., 2002; Barkho et al., 2006; Lu and Kipnis, 2010; Terrillion et al., 2017; Wilhelmsson et al., 2019; Asrican et al., 2020). This property seems to be restricted to astrocytes localized at neurogenic niches, indicating that these cells might provide regionally-specific signals that allow certain brain areas to maintain its capability of generating new cells (Song et al., 2002). Besides, Sultan et al. (2015) demonstrated that astrocytes are essential for regulating the survival and integration of newly-born neurons into the adult hippocampal synaptic circuitry. CBD has been shown to increase the proliferation, survival, differentiation, and migration of precursor cells in the subgranular zone of the dentate gyrus of the hippocampus (Esposito et al., 2011; Campos et al., 2013a; Schiavon et al., 2016; Fogaça et al., 2018; Luján et al., 2018). Nevertheless, it is still unknown whether CBD acts directly at progenitor cells and neuroblasts or indirectly by modulating the function of local cells that control the neurogenic process.

Neural stem cells located in the neurogenic niches of the adult brain are multipotent and can give rise to astrocytes, even though the generation of new-astrocytes in the subgranular zone is usually underestimated and very rarely evaluated. In fact, Bonaguidi et al. (2011), using a genetic non-invasive approach to evaluate the lineage tracing of nestin-positive radial glia-like precursors and showed that the number of newly-born astrocytes is similar to that of new neurons in the adult dentate gyrus. Nevertheless, none of the studies that evaluated CBD effects in the subgranular zone of the dentate gyrus addressed astrogliogenesis. Some works used as the sole measure of neurogenesis the number of cells that express doublecortin (DCX) in the dentate gyrus (Esposito et al., 2011; Mori et al., 2017). Even though DCX is widely proposed as a marker of cells compromised with the neuronal phenotype, it has been shown that glial cells, especially some polydendrocytes, can also express DCX (Boulanger and Messier, 2017). Besides, when the fate of newly born cells was evaluated, only markers of the neuronal phenotype were used (Campos et al., 2013a; Fogaça et al., 2018; Luján et al., 2018).

The potential impact of CBD on astrogliogenesis in the dentate gyrus remains unknown. Campos et al. (2013a) showed that two weeks of treatment with CBD (30 mg/kg) increased the proliferation of precursor cells in the dentate gyrus of wild-type animals. The drug, however, failed to change this proliferation in the hippocampus of ganciclovir-treated mice expressing the thymidine kinase (TK) under the control of the GFAP promoter (GFAP-TK). The dampened pro-proliferative effect of CBD prevented its anxiolytic-like effect in chronically stressed GFAP-TK mice. The study concluded that an intact adult hippocampal neurogenesis capacity is needed for the anxiolytic response generated by CBD in animals exposed to chronic stress. Nonetheless, in transgenic GFAP-TK mice treated with ganciclovir there is a depletion of radial glia-like GFAP-positive precursor cells, which could potentially impact not only neurogenesis but also the astrogliogenesis in the dentate gyrus. Whether or not astrogliogenesis could play a role in the anxiolytic-like response triggered by CBD in chronically stressed mice, remains to be investigated.

Another approach used to address the relevance of neurogenesis in CBD effects is the pharmacological inhibition of cell division. Luján et al. (2020) showed that a 10-days treatment with CBD (20 mg/kg) reduces cocaine self-administration, and this effect was blocked in mice previously treated systemically with the chemotherapy drug temozolomide. The study proposed that the alkylating agent would affect the DNA replication and mitosis of cells with low proliferative profile, like neural precursor cells. Indeed, the chemotherapy drug reduced the number of new neurons generated in the dentate gyrus after CBD treatment. The authors concluded that adult neurogenesis could be essential for the reduction of cocaine intake induced by CBD. Again, this study does not address astrogliogenesis. Besides, outside neurogenic niches of the adult brain, polydendrocytes (NG2 cells) are the CNS main proliferative cells (Geha et al., 2010). These cells, apart from being oligodendrocytes precursors, control ion homeostasis, remyelination, receive synaptic inputs from glutamatergic and GABAergic neurons, and might even be able to differentiate into neurons (Nishiyama et al., 2009; Nishiyama et al., 2014). A pharmacological protocol that interferes with cell division could affect the proliferation and function of NG2 cells. No study so far, nonetheless, has addressed the role of NG2 cells on the effects of CBD.

Stress is as a common risk factor for most of the psychiatric disorders for which CBD is proposed to be effective. In animal models, repeated CBD treatment counteracts the effects of chronic stress exposure. Fogaça et al. (2018) demonstrated that two weeks of treatment with CBD (30 mg/kg) prevented stress-induced impairment in synaptic plasticity, represented by a decrease in dendritic arborization and the number of dendritic spines density in granular neurons of the dentate gyrus. Chronic stress alters glial function and, just like CBD, pharmacological or genetic targeting these stress-induced changes modify its behavioral and neuroplastic consequences. Chronic stress increases astrocyte number and microglial activation in the dentate gyrus of the hippocampus (Machado-Santos et al., 2019; Du Preez et al., 2020). Yu et al. (2019) showed that chronic stress decreases the expression of the astroglial glutamate transporter-1 (GLT-1) in the hippocampus after ischemic stroke, which was accompanied by impaired synaptic plasticity and depressive-like behavior. Ceftriaxone, an antibiotic known to increase GLT-1 expression, counteracted the deleterious behavioral and neuroplastic effects of stress exposure (Yu et al., 2019). Besides, Hao et al. (2020) demonstrated that astrocyte chemogenetic inhibition in the hippocampus and prefrontal cortex, and microglial depletion reverse the behavioral consequences of a ten-day exposure to social defeat stress.

The brain-derived neurotrophic factor (BDNF) has been implicated in several brain functions. Decreased levels of BDNF is commonly associated with stress-related disorders, including depression and anxiety. BDNF modulates neuronal as well as glial functions. Ye et al. (2011) showed that the intrahippocampal infusion of BDNF restored the levels of the astrocytic proteins GFAP and S100b in stressed rats. Moreover,

BDNF overexpression in hippocampal astrocytes increased neurogenesis and induced an anxiolytic-like response in the novelty suppressed feeding test (Quesseveur et al., 2013). Besides, the antidepressant fluoxetine has been shown to induce an ATP-mediated increase in BDNF in hippocampal astrocytes. This ATP-dependent mechanism is directly related to the antidepressant-like effect triggered by this drug (Kinoshita et al., 2018). CBD also seems to affect BDNF levels, although the cell types involved in this effect are unknown. Sales et al. (2018) showed that acute CBD treatment induces a rapid antidepressant-like effect accompanied by an increase in BDNF levels in the hippocampus and prefrontal cortex. CBD antidepressant-like effect was blocked by the intracerebroventricular administration of K252a, an antagonist of BDNF receptor TrkB. Furthermore, chronic CBD treatment attenuates the decreases in BDNF and the astroglial protein GFAP observed in the hippocampus of diabetic animals submitted to a model of chronic cerebral hypoperfusion (Santiago et al., 2019).

As discussed before, the serotonergic system is also frequently associated with CBD effects. Treatment with this drug increased serotonin levels in the prefrontal cortex (Linge et al., 2016). The pretreatment with an inhibitor of serotonin synthesis abolished the antidepressant-like effect of acute CBD treatment (Sales et al., 2018). This increase in serotonin levels induced by CBD has been attributed to its action at serotonergic 5-HT1A receptors, once CBD acts as an agonist of 5-HT1A receptors (Russo et al., 2005). CBD action at 5-HT1A receptors, however, could be also indirect, by acting as an allosteric modulator (Rock et al., 2012).

Apart from its antidepressant-like properties, other actions of CBD seem to depend on 5HT1A receptors. Administration of WAY100635, a 5-HT1A antagonist, prevented the antipsychotic-like effects of CBD in a mouse model of schizophrenia based on chronic NMDA receptor antagonism (Rodrigues-da-Silva et al., 2020). Serotonin-dependent synaptic plasticity might depend on 5-HT1A glial receptors, once serotonin modulates the density of synaptic connections in the dentate gyrus of the hippocampus via astroglial 5-HT1A receptors (Wilson et al., 1998). Also, glial cells are able to modulate extracellular serotonin levels by expressing the serotonin transporter (Inazu et al., 2001). Other serotonin receptors might modulate astrocytic calcium signaling (Schipke et al., 2011) and microglial exosome release (Glebov et al., 2015).

In addition to its interaction with membrane-associated receptors and related downstream signaling cascades, CBD can also bind to nuclear receptors. Converging evidence obtained over the last decade indicate that the peroxisome proliferator-activated receptor-γ (PPAR-γ) is a nuclear target to CBD. This receptor modulates the expression of genes related to the control of central and peripheral inflammation and immune responses (Bensinger and Tontonoz 2008; Wang et al., 2013). Activation of this receptor by CBD could interfere with transcriptional pathways responsible for inflammatory responses, eg, modulation of NF-κB signaling (Esposito et al., 2007). Therefore, PPAR-γ is frequently associated with CBD neuroimmune effects. This receptor is expressed by astrocytes, microglial cells, oligodendrocytes, NG2 cells, and neurons (Bernardo et al., 2000; Cristiano et al., 2001; Gray et al., 2012; Ke et al., 2014; Ding et al., 2020).

CBD might also exert its protective effects by reducing the permeability of the blood-brain barrier (BBB). Mecha et al. (2013) showed that in animal model of MS, CBD neuroprotective effects were mediated by adenosine A2, another receptor frequently linked to its neuroimmune modulatory action (Castillo et al., 2010; Mecha et al., 2013).

Glial cells respond to neuronal stimulation, releasing gliotransmitters (like glutamate, prostaglandins, and ATP) and actively affecting neuronal firing rate and synaptic plasticity in the developing and adult brain (Haydon, 2001). The description of the tripartite synapse, a concept that could even be expanded for a quadpartite or even a pentapartite synapse, highlighted that the normal brain requires proper functioning of glial cells that ultimately maintain the homeostasis of the system (Perea et al., 2009; Schafer et al., 2013; De Luca et al., 2020). This new perspective also impacts how psychopharmacology looks at the action of psychoactive drugs like CBD. Investigating the non-neuronal cells involved in the actions of CBD might be as relevant as identifying the receptors targeted by this phytocannabinoid. As discussed above, several of the receptors target by CBD are present in astrocytes, microglial cells, oligodendrocytes, and NG2 cells. Very few studies, however, have investigated whether glial cells play a role in CBD potential therapeutic effects. **Figure 1** summarizes the main receptors and enzymes present in glia that have been shown to participate in CBD actions.

CURRENT EVIDENCE SHOWING THAT CBD ACTS BY MODULATING GLIAL CELLS FUNCTIONS

CBD and Astrocytes

In studies evaluating CBD effects in astrocytes, GFAP and S100β are the most commonly used astroglial markers. di Giacomo et al. (2020) showed that astrocytes treated for 48 h with 1 μM of CBD present increased cell proliferation. The same concentration of CBD protected astrocytes from oxidative damage and apoptosis after exposure to hydrogen peroxide *in vitro*. In human astrocytes in co-culture with human brain microvascular endothelial cells submitted to oxygen-glucose deprivation, CBD increased cell survival, evidenced by a reduced lactate dehydrogenase (LDH) release and decreased VCAM-1 expression (Hind et al., 2016). In human astrocytes in monoculture, however, CBD increased cell damage levels, with increased LDH levels, at the concentration of 10 μM (Hind et al., 2016). Auzmendi et al. (2020) used a primary astrocyte culture to show that CBD treatment, in a concentration-dependent manner, inhibits the active efflux of the P-glycoprotein substrate rhodamine-123.

Lafuente et al. (2011) showed that a hypoxic-ischemic lesion in newborn piglets led to a decrease in cortical GFAP-positive cells and an increase in the levels of S100β in the cerebrospinal fluid, indicating astrocytic damage. CBD treatment attenuated the alterations in astrocytic markers, indicating that it protects astrocytes from ischemic injury. Mori et al. (2017) showed that 21 days after bilateral carotid artery occlusion in adult mice, there was an increase in immunoreactivity for GFAP in

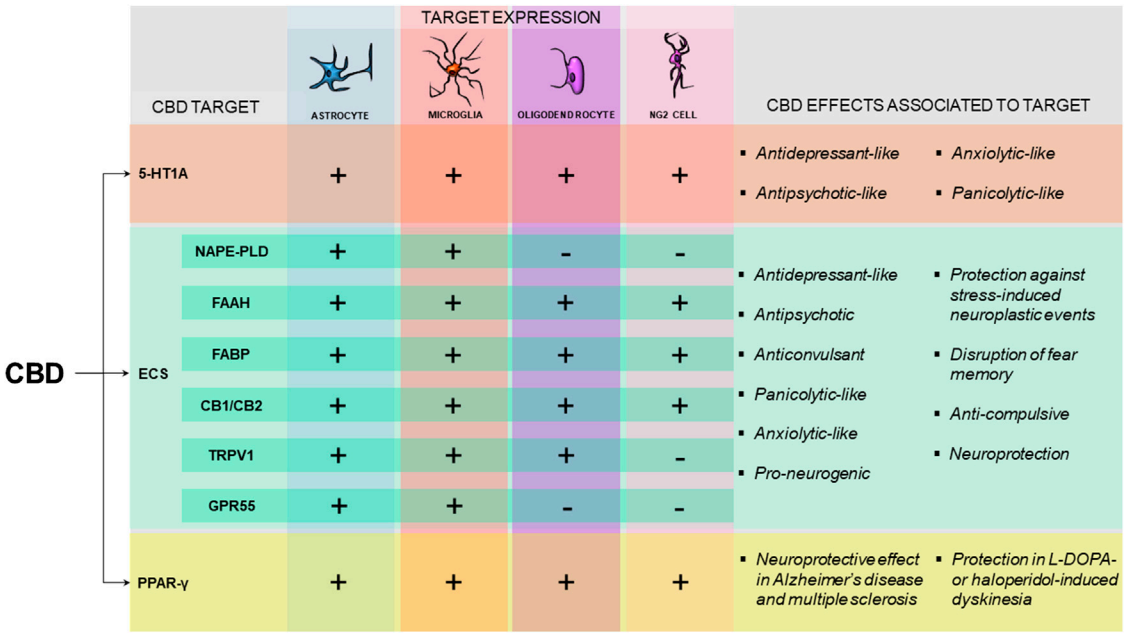

FIGURE 1 | Pattern of expression and effects associated with the molecular targets potentially involved in CBD mechanism of action in glial cells. Schematic representation pointing the expression of 5-HT1A receptor, proteins of the endocannabinoid system (ECS) and the PPAR-γ receptor in astrocytes, microglia, oligodendrocytes and NG2 cells (Azmitia et al., 1996; Bernardo et al., 2000; Egertová et al., 2003; Zhang et al., 2011; Gonzalez-Reyes et al., 2013; Graves, 2013; Sharifi et al., 2013; Young et al., 2013; Ke et al., 2014; Fan and Agid, 2018; Duffy et al., 2017; Viudez-Martínez et al., 2018; Kong et al., 2019; Yang et al., 2019; Ding et al., 2020) (+) indicates that there is evidence that the protein has been found in the specific cell type (–) indicates the absence of evidence for the expression of the molecular target in the specific cell type. For the ECS, NAPE-PLD, FAAH and FABP are considered as the potential targets involved in the increased AEA availability induced by CBD treatment. The effects of CBD that have been shown to be dependent on 5-HT1A (Campos and Guimarães, 2008; Zanelati et al., 2010; Campos et al., 2013a; Rodrigues da Silva et al., 2020), on the ECS (Casarotto et al., 2010; Leweke et al., 2012; Stern et al., 2012; Campos et al., 2013b; Pazos et al., 2013; Sartim et al., 2016; Fogaça et al., 2018; Khan et al., 2020) or on PPAR-γ receptors (Esposito et al., 2011; dos-Santos-Pereira et al., 2016; Giacoppo et al., 2017; Sonego et al., 2018) are highlighted. *CBD*, Cannabidiol; *5-HT1A*, 5-hydroxytryptophan 1 A receptor; *ECS*, endocannabinoid system; *NAPE-PLD*, N-acyl phosphatidylethanolamine-specific phospholipase D; *FAAH*, fatty-acid amide hydrolase; *FABP*, fatty acid binding protein; *CB1/CB2*, cannabinoid receptor one/cananbinoid receptor two; *TRPV1*, transient receptor potential vanilloid one; *GPR55*, G coupled receptor 55; PPAR-γ, Peroxisome proliferator-activated receptor-γ.

the CA1 and CA3 hippocampal regions, with augmented total levels of GFAP in the hippocampus. CBD prevented these alterations. Furthermore, in newborn rats exposed to collagenase-induced germinal matrix hemorrhage, CBD treatment reduced the number of reactive astrocytes (GFAP-positive) and caspase-3 positive-astrocytes in the perilesional area (Abrantes de Lacerda Almeida et al., 2019).

Esposito et al. (2011) showed that in cultured astrocytes, CBD treatment decreased the ß-amyloid-induced release of pro-inflammatory mediators such as nitric oxide, TNF-α, S100B, and IL-1β. CBD effects were abolished by the PPAR-γ antagonist, GW9662. *In vivo* data also showed that CBD, via a PPAR-γ-dependent mechanism, diminished the pro-inflammatory response triggered by the intrahippocampal injection of ß-amyloid (Esposito et al., 2011). Through this PPAR-γ-mediated action, CBD is proposed to reduce neuroinflammation and protect neurons from neurodegeneration in AD (Valée et al., 2017). Also, CBD systemic administration dose-dependently reduced the increased hippocampal levels of GFAP mRNA and S100β caused by local injection of ß-amyloid (Esposito et al., 2007, 2011).

Hind et al. (2016) showed in an *in vitro* model using a co-culture of human brain microvascular endothelial cells and astrocytes that CBD decreased BBB permeability via PPAR-γ-dependent mechanism. They propose that this mechanism contributes to the protective effects of CBD in ischemic stroke.

In a rat model of epilepsy based on the chronic treatment with the GABAergic antagonist pentylenetetrazol, co-treatment with CBD prevented the increase in the number of GFAP-positive cells in the CA1 and CA3 hippocampal areas (Mao et al., 2015). Similarly, Gomes et al. (2015) showed that repeated CBD treatment attenuated the increased GFAP-positive cell number in the medial prefrontal cortex in a mouse model of schizophrenia.

Interestingly, although the studies described so far focused on brain glial cells, enteric astrocytes might also be affected by CBD treatment. De Filippis et al. (2011) showed that LPS administration in mice increased intestinal S100β, an effect blocked by CBD. Moreover, CBD attenuate increased S100β levels observed in cultures generated from intestinal biopsies obtained from patients with ulcerative colitis (De Filippis et al., 2011).

CBD and Oligodendroglia

Oligodendrocytes have been associated with white matter dysfunction in neurodegenerative and psychiatric disorders such as schizophrenia (Hof et al., 2002; Flynn et al., 2003).

In this sense, cannabinoids counteract demyelination in some conditions (Molina-Holgado et al., 2002; Mecha et al., 2013; Tomas-Roig et al., 2015). Therefore, CBD and analogs might represent a useful neuroprotective candidate to manage neuropsychiatric white matter-associated deficits. Several lines of evidence highlight CBD benefits toward glial damage in diverse models ranging from pediatric conditions, such as demyelination induced by neonatal hypoxia (Ceprián et al., 2017), to age-related diseases, such as PD and AD, for instance (Benito et al., 2003; García-Arencibia et al., 2009; Fernandez-Ruíz, 2019). In spite of the remarkable therapeutic potential of CBD to demyelinating diseases, more studies are needed to produce a deep understanding of the machinery involved in CBD anti-inflammatory and antioxidant mechanisms are still unknown.

Mecha and colleagues (2012) reported anti-inflammatory effects of CBD (1uM) in OPCs in an independent manner of CB_1, CB_2, TRPV1, and PPAR-γ. This effect was intracellularly mediated by a decrease in the phosphorylation of proteins that coordinates endoplasmic reticulum apoptotic pathway, the RNA-activated serine/threonine kinase (PKR) and translation initiation factor 2α (eIF2α) (Mecha et al., 2012). However, in adult oligodendrocytes derived from the rat optic nerve, CBD (1uL) promoted disruption of mitochondrial membrane potential along with elevation of intracellular calcium and increase of ROS production. This effect leads to a decrease in oligodendrocyte viability via a mechanism of CB_1, CB_2, and TRPV1, but mediated by the activation of both caspase-dependent and independent cell death pathways (Mato et al., 2010). Considering that both studies have used the same concentration of CBD, the divergences in CBD effects might be related to differences in the stage of cells maturation (OPCs *vs.* mature oligodendrocytes). Ceprián et al. (2017) first reported maturation stage-dependency of CBD effects in oligodendrocytes. In the ipsilateral cortex, but not in the white matter, CBD restored mature oligodendrocyte cell density after hypoxic brain injury. In the white matter, CBD protected the axons, preserving appropriate myelination after injury (Ceprián et al., 2017). The authors conclude that the differences between CBD effects in the white matter and ipsilateral cortex could be explained by the distinct maturational stages of oligodendrocytes in these areas. Maturation from OPCs to immature oligodendrocytes in the white matter occurs first than in the ipsilateral cortex (Ceprián et al., 2019).

CBD and Microglial Cells

The hypothesis of activated microglia as a key feature in neurodegenerative diseases and possibly in psychiatric disorders suggests that these cells may represent a new therapeutic approach.

Many lines of evidence suggest that cannabinoids are neuroprotective by promoting anti-inflammatory mechanisms. The effects of CBD have been related to the control of microglial migration, microglia activation, and the toxicity exerted by these cells by producing pro-inflammatory mediators (Rahimi et al., 2015)

A study by Hayakawa et al. (2008) demonstrated that the administration of CBD (3 mg/kg, i.p) immediately before and 3 h after cerebral artery occlusion prevented glial activation, as indicated by the reduction of Iba-1 expression in the infarcted area (Hayakawa et al., 2008). CBD treatment also diminished the infiltrate of immune cells such as neutrophils, macrophages, and monocytes, and decreased the infarct size in a CB_1 and CB_2 independent manner (Hayakawa et al., 2008).

The most straightforward association between CBD actions and microglial cells is the murine model of EAE, which resembles MS-like conditions. CBD ameliorated the disease progression while decreasing the activation of microglial cells in the spinal cord (Kozela et al., 2011). A decrease in microglial activation is also proposed as a possible mechanism of the reduced neuroinflammation and improved cognitive performance observed in mice submitted to a model of AD treated with CBD (Martín-Moreno et al., 2011; Watt et al., 2020).

Recent work by Sonego et al. (2018) has shown in a primary microglial culture that a PPAR-γ antagonist, GW9662, was able to block the protective effects of CBD on the enhancement of Iba-1 expression, the microglial production of ROS, and the NF-κB translocation to the nucleus induced by LPS (Sonego et al., 2018). The intracellular machinery responsible for CBD anti-inflammatory properties remains under investigation, although some mechanisms have been proposed. For instance, CBD was shown to be transported intracellularly by FABPs, which might explain the mechanism for nuclear receptors activation (Elmes et al., 2015). Moreover, CBD was also shown to be able to regulate inflammatory signaling of NF-κB by promoting inhibitory control of phosphorylation of specific kinases (eg p38 MA P kinase, PI3K), preventing the activation of pro-inflammatory genes (Esposito et al., 2007).

CONCLUSIONS AND PERSPECTIVES

Its wide range of putative therapeutic applications, safety profile, and still not very clear action mechanism makes CBD one of the most intriguing phytocannabinoid. Although several groups, including ours, have pointed to the involvement of different receptors (5-HT1A, CB1, CB2, PPAR-γ, Adenosine, TRPV1) and enzymes (FAAH, NAPE-PLD, enzymes related to oxidative stress process) in the effects of CBD, the contribution of specific neural cell types remains poorly understood.

In the present review we highlight the possibility that, in addition to neurons, glial changes could help to explain the complex pharmacology of CBD. Corroborating this proposal, acute or chronic administration of this drug can modify the expression of glial cell markers or induce changes in their morphology. On the other hand, *in vitro* studies using primary or immortalized culture of astrocytes and microglia have demonstrated that CBD can interfere with their function, especially during cell insults, such as inflammation (caused by LPS for instance). New studies using more sophisticated models (mini-brains, *in vivo* transgenic models) and aimed at observing the effects of CBD in the absence of specific glial cell responses (inhibition by Designer Receptors Exclusively Activated by Designer Drugs (DREADD)-based chemogenetics, or optogenetics) or their receptors (specific KO mice in glial cell populations) are needed to fully address this possibility.

It is unlikely, in our opinion, that CBD shares the same pharmacological mechanism in different brain disorders. Therefore, an important step to fully understand CBD mechanisms and potential role in the treatment of

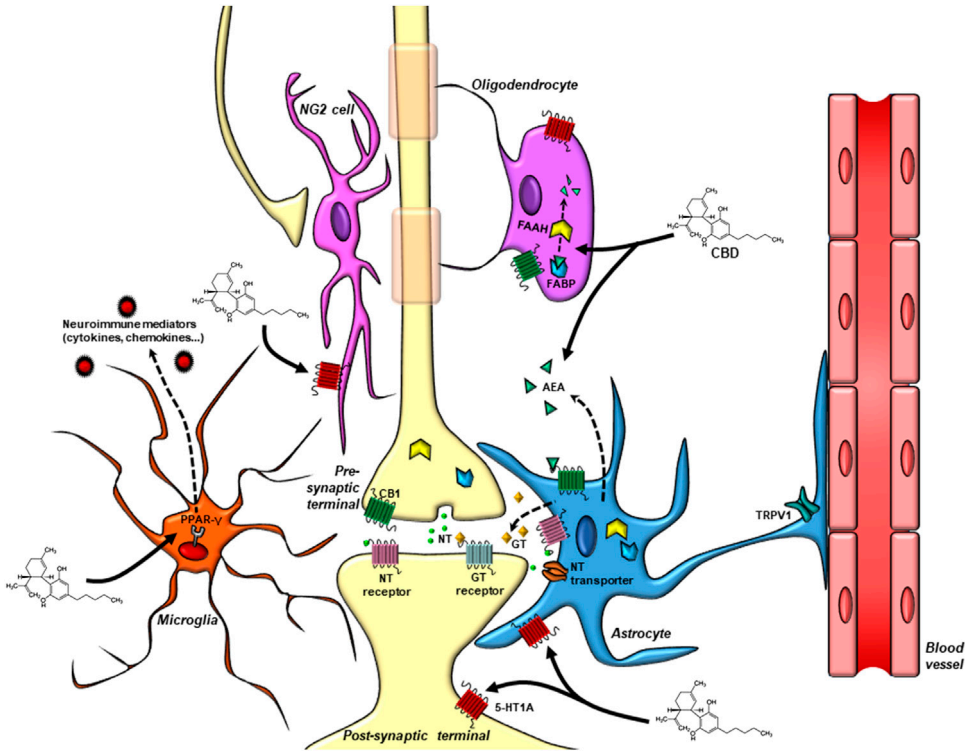

FIGURE 2 | Drug targets for CBD in the "pentapartite" synapse. Glial cells display important roles in synaptic signaling and maintenance. They are in close proximity to the pre and postsynaptic terminals, express neurotransmitter receptors, control the content of neurotransmitters in the synaptic cleft via the expression of transporter proteins, and modulate synaptic activity by releasing gliotransmitters that act in neuronal receptors. Astrocytes also regulate the neurovascular coupling and the blood-brain barrier permeability. Microglial cells control the maintenance of synaptic connections, eliminate unwanted synaptic contacts and release neuroimmune modulators that regulate neuronal function. NG2 cells receive direct synaptic contacts from glutamatergic and GABAergic neurons. Oligodendrocytes maintain the myelin sheets necessary for proper impulse propagation in neurons. In this complex cellular dynamic, CBD targets are found not only in neurons, but also in glial cells. Astrocytes and microglial cells can synthetase and release AEA. Endocannabinoid levels are regulated by FABP and FAAH present both in neuronal and glial cells. Cannabinoid, TRPV1, PPAR-γ, and the serotonin 5-HT1A receptors, proposed to participate in CBD actions, are also present in glial cells and neurons. *CBD*, Cannabidiol; *NT*, neurotransmitter; *GT*, gliotransmitter; *AEA*, anandamide; *FAAH*, fatty-acid amide hydrolase; *FABP*, fatty acid binding protein; *CB_1*, cannabinoid receptor one; *TRPV1*, transient receptor potential vanilloid 1; *5-HT1A*, 5-hydroxytryptophan 1 A receptor; PPAR-γ, Peroxisome proliferator-activated receptor-γ.

neuropsychiatric disorders is to unveil its interference in specific cellular subpopulations present in the CNS.

The complex pharmacokinetics of CBD poses another problem for its therapeutic use. Macrophages have been studied as possible alternatives for drug delivery (Jain et al., 2013). This opens the possibility of using microglial cells to delivery CBD to specific brain areas where their density would be more prominent due to pathological conditions.

Therefore, the investigation of CBD effects on glial cells opens a new route of scientific opportunities. Understanding the role of "pentapartite" synapses (**Figure 2**) on CBD actions could be the "rosetta stone" to decipher the complexity behind its pharmacology.

AUTHOR CONTRIBUTIONS

All authors have approved this manuscript and have agreed to the Frontiers in Pharmacology's submission policies. We state that we are entirely responsible for the scientific content of the present work. We declare that this manuscript has not been published or is being considered for publication elsewhere.

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BRIEF RESEARCH REPORT
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Cannabidiol in Pharmacoresistant Epilepsy: Clinical Pharmacokinetic Data From an Expanded Access Program

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Background and Aim: Data on the clinical pharmacokinetics of cannabidiol (CBD) are scanty. We explored the effect of demographic and clinical variables on plasma concentrations of purified CBD in patients with Dravet (DS) and Lennox–Gastaut syndrome (LGS).

Methods: The study design was an open, prospective, multicenter expanded access program (EAP). Venous blood samples were drawn from patients between 8 and 9 am, before the CBD morning dose, 12 h apart from the last evening dose, and then 2.5 h after their usual morning dose.

Results: We collected 127 plasma samples (67-morning pre-dosing and 60 post-dosing) from 43 patients (24 females, 19 males), 27 with LGS and 16 with DS. Mean ± standard deviation age was 26 ± 15 years. Duration of CBD treatment averaged 4.2 ± 2.9 months at 13.2 ± 4.6 mg/kg/day. CBD median trough plasma concentration was 91 ng/ml; it doubled to 190 ng/ml 2.5 h post-dosing ($p < 0.001$). Cannabidiol trough plasma concentrations were linearly related to daily doses ($r = 0.564$, $p < 0.001$). Median trough CBD plasma concentration-to-weight-adjusted dose ratio (C/D) was 32% higher ($p < 0.02$) in plasma samples from subjects aged 18 and over than in those under 18. Sex and concomitant antiseizure medications (ASMs) were not associated with significant variations in CBD C/D, but caution is required due to the potential influence of confounders.

Conclusion: These are the first data on CBD pharmacokinetics in children and adults with LGS or DS in a real-world setting. The most relevant finding was the higher CBD C/D in adults. In practice, reduced weight-normalized doses might be required with aging to achieve the same CBD plasma levels.

Keywords: cannabidiol, pharmacokinetics, antiseizure medication, epilepsy, Dravet syndrome, Lennox–Gastaut syndrome

INTRODUCTION

Cannabidiol (CBD) is a nonpsychoactive cannabis-derived compound claimed to possess a variety of pharmacological properties (Amin and Ali, 2019). It is currently being investigated in the treatment of several disorders (Fraguas-Sánchez and Torres-Suárez, 2018), including epilepsy (Franco and Perucca, 2019). Despite the huge number of published studies, both clinically controlled and observational data on the pharmacokinetics of CBD are scanty (Millar et al., 2018). A recent review on CBD dosing in clinical populations, examining 35 studies in 13 different medical contexts, pinpointed that none provided CBD plasma concentrations (Millar et al., 2019).

CBD shows challenging pharmacokinetic characteristics, including very low and variable oral bioavailability and high drug-drug interaction potential (Franco and Perucca, 2019; Landmark and Brandl, 2020; Lattanzi et al., 2020a; Patsalos et al., 2020; Perucca and Bialer, 2020). Published data, mostly from healthy volunteers, show remarkable intersubject variability in CBD plasma concentrations after oral dosing (Millar et al., 2019). No data are available on the potential effects of variables such as age and sex on CBD bioavailability, and knowledge of the effects of concomitant therapies on CBD plasma levels is limited (Franco and Perucca, 2019; Landmark and Brandl, 2020).

A highly purified plant-based form of oral CBD formulation was approved by the United States (US) Food and Drug Administration (FDA) in 2018 and the European Medicines Agency (EMA) in 2019 for the treatment of seizures associated with Dravet (DS) and Lennox–Gastaut syndrome (LGS).

We aimed to explore the effect of dose, age, sex, and concomitant antiseizure medications (ASMs) on steady-state plasma concentrations of CBD in a cohort of patients with highly treatment-resistant DS and LGS receiving this FDA/EMA-approved oral formulation of CBD in the context of an expanded access program (EAP) in Italy. Data were also collected on the potential correlation between CBD plasma concentrations and evidence of both tolerability and seizure control.

MATERIALS AND METHODS

EAP Study Design and Patients

The study design was an open, prospective, multicenter EAP. Thirty Italian epilepsy centers were involved in the study. Inclusion and exclusion criteria for patients' enrollment are reported in **Supplementary Table S1**. The study protocol was approved by each site (described in MD September 07, 2017, published in the Official Gazette on November 2, 2017), and written informed consent was obtained from patients or parents/caregivers. Overall data collection was approved by the Ethics Committee "Regione Calabria Area Centro", Catanzaro (Italy), protocol number 115/19.

During a 4-week baseline period, diaries of all countable seizures were provided by patients and/or parents/caregivers. Afterward, patients received an oral solution of purified CBD (100 mg/ml; Epidyolex GW Research Ltd.), at a starting dosage ranging from 2 to 5 mg/kg/day up to a maximum of 18–25 mg/kg/day.

Follow-up visits to assess seizure control were programmed at 3, 6, 9, and 12 months. Patients with a percentage change in seizure frequency ≥50 compared to a 4-week baseline were classified as responders. Percentage change in seizure frequency for each patient was calculated as [(seizure frequency per 28 days) − (seizure frequency at baseline)]/(seizure frequency at baseline) × 100. Assessment of adverse effects (AEs) and clinical laboratory parameters, including liver tests, was performed at baseline, after 2 weeks, 1, 3, and 6 months of treatment, and then periodically.

Concomitant ASMs were recorded at baseline and during the treatment period. CBD and ASMs doses modification, as well as adding/removing coadministered ASMs, were allowed as clinically indicated.

The collection of clinical data was harmonized among different centers by adopting a standardized case report form.

Procedures for CBD Plasma Specimen Collection and Quantitation

Inclusion criteria for CBD plasma specimen collection and quantitation were chronic CBD therapy for at least 1 month and no change in dosage of CBD or concomitant ASMs over the preceding 4 weeks. Venous blood samples (3 ml) were drawn from patients between 8 and 9 am, 12 h apart from the last evening dose, and 2.5 h after ingestion of their usual morning dose, taken after breakfast (basically, milk and biscuits for children; milk, or milk and coffee, or coffee, or tea with a pastry for adults). Some patients were sampled on different occasions during their follow-up.

Blood samples were transferred into heparinized tubes and immediately centrifuged at 1,500 × g for 10 min, at 4°C. Plasma was separated and stored at −80°C until analysis, within 6 months from the collection (Andrenyak et al., 2017). Plasma concentrations of CBD were measured by ultra-high-pressure liquid chromatography-mass spectrometry (Dulaurent et al., 2014). All the analyses were performed at the Laboratory of Clinical Neuropharmacology of the Institute of Neurological Sciences of Bologna. The lower limit of quantification (LLOQ) and limit of detection (LOD) were 0.5 and 0.2 ng/ml, respectively. Intra- and interassay imprecision and inaccuracy were ≤15%.

Data and Statistical Analysis

The sample size was based on patient's enrollment on each study site and not precalculated. The main study outcome was morning trough CBD plasma concentration-to-weight-adjusted daily dose ratio (C/D) [(ng/ml)/(mg/kg/day)].

ASM comedications were classified as strong enzyme inducers (I), including carbamazepine (CBZ), phenobarbital, and phenytoin (PHT); not strong enzyme inducers/not inhibitors (notI/notInhib), such as brivaracetam, clobazam (CLB), felbamate, lacosamide, lamotrigine, levetiracetam, oxcarbazepine, perampanel, topiramate, rufinamide, zonisamide; and enzyme inhibitors (Inhib), stiripentol (STP), and valproic acid (VPA).

The statistical significance of differences between the two groups was assessed by the Student's *t*-test or the Mann-Whitney rank-sum test, whenever appropriate. Intrasubject comparisons were performed by the paired *t*-test or the signed-rank test. Correlations between variables were assessed by Pearson's product-moment coefficient. Clinical variables distribution was compared between patients' subgroups by chi-square test. Comparisons of CBD C/D ratios among ASM comedication subgroups were carried out by one-way analysis of variance (ANOVA). Pairwise comparisons were performed by the Holm-Sidak method when ANOVAs indicated a significant difference among subgroups. Significance was set at $p < 0.05$. Analyses were carried out by SigmaPlot 13.0 software (Systat Software, San Jose, CA, United States).

RESULTS

From December 2018 to December 2019, a total of 110 patients were enrolled in the EAP, 93 with complete data available. Between January 29, 2019, and March 19, 2020, we collected 127 plasma samples (67-morning pre-dosing and 60 post-dosing) from 43 patients (24 females, 19 males), 27 with LGS and 16 with DS, enrolled by 13 clinical centers. Mean ± standard deviation (SD) age was 26 ± 15 years (range 5–56 years, <18 years, n = 17). Duration of CBD treatment averaged 4.2 ± 2.9 months (range 1–12 months) at a mean daily dose of 13.2 ± 4.6 mg/kg (range 4.6–22.8 mg/kg/day), in two divided doses (approximately 8 am–8 pm) in all subjects.

CBD median trough plasma concentration was 91 ng/ml (25–75%, 65–153 ng/ml); overall CBD trough plasma concentrations were linearly related to daily doses (r = 0.564, $p < 0.001$, **Figure 1**). Median trough drug plasma levels doubled to 190 ng/ml (95–322 ng/ml) 2.5 h post-dosing, $p < 0.001$ (**Figure 2**). Intrasubject CBD concentration-dose relationship obtained in a subset of patients sampled on different occasions during the follow-up is depicted in **Supplementary Figure S1**. Values of plasma CBD increased almost proportionally with dose in the majority of subjects.

Median trough CBD C/D ratio was 32% higher in plasma samples from patients ≥18 years (mean age 35 ± 11 years, range 18–56 years) compared with those <18 years (10 ± 4 years, range 5–17 years): 7.97 vs. 6.02, $p < 0.02$ (**Figure 3A**). The two age groups were comparable for sex and ASM cotherapies (**Supplementary Table S2A**).

No significant difference in median trough CBD C/D ratio was observed in plasma samples from males (6.84) vs. females (7.29) (**Figure 3B**). The female subgroup was significantly older than the male one ($p < 0.001$); strong inhibitor ASMs were more frequently coprescribed in males (**Supplementary Table S2B**).

As far as concomitant ASMs are concerned, 7 samples were associated with I prescription, 9 with I + Inihib, 8 with notI/notInhib, 26 with notI/notInhib + Inhib, and 17 with Inhib. The name, number, and daily doses of concomitant ASMs are specified in **Supplementary Table S3**. From separate analyses, median CBD C/Ds were comparable among notI/notInhib, notI/notInhib + Inhib, and Inhib ASM subgroups, which were pooled together for subsequent analyses. Similarly, I and I + Inhib ASM subgroups did not differ and were pooled together. No significant difference was observed in median C/D ratios of CBD segregated in two main categories, with (n = 16) and without (n = 51) concomitant strong enzyme-inducing ASMs: 9.63 vs. 6.84 (**Figure 4**). These two groups were comparable for sex distribution but different for age, which was significantly older in patients cotreated with strong enzyme inducers (38 ± 14 vs. 22 ± 13 years, $p < 0.001$).

Thirty-six plasma samples (54%) were matched to CBD responder patients. Both median CBD plasma concentrations (106 vs. 87 ng/ml) (**Supplementary Figure S2A**) and C/Ds (7.69 vs. 6.33) (**Supplementary Figure S2B**) did not differ between responders and nonresponders. Clinical and therapeutic

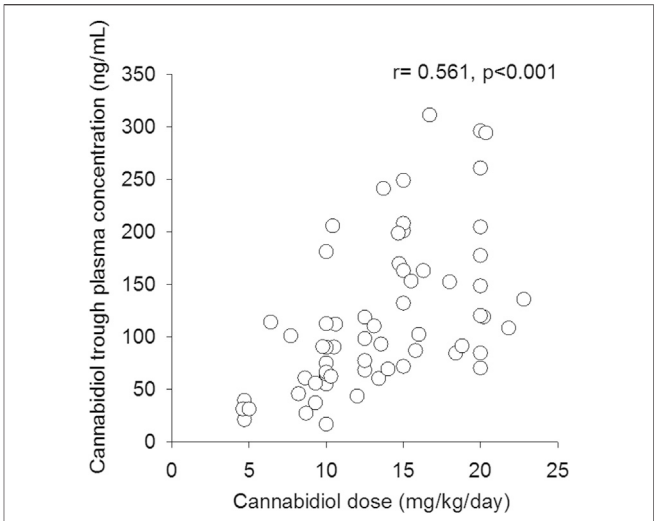

FIGURE 1 | Correlation between cannabidiol trough plasma concentrations and related weight-adjusted daily doses (n = 67).

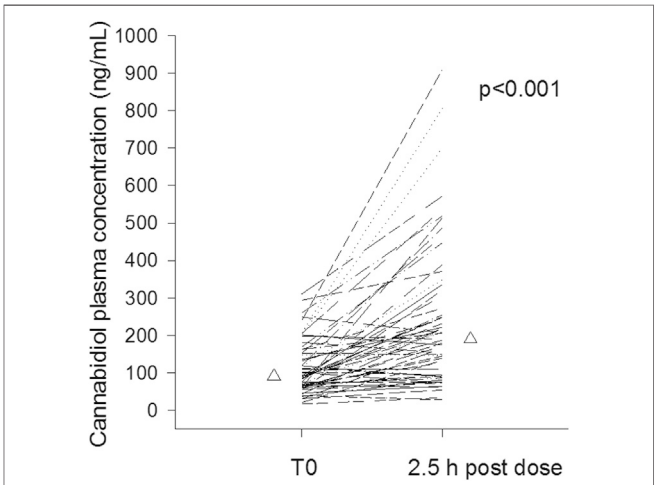

FIGURE 2 | Intrasubject cannabidiol morning trough and 2.5 post-dosing plasma concentrations of cannabidiol (n = 60). Median values are represented by triangles.

characteristics were comparable between these two subgroups, except for age and CBD treatment duration, which were, respectively, older ($p < 0.01$) and shorter ($p < 0.02$) in responders (**Supplementary Table S4A**). In particular, as far as ASM cotherapy is concerned, distribution of CLB cotherapy did not differ between the two groups (16 out of 36 from responder plasma samples and 20 out of 31 from nonresponders, $p = 0.162$).

Twenty-nine plasma samples (43%) were associated with AE reports. They included mainly gastrointestinal disorders (32%), namely, appetite loss, diarrhea, followed by somnolence (18%), increase in transaminase levels (13%), and behavioral changes (11%), such as agitation or irritability. Increase in transaminase levels was observed in all patients receiving VPA.

No difference was found either in CBD plasma concentrations (93.1 vs. 90.4) (**Supplementary Figure S2A**) or in C/D ratio (7.87 vs. 6.74) (**Supplementary Figure S2B**) between the two groups with or without evidence of CBD-related AEs. The AE subgroup was characterized by older age ($p < 0.02$), lower CBD daily dose ($p < 0.01$), shorter treatment duration (<0.008), and higher frequency of strong enzyme-inducing ASM cotherapy ($p < 0.008$) (**Supplementary Table S4B**). Frequency of CLB cotreatment was similar between the two subgroups (14 out of 29 with EAs vs. 22 out of 38 without AEs, $p = 0.593$).

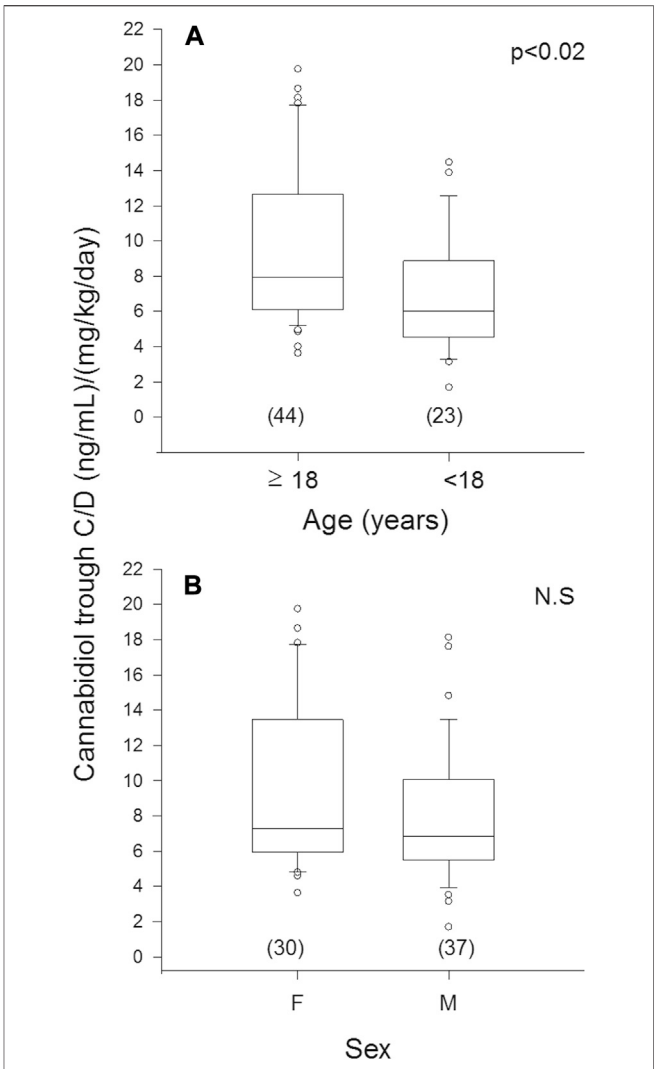

FIGURE 3 | Plasma concentration to weight-adjusted daily dose ratio of cannabidiol (C/D) from patients' specimens (n) grouped by age **(A)** and sex **(B)**. Box plots depict the range between the 25th and 75th percentiles of the data. The horizontal line marks the median value; capped bars indicate 10th–90th percentiles. Black circles represent outlying values. *p*, significance of comparison between age and sex groups by Mann-Whitney rank sum test; N.S., not significant ($p \geq 0.05$).

DISCUSSION

To the best of our knowledge, these results are the first on the effect of demographic and clinical variables on CBD plasma concentrations in *"real"* children and adults with LGS and DS. In our cohort, plasma concentrations of CBD were linearly related to matched daily dose, expressed as mg/kg/day, over a range of 5–23 mg/kg/day. This result is in keeping with findings obtained in pediatric patients by Devinsky et al. (2018a), over a 5–20 mg/kg/day dose range, and Wheless et al. (2019), over 5–40 mg/kg/day doses, and in healthy adult volunteers after multiple doses (750–1,500 mg/day) (Taylor et al., 2018). Plasma concentration-dose linearity is an important drug characteristic in clinical practice, as it may help physicians in patients' dose adjustments. However, it was characterized by a large, up to 10-fold, intersubject variation in drug trough plasma concentrations at a given dosage. High intersubject variability in CBD bioavailability has been reported in patients (Devinsky et al., 2018a; Wheless et al., 2019) and in healthy subjects (Taylor et al., 2018; Crockett et al., 2020), partly ascribed to CBD incomplete oral absorption and large pre-systemic elimination (Perucca and Bialer, 2020). Moreover, food intake, especially high fat/high caloric meals, has a marked effect on CBD exposure, increasing drug bioavailability up to 4-5-fold (Taylor et al., 2018; Crockett et al., 2020). All our patients took their morning CBD dose in a fed state, but breakfast type was not standardized.

Notably, up to 8-fold fluctuations in intrasubject CBD plasma concentrations were observed in most patients' samples between the morning trough and 2.5 h post-dosing, in line with previous evidence (Devinsky et al., 2018a). Reported times to peak of plasma CBD oral formulations are highly variable, mostly in the range of 1–4 h (Millar et al., 2018). We established the time of post-dosing blood sampling based on the clinical trial of Devinsky et al. (2018a), using the same CBD oral solution.

A novel finding was the significant effect of age on median trough CBD C/D ratio, which was higher in subjects aged 18 and over than in those under 18. Cannabidiol undergoes both an extensive first-pass effect and metabolism in the liver (Franco and Perucca, 2019; Perucca and Bialer, 2020), and age-mediated reduction in both these processes may partly explain this observation (Van den Elsen et al., 2014). The only data reported so far on the potential influence of age on CBD pharmacokinetics were confined within a cohort of pediatric

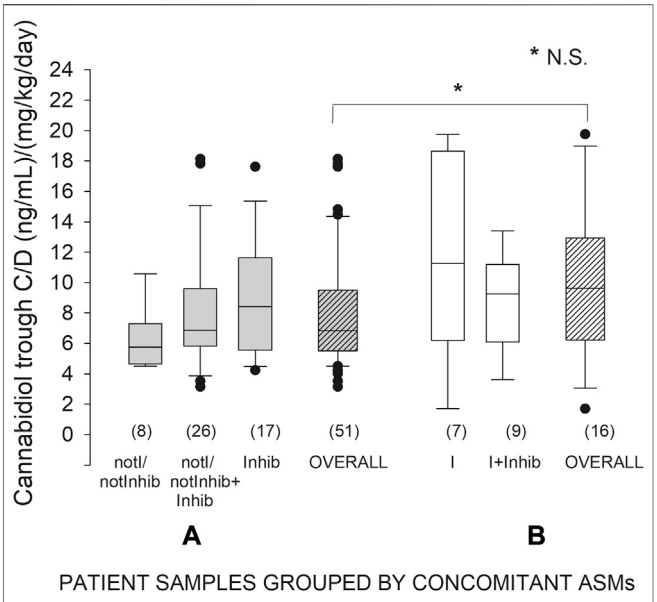

FIGURE 4 | Plasma concentration to weight-adjusted daily dose ratio of cannabidiol (C/D) from patients' specimens (n) grouped by concomitant antiseizure medication (ASM): **(A)** patients not receiving strong inducers; **(B)** patients receiving strong inducers. Box plots depict the range between the 25th and 75th percentiles of the data. The horizontal line marks the median value; capped bars indicate 10th–90th percentiles. Black circles represent outlying values. *p*, significance of comparison between the two overall treatment groups by Mann-Whitney rank sum test; N.S., not significant ($p \geq 0.05$). NotI/notInhib, not strong enzyme inducers/not inhibitors; I, strong enzyme inducers; Inhib, enzyme inhibitors.

patients (Wheless et al., 2019). At any given dosage, plasma CBD concentrations were lower in infants (aged 1 to <2 years) compared to children (2 to <12 years) and adolescents (12 ≤17 years).

Sex did not affect median trough C/D ratio of CBD in our patients. This observation should be considered cautiously as potentially influenced by the older age of the women group. Of note, no study has explored so far the potential differences between males and females in cannabinoids pharmacokinetics (Millar et al., 2018), which might contribute to observed sex-dependent differences in some of their effects (Fattore and Fratta, 2010). From a theoretical point of view, it has been hypothesized that the larger percentage of body fat in women might result in an increased volume of distribution of lipophilic compounds such as CBD, with a higher proportion of drug concentration sequestered in fat tissue and reduced drug plasma concentrations (Fattore and Fratta, 2010). This should also be linked to the peculiar half-life of CBD being initially shorter and then longer according to the possibility of compartmentalization of the drug in some not defined deep compartments (e.g., adipose tissue) (Lattanzi et al., 2020a).

No significant differences emerged in CBD C/D ratio from patients' samples grouped based on metabolism inducing or inhibiting properties of concomitant ASMs. The interpretation of these results is limited by the small sample size per ASM cotherapy groups coupled with high within-group intersubject variability in CBD C/Ds, especially in patients taking strong enzyme inducers. Moreover, the influence of confounders, such as older age of the subgroup on inducers, cannot be ruled out. CBD is metabolized by the cytochrome P450 isoenzyme CYP2C19 to the active metabolite 7-hydroxy-CBD and further to inactive metabolites through CYP3A4 and uridine 5′-diphospho-glucuronosyltransferases (UGTs) (Landmark and Brandl, 2020). Enzyme-inducing ASMs, especially CBZ and PHT, would be expected to reduce CBD C/D ratio (Franco and Perucca, 2019), but no formal study has explored so far this potential interaction. Data on the effect of concomitant ASMs on CBD pharmacokinetics are scanty. From a phase I, open-label healthy volunteer trial (Morrison et al., 2019), concomitant intake of metabolism inhibitors such as STP (750 mg b.i.d., for 14 days) and VPA (500 mg b.i.d., for 14 days) had no significant effect on CBD bioavailability; 7-hydroxy-CBD exposure was decreased by 29% by STP, but the underlying mechanism is unknown. Clobazam (5 mg b.i.d., for 21 days) did not affect CBD exposure, while 7-hydroxy-CBD increased 1.5-fold, possibly by inhibition of UGTs (Morrison et al., 2019).

Dose-dependency for both efficacy and tolerability was not evidenced by our data. Attempts to find out a relationship between CBD plasma concentrations and both seizure control and AEs yielded no significant results. Plasma CBD values associated with therapeutic efficacy or AEs were overlapping. These findings might partly reflect high intersubject variability in CBD bioavailability, patients' different clinical characteristics, and the heterogeneous contribution of different types and doses of concomitant ASMs.

The observed 54% responder rate was in line with the 38–52% previously reported in open-label studies (Devinsky et al., 2016; Thiele et al., 2019), a EAPs (Szaflarski et al., 2018; Laux et al., 2019) and randomized controlled trials (Devinsky et al., 2018b) involving patients with pharmacoresistant epilepsies treated with the same oral solution of purified CBD. Of note, distribution of CLB cotherapy did not differ between responders and nonresponders, in line with the evidence coming from randomized controlled studies that CBD has antiseizure activities irrespective of CLB coadministration (Bialer and Perucca, 2020; Lattanzi et al., 2020b).

The AE rate of 43% was lower than 79–94% reported in the abovementioned studies; the most common registered AEs, appetite loss, diarrhea, and somnolence were in line with the literature.

All these observations should be taken with caution due to the limited number of patients, the uncontrolled design of EAP protocol, and possible intersite variability in reporting methods, among others. Further studies in larger cohorts of patients are needed to confirm these findings.

CONCLUSION

We provide for the first time a picture of CBD pharmacokinetics in patients with LGS and DS under an EAP, a study condition that is closer to "*real*" patients compared with controlled clinical trials. The most relevant finding was the evidence of a significant

increase in CBD plasma concentrations with aging. Age may be added to the variables contributing to the wide intersubject variability observed in plasma CBD at the same dosages. From a practical point of view, reduced weight-normalized doses might be required with aging to achieve the same CBD plasma levels.

CBD LICE ITALY STUDY GROUP

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DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee "Regione Calabria Area Centro," Catanzaro (Italy), protocol number 115/19. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MC, MS, MAML, ER, and OM contributed to the conception and design of CBD pharmacokinetic study protocol. MC and SM acquired the multicenter study data. SM organized the database and performed the quantitation analyses of plasma cannabidiol. MC performed the statistical analysis. MC wrote the first draft of the manuscript. MS, SM, MAML, ER, and OM contributed to interpretation of data and manuscript critical revision.

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SUPPLEMENTARY MATERIAL

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Cannabidiol and Amisulpride Improve Cognition in Acute Schizophrenia in an Explorative, Double-Blind, Active-Controlled, Randomized Clinical Trial

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Cannabidiol (CBD), a principal phytocannabinoid constituent, has demonstrated antipsychotic properties in recent clinical trials. While it has also been suggested a promising candidate for the treatment of neurodegenerative disorders, it failed to demonstrate efficacy in cognitive impairments associated with schizophrenia as an add-on treatment (600 mg/day for 6 weeks) in 18 chronically ill patients co-treated with a variety of psychopharmacologic drugs. Here, we report on the results of parallel-group, active-controlled, mono-therapeutic, double-blind, randomized clinical trial (CBD-CT1; ClinicalTrials.gov identifier: NCT00628290) in 42 acute paranoid schizophrenic patients receiving either CBD (up to 800 mg/day) or amisulpride (AMI, up to 800 mg/day) for four weeks in an inpatient setting with neurocognition as a secondary objective. Twentynine patients (15 and 14 in the CBD and AMI group, respectively) completed two cognitive assessments at baseline and the end of the treatment period. We investigated the following cognitive domains: pattern recognition, attention, working memory, verbal and visual memory and learning, processing speed, and verbal executive functions. When applying the Bonferroni correction for multiple testing, $p < 0.0004$ would indicate statistical significance. There was no relevant difference in neurocognitive performance between the CBD and the AMI group at baseline, and we observed no post-treatment differences between both groups. However, we observed improvements within both groups from pre- to post-treatment (standardized differences reported as Cohen's *d*) in visual memory (CBD: 0.49, $p = 0.015$ vs. AMI: 0.63, $p = 0.018$) and processing speed (CBD: 0.41, $p = 0.004$ vs. AMI: 0.57, $p = 0.023$). Furthermore, CBD improved sustained attention (CBD: 0.47, $p = 0.013$, vs. AMI: 0.52, $p = 0.085$), and visuomotor coordination (CBD: 0.32, $p = 0.010$ vs. AMI: 0.63, $p = 0.088$) while AMI led to enhanced working memory performance in two different paradigms (Subject Ordered Pointing Task–AMI: 0.53, $p = 0.043$ vs. CBD: 0.03, $p = 0.932$ and Letter Number Sequencing–AMI: 0.67, $p = 0.017$ vs. CBD: 0.08 $p = 0.755$).

There was no relevant correlation between changes in neurocognitive parameters and psychotic symptoms or anandamide serum levels. This study shows that both CBD and AMI improve neurocognitive functioning with comparable efficacy in young and acutely ill schizophrenia patients via an anandamide-independent mechanism.

Keywords: cognition, neuropsychological function, cannabidiol, schizophrenia, endocannabinoids, RCT, human

INTRODUCTION

Schizophrenia is a complex psychiatric syndrome including positive symptoms (delusions, hallucinations, thought disorder) and negative symptoms (anhedonia, blunted affect, social withdrawal) as well as cognitive impairment (American Psychiatric Association, 2013). The disease concept has been originally described by Emil Kraepelin as "dementia praecox" or premature dementia. Originally, dementia was synonymous with insanity and not related to age, cognitive status, or reversibility (Adityanjee et al., 1999). In contrast, Kraepelin used it in the more modern sense of the word, including the cognitive decline he observed. Eugen Bleuler pointed to a temporary remission and even recovery of the "dementia praecox" syndrome he named "schizophrenia" (Bleuler, 1908). Nevertheless, more than a century later, the cognitive impairment in a large number of patients remains among the most difficult to influence aspects of schizophrenia.

The fundamental dimensions of cognitive deficits in schizophrenia encompass memory, attention, working memory, problem-solving, processing speed, and social cognition (Nuechterlein et al., 2004; Keefe and Fenton, 2007). Despite a considerable heterogeneity in cognitive symptoms, approximately 65–80% of patients show clinically significant impairments and perform one to two standard deviations (SD) below the population mean (Kremen et al., 2000; Keefe and Fenton, 2007; Mesholam-Gately et al., 2009; Uren et al., 2017; Islam et al., 2018; McCleery and Nuechterlein, 2019). However, it has been suggested that even those individuals who had been rated to perform as "within normal limits" exhibit a cognitive decline compared with what their cognitive functions would have been if they had never developed the illness (Kremen et al., 2000; Keefe and Fenton, 2007; Vaskinn et al., 2014). Another study reported that even patients with overall normal cognitive and intellectual functioning showed impairments in processing speed-dependent domains (Bechi et al., 2019).

Cognitive deficits are already profound early in the course of the illness (Riley et al., 2000; Mesholam-Gately et al., 2009), only modestly related to negative symptoms, essentially independent of positive symptom severity (Keefe et al., 2006), and exist already prior to the initiation of antipsychotic treatment (Saykin et al., 1994), indicating that they are not merely the result of other schizophrenia symptoms or psychotropic treatments. Furthermore, the presence of cognitive deficits or cognitive decline during adolescence has been found to predict the conversion to schizophrenia (Cannon et al., 2002; Reichenberg et al., 2005; Keefe et al., 2006; Keefe and Fenton, 2007; Seidman et al., 2016; Lam et al., 2018), supporting the view that significant cognitive deficits precede the onset of psychotic symptoms (McCleery and Nuechterlein, 2019).

The various cognitive deficits have been shown to contribute to functional outcomes, such as interpersonal relationships, social problem-solving, participation in recreational and community activities, occupational and vocational functioning, and self-care (Green et al., 2000; Bryson and Bell, 2003; Mohamed et al., 2008; Fervaha et al., 2014; Chang et al., 2016). Consequently, cognition is an important treatment target. Currently available antipsychotic medications can yield modest beneficial effects on cognitive functioning, although the findings have been inconsistent regarding whether atypical antipsychotics confer greater effects than typical antipsychotics (Woodward et al., 2005; Keefe et al., 2007; McCleery and Nuechterlein, 2019). Notably, detrimental effects of antipsychotics on cognition are also possible. This has been associated with very high dopamine D_2 receptor occupancy level, very high dosing, polypharmacy, and concomitant use of anticholinergic medications (Hori et al., 2006; Sakurai et al., 2013; McCleery and Nuechterlein, 2019).

Due to the lack of marked cognitive benefits of standard antipsychotics, alternative pharmacological treatments with different mechanisms of action are currently investigated, including cholinergic agents, dopamine D_1 agonists, and glutamatergic agents (Buchanan et al., 2007).

Another promising novel antipsychotic agent is cannabidiol (CBD), a major ingredient of *Cannabis sativa*. CBD has demonstrated antipsychotic properties in small case studies (Zuardi et al., 1995; Zuardi et al., 2006; Zuardi et al., 2009; Makiol and Kluge, 2019) and randomized, placebo-controlled clinical trials in acutely and non-acutely ill schizophrenia patients (Leweke et al., 2012; McGuire et al., 2018). Moreover, first placebo-controlled, double-blind studies showed that CBD might also have beneficial effects in individuals in a Clinical-High-Risk (CHR) mental state for psychosis (Bhattacharyya et al., 2018; Appiah-Kusi et al., 2020).

However, while CBD has also been suggested to be a promising therapeutic candidate for treating neurodegenerative diseases through multifaceted molecular mechanisms (Cassano et al., 2020), it failed to demonstrate efficacy in ameliorating cognitive impairments in schizophrenia patients. In a small double-blind, placebo-controlled, three-parallel-arm clinical trial with non-acutely ill schizophrenia patients, a single dose of 300 (N = 9) or 600 mg (N = 9) CBD did not improve cognitive performance–more precisely selective attention–compared to placebo (N = 10) (Hallak et al., 2010). Furthermore, in comparison to placebo (N = 18), a six-week treatment with CBD (600 mg/day; N = 18) did not improve the cognitive performance (assessed with the MATRICS Consensus Cognitive Battery) of stable antipsychotic-treated patients diagnosed with chronic schizophrenia (Boggs et al., 2018). At the same time, a double-blind, randomized, placebo-controlled,

TABLE 1 | Characteristics of the patient sample.

	Full analyis set			Subset with full neurocognitive assessment		
	CBD (n = 20)	**AMI (n = 19)**	**CBD vs. AMI P-Value[a]**	**CBD (n = 15)**	**AMI (n = 14)**	**CBD vs. AMI P-Value[a]**
Demographic characteristics						
Age, years (mean ± SD)	29.7 ± 8.3	30.6 ± 9.4	0.966	28.8 ± 7.7	30.3 ± 9.7	0.844
Male gender, count (%)	15 (75.0)	17 (89.5)	0.407	12 (80.0)	13 (92.9)	0.598
Baseline severity of illness scores, mean ± SD						
PANSS Total	91.2 ± 14.0	99.5 ± 17.1	0.736	89.9 ± 15.9	98.9 ± 16.7	0.347
PANSS Positive	24.6 ± 5.6	22.5 ± 6.2	0.205	23.5 ± 5.3	23.4 ± 6.3	0.678
PANSS Negative	23.7 ± 5.4	25.3 ± 5.6	0.573	23.8 ± 5.8	25.7 ± 6.1	0.511
PANSS General	42.9 ± 8.6	47.7 ± 11.4	0.155	42.5 ± 9.4	49.9 ± 11.1	0.063
BPRS	58.1 ± 9.7	57.7 ± 10.3	0.764	56.1 ± 9.8	59.4 ± 8.9	0.541
CGI	6.3 ± 0.7	6.8 ± 0.4	**0.011**	6.3 ± 0.6	6.7 ± 0.5	**0.037**
Other, mean ± SD						
Lorazepam, mg/day	2.2 ± 1.6	4.2 ± 2.4	**0.006**	2.0 ± 1.7	3.8 ± 2.3	0.055
SAS	36.4 ± 7.7	36.9 ± 8.1	0.800	35.7 ± 6.8	37.6 ± 8.5	0.584
EPS	0.1 ± 0.2	0.0 ± 0.1	0.485	0.1 ± 0.2	0.0 ± 0.0	0.682
Changes in PANSS after 28 days of treatment, mean ± SD; n [all changes significant compared to baseline, $p < 0.001$ in the full analysis set Leweke et al. (2012)]						
PANSS Total	−30.5 ± 16.4; 17	−30.1 ± 24.7; 18	0.843	−31.3 ± 16.8	−37.0 ± 21.4	0.332
PANSS Positive	−9.0 ± 6.1; 17	−8.4 ± 7.5; 18	0.519	−8.9 ± 6.3	−10.1 ± 7.3	0.903
PANSS Negative	−9.1 ± 4.9; 17	−6.4 ± 6.0; 18	0.234	−9.6 ± 5.1	−7.9 ± 5.7	0.527
PANSS General	−12.5 ± 10.4, 17	−15.3 ± 14.3; 18	0.457	−12.8 ± 10.1	−19.0 ± 13.0	0.159

CBD, cannabidiol; AMI, amisulpride; BPRS, brief psychiatric rating scale; CGI, clinical global impression scale; EPS, extrapyramidal symptoms rating scale; PANSS, positive and negative syndrome scale; SAS, social anxiety scale. Full analysis set (Leweke et al., 2012) and subset of patients who completed neuropsychological assessments prior to initiation of treatment. Please note, the p-values for changes in PANSS slightly differ from the ones given in the main article (Leweke et al., 2012), since therein results based on a mixed model for repeated measures with baseline adjustment are reported. However, both approaches support the same conclusions.

[a]*The Kruskal-Wallis test (continuous data) or Fisher's exact (nominal data) test. Statistical significance between groups ($p \leq 0.05$) is indicated in bold.*

parallel-group clinical trial investigating the efficacy of a higher CBD dosage (1000 mg/day, over eight weeks) as an add-on to stable antipsychotic medication in sub-acute schizophrenia spectrum patients (n = 43), observed a slightly improved cognitive performance (Brief Assessment of Cognition in Schizophrenia (BACS) composite score and subdomain "executive functions") compared to those who received placebo (n = 45) as a secondary outcome. Although these differences did not reach statistical significance, the motor speed improvements were significantly greater in the CBD than in the placebo group (McGuire et al., 2018).

In comparison to these previously published studies, the present study compared the effects of a mono-therapeutic approach with CBD or the second-generation antipsychotic AMI in earlier stages of schizophrenia on six neurocognitive domains (pattern recognition; sustained attention; working memory; verbal and visual memory and learning; processing speed; verbal executive functions) in 42 acute paranoid schizophrenic patients as a secondary objective. AMI has been chosen as a comparator because of its clear dopamine $D_{2/3}$-receptor antagonistic mechanism of action. In contrast, CBD's antipsychotic potential has been found to be substantially linked to an increase in anandamide levels (Leweke et al., 2012).

METHODS

Subjects

This therapeutic-exploratory (phase II), double-blinded, monocenter, randomized, parallel-group, controlled clinical trial (RCT) of CBD vs. AMI (CBD-CT1; ClinicalTrials.gov. Identifier: NCT00628290) was approved by the Ethics Committee of the University of Cologne and the BfArM (Federal Institute for Drugs and Medical Devices). Initially, an independent psychiatrist assessed patients to confirm their ability to provide informed consent. After a detailed explanation of study procedures, written informed consent was obtained from each patient.

Details on the patient samples have been previously provided elsewhere (Leweke et al., 2012) and are summarized in **Table 1**. In brief, men and women aged 18–50 years and diagnosed with schizophrenia or schizophreniform psychosis, with a total Brief Psychiatric Rating Scale (BPRS) score ≥36 and a BPRS thought disorder score ≥12, were eligible to participate in the study. Exclusion criteria comprised a positive urine drug screening for illicit drugs, other relevant psychiatric disorders, treatment with a depot antipsychotic within three months before participation in the study, history of treatment resistance, a relevant and/or unstable medical condition, and insufficient contraception, pregnancy, or breast-feeding in female patients. The consort diagram (**Figure 1**) demonstrates the participants' flow. Out of 42 inpatients with acute paranoid schizophrenia, 33 completed the study per protocol (participation in the study throughout the planned course). After a screening period of up to 7 days and a minimum period of three antipsychotic-free days (the vast majority of patients was antipsychotic-naïve or hospitalized for acute exacerbation after discontinuing antipsychotic treatment and therefore off antipsychotic well before inclusion in our study), patients were randomized (1:1) to receive either CBD or AMI starting with 200 mg per day each

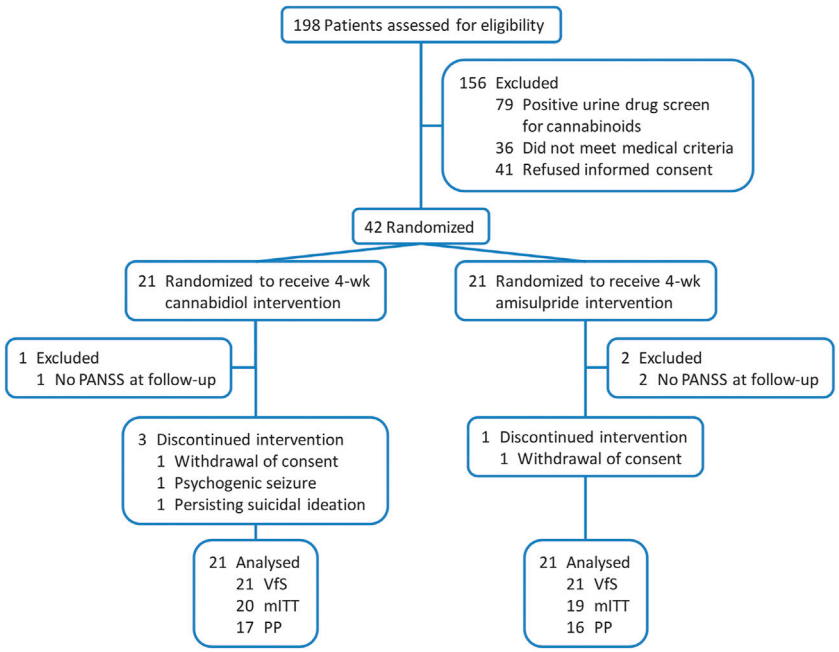

FIGURE 1 | CONSORT flow diagram (Leweke et al., 2012). Analyses sets: VfS, valid for safety; mITT, modified intention-to-treat and PP, per protocol.

and increased stepwise by 200 mg per day to a daily dose of 200 mg four times daily (total 800 mg per day) each within the first week that was maintained for another three weeks. A reduction of each treatment to 600 mg per day was allowed for clinical reasons, such as unwanted side effects after week 2, which was the case for three patients in the CBD and five patients in the AMI arm. The assessment of the change of neurocognitive performance was a secondary objective of this RCT. Fifteen patients of the CBD and 14 patients of the AMI group were able to complete neuropsychological assessments prior to initiation of treatment (day -8 to -1) and on the last day of active treatment with either CBD or AMI (day 28). However, only 12 and 11 patients treated with CBD and AMI respectively completed all neurocognitive tests.

Neurocognitive Assessment

The neurocognitive test battery took approximately 2 h to complete and was conducted by fully qualified neuropsychologists. Furthermore, training effects were diminished by using parallel versions of each test at t_0 and t_1. Premorbid verbal IQ was tested with the German version of the Multiple Choice Vocabulary Test (Lehrl, 1995), which is highly correlated with total IQ.

The test battery comprised 10 validated neuropsychological tests with good test-retest reliability (Delaney et al., 1992; Keefe et al., 2004; Ross et al., 2007; Nuechterlein et al., 2008) to assess the following neurocognitive domains:

(1) Pattern recognition. A computerized version of a *visual backward masking task* with letters F, H, or T as target stimuli and one of four masking conditions [random dot pattern or letter pattern masking stimulus after short (42 ms) or long (104 ms) interstimulus intervals] provided a measure of visual information-processing (number of hits). The session consisted of three blocks of 30 trials, each including six trials of each masking condition and six no-mask control trials presented in random order. To enhance reliability for the measurement of pattern recognition, aggregated scores across the different masking conditions were calculated.

(2) Attention. The *Continuous Performance Test* (identical pairs version (Cornblatt, 1996)) provided a measure of sustained attention. The signal detection parameter d' was calculated across 150 trials with 4-digits stimuli (emphasis on left hemispheric processing) and 150 trials with meaningless symbols stimuli (emphasis on right-sided processing).

(3) Working memory. The *Letter Number Sequencing* (Gold et al., 1997) requires subjects to sort letters from numbers within a sequence of alternating letters and numbers read to them and recall the letters and numbers in ascending orders separately. During each trial of a computerized version of the *Subject Ordered Pointing Task* (Petrides and Milner, 1982), subjects had to point to 1 of 12 objects, and the relative positions of the objects varied randomly across trials. Across three sessions of 12 trials, the number of errors (pointing to an object already chosen on a previous trial) was calculated. During each trial of the *Delayed Response Task* (Park and Holzman, 1992; Pukrop et al., 2003) for spatial working memory, a black dot was presented for 200 ms at 1 of 16 possible positions of a circle followed by a specific delay (5 s or 15 s). During the

delay period, subjects had to solve arithmetic distractor tasks. After the delay period, subjects were required to indicate the position of the previously presented dot on a touch-sensitive monitor to determine the Euclidean distance to the target. To enhance reliability for the measurement of the spatial working memory, aggregated scores across the two different delay periods were calculated.

(4) Verbal and visual memory and learning. The *Auditory Verbal Learning Test* (Lezak et al., 2012) provided measures for the immediate recall capabilities after one to five learning trials of word lists and delayed recall performance. A measure of visual memory was provided by the *Rey-Osterrieth Complex Figure Test* (Rey, 1941), calculating the delayed recall performance by a standardized scoring procedure.

(5) Processing speed. The *Digit-Symbol-Test* (Kaplan et al., 1991) and *Trail-Making Test A and B* (Reitan and Wolfson, 1985) provided measures for the speed of visual information-processing and visuomotor coordination. The ratio of TMT-B to TMT-A can be interpreted as an indicator of the cognitive component independent of the motor component.

(6) Verbal executive functions. A *verbal fluency task* (sum of four lexical and semantic category tasks) was used to measure verbal executive functions (Turek et al., 2017; Palsetia et al., 2018).

These domains of cognitive functioning are those found to be consistently impaired and related to outcome in schizophrenia and are assessed by the BACS as well (Keefe et al., 2004) that was not available at the time the trial started.

Determination of Anandamide Levels

For a subgroup of patients, serum anandamide levels were determined before (baseline) and after CBD (N = 14) or AMI (N = 8) treatment (day 28) by liquid chromatography/electrospray tandem mass spectrometry (LC/ESI-MS-MS) using a method following international guidelines and requirements for the validation of a method and the quantitative evaluation of the compounds as described previously in detail (Giuffrida et al., 2000; Schreiber et al., 2007; Leweke et al., 2012).

Statistical Analysis

Since observed distributions of all neuropsychological characteristics could be well approximated by normal distributions, parametric methods (from the t-test family) were used to evaluate differences in location. Due to the explorative character of the study, the type I error (alpha) was not adjusted for multiplicity. Thus, the results need to be interpreted carefully. When applying the Bonferroni correction, $p < 0.0004$ (i.e., 0.05/120) would indicate statistical significance. Throughout the manuscript, we added the Bonferroni-corrected p-values (p_{corr}) in square brackets. Alternatively, also based on given p values, readers may prefer to apply the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) to control the false discovery rate: The p values need to be ranked; find the maximum rank k with $p_{(k)} \leq i/120*0.05$; reject all null hypotheses with p values of rank $i \leq k$.

Standardized differences for neuropsychological performance changes from t0 to t1 in both groups are reported as Cohen's d (within groups over time, i.e., standardized mean gain (t_0 minus t_1), and between groups, i.e., standardized mean difference (AMI minus CBD), respectively) (Lipsey and Wilson, 2001). Thus, in both cases, Cohen's d was calculated based on the pooled standard deviation (either pooled over time or pooled over groups). Associations between neuropsychological performance and psychopathological symptoms were described by Pearson's correlation coefficient. Moreover, for a subgroup of patients, serum anandamide levels were available (Leweke et al., 2012). Thus, associations between changes of the neurocognitive performance and serum anandamide levels were assessed by a median slope analysis (Wilcoxon signed-rank test of the null hypothesis that the distribution of slopes is symmetric about zero). This type of analysis is consistent with our previous analysis to assess the association of the change of anandamide levels in serum and the change in the PANSS total score (Leweke et al., 2012). Calculations were done with the software SPSS Statistics (IBM Corp., Armonk, NY, United States) and Stata/SE (StataCorp LLC, College Station, TX, United States).

RESULTS

Both treatment groups improved on all neuropsychological functions from pre-to post-treatment (except for slight non-significant deteriorations of verbal working memory performance assessed by AVLT–delayed recall (p = 0.881 [p_{corr} = 1], d = -0.03) and the Letter Number Sequencing (p = 0.366 [p_{corr} = 1], d = 0.08) in the CBD group, and of working memory performance assessed by the delayed response task in the AMI group (p = 0.066 [p_{corr} = 1], d = -0.81); **Table 2**). In the AMI group, t-tests (**Table 2**) revealed improvements from t0 to t1 on the Letter Number Sequencing Test and Subject Ordered Point Task (both working memory tests), as well as the Rey-Osterrieth Complex Figure Test (ROFT, visual memory) and the Digit-Symbol Test (processing speed). Patients treated with CBD showed improvements on the Continuous Performance Test–symbol stimuli (sustained attention), the ROFT (visual memory), Digit-Symbol Test (processing speed), and Trail-Making Test B (visuomotor coordination). Effect sizes (**Table 3**'; **Figure 2**) for all improvements ranged from 0.21 (visuomotor coordination) to 0.67 (verbal working memory) in the AMI group and from 0.03 (working memory) to 0.49 (visual memory) in the CBD group. We did not find significant differences between treatment groups.

Changes in neurocognitive performance were not systematically correlated with psychopathological improvements (PANSS total, general, positive, and negative symptom scores). Only 4 out of 104 (13 neuropsychological and 4 psychopathological parameter per treatment group) correlation coefficients between difference scores became "significant" (note, that about 5 (i.e., 5%) coefficients can be

TABLE 2 | Changes in neurocognitive performance (raw score means ± SD) before (t_0) and after (t_1) intervention. Statistical significant changes ($p \leq 0.05$) are indicated in bold.

	AMI			CBD		
	t_0 (mean ± SD; N)	**t_1 (mean ± SD; N)**	**paired t-test (t_0-t_1), [95% CI]**	**t_0 (mean ± SD; N)**	**t_1 (mean ± SD; N)**	**paired t-test (t_0-t_1) [95% CI]**
VBM %Hits	71.79 ± 17.20; 14	80.34 ± 12.27; 14	$t_{(13)} = -1.75$, $p = 0.105$ [$p_{corr} = 1$] [–19.14, 2.04]	70.57 ± 16.79; 15	77.15 ± 18.35; 15	$t_{(14)} = -1.45$, $p = 0.170$ [$p_{corr} = 1$] [–16.35, 3.19]
CPT d' figures	1.04 ± 0.55; 13	1.32 ± 0.68; 13	$t_{(12)} = -1.68$, $p = 0.119$ [$p_{corr} = 1$] [–0.65, 0.09]	1.49 ± 1.08; 15	1.73 ± 0.79; 15	$t_{(14)} = -1.31$, $p = 0.213$ [$p_{corr} = 1$] [–0.63, 0.152]
CPT d' symbols	1.31 ± 0.84; 13	1.68 ± 0.75; 13	$t_{(12)} = -1.88$, $p = 0.085$ [$p_{corr} = 1$] [–0.80, 0.06]	1.91 ± 0.82; 15	2.30 ± 0.85; 15	$t_{(14)} = -2.85$, $\mathbf{p = 0.013}$ [$p_{corr} = 1$] **[–0.69, –0.10]**
LNS-# correct	14.36 ± 3.30; 11	16.36 ± 2.73; 11	$t_{(10)} = -2.85$, $\mathbf{p = 0.017}$ [$p_{corr} = 1$] **[–3.56, –0.44]**	16.23 ± 3.11; 13	15.92 ± 4.43; 13	$t_{(12)} = 0.32$, $p = 0.755$ [$p_{corr} = 1$] [–1.80, 2.41]
SOPT # Errors	5.75 ± 2.83; 12	4.33 ± 2.15; 12	$t_{(11)} = 2.28$, $\mathbf{p = 0.043}$ [$p_{corr} = 1$] **[0.05, 2.78]**	4.58 ± 3.09; 12	4.50 ± 2.39; 12	$t_{(11)} = 0.09$, $p = 0.932$ [$p_{corr} = 1$] [–2.02, 2.19]
DRT Euclidian distance	9.41 ± 18.76; 14	21.41 ± 11.08; 14	$t_{(13)} = -2.00$, $p = 0.066$ [$p_{corr} = 1$] [–24.93, 0.94]	15.05 ± 23.47; 15	11.81 ± 16.06; 15	$t_{(14)} = 0.60$, $p = 0.563$ [$p_{corr} = 1$] [–8.49, 14.98]
AVLT immediate recall # correct	8.64 ± 3.63; 14	9.86 ± 4.11, 14	$t_{(13)} = -1.30$, $p = 0.216$ [$p_{corr} = 1$] [–3.23, 0.80]	9.40 ± 3.54; 15	9.53 ± 3.09; 15	$t_{(14)} = -0.15$, $p = 0.881$ [$p_{corr} = 1$] [–2.02, 1.75]
AVLT delayed recall # Correct	8.07 ± 4.39; 14	9.29 ± 4.14; 14	$t_{(13)} = -1.09$, $p = 0.296$ [$p_{corr} = 1$] [–3.62, 1.20]	8.80 ± 3.78; 15	8.67 ± 3.958; 15	$t_{(14)} = 0.15$, $p = 0.881$ [$p_{corr} = 1$] [–1.75, 2.02]
ROFT delayed recall standardized score	18.54 ± 7.19; 13	24.19 ± 8.83; 13	$t_{(12)} = -2.74$ $\mathbf{p = 0.018}$ [$p_{corr} = 1$] **[–10.16, –1.15]**	21.14 ± 7.17; 14	24.68 ± 7.17; 14	$t_{(13)} = -2.80$ $\mathbf{p = 0.015}$ [$p_{corr} = 1$] **[–6.27, –0.80]**
Digit-symbol coding # Correct	42.46 ± 9.37; 11	46.36 ± 7.41; 11	$t_{(10)} = -2.68$, $\mathbf{p = 0.023}$ [$p_{corr} = 1$] **[–7.17, –0.65]**	52.15 ± 13.25; 13	57.46 ± 12.82; 13	$t_{(12)} = -3.60$, $\mathbf{p = 0.004}$ [$p_{corr} = 0.480$] **[–8.52, –2.10]**
TMT-B time in sec	120.73 ± 64.31; 11	83.64 ± 27.78; 11	$t_{(10)} = 1.89$, $p = 0.088$ [$p_{corr} = 1$] [–6.58, 80.76]	88.79 ± 49.70; 13	69.31 ± 32.24; 13	t(12) = 3.05, $\mathbf{p = 0.010}$ [$p_{corr} = 1$] **[5.55, 33.42]**
Ratio TMT-B/TMT-A time in sec	3.17 ± 1.19; 11	2.82 ± 1.38; 11	$t_{(10)} = 0.65$, $p = 0.531$ [$p_{corr} = 1$] [–0.84, 1.53]	2.74 ± 0.94; 13	2.65 ± 0.61; 13	$t_{(12)} = 0.36$, $p = 0.728$ [$p_{corr} = 1$] [0.65, 0.36]
Verbal fluency # Correct	11.28 ± 4.16; 12	12.21 ± 2.63; 11	$t_{(10)} = -0.92$, $p = 0.376$ [$p_{corr} = 1$] [–3.16, 1.29]	11.90 ± 2.48; 12	12.64 ± 3.46; 12	$t_{(11)} = -0.94$, $p = 0.366$ [$p_{corr} = 1$] [2.5, 0.98]

CBD, cannabidiol; AMI, amisulpride; AVLT, auditory verbal learning test; d', signal detection parameter; DRT, delayed response task, LNS, letter number sequencing; ROFT, Rey-Osterrieth complex figure test; SOPT, subject ordered pointing task; TMT-A, trail-making test A; TMT-B, trail-making test B; VBM, visual backward masking. Descriptive statistics, and paired t-test results (intention-to-treat set). Improvements are indicated by negative t-values except for SOPT (#error), DRT (Euclidian distance), TMT-B, and ratio TMT-B/TMT-A (both time in s).

expected by chance alone): In the AMI group, the Letter Number Sequencing performance correlated with PANSS total (r = -0.69; $p = 0.026$ [$p_{corr} = 1$], N = 10), and PANSS general scores (r = -0.81; $p = 0.005$ [$p_{corr} = 0.600$], N = 10), while in the CBD group the verbal fluency performance was significantly associated with PANSS total (r = -0.65; $p = 0.024$ [$p_{corr} = 1$], N = 12) and PANSS general (r = -0.61; $p = 0.037$ [$p_{corr} = 1$], N = 12). The association with PANSS positive did not reach significance (r = -0.52; $p = 0.085$ [$p_{corr} = 1$], N = 12).

In addition, changes in neurocognitive performance were also not systematically associated with changes in serum anandamide levels (**Table 4**). Solely improvements on the Continuous Performance Test–figure stimuli (sustained attention, median slope = 0.99, 95% CI [0.03, 4.21]; $p = 0.046$ [$p_{corr} = 1$]) and the Digit-Symbol Test (processing speed, median slope = 3.31, 95% CI [1.72, 8.95], $p = 0.012$ [$p_{corr} = 1$]) in the AMI and CBD group respectively, were associated with higher anandamide levels.

DISCUSSION

This study shows that both CBD and AMI improve neurocognitive functioning with comparable efficacy in young, acutely ill schizophrenia patients. Interestingly, AMI improved working and visual memory performance as well as the processing speed, while CBD treatment led to improvements in processing speed, visual memory, visuomotor coordination, and sustained attention. However, all effect sizes were similar for both treatment groups.

In consideration of previous studies showing limited (McGuire et al., 2018) to no (Boggs et al., 2018) effect of CBD on neurocognitive functioning as an add-on medication in sub-acutely and chronically ill schizophrenia patients, our results indicate that CBD may be more beneficial when administered during the earlier acute phases of the illness. Neuroprotective properties of CBD have been described earlier (Hampson et al., 1998), and more recent data suggests anti-inflammatory effects of CBD as well (Mori et al., 2017), potentially contributing to an effectiveness at earlier stages of the disease. In addition, the influence of an add-on treatment is not clear, and potential pharmacodynamic interactions need to be considered. McGuire et al. (McGuire et al., 2018) accepted a stable treatment with antipsychotics only and observed a trend in favor of CBD (at a dosage of 1 g per day) in improving cognitive symptoms as assessed by the Brief Assessment of Cognition in Schizophrenia (BACS), an instrument basically

TABLE 3 | Effect size (Cohen's d) for changes in neurocognitive test scores (t_0-t_1) and independent t-test results, to assess the equality of the effect sizes. Improvements are indicated by negative effect sizes except for SOPT (#error), DRT (Euclidian distance), TMT-B, and ratio TMT-B/TMT-A (both times in s).

	Cohen's d		
	AMI d (t_0 vs. t_1) [95% CI]	**CBD d (t_0 vs. t_1) [95% CI]**	**AMI vs. CBD (t_0-t_1) $t_{(df)}$, p, d [95% CI]**
VBM %hits	−0.59 [−1.23, 0.05]	−0.37 [−0.90, 0.15]	$t_{(27)}$ = −0.30, p = 0.770 [p_{corr} = 1], d = −0.11 [−0.84, 0.62]
CPT d' figures	−0.55 [−1.11, 0.02]	−0.23 [−0.60, 0.13]	$t_{(26)}$ = −0.19, p = 0.850 [p_{corr} = 1], d = −0.07 [−0.81, 0.67]
CPT d' symbols	−0.52 [−1.02, −0.02]	−0.47 [−0.83, −0.11]	$t_{(26)}$ = −0.10, p = 0.923 [p_{corr} = 1], d = 0.04 [−0.71, 0.78]
LNS # correct	−0.67 [−1.72, −0.17]	−0.08 [−0.40, 0.55]	$t_{(22)}$ = −1.87, p = 0.074 [p_{corr} = 1], d = −0.77 [−1.60, 0.07]
SOPT # errors	0.53 [0.06, 1.01]	0.03 [−0.64, 0.71]	$t_{(22)}$ = 1.17, p = 0.255 [p_{corr} = 1], d = 0.48 [−0.34, 1.28]
DRT mean Euclidian distance	−0.81 [−1.58, −0.03]	0.16 [−0.36, 0.68]	$t_{(27)}$ = −1.88, p = 0.071 [p_{corr} = 1], d = −0.70 [−1.45, 0.06]
AVLT immediate recall # correct	−0.33 [−0.79, 0.11]	−0.04 [−0.56, 0.48]	$t_{(27)}$ = −0.85, p = 0.406 [p_{corr} = 1], d = −0.31 [−1.04, 0.42]
AVLT delayed recall # correct	−0.29 [−0.77, 0.19]	0.03 [−0.41, 0.48]	$t_{(27)}$ = −0.96 p = 0.347 [p_{corr} = 1], d = −0.36 [−1.09, 0.38]
ROFT delayed recall standardized score	−0.63 [−1.14, −0.12]	−0.49 [−0.88, −0.10]	$t_{(25)}$ = −0.89 p = 0.383 [p_{corr} = 1], d = −0.34 [−0.54, 1.08]
Digit-symbol coding # correct	−0.57 [−1.01, −0.13]	−0.41 [−0.68, −0.14]	$t_{(22)}$ = 0.67 p = 0.511[p_{corr} = 1], d = 0.27 [−0.71, 0.78]
TMT-B time in s	0.63 [−0.08, 1.34]	0.32 [0.08, 0.56]	$t_{(22)}$ = 0.91 p = 0.371 [p_{corr} = 1], d = 0.37 [−0.44, 1.18]
Ratio TMT-B/TMT-A time in s	0.21 [−0.56, 0.98]	0.11 [−0.51, 0.73]	$t_{(22)}$ = 0.45 p = 0.655 [p_{corr} = 1], d = 0.19 [−0.62, 0.99]
Verbal fluency # correct	−0.34 [−0.89, 0.21]	−0.24 [−0.73, 0.26]	$t_{(22)}$ = −0.15 p = 0.880 [p_{corr} = 1], d = −0.06 [−0.86, 0.74]

CBD, cannabidiol; AMI, amisulpride; AVLT, auditory verbal learning test; d', signal detection parameter; CPT, continous performance task; DRT, delayed response task, LNS, letter number sequencing; ROFT, Rey-Osterrieth complex figure test; SOPT, subject ordered pointing task; TMT-A, trail-making test A; TMT-B, trail-making test B; VBM, visual backward masking.

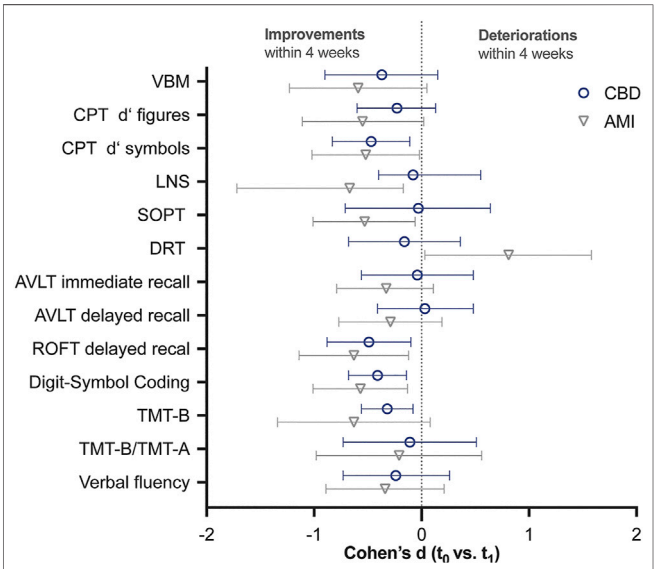

FIGURE 2 | Effect size [Cohen's d (95% CI)] for changes in neurocognitive test scores (t_0-t_1 mean). Cohen's d for SOPT (#error), DRT (Euclidian distance), TMT-B, and ratio TMT-B/TMT-A (both times in s) have been inverted to allow for a better comparison. Thus, improvements of neurocognitive functioning within four weeks of treatment are indicated by negative effect sizes. 95% CI that do not contain 0, indicate significant improvements at the 5% level. CBD, cannabidiol; AMI, amisulpride; AVLT, auditory verbal learning test; d′, signal detection parameter; DRT, delayed response task, LNS, letter number sequencing; ROFT, Rey-Osterrieth complex figure test; SOPT, subject ordered pointing task; TMT-A, trail-making test A; TMT-B, trail-making test B; VBM, visual backward masking.

covering the cognitive domains assessed in our study. In the study of Boggs et al. (Boggs et al., 2018), in which no beneficial CBD effects were observed, the patients were allowed to take a much broader spectrum of concomitant medication, including antipsychotics, antidepressants, and mood stabilizers and combined administration of these drugs. In addition, the daily dosage of CBD was limited to 600 mg.

Interestingly, data on the effects of AMI on cognitive function in schizophrenia patients is limited to a few open-label studies and randomized controlled trials (Tyson et al., 2004; Wagner et al., 2005; Mortimer et al., 2007; Wang et al., 2008; Kumar and Chaudhury, 2014). Consistent with our findings, AMI ameliorated cognitive impairments in all studies. Furthermore, the reported effect size of 0.4 for the global cognitive index after an 8-weeks treatment (Wagner et al., 2005) is comparable to the median effect size of 0.53 observed in the current study after four weeks of AMI treatment.

It has been suggested that the combined serotonin ($5\text{-}HT_{2A}$) and dopamine-2 (D_2) receptor blockade of second-generation antipsychotics is relevant for their ameliorating effects on neurocognitive impairments (Wagner et al., 2005). However, AMI is a second-generation antipsychotic with almost no affinity to $5\text{-}HT_{2A}$ receptors but a high affinity to block dopamine-3 (D_3) receptors (Schoemaker et al., 1997). Nevertheless, AMI reduces cognitive impairments with at least similar efficacy as high $5\text{-}HT_{2A}$ receptor affine second-generation antipsychotics such as olanzapine or risperidone (Tyson et al., 2004; Wagner et al., 2005; Tyson et al., 2006; Mortimer et al., 2007; Wang et al., 2008; Kumar and Chaudhury, 2014). Furthermore, significant improvements in attention (Wagner et al., 2005; Tyson et al., 2006), executive function (Wagner et al., 2005), and auditory verbal learning (Mortimer et al., 2007) were only observed in patients treated with AMI. These findings suggest that low or no $5\text{-}HT_{2A}$ affinity may be more beneficial for cognition than high affinity (Tyson et al., 2004; Wagner et al., 2005). Although AMI and other second-generation antipsychotics have a considerable affinity to D_3 receptors, little is known about the role of D_3 receptor antagonism in ameliorating positive, negative, and cognitive symptoms (Gross and Drescher,

TABLE 4 | Association of change in serum anandamide levels and change in neurocognitive performance. Wilcoxon signed-rank test of the null hypothesis that the distribution of slopes is symmetric about zero. Statistical significant changes ($p \leq 0.05$) are indicated in bold.

	Amisulpride		CBD	
	Slope (mean ± SD; N)	***Z*, *P* [P_{corr}], *median slope* [95% CI]**	**Slope (mean ± SD; N)**	***Z*, *P* [P_{corr}], *median slope* [95% CI]**
VBM %hits	40.10 ± 123.77; 6	0.11, 0.917 [1], 2.94 [–30.87, 148.23]	15.36 ± 34.71; 11	1.16, 0.248 [1], 8.82 [–7.10, 42.98]
CPT d' figures	1.72 ± 2.41; 6	1.99, **0.046** [1], **0.99 [0.03, 4.21]**	0.26 ± 1.66; 11	0.80, 0.424 [1], 0.19 [–0.81, 1.43]
CPT d'	1.71 ± 3.81; 6	1.15, 0.249 [1], 0.36 [–0.19, 4.98]	0.30 ± 2.02; 9	1.51, 0.131 [1], 0.24 [–0.21, 1.95]
LNS # correct	6.15 ± 12.33; 6	1.48, 0.138 [1], 1.85 [–0.58, 17.38]	–0.12 ± 2.51; 9	0.00, 1,000 [1], 0.14 [–2.20, 2.20]
SOPT # errors	–0.23 ± 3.29; 7	–0.37, 0.715 [1], 0.00 [–3.61, 3.45]	1.47 ± 2.70; 11	1.72, 0.086 [1], 1.57 [–0.63, 3.33]
DRT mean euclidian distance	–21.11 ± 86.48; 6	–0.31, 0.753 [1], –5.58 [–113.38, 53.76]	–15.10 ± 28.01; 11	–1.25, 0.213 [1], –9.89 [–37.10, 2.78]
AVLT immediate recall # correct	4.6 ± 11.84; 7	0.73, 0.465 [1], 0.00 [–4.23, 15.78]	0.53 ± 4.93; 12	–0.05, 0.959 [1], 0.00 [–1.56, 4.04]
AVLT delayed recall # correct	5.85 ± 14.78; 7	0.41, 0.686 [1], 0.00 [–5.24, 22.10]	–1.33 ± 4.85; 11	–1.07, 0.285 [1], –1.30 [–4.76, 1.65]
ROFT delayed recall standardized score	22.15 ± 47.90; 6	0.11, 0.917 [1], 14.41 [–11.07, 78.03]	–1.22 ± 15.39; 11	0.45, 0.657 [1], 1.28 [–9.81, 6.52]
Digit-symbol coding # correct	8.02 ± 17.04; 6	1.214, 0.225 [1], 3.61 [–3.45, 24.41]	4.30 ± 4.31; 9	2.52, **0.012** [1], **3.31 [1.72, 8.95]**
TMT-B time in s	–6.73 ± 175.76; 5	–0.14, 0.893 [1], –4.19 [–286.24, 156.87]	–13.78 ± 30.78; 9	–1.60, 0.110 [1], –14.90 [–37.32, 14.04]
Ratio TMT-B/TMT–A time in s	0.43 ± 3.47; 5	0.41, 0.686 [1], 0.88 [–5.10, 3.99]	0.44 ± 2.94; 9	–0.53, 0.594 [1], –0.19 [–1.52, 3.67]
Verbal fluency # correct	–3.54 ± 12.32; 7	–0.11, 0.917 [1], 0.14 [–18.94, 6.71]	5.88 ± 14.20; 9	1.24, 0.214 [1], 1.26 [–0.30, 21.59]

AVLT, auditory verbal learning test; d', signal detection parameter; DRT, delayed response task, LNS, letter number sequencing; P, p-value; ROFT, rey-osterrieth complex figure test; SOPT, subject ordered pointing task; TMT-A, trail-making test A; TMT-B, trail-making test B; VBM, visual backward masking; Z, Wilcoxon signed-rank standardized test statistic.

2012; Sokoloff and Le Foll, 2017). In preclinical studies, D_3 receptor antagonists improved cognitive function, emotional processing, executive function, flexibility, and social behavior, but the few clinical studies with compounds of high affinity for D_3 receptors and different degrees of selectivity over D_2 receptors give only limited insight into the therapeutic potential of selective D_3 antagonism (Gross and Drescher, 2012; Sokoloff and Le Foll, 2017).

Although current antipsychotics can yield modest beneficial effects on neurocognitive functioning (McCleery and Nuechterlein, 2019), cognitive impairments continue to pose a burden on patients. Therefore, novel compounds with a different mechanism of action are currently investigated, such as cholinergic agents, dopamine D_1 agonists, glutamatergic agents (Buchanan et al., 2007), and CBD (Boggs et al., 2018). CBD seems to mediate its antipsychotic effects by modulating the endocannabinoid system (Leweke et al., 2012; Rohleder et al., 2016; Leweke et al., 2018). More precisely, its antipsychotic actions have been found to be related to increased levels of the endocannabinoid anandamide (Leweke et al., 2012), e.g., by blocking the anandamide degrading enzyme fatty acid amide hydrolase (FAAH) (Leweke et al., 2012) or the fatty amide binding proteins (FABPs) that transport anandamide to the FAAH enzyme (Elmes et al., 2015). Elevated anandamide levels can, in turn, interact with other neurotransmitter (e.g., dopamine) systems via cannabinoid type 1 receptors (CB_1R) (Giuffrida et al., 1999; Giuffrida et al., 2004; Leweke, 2012), enhance glucose metabolism via peroxisome proliferator-activated receptor-γ (PPARγ) (Bouaboula et al., 2005) or modulate the immune function via cannabinoid type 2 receptors (CB_2R). Other suggested pharmacological effects of CBD include: the activation of the vanilloid receptor 1 (TRPV1, transient receptor potential cation channel subfamily V member 1) (Bisogno et al., 2001; De Petrocellis et al., 2011), negative allosteric modulation of CB_1R (Laprairie et al., 2015; Sabatucci et al., 2018), the facilitation of serotonergic neurotransmission via allosteric 5-HT_{1A} receptor modulation (Rock et al., 2012; Hind et al., 2016; Sonego et al., 2016), and modulation of glucose homeostasis and inflammatory processes by PPARγ activation (Esposito et al., 2011; Hind et al., 2016; Sonego et al., 2018).

However, further research is needed to clarify which of these pharmacological mechanisms contribute to CBD's beneficial effects on cognition in acutely ill patients. It may be that CBD reduces cognitive impairments by reducing the proposed synaptic dopaminergic excess indirectly via CB_1R activation by anandamide (Giuffrida et al., 1999; Giuffrida et al., 2004; Leweke, 2012). In this study, the neurocognitive performance changes were not systematically associated with changes in serum anandamide levels, indicating that CBD's effects on cognition are mediated via different mechanisms, in particular given the fact that in the same patients, the significant increase in serum anandamide levels has been shown to be significantly associated with clinical improvement (Leweke et al., 2012). It has been suggested that the stimulation of 5-HT_{1A} receptors may improve cognition in schizophrenia (Meltzer and Sumiyoshi, 2008). Thus, the allosteric 5-HT_{1A} receptor modulation by CBD may also be an additional relevant mechanism of action. This hypothesis is consistent with the preclinical finding that CBD attenuates cognitive impairments in a schizophrenia-like animal mode and that these effects can be blocked by a 5-HT_{1A} receptor antagonist but not by CB_1 and CB_2 receptor antagonists (Rodrigues da Silva et al., 2020). Thus, the allosteric 5-HT_{1A} receptor modulation by CBD may also be an additional relevant mechanism of action. Notably, none of these mechanisms alone or a combination of different mechanisms seem to be more

effective than the D_2/D_3 receptor blockade by AMI, as the efficacy of CBD and AMI was comparable in our study.

Changes in neurocognitive performance were also not systematically correlated with psychopathological improvements. Only PANSS total and PANSS general score were associated with the performance in one of three working memory tests (Letter Number Sequencing) in the AMI group and with the verbal learning performance in the CBD group. This finding supports the view that cognitive deficits are not merely the result of other schizophrenia symptoms (Keefe et al., 2006). In particular, the independence of cognitive deficits from positive symptoms has been shown previously (Green, 1996; Mohamed et al., 1999; Hughes et al., 2003; Gladsjo et al., 2004; Wagner et al., 2005; Keefe et al., 2006). On the other hand, a relationship between negative symptom severity and cognitive performance has been observed (Mohamed et al., 1999; Gladsjo et al., 2004; Keefe et al., 2006; Üçok et al., 2020). Furthermore, improvements in negative symptoms have been found to be associated with amelioration of cognitive deficits in people with schizophrenia treated with olanzapine or AMI for eight weeks (Wagner et al., 2005). The authors suggested that the same mechanisms may partly mediate both improvements in negative symptoms and cognitive performance. However, this hypothesis is not supported by our data. It may be that the treatment duration was not long enough to detect an association in this group. Furthermore, as discussed above, our findings indicate that CBD affects psychopathology via an anandamide-dependent pathway (Leweke et al., 2012), while its effects on cognitive performance seem to be mediated by another mechanism. This hypothesis is supported by the absence of a systematic correlation between the changes in neurocognitive performance and psychopathological improvements.

Strengths and Limitations

The major strength of our study is the monotherapeutic, parallel-group design (Leweke et al., 2012). In contrast to previous add-on studies (Boggs et al., 2018; McGuire et al., 2018), our study design allows for investigating the therapeutic potential of the substance more precisely as no pharmacodynamic interactions need to be considered when assessing the effects of CBD. However, the lack of a placebo condition does not allow for an estimate of a potential placebo response to cognitive functioning. Nevertheless, these tests are generally considered robust for rater bias because of their objective character. While placebo-effects in cognitive trials in schizophrenia have been considered fairly small (Keefe et al., 2017), we cannot rule out practice effects on the improvements. Parallel versions of each cognitive test were used at t_0 and t_1, and all tests have been reported to have a good test-retest reliability and low item-specific learning. While we acknowledge the potential development of test-taking strategies and/or procedural learning as potential confounders in our trial, their contribution to the observed improvement in cognitive scores after four weeks of treatment is likely quite limited (Goldberg et al., 2010).

Further, the administration of lorazepam as a co-medication (up to 7.5 mg per day) may have influenced results as initial use of lorazepam was higher in the AMI than in the CBD group (Leweke et al., 2012) with a similar mean dosage at the end of the trial. This may have caused a cognitive improvement due to reduction in lorazepam favoring the AMI group, although the effect of short-term benzodiazepines on cognitive performance in schizophrenia is not well investigated (Baandrup et al., 2017; Fond et al., 2018). Furthermore, we included only acutely ill patients in earlier phases of the disease with a mean age of 29.7 ± 8.3 and 30.6 ± 9.4 years in the CBD and AMI group, respectively. In this group of patients, CBD seems to be more effective than in older sub-acutely (40.9 ± 12.5 years (McGuire et al., 2018)) and chronically (48.4 ± 9.3 years (Boggs et al., 2018)) ill patients. However, further studies investigating CBD's therapeutic effects in first-episode psychosis or the prodromal phase are needed to confirm this hypothesis and investigate the contributing factors.

The current study is limited by the small sample size and comparatively short treatment duration. The actual sample size of n = 14–15 per treatment group is sufficient to detect large effect sizes (i.e., Cohen's d) of 1.1 with a power of 80% at two-sided alpha 5%. However, the calculated 95% confidence intervals still give useful ranges for true differences that are compatible with the data obtained. Likewise, differences outside the intervals are not compatible with the data. Furthermore, as above mentioned, we did not make any multiplicity adjustments due to the exploratory character of the study, and none of the p-values would be significant after Bonferroni correction. Consequently, our exploratory finding that CBD improves neurocognitive functioning needs to be confirmed in a larger cohort of acutely ill schizophrenia patients (i.e., in a large RCT with neurocognitive functioning as primary objective). Furthermore, it needs to be investigated whether the maximal effect of CBD had already been achieved after four weeks of treatment or whether an extended treatment duration will lead to larger effects.

CONCLUSION

This exploratory study shows that both CBD and AMI improve neurocognitive functioning with comparable efficacy in younger and acutely ill schizophrenia patients. However, larger RCTs are needed to confirm this explorative finding. Furthermore, our data indicates that CBD may affect psychopathology and cognitive performance via different physiological mechanisms. While improvements in psychopathology were significantly associated with an increase in serum anandamide levels (Leweke et al., 2012), cognitive improvements (if at all present) seemed to be induced via anandamide-independent pathways. Several alternative mechanisms of action have already been suggested for CBD, including an allosteric 5-HT_{1A} receptor modulation that may be relevant for CBD's effects on neurocognitive functioning. However, the actual involvement of 5-HT_{1A} receptor modulation and other postulated mechanisms of action need to be examined explicitly in future studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because they are available only to approved collaborators and competent authorities. Requests to access the datasets should be directed to markus.leweke@zi-mannheim.de.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the University of Cologne, Cologne, Germany. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FML conceived and designed the study with input from MH. FML, CG, and DK treated the majority of patients in the trial, and RP performed the neuropsychological experiments; RP, MH and CR analyzed the data with input from FML; FML and CR drafted the manuscript with input from RP and MH. All authors contributed to final manuscript preparation and have read and approved the final manuscript.

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REVIEW
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Role of Cannabidiol in the Therapeutic Intervention for Substance Use Disorders

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Drug treatments available for the management of substance use disorders (SUD) present multiple limitations in efficacy, lack of approved treatments or alarming relapse rates. These facts hamper the clinical outcome and the quality of life of the patients supporting the importance to develop new pharmacological agents. Lately, several reports suggest that cannabidiol (CBD) presents beneficial effects relevant for the management of neurological disorders such as epilepsy, multiple sclerosis, Parkinson's, or Alzheimer's diseases. Furthermore, there is a large body of evidence pointing out that CBD improves cognition, neurogenesis and presents anxiolytic, antidepressant, antipsychotic, and neuroprotective effects suggesting potential usefulness for the treatment of neuropsychiatric diseases and SUD. Here we review preclinical and clinical reports regarding the effects of CBD on the regulation of the reinforcing, motivational and withdrawal-related effects of different drugs of abuse such as alcohol, opioids (morphine, heroin), cannabinoids, nicotine, and psychostimulants (cocaine, amphetamine). Furthermore, a special section of the review is focused on the neurobiological mechanisms that might be underlying the 'anti-addictive' action of CBD through the regulation of dopaminergic, opioidergic, serotonergic, and endocannabinoid systems as well as hippocampal neurogenesis. The multimodal pharmacological profile described for CBD and the specific regulation of addictive behavior-related targets

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Abbreviations: 2-AG, 2-arachidonoylglycerol; 3H-DAMGO, D-Ala^2, NMePhe^4, Gly ol; 5HT1a, serotonin 1a receptor; 5HTT, serotonin transporter; AEA, anandamide; Amy, amygdala; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; AUD, alcohol use disorder; BDNF, brain-derived neurotrophic factor; BPN, buprenorphine; CB1R, cannabinoid receptor 1; CB2R, cannabinoid receptor 2CBD, cannabidiol; CBT, cognitive behavioral therapy; CNS, central nervous system; CPA, conditioned place aversion; CPP, conditioned place preference; CREB, cAMP response element-binding protein; CUD, cannabis use disorder; DAGL, diacylglycerol; DALYs, disability-adjusted life years; DAT, dopamine transporter; DG, dentate gyrus; DR, dorsal raphe; DSM-5, diagnostic and statistical manual of mental disorders, 5th version; ECS, endocannabinoid system; EMA, European Medicines Agency; EtOH, Ethanol; FAAH, fatty acid amide hydrolase; FDA, Food and Drug Administration; FR1, fixed-ratio 1; FR3, fixed-ratio 3; FMRI, functional Magnetic Resonance Imaging; GPCRs, Gq protein-coupled receptors; Glu, glutamate; HIPP, hippocampus; ICD-11, International Classification of Diseases; ICSS, intracranial self-stimulation; ICV, intracerebroventricular; MAGL, monoacylglycerol lipase; MAPK, mitogen-activated protein kinase; MET, motivational enhancement therapy; MOr, mu-opioid receptor; NAcc, nucleus accumbens; NAPE-PLD, N-acylphosphatidylethanolamine specific phospholipase D; NMDAr, N-methyl-d-aspartate receptors; NTX, naltrexone; OUD, opioid use disorder; PFC, prefrontal cortex; PTSD, post-traumatic stress disorder; RCT, randomized controlled trial; RT-PCR, real time polymerase chain reaction; SA, self-administration; SUD, substance use disorder; SVZ, subventricular zone; THC, delta-9-tetrahydrocannabinol; THC-COOH, 11-nor-9-carboxy-δ-9-tetrahydrocannabinol; TH, tyrosine hydroxylase; TNF-α, tumor necrosis factor α; VEC, voluntary ethanol consumption; VTA, ventral tegmental area; YLDs, years lived with disability.

explains, at least in part, its therapeutic effects on the regulation of the reinforcing and motivational properties of different drugs of abuse. Moreover, the remarkable safety profile of CBD, its lack of reinforcing properties and the existence of approved medications containing this compound (Sativex®, Epidiolex®) increased the number of studies suggesting the potential of CBD as a therapeutic intervention for SUD. The rising number of publications with substantial results on the valuable therapeutic innovation of CBD for treating SUD, the undeniable need of new therapeutic agents to improve the clinical outcome of patients with SUD, and the upcoming clinical trials involving CBD endorse the relevance of this review.

Keywords: cannabidiol, substance use disorder, alcohol, cocaine, cannabis, psychostimulant, neurobiology

INTRODUCTION

Substance Use Disorders (SUD) are chronic and relapsing clinical conditions meeting the diagnostic criteria for drug dependence defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (APA, 2013) and the World Health Organization's International Classification of Diseases (ICD-11) (World Health Organization, 2018). SUD are one of the most important health problems globally. In 2017, it was estimated that over 30 million individuals present an SUD leading to more than 31 thousand years lived with disability (YLDs) with a worrying increase (16.7%) over the previous decade (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018). Furthermore, substance use was indirectly and directly responsible for 11.8 million deaths which implies one in five deaths worldwide (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018).

Despite the range of the psychosocial and pharmacological therapeutic approaches for substance use treatment, relapse prevalence into drug consumption is estimated between 40 and 75% (Sinha, 2011; Pasareanu et al., 2016; Andersson et al., 2019). This high rate of recurrence is largely due to the ineffectiveness of the available drugs or the lack of specific treatments (e.g., cannabis, cocaine, or amphetamine-type use disorders). Thus, there is a growing need to significantly improve our knowledge about the underlying mechanisms involved in the development of drug dependence to finally design new pharmacological tools with higher efficacy and safety. In this sense, the manipulation of the endocannabinoid system (ECS) by administering cannabinoid compounds has raised much interest due to its close functional involvement in the regulation of emotion, cognition, and reward (Solinas et al., 2008; Marco et al., 2011; Campolongo and Trezza, 2012; Marco and Laviola, 2012; Manzanares et al., 2018; Navarrete et al., 2020).

Cannabis sativa plant contains numerous chemical entities including cannabinoids, terpenes, and phenolic compounds (Andre et al., 2016). To date, over 120 cannabinoids have been isolated from the plant (Morales et al., 2017). From these, delta-9-tetrahydrocannabinol (THC) is the main psychotomimetic or hallucinogenic component and the first cannabinoid to be identified and studied. First described and synthesized by Roger Adams in 1942 (Adams, 1942), and then isolated for the first time by Gaoni and Mechoulam in 1964 (Gaoni and Mechoulam, 1964), THC mediates the rewarding properties of cannabis (Zhang et al., 2004). Along with THC, cannabidiol (CBD) is the other most abundant phytocannabinoid in the *Cannabis sativa* plant. It was first synthesized by Roger Adams (Adams, 1942) and isolated by Mechoulam and Shvo in 1963 (Mechoulam et al., 1963), from which a growing interest in its pharmacological actions began to emerge. The results from basic and clinical studies suggested that CBD may present beneficial effects for the management of neurological disorders such as epilepsy (Carlini and Cunha, 1981; Devinsky et al., 2014; Devinsky et al., 2016), multiple sclerosis (Kozela et al., 2011; Giacoppo et al., 2015; Jones and Vlachou, 2020), Parkinson's (Zuardi et al., 2009; Chagas et al., 2014) or Alzheimer's diseases (Martín-Moreno et al., 2011; Cheng et al., 2014). Moreover, there is a growing body of evidence suggesting that CBD improves cognition (Osborne et al., 2016) and neurogenesis (Liput et al., 2013; Schiavon et al., 2016), and presents antipsychotic (Zuardi et al., 1991; Moreira and Guimarães, 2005; Long et al., 2006; Leweke et al., 2012; Leweke et al., 2016; Peres et al., 2016), anxiolytic (Guimarães et al., 1990; Moreira et al., 2006; Resstel et al., 2006; Blessing et al., 2015) and antidepressant-like effects (Zanelati et al., 2010; Linge et al., 2016; Sartim et al., 2016). All these potential therapeutic actions of CBD are due to its multiple pharmacological mechanisms. CBD was proposed to directly or indirectly modulate the function of more than 65 targets in the central nervous system (CNS) (Ibeas Bih et al., 2015), including cannabinoid receptors (CB1, CB2), GPR55 receptor, vanilloid receptor TRPV1, serotonin 5HT1a receptor (Bisogno et al., 2001; Russo et al., 2005; Ryberg et al., 2007; Thomas et al., 2007; Campos et al., 2012), the anandamide (AEA) hydrolyzing enzyme (fatty acid amide hydrolase, FAAH) or the adenosine transporter (Carrier et al., 2006; Massi et al., 2008). However, additional studies are needed to precisely determine the target engagement profile of CBD.

Importantly, CBD lacks addictive potential in contrast to THC. Several studies in animals and humans demonstrated the absence of rewarding properties (Parker et al., 2004; Katsidoni et al., 2013; Babalonis et al., 2017; Schoedel et al., 2018). Indeed, recent studies carried out in mice in our laboratory further demonstrate that CBD is not an addictive substance. A range of CBD doses were evaluated in different animal models of addiction commonly used to assess the reinforcing and motivational properties of drugs (conditioned place preference

(CPP) and oral self-administration (SA)). Also, withdrawal-related signs were analyzed after the abrupt cessation of CBD chronic administration. Interestingly, CBD did not induce CPP, oral SA or withdrawal-related signs, findings that suggested the lack of rewarding effects of CBD (Viudez-Martínez et al., 2019). Moreover, CBD presents an excellent safety profile supported by both animal and clinical studies (Bergamaschi et al., 2011; Iffland and Grotenhermen, 2017; Taylor et al., 2018). Proof of this is the recent marketing of the drug Epidiolex®, a 99% pure oral CBD extract for the treatment of refractory childhood epilepsies (Lennox-Gastaut and Dravet syndrome) (Sekar and Pack, 2019; Raucci et al., 2020). Likewise, nabiximols is another marketed formulation containing CBD and THC (25 and 27 mg/ml, respectively) under the trade name Sativex®. Nabiximols is an oromucosal spray widely employed for the treatment of muscle spasticity in multiple sclerosis patients (Patti et al., 2016; Giacoppo et al., 2017).

Therefore, the versatile pharmacological profile and safety of CBD support its therapeutic potential in the management of SUD. This review focuses on collecting all the available evidence about the effects of CBD on the different aspects that accompany drug dependence (reinforcement, motivation, contextual conditioning, relapse, withdrawal syndrome or motor sensitization). Also, it covers all the mechanisms proposed to mediate the CBD actions on drug addiction.

METHODS

The literature review consisted of an exhaustive search for scientific information in the Medline database (PubMed). A total of 7 search boxes were employed according to the total of drugs included in the review: cannabis, alcohol, morphine, heroin, amphetamine/methamphetamine, cocaine, and nicotine. These terms were combined with the term "cannabidiol" by the Boolean operator "AND". All the results for each search were critically analyzed by all the authors to decide the selection of each reference according to the adequacy of its content with the subject matter of the study. No PubMed filters were applied to maximize the selection of all the available and appropriate information. All original articles, systematic reviews or meta-analyses focusing on the effects of CBD on drug addiction were accepted. Those articles not related to the topic of interest, not written in English or to which access was not possible were discarded. In addition, the same searches were performed on the ClinicalTrials.gov database to retrieve all the ongoing clinical studies.

CBD AS A POTENTIAL NEW PHARMACOLOGICAL TOOL FOR THE TREATMENT OF SUD

This section details all the available evidence, both pre-clinical and clinical, about the therapeutic potential of CBD in the management of various SUD.

CBD and Cannabis

Cannabis is the second smoked substance of abuse after tobacco (Hasin et al., 2016) and the most consumed illicit drug worldwide (World Drug Report, 2020). The use of cannabis is growing due to the increasing legalization trend for medicinal and recreational purposes. Furthermore, according to recent studies, THC concentrations in cannabis doubled in the past decade and consequently the content of CBD substantially dropped to an almost non-detectable level (Chandra et al., 2019; Freeman et al., 2019). This scenario facilitates cannabis consumption and may lead to the development of dependence criteria in the context of cannabis use disorder (CUD), affecting approximately 22 million people (Degenhardt et al., 2018). CUD is associated with disruptions in social, occupational, recreational activities and mental health problems. The latter includes impaired cognition abilities and motor coordination, euphoria, depression, psychosis, dependence and withdrawal syndrome (Patel and Marwaha, 2020). Although not medically serious, cannabis withdrawal should be a focus of treatment because one-half of the patients in treatment for CUD report withdrawal-related symptoms and it may serve as a negative reinforcement for relapse to cannabis use in individuals trying to abstain (Budney and Hughes, 2006; Levin et al., 2010; Gorelick et al., 2012).

Nowadays there is no official drug approved for the treatment of CUD by the main drug regulatory authorities (i.e., European Medicine Agency (EMA) or US Food and Drug Administration (FDA)). Many studies were carried out to find out new pharmacotherapies with two main aims: 1) to identify medications to attenuate symptoms of cannabis withdrawal, and 2) to identify medications to reduce subjective and reinforcing effects of cannabis. Some off-label pharmacological interventions targeting distinct neurotransmitter systems involved in drug dependence were investigated (for a recent review see (Sabioni and Le Foll, 2019; Brezing and Levin, 2018)). Recently, the pharmacological modulation of the cannabinoid system gained great interest as a potential therapeutic approach for CUD. Particularly, in the last years CBD attracted much attention as a pharmacological tool for the treatment of CUD due to its safety and multimodal pharmacological profile (García-Gutiérrez et al., 2018) (**Table 1**). Also, it has been proposed that CBD may reduce the negative psychotropic effects of THC (for a recent review see (Niesink and van Laar, 2013; Freeman et al., 2019)) and might potentiate its positive therapeutic actions (Russo and Guy, 2006; McPartland and Russo, 2014).

Several studies carried out with cannabis users classified them according to the higher or lower CBD:THC ratio of their smoked cannabis. Interestingly, CBD reduces the rewarding effects of THC since cannabis smokers (n = 94) with high CBD:THC ratio showed reduced attentional bias to drug stimuli and lower self-rated liking of cannabis (Morgan et al., 2010). Another study recruited cannabis users (n = 134) that were classified based on levels of CBD in their own chosen cannabis, low (0.14%) vs. high (0.75%). CBD-enriched cannabis did not cause the deficits of immediate and delayed prose recall that were caused by CBD-poor cannabis (Morgan et al., 2010), and users habitually exposed

TABLE 1 | Main findings from human and animal studies aimed to evaluate the therapeutic potential of CBD for the treatment of cannabis use disorder.

CBD and cannabis					
Treatment	**Doses, route of administration, and treatment duration**	**Study design/model**	**Subjects, samples, and gender**	**Main outcomes**	**References**
Clinical studies					
Nabiximols (CBD:THC)	80 mg CBD:84.6 mg THC/day (maximum daily doses), oromucosal spray, 6 days	2-Site, inpatient, double-blind RCT	Cannabis-dependent treatment seekers *N* = 51 (39 M and 12 F)	↓ CWS = Weekly cannabis use ↑ withdrawal treatment retention	Allsop et al. (2014)
Sativex (CBD:THC)	100 mg CBD:108 mg THC/day (maximum daily doses), oromucosal spray, 8 weeks	Double-blind placebo-controlled trial	Community-recruited cannabis dependent patients *N* = 9 (8 M and 1 F)	↓ CWS = Craving	Trigo et al. (2016)
Sativex (CBD:THC) + MET/CBT	105 mg CBD:113,4 mg THC/day (maximum daily doses), oromucosal spray, 12 weeks	Open-label trial	Treatment-seeking community-recruited cannabis-dependent patients *N* = 4 (2 M and 2 F)	↓ cannabis use = CWS	Trigo et al. (2016)
Nabiximols (CBD/THC)	100 mg CBD:108 mg THC/day, oromucosal spray, 8 weeks	Double-blind RCT	Treatment-seeking cannabis-dependent patients *N* = 40 (29 M and 11 F)	↓ cannabis use ↓ craving = CWS	Trigo et al. (2018)
Nabiximols (CBD/THC) + CBT	80 mg CBD:86,4 mg THC/day (maximum daily doses), oromucosal spray, 12 weeks	Multi-site, outpatient, double-blind RCT	Treatment-seeking cannabis-dependent patients *N* = 128 (98 M and 30 F)	↓ cannabis use = Craving = CWS	Bhardwaj et al. (2018); Lintzeris et al. (2019)
CBD	300–600 mg/day, capsules, p.o., 11 days	Case report	19 years-old F with cannabis dependence	↓ CWS ↓ frequency of relapse	Crippa et al. (2013)
CBD	18–24 mg/day, oromucosal spray, 5 months	Case report	27 years-old M with bipolar disorder and cannabis dependence	↓ anxiety levels ↓ sleep disturbances Cessation of cannabis use	Shannon & Opila-Lehman (2015)
CBD	0, 200, 400, 800 mg/day, capsules, p.o., 8 outpatient sessions	Multi-site, double-blind, within-subject RCT	Non-treatment seeking healthy cannabis users *N* = 31 (17 M and 14 F)	= Cannabis self-administration = Subjective effects = Cannabis ratings	Haney et al. (2016)
CBD	0, 200, 400, 800 mg/day, capsules, p.o., 4 weeks	Phase 2a, double-blind RCT	Participants meeting CUD criteria *N* = 82 (59 M and 23 F)	↓ cannabis use ↓ urinary THC-COOH: creatinine ratio	Freeman et al. (2020)
CBD	200 mg/day, capsules, p.o., 10 weeks	Open-label trial	Regular cannabis users *N* = 18 (14 M and 4 F)	↓ cannabis-induced hippocampal disturbances	Beale et al. (2018)
CBD	200 mg/day, capsules, p.o., 10 weeks	Open-label trial	Regular cannabis users *N* = 16 (M)	↓ cannabis-induced euphoria ↓ depressive and psychotic-like symptoms ↑ attentional switching, verbal learning, and memory	Solowij et al. (2018)
Epidiolex (CBD)	800 mg/day (maximum daily dose), solution, p.o., 6 weeks	Double-blind RCT	Cannabis-dependent patients *N* = 10 (4 M and 6 F)	↑ cannabis use	ClinicalTrials.gov ID: NCT03102918
CBD	300–600 mg/day, capsules, p.o., 6 weeks	Double-blind RCT	Patients with psychosis and cannabis abuse *N* = 130 (M/F)	- Cannabis cessation - Psychotic symptoms (no results posted yet)	ClinicalTrials.gov ID: NCT04105231
CBD	600 mg/day, p.o., 12 weeks	Double-blind RCT	Regular cannabis users with recent-onset psychosis *N* = 84 (M/F)	- Change in BPRS score - Change in MATRICS score - Change in serum [THC-COOH] (no results posted yet)	ClinicalTrials.gov ID: NCT03883360
Animal studies					
CBD	5, 10, 30 mg/kg, i.p., acute treatment	Spontaneous cannabinoid withdrawal	C57BL/6J mice *N* = 180 (M)	↓ anxiety level ↓ hyperactivity ↓ withdrawal somatic signs	Navarrete et al. (2018)
CBD	0–20 mg/kg, i.p., chronic treatment	Precipitated cannabinoid withdrawal	C57BL/6J mice *N* = 335 (M)	= Withdrawal somatic signs ↓ anxiety level	Myers et al. (2019)

CBD, cannabidiol; THC, tetrahydrocannabinol; RCT, randomized clinical trial; CWS, cannabis withdrawal syndrome; MET, motivational enhancement therapy; CBT, cognitive behavioral therapy; BPRS, Brief Psychiatric Rating Scale; MATRICS, MATRICS Consensus Cognitive Battery; CUD, cannabis use disorder; M, male; F, female; p.o., per os (oral administration); i.p., intraperitoneal injection; ↑, increase; ↓, decrease; = , no effect.

to CBD-rich cannabis relatively preserved recognition memory vs. CBD-poor cannabis users (Morgan et al., 2012). Likewise, the analysis of cannabinoids in hair samples collected from 140 individuals allowed the comparison between "THC only", "THC + CBD" and "no cannabinoid" groups in terms of schizophrenia-like symptoms. The "THC + CBD" group showed lower levels of positive psychotic symptoms compared with the "THC only" and "no cannabinoid" groups (Morgan and Curran, 2008). These findings are relevant for the therapeutic and public health implications, suggesting that for recreational cannabis users and for those patients taking medicinal cannabis, a more balanced CBD to THC concentration would improve therapeutic endpoints while minimizing side effects.

In a recent clinical trial with healthy volunteers (n = 17) experienced with cannabis (not regular users), functional Magnetic Resonance Imaging (fMRI) studies were performed to investigate the effects of THC (8 mg) and THC + CBD (8 mg + 10 mg) on resting-state brain functional connectivity. CBD restored the THC-induced disruption of the salience network, effect that authors associated with its potential to treat disorders of salience such as psychosis and addiction (Wall et al., 2019). Likewise, another study enrolling frequent and infrequent cannabis users (n = 36) evaluated the effects of THC alone (8 mg) and THC combined with low (4 mg) or high (400 mg) doses of CBD. The results showed that only the high dose of CBD reduced the intoxicating effects of THC (Solowij et al., 2019). In addition, the cannabinoid spray Sativex (1:1 ratio of CBD:THC) at low doses reduces some of the effects produced by THC, including subjective ratings of intoxication and abuse/dependence (Robson, 2011; Schoedel et al., 2011). Also, CBD:THC (1:1 or 1:10 ratios) reversed the conditioned place aversion (CPA) induced by the acute injection of THC (10 mg/kg) in Long Evans rats (Vann et al., 2008).

The protective effects of CBD alone on THC-induced impairments were extensively explored in preclinical and clinical studies. For instance, the administration of CBD (0.5 mg/kg) to rhesus monkeys challenged with THC (0.2, 0.5 mg/kg) significantly attenuated THC-induced cognitive disturbances (Wright Jr. et al., 2013). CBD reduced anxiety and improved fear-related responses induced by THC in male Sprague Dawley rats via a bidirectional control of ERK1-2 phosphorylation (Hudson et al., 2019). In C57BL/6J mice, CBD (3 mg/kg) significantly blunted the cognitive alterations induced by THC (1 mg/kg) administration in an object recognition task (Aso et al., 2019). In the clinical setting, CBD (1 mg/kg) blocked the anxiety induced by THC (0.5 mg/kg) (Zuardi et al., 1982). Furthermore, CBD pre-treatment (600 mg) inhibited THC (1.5 mg)-induced paranoia, inhibited the detrimental effects of THC on episodic memory and decreased the proportion of participants experiencing clinically significant acute THC psychosis (Englund et al., 2013). Importantly, the restorative properties of CBD were also explored in 18 regular cannabis users (heavy and light users) enrolled in a 10 weeks open-label pragmatic trial. Authors measured baseline and post-CBD hippocampal subregions volumes by structural fMRI. CBD restored cannabis-induced anatomical disturbances in the subicular and CA1 subfields of the hippocampus (HIPP) in current cannabis users, especially in those with greater lifetime exposure (Beale et al., 2018). In the same study, CBD improved psychological symptoms (depressive and psychotic-like traits) and cognition (attentional switching, verbal learning, and memory) in dependent cannabis users (Solowij et al., 2018).

Considering the significant CBD-mediated attenuation of the negative outcomes induced by THC, as well as the promising effects of cannabinoid agonist substitution approaches employing synthetic derivatives of THC (e.g., dronabinol, nabilone) (Haney et al., 2004; Budney et al., 2007; Haney et al., 2008; Haney et al., 2013; Vandrey et al., 2013), there has been a growing interest in the therapeutic potential of the combination CBD:THC for the treatment of distinct aspects of CUD (Allsop et al., 2015). Cannabis-dependent treatment seekers (n = 51) received nabiximols (maximum daily doses: 80 mg CBD/86.4 mg THC, oromucosal spray) or placebo with standardized psychosocial interventions. Nabiximols significantly reduced the severity of cannabis withdrawal and prolonged the retention in withdrawal treatment (Allsop et al., 2014). Later, Trigo et al. first explored the effects of fixed or self-titrated dosages of Sativex (maximum daily doses: 100 mg CBD:108 mg THC, oromucosal spray) on cannabis withdrawal and craving. High fixed Sativex doses were well tolerated and significantly attenuated cannabis withdrawal while craving was similar compared to placebo (Trigo et al., 2016). Second, the effects of self-titrated Sativex doses combined with motivational enhancement therapy and cognitive behavioral therapy (MET/CBT) on cannabis withdrawal, use and craving were evaluated. Self-titrated Sativex (maximum daily doses: 105 mg CBD/113.4 mg of THC, oromucosal spray) with MET/CBT significantly decreased cannabis use and prevented cannabis withdrawal under abstinence conditions in these case series (Trigo et al., 2016). Third, the same previous experimental design was employed to evaluate the tolerability, safety, and efficacy of nabiximols (maximum daily doses: 100 mg CBD: 108 mg THC, oromucosal spray). Cannabis use as well as craving were reduced in nabiximols-treated patients compared with placebo, although no differences were found on withdrawal scores (Trigo et al., 2018). Finally, a clinical trial examined the safety and efficacy of nabiximols treatment (up to 32 oromucosal sprays containing 86.4 mg THC/80 mg CBD), combined with individual CBT (Bhardwaj et al., 2018). Interestingly, the nabiximols group reported significantly less days using cannabis than the placebo group while both groups improved to a comparable degree on a range of secondary cannabis-related and general health and psychosocial outcomes (Lintzeris et al., 2019).

One of the major concerns of the cannabinoid replacement therapy is whether the presence of THC in nabiximols could be problematic, especially in the still unexplored long-term treatment of CUD. For this reason, special attention has been paid to the evaluation of the clinical efficacy of CBD alone. The potential therapeutic usefulness of CBD for the treatment of CUD was investigated in some case report clinical studies. Crippa et al. administered CBD for 11 days (300 mg on day 1, 600 mg on days 2–10, and 300 mg on day 11, capsules, p.o.) to a 19 year-old female with cannabis dependence who experienced withdrawal

syndrome when she tried to cease cannabis use. Daily assessments showed a rapid decrease in withdrawal symptoms leading to a score of zero in all tests by day 6. A 6 months follow-up showed a relapse in cannabis use but at a lower frequency (once or twice a week vs. 7 days a week) (Crippa et al., 2013). Another case report study evaluated the use of a CBD oil in a 27 year-old male presenting a long-standing diagnosis of bipolar disorder and a daily addiction to cannabis use. After initiating the treatment with CBD oil (18–24 mg/day, oromucosal spray), the patient reported a decrease in the anxiety level and sleep disturbances, as well as a complete cessation of cannabis use (Shannon and Opila-Lehman, 2015). A multi-site clinical study analyzed the effects of oral CBD (0, 200, 400, 800 mg, capsules, p.o.) on the reinforcing, subjective, cognitive, and physiological effects of smoked cannabis. CBD was administered 90 min prior to smoking half of a cannabis cigarette by non-treatment-seeking healthy cannabis users ($n = 31$) during 8 outpatient sessions. No difference was found in comparison with placebo-treated patients (Haney et al., 2016). This may be due to the acute CBD treatment, the study population (non-treatment-seeking patients) or the poor bioavailability of oral CBD. Recently, a phase 2a, double-blind, placebo-controlled, randomized clinical trial was carried out to identify efficacious doses of CBD (200, 400 and 800 mg, capsules, p.o., 4 weeks) for the treatment of CUD. Following a 2-stages design with 82 participants meeting CUD criteria from DSM-5 (48 in stage 1 and 34 in stage 2), CBD efficacy was determined according to urinary 11-nor-9-carboxy-δ-9-tetrahydrocannabinol (THC-COOH):creatinine ratio and/or increased days per week with abstinence from cannabis during treatment. CBD 400 and 800 mg doses were well tolerated and more efficacious than placebo at reducing cannabis use (Freeman et al., 2020). Another recent clinical study also explored the effects of CBD (Epidiolex, up to 800 mg, solution, p.o., 6 weeks treatment period) in cannabis dependent subjects ($n = 10$). Although no significant differences were found, cannabis consumption was higher in the CBD-treated group. However, as stated by the authors, more participants are necessary to draw definitive conclusions from this study (Clinicaltrials.gov identifier: NCT03102918). Interestingly, two clinical trials have been recently registered (Clinicaltrials.gov identifiers: NCT04105231 and NCT03883360) to explore the effects of long-term administration of CBD (up to 600 mg, capsules, p.o., 6 or 12 weeks, respectively) on psychiatric symptoms, cognition, and cannabis consumption in patients with recent-onset psychosis and comorbid cannabis use.

Apart from the valuable information provided by clinical studies, it is essential to analyze the effects of CBD on behavioral and neurobiological alterations related with cannabis dependence at the preclinical level. For that purpose, our laboratory was the first to explore CBD actions (5, 10 and 20 mg/kg, i.p.) in an animal model of spontaneous cannabinoid withdrawal syndrome developed after 7 days of treatment with CP-55,940 (a 45-fold more potent cannabinoid 1 receptor (CB1R) agonist compared to THC) (Aracil-Fernández et al., 2013). Withdrawal-related behavioral signs were evaluated by measuring motor activity, somatic signs, and anxiety-like behavior in abstinent C57BL/6J mice treated with CBD or its corresponding vehicle. In addition, real-time PCR (RT-PCR) analyses were performed to evaluate changes in the gene expression of relevant targets of the cannabinoid, dopaminergic, and opioidergic systems. Interestingly, CBD administration significantly blocked the increase in motor-activity, number of rearings, rubbings, and jumpings associated with spontaneous cannabinoid withdrawal, and normalized the decrease in the number of groomings. Furthermore, the anxiogenic-like effect observed in abstinent mice was completely abolished by CBD. These effects were associated with a CBD-induced up-regulation of tyrosine hydroxylase (TH) in the ventral tegmental area (VTA) and cannabinoid 2 receptor (CB2R) in the nucleus accumbens (NAcc), whereas a down-regulation of mu-opioid receptor (MOR) and CB1R in the NAcc (Navarrete et al., 2018). Also, a recent study was aimed to evaluate if CBD (0–20 mg/kg, i.p.) improves cognitive deficits and withdrawal signs induced by cannabinoid CB1/CB2 receptor agonists such as THC. CB1R antagonist (SR141716) administration precipitated withdrawal signs in chronically THC-treated C57BL/6J mice and they were not attenuated by CBD. However, the lack of CBD-induced withdrawal signs or cognitive performance impairment, together with the robust anxiolytic effect led the authors to conclude that CBD as a monotherapy might be a safer pharmacological agent for the treatment of several disorders (Myers et al., 2019).

According to the previous evidence, it seems that CBD could play a crucial role in the management of CUD. The clinical studies that are underway as well as future investigations will be decisive to determine the therapeutic application of CBD to treat cannabis addiction.

CBD and Alcohol

Problematic alcohol use is an important risk factor for many health problems significantly contributing to the global burden of disease (Collaborators, 2018). In 2016, harmful alcohol use caused 3 million of deaths worldwide and 132.6 million disability-adjusted life years (DALYs) (OMS, 2019). Alcohol Use Disorder (AUD) is one of the most common addictive disorders with a greatest health and socioeconomic impact. The prevalence of AUD varies from 13 to 30% in most western countries (Grant et al., 2015; WHO, 2018). Current options for AUD treatment are scarce and have limited efficacy. To date, there are only four drug-based treatments approved for AUD by the FDA and EMA: naltrexone, nalmefene, acamprosate, and disulfiram (Soyka and Müller, 2017). Despite the optimization of pharmacological and psychosocial interventions for the management of AUD, at least 60% of alcoholic patients usually relapse during the first 6 months after dishabituation treatment (Maisto et al., 2006; Kirshenbaum et al., 2009; Witkiewitz, 2011; Durazzo and Meyerhoff, 2017). Thus, the need for new pharmacological approaches proving higher efficacy in alcohol relapse avoidance and maintenance of abstinence is evident. In this sense, CBD has recently attracted attention because of its ability to modulate the reinforcing and motivational effects of alcohol, as well as to improve the damage produced by alcohol in

TABLE 2 | Main findings from clinical and animal studies aimed to evaluate the therapeutic potential of CBD for the treatment of alcohol use disorder.

CBD and alcohol					
Treatment	**Doses, route of administration, and treatment duration**	**Study design/ model**	**Subjects, samples, and gender**	**Main outcomes**	**References**
Clinical studies					
CBD	600 and 1,200 mg/day, p.o., 4 + 4 weeks	Double-blind RCT	Patients with moderate or severe AUD (DSM-5) *N* = 40 (M/F)	- TLFB assessment of alcohol consumption in serum - Change in % CDT assessment of alcohol consumption in serum (no results posted yet)	ClinicalTrials.gov ID: NCT03252756
CBD	600 mg/day, p.o., 6 weeks	Double-blind RCT	Patients with AUD and PTSD comorbidity *N* = 48 (M/F)	- Number of drinks per day with TLFB (no results posted yet)	ClinicalTrials.gov ID: NCT03248167
CBD	800–1,200 mg/day, capsules, p.o., 4 days	Double-blind RCT	Patients with AUD undergoing alcohol withdrawal *N* = 52 (M/F)	- diazepam use over the 5 days withdrawal period (no results posted yet)	ClinicalTrials.gov ID: NCT04205682
Animal studies					
CBD	30, 60, 120 mg/kg, i.p., 30 mg/kg/day, s.c. (continuous controlled release), chronic treatment	VC, ESA	C57BL/6J mice *N* = 40 (M)	↓ ethanol intake and preference ↓ motivation to ethanol consumption ↓ ethanol relapse	Viudez-Martínez et al. (2018a)
CBD	15 mg/kg/day, t.d., 7 days	ESA, DRT	Wistar rats *N* = 52 (M)	↓ context-induced and stress-induced reinstatement ↓ impulsivity level in rats with alcohol dependence history	Gonzalez-Cuevas et al. (2018)
CBD CBD + THC	2.5 mg/kg CBD ±2.5 mg THC, i.p., acute treatment	Ethanol-induced locomotor sensitization	DBA/2 mice *N* = 84 (M)	↓ motor sensitization to ethanol	Filev et al. (2017)
CBD ± NTX WAY	20 mg/kg/day CBD, s.c. (continuous controlled release) ± 0.7 mg/kg NTX; p.o., 0.3 mg/kg WAY, i.p., chronic treatment	ESA	C57BL/6J mice *N* = 140 (M)	↓ motivation to ethanol consumption (CBD + NTX) → abolished by WAY	Viudez-Martínez et al. (2018b)
CBD	15, 30, 60, 90 mg/kg, i.p., chronic treatment	Binge drinking	C57BL/6J mice *N* = 120 (60 M and 60 F)	↓ ethanol intake (30, 60 and 90 mg/kg, repeated administration, M) ↓ ethanol intake (90 mg/kg, acute and repeated administration, F)	Viudez-Martínez et al. (2020)
CBD	20, 40 mg/kg, i.p., repeated treatment	Binge ethanol exposure	Sprague-dawley rats (M)	↓ ethanol-induced hippocampal and entorhinal cortical neurodegeneration	Hamelink et al. (2005)
CBD	1, 1.0, 2.5 and 5.0% gel, t.d., 40 mg/kg, i.p., repeated treatment	Binge ethanol exposure	Sprague-dawley rats (M)	↓ FJB + cells in the entorhinal cortex	Liput et al. (2013)
CBD	5 mg/kg, i.p., 5 days	Binge ethanol exposure	C57BL/6J mice (M)	↑ alcohol-induced liver steatosis ↓ alcohol-mediated oxidative stress ↓ JNK MAPK activation ↑ autophagy	Yang et al. (2014)
CBD	5, 10 mg/kg, i.p., 11 days	Chronic ethanol exposure	C57BL/6J mice (M)	↓ alcohol feeding-induced serum transaminase elevations ↓ hepatic inflammation ↓ oxidative/nitrative stress	Wang et al. (2017)

CBD, cannabidiol; THC, tetrahydrocannabinol; RCT, randomized clinical trial; AUD, alcohol use disorder; DSM-5, Diagnostic and Statistical Manual of Mental Disorders; TLFB, Time-line Follow-back scale; CDT, carbohydrate deficient transferrin; VC, voluntary consumption; ESA, ethanol self-administration; DRT, delayed reinforcement task; WAY, WAY-100635 (5HT1a selective antagonist); M, male; F, female; p.o., per os (oral administration); i.p., intraperitoneal injection; t.d., transdermal; ↑, increase; ↓, decrease; = , no effect.

the liver or CNS (De Ternay et al., 2019; Turna et al., 2019) (**Table 2**).

Our laboratory was the first to publish relevant data regarding the effects of CBD on ethanol reinforcement, motivation, and relapse in C57BL/6J male mice. Voluntary ethanol consumption (VEC) and oral ethanol SA procedures were employed. First, VEC was evaluated in a two-bottle choice paradigm in which mice were repeatedly administered with different doses of CBD (30, 60 and 120 mg/kg, i.p.). Ethanol consumption and preference were significantly reduced by CBD in a dose-dependent manner. Second, oral ethanol SA was carried out in operant skinner boxes to evaluate the effects of a single administration of CBD in a microparticle formulation providing a constant release (30 mg/kg/day, s.c.). Interestingly, CBD significantly reduced the number of active lever presses and ethanol intake under fixed-ratio 1 (FR1) and fixed-ratio 3 (FR3) schedules, as well as

the breaking point that measures the motivation to drink alcohol. Third, the effects of CBD on alcohol relapse were also analyzed in the oral ethanol SA paradigm with some modifications. The administration of CBD (120 mg/kg, i.p.) significantly reduced the number of active lever presses and ethanol intake during relapsing conditions. Importantly, these effects were accompanied by changes on the relative gene expression (RT-PCR) of selected dopaminergic, opioidergic and cannabinoid targets. Briefly, CBD induced a down-regulation of TH in the VTA and MOR, CB1R and G-protein coupled receptor 55 (GPR55) gene expressions in the NAcc whereas CB2R mRNA levels were increased in the NAcc (Viudez-Martínez et al., 2018a). Shortly thereafter, Gonzalez-Cuevas et al. demonstrated that CBD transdermal administration (fast-drying 2.5% hydroalcoholic gel formulation, 15 mg/kg/day, 7 days) significantly attenuated the context- and stress-induced reinstatement for ethanol seeking, and this effect lasted up to 5 months. In addition, CBD fully reversed the high impulsivity level showed by rats with an EtOH dependence history (Gonzalez-Cuevas et al., 2018). On the other hand, the effects of CBD alone or in combination with THC (2.5 mg/kg each, i.p., 4 days) on ethanol-induced locomotor sensitization were also evaluated in DBA/2 mice. THC alone or combined with CBD, but not CBD alone, significantly inhibited the expression of sensitization to ethanol in this paradigm (Filev et al., 2017).

The combination of different drugs is a commonly used procedure for the treatment of AUD. This strategy usually provides a greater effect and prevents certain dose-related side effects by using lower doses of each drug than the ones employed in monotherapy. Taking into consideration this approximation, our group was also aimed to explore whether the combination of CBD with naltrexone (NTX) might reduce alcohol consumption and motivation to drink in C57BL/6J mice to a higher extent. For that purpose, the effects of a sub-effective dose of NTX (0.7 mg/kg, p.o.), CBD (20 mg/kg/day, s.c., microparticles formulation for continuous controlled release for 3 weeks) or their combination were evaluated. Interestingly, the administration of CBD plus NTX was the only treatment able to reduce motivation and ethanol intake in the oral ethanol SA. Also, these effects were associated with a down-regulation in the gene expression of TH in the VTA, MOR in the NAcc, and serotonin 1a receptor (5HT1a) in the dorsal raphe. To elucidate the role of 5HT1a receptors in the mechanisms that could underlie CBD plus NTX effects on ethanol reinforcement and motivation, the 5HT1a antagonist WAY 100635 was concomitantly administered. Pretreatment with this compound significantly blocked the effects of CBD plus NTX, a finding that supports the involvement of 5HT1a receptors (Viudez-Martínez et al., 2018a).

One of the major concerns of harmful ethanol consumption is the binge drinking pattern that has become a major public health problem in modern societies (Lannoy et al., 2019). Nevertheless, the available pharmacological options for binge drinking management are scarce and limited (Rolland and Naassila, 2017). In this respect, therapeutic usefulness of CBD for the treatment of binge drinking patterns was analyzed also by our group taking into consideration gender differences. The effects of CBD on ethanol binge drinking were explored in male and female C57BL/6J mice by using the drinking in the dark procedure. Repeated CBD administration (15, 30 and 60 mg/kg, i.p.) significantly reduced ethanol intake only in males and was associated with a down-regulation of TH gene expression in the VTA, and MOR and CB1R gene expressions in the NAcc. Interestingly, a higher CBD dose (90 mg/kg, i.p.) significantly reduced ethanol intake under acute and repeated administration patterns not only in males but also in females (Viudez-Martínez et al., 2020). Except for these findings, previous studies provided evidence of CBD neuroprotective actions in rodent models of ethanol binge intoxication. In 2005, Hamelink et al. demonstrated that CBD (40 mg/kg, i.p.) significantly reduced the number of degenerated argyrophilic neurons in the dentate gyrus of the HIPP and the entorhinal cortex of Sprague Dawley rats exposed to a 4 days ethanol binge administration (Hamelink et al., 2005). Also, Liput and cols followed a similar procedure showing a significant CBD-mediated reduction in the neurodegeneration induced by ethanol binge treatment reflected in a lower number of Fluoro-Jade B positive cells in the entorhinal cortex (Liput et al., 2013). Finally, it is worth to mention that CBD might also present protective actions against alcohol-induced liver disease, attenuating hepatic steatosis and metabolic dysregulation by anti-inflammatory and antioxidant mechanisms in animal models of repeated ethanol exposure (Yang et al., 2014; Wang et al., 2017).

Taking into consideration the promising preclinical data pointing out CBD as a potential therapeutic tool for AUD, clinical studies were recently initiated. In 2019, a randomized, double blinded and proof-of-concept clinical trial was started in United States (New York) to assess the effects of extended treatment with CBD (600 and 1,200 mg/day, 4 weeks for each dosing, p.o.) compared to placebo in 40 patients with severe AUD (NCT03252756). In the same year, another randomized, double-blind and placebo-controlled clinical trial began also in United States (New York) to determine whether CBD (600 mg, 6 weeks, p.o.) is effective in treating AUD in individuals (48 participants) with moderate or severe AUD and comorbid posttraumatic stress disorder (PTSD) (NCT03248167). Finally, another randomized, double-blind, and placebo-controlled clinical trial to explore the effectiveness and tolerability of CBD (1,200 mg/day 1, 800 mg/days 2–4, p.o.) in the treatment of alcohol withdrawal symptoms in an inpatient setting (52 participants) in Australia (NCT04205682) is expected to start in 2020.

Thus, the great and growing interest in CBD as a new drug for AUD management is more than evident. However, further studies are warranted to shed light on the underlying brain mechanisms involved as well as on pharmacokinetics aspects such as dose, treatment duration, route of administration or pharmaceutical formulation.

CBD and Opioids

Opioid use disorder (OUD) could be defined as a chronic, relapsing illness, associated with significantly increased rates of morbidity and mortality. In the United States, 5.1 million people (1.9 percent of persons age 12 or older) were estimated in 2015 to

TABLE 3 | Main findings from clinical and animal studies aimed to evaluate the therapeutic potential of CBD for the treatment of opioid use disorder.

CBD and opioids					
Treatment	**Doses, route of administration, and treatment duration**	**Study design/ model**	**Subjects, samples, and gender**	**Main outcomes**	**References**
Clinical studies					
Epidiolex (CBD)	400 or 800 mg/day, p.o., 3 days	Double-blind RCT	Patients with heroin use disorder *N* = 42 (35 M and 7 F)	↓ craving and anxiety after acute, short term and long-term evaluation ↓ heart rate after acute and short-term evaluation ↓ cortisol levels	Hurd et al. (2019)
APH-1501 (>98.5% CBD, <0.3 THC)	400, 600, 800 mg/day, capsules, p.o., 28 days	Triple-blind RCT	Opioid-dependent patients *N* = 32 (M/F)	- Incidence of treatment adverse effects - Pharmacokinetics of APH-1501 (no results posted yet)	ClinicalTrials.gov ID: NCT03813095
Epidiolex (CBD)	800 mg/day, oral solution, p.o., 2 days	Open-label	Methadone-maintained participants undergoing spontaneous withdrawal *N* = 50 (M/F)	- Safety as assessed by number of adverse events - Number of participants whose AST/ ALT levels >3x upper limit of normal - Feasibility of spontaneous withdrawal model as assessed by change in withdrawal scores (no results posted yet)	ClinicalTrials.gov ID: NCT04238754
Animal studies					
CBD ± THC	10 mg/kg CBD ±2 mg/kg THC, i.p., acute treatment	Naloxone-induced morphine abstinence	Sprague-dawley rats *N* = 33 (M)	↓ morphine withdrawal signs (CBD + THC combination)	Hine et al. (1975)
CBD	5, 10, 20 mg/kg, i.p., acute treatment	Naloxone-induced morphine withdrawal	Swiss-webster mice (M)	↑ dose of naloxone needed to induce morphine withdrawal jumping in 50% of the animals (ED50) ↓ jumping, defecations, and rearing behaviors	Bhargava. (1976)
CBD	5, 20, 80 mg/kg, i.p., acute treatment	Quasi-morphine withdrawal syndrome	Sprague-dawley rats (M)	= Withdrawal score	Chesher & Jackson. (1985)
CBD	5 mg/kg, i.p., acute treatment	Morphine-induced ICSS	Sprague-dawley rats (M)	⊗ reward-facilitating morphine effects → abolished by WAY	Katsidoni et al. (2013)
CBD	5, 10 mg/kg, s.c., acute treatment	Morphine-induced CPP, naltrexone-induced CPA	Wistar rats N = 295 (M)	↓ CPP ⊗ morphine priming- or stress-induced CPP reinstatement ⊗ naltrexone-induced CPA	de Carvalho & Takahashi. (2017)
CBD	2.5, 5, 10, 20 mg/kg, i.p., acute treatment	Morphine-induced CPP	C57BL/6 mice *N* = 100 (M)	↓ CPP	Markos et al. (2018)
CBD	5, 10, 20 mg/kg, i.p., acute treatment	Heroin-induced ISA	Long-evans rats (M)	= Heroin ISA = priming-induced heroin seeking ↓ cue-induced heroin seeking	Ren et al. (2009)

CBD, cannabidiol; THC, tetrahydrocannabinol; RCT, randomized clinical trial; CPP, conditioned place preference; CPA, conditioned place aversion; ISA, intravenous self-administration; WAY, WAY-100635 (5HT1a selective antagonist); M, male; F, female; p.o., per os (oral administration); i.p., intraperitoneal injection; ↑, increase; ↓, decrease; = , no effect; ⊗, blockade.

have used heroin at some point in their lives (Hser et al., 2015; Substance Abuse and Mental Health Services Administration, 2016). Patients may develop OUD by acquiring illegal opioid drugs (e.g., heroin), by obtaining them legally but use them for not legitimate medical purposes (morphine, fentanyl, oxycodone, etc.), or at doses in excess to the needed for the medical condition (APA, 2013). One of the major concerns associated with OUD is the occurrence of opioid overdose with high rates of mortality, especially in United States were recent data show a significant increase (Rudd et al., 2016). Currently, the FDA and the EMA authorize the marketing of three classes of medications: 1) the short-acting opioid antagonist naloxone employed to reverse the life-threatening effects of opioid overdose, 2) oral opioid agonists methadone and buprenorphine, highly effective and widely employed in opioid maintenance programs to achieve abstinence and avoid relapse, and 3) the alpha 2-adrenergic agonist lofexidine, recently approved by the FDA representing the first non-opioid medication indicated for mitigation of symptoms associated with acute opioid withdrawal and for facilitation of the completion of opioid discontinuation treatment (Gorodetzky et al., 2017; Guo et al., 2018). Nowadays, buprenorphine (BPN) has been proposed as one of the first-line treatments for OUD management due to its low abuse potential, reduced risk of overdose and flexible dosing in comparison with methadone (Li et al., 2014). However, recent evidence points out poor patient retention in BPN maintenance (Bell, 2014; Hser et al., 2014; Mattick et al., 2014; Burns et al., 2015). This fact together with the limited efficacy of current

options for the treatment of OUD motivates the development of new mechanistically based pharmacological strategies that go beyond treating symptoms associated with opioid withdrawal syndrome to relapse. CBD may serve as a new therapeutic strategy for the treatment of OUD, not simply for withdrawal symptomatic relief partly due to its anxiolytic properties, but also to reduce craving and avoiding relapse (**Table 3**).

The first evidence on the possible therapeutic utility of CBD in the regulation of pharmacologically induced morphine withdrawal was reported in 1975. The effects of CBD (10 mg/kg, i.p.), alone or combined with THC (2 mg/kg, i.p.), on naloxone-induced morphine abstinence were evaluated in male Sprague-Dawley rats. THC and especially CBD plus THC combination significantly attenuated morphine withdrawal signs whereas no effects were found with CBD alone (Hine et al., 1975). Shortly another study evaluated the effects of some cannabinoid compounds on naloxone-precipitated abstinence signs in Swiss-Webster male mice. Interestingly, CBD (5 and 10 mg/kg, i.p.) significantly increased the dose of naloxone needed to induce morphine withdrawal jumping in 50% of the animals (ED50), although it was not the most effective cannabinoid (Bhargava, 1976). To further elucidate the therapeutic potential of cannabinoid compounds to modulate morphine withdrawal, Chesher and Jackson analyzed whether cannabinol, CBD or THC attenuate the signs associated with the quasi-morphine withdrawal syndrome in male Sprague-Dawley rats. THC and cannabinol significantly reduced the withdrawal score whereas CBD was without effect at the dosage levels used (5, 20 and 80 mg/kg, i.p.) (Chesher and Jackson, 1985).

More recently, it was reported that CBD might interfere with brain reward mechanisms responsible for the expression of the acute reinforcing properties of opioids such as morphine. Indeed, authors showed that CBD inhibited the reward-facilitating effect of morphine employing the intracranial self-stimulation (ICSS) paradigm. Interestingly, pre-treatment with an intra-dorsal raphe injection of the selective 5HT1a receptor antagonist WAY-100635 reversed the effects of CBD, suggesting the involvement of these receptors in the CBD-mediated inhibition of morphine-induced reward (Katsidoni et al., 2013). Also, the efficacy of CBD to regulate morphine-induced CPP was investigated by two independent studies. First, in male Wistar rats the administration of CBD (10 mg/kg, i.p.) blocked place conditioning behavior and reinstatement induced by a priming dose of morphine or stress exposure (de Carvalho and Takahashi, 2017). Second, in male C57BL/6J mice the same dose of CBD also significantly attenuated morphine-induced CPP (Markos et al., 2018).

Heroin is a morphine derivative with a higher addictive power and is usually consumed first by patients starting the use of opioids (Cicero et al., 2017). To evaluate if the administration of CBD could modify the reinforcing and motivational properties of heroin, Ren et al. employed an animal model of heroin intravenous SA. They assessed the actions of CBD on heroin SA and relapse induced by a heroin prime injection or the exposure to conditioned contextual cues. The administration of CBD (5 or 20 mg/kg, i.p.) was without effect on heroin consumption and did not prevent relapse by a priming dose of heroin. However, it significantly attenuated the reinstatement of cue-induced heroin seeking. Interestingly, CB1R and glutamatergic mGluR5 and GluR1 gene and/or protein alterations were normalized with CBD treatment (Ren et al., 2009). A few years later, a double-blind randomized placebo-controlled clinical trial evaluated the utility of CBD (400 or 800 mg) to reduce cue-induced craving and anxiety in drug-abstinent patients with heroin use disorder. The results showed that the administration of CBD reduces both craving and anxiety induced by the presentation of heroin-related salient drug cues. Furthermore, CBD also attenuated drug cue-induced physiological measures of heart rate and salivary cortisol levels in heroin abstinent patients. Remarkably, these effects were maintained one week after the end of the CBD short-term administration (Hurd et al., 2019). Finally, it is relevant to mention that an exploratory dose ranging study was recently posted in ClinicalTrials.gov to assess the safety, efficacy, and tolerability of APH-1501 (>98.5% CBD, <0.3 THC) for the treatment of opioid dependence. This clinical trial will target opioid-dependent patients completing detoxification in a treatment facility. These will be randomized into 4 treatment groups receiving APH-1501 (400, 600 or 800 mg/m^2) or placebo over a 30 day period (NCT03813095). Also, another pilot study sponsored by the Johns Hopkins University has been proposed to examine the safety of CBD (Epidiolex) in a human laboratory model of clinically relevant opioid withdrawal. In a residential, randomized and within-subject comparison design, authors will evaluate the effects of placebo and CBD (800 mg) in methadone-maintained patients undergoing spontaneous withdrawal (NCT04238754).

In summary, to date few studies have attempted to demonstrate the efficacy of CBD in opioid addiction. The achievement of promising results lately has motivated further research to evaluate the potential utility of CBD in the management of OUD.

CBD and Psychostimulants

Stimulant use disorder is defined by the DSM-5 as the continued use of amphetamine-type substances, cocaine, or other stimulants leading to clinically significant impairment or distress, from mild to severe (APA, 2013). The global prevalence of stimulant use has increased over the past decade with a worrying rise in the use of amphetamine-type stimulants and cocaine (United Nations Office on Drugs and Crime, 2019). Amphetamine-type stimulants include substances with a similar chemical structure, such as amphetamine and methamphetamine, and other structurally different but with similar effects, such as methylphenidate. Amphetamine-type stimulants as well as cocaine are highly addictive substances. One of the main concerns is the lack of specific pharmacological tools for the treatment of amphetamine-type or cocaine use disorder. Although psychostimulants have shown some favorable results, high quality clinical trials and meta-analyses are needed to determine their clinical utility (Ronsley et al., 2020). Thus, it is essential to search for new therapeutic approaches. In the last years, many authors evaluated the therapeutic utility of CBD to

TABLE 4 | Main findings from clinical and animal studies aimed to evaluate the therapeutic potential of CBD for the treatment of stimulant use disorder.

CBD and psychostimulants					
Treatment	**Doses, route of administration, and treatment duration**	**Study design/ model**	**Subjects, samples, and gender**	**Main outcomes**	**References**
Amphetamine/methamphetamine					
CBD	5 mg/kg, i.p., 4 days (conditioning pase of CPP) or 1 day (extinction trial)	AMPH-induced CPP	Sprague-dawley rats (M)	= Conditioning score ↑ CPP extinction	Parker et al. (2004)
CBD	10 μg/5 μl, ICV, acute treatment	METH-induced CPP	Wistar rats (M)	↓ METH-induced CPP reinstatement (high priming dose) ↓ METH-induced CPP reinstatement (low priming dose in REM sleep deprived rats)	Karimi-Haghighi & Haghparast. (2018)
CBD	10, 20, 40, 80 mg/kg, i.p., repeated treatment (METH-paired conditioning sessions)	METH-induced CPP	Sprague-dawley rats (M)	↓ METH-induced CPP (dose-dependently)	Yang et al. (2020)
CBD	0, 20, 40 and 80 mg/kg, i.p., acute treatment	METH-induced ISA	Male sprague-dawley rats *N* = 32 (M)	↓ motivation to self-administer METH ↓ METH-primed relapse after extinction	Hay et al. (2018)
CBD	32 and 160 nmol, ICV, 10 days (abstinence)	Chronic exposure to METH	Wistar rats *N* = 62 (M)	↑ long-term memory in the NOR test	Razavi et al. (2020)
Cocaine					
CBD	5 mg/kg, i.p., 4 days (conditioning phase of CPP) or 1 day (extinction trial)	Cocaine-induced CPP	Sprague-dawley rats (M)	= Conditioning score ↑ CPP extinction	Parker et al. (2004)
CBD	10 mg/kg, i.p., acute treatment	Cocaine-induced CPP	Wistar rats *N* = 295 (M)	↓ reconsolidation of cocaine-induced CPP	de Carvalho & Takahashi. (2017)
CBD	10 mg/kg, i.p., acute and repeated administration	Cocaine-induced CPP	C57BL/6J mice (M)	↓ preference for the cocaine context ↓ consolidation of cocaine memory = cocaine-induced CPP = Rate of extinction of cocaine memory = cocaine-primed reinstatement	Chesworth & Karl. (2020)
CBD	30, 60 mg/kg, i.p., acute treatment	Cocaine-induced CPP	CD1 mice *N* = 120 (M)	↓ cocaine-primed reinstatement ↓ social defeat-induced reinstatement	Calpe-Lopez et al. (2020)
CBD	10, 20 mg/kg, i.p., 10 days	Cocaine-induced ISA	CD1 mice (M)	↓ cocaine self-administration and motivation → abolished by hippocampal neurogenesis blockade (temozolomide) = cocaine-induced reinstatement	Calpe-Lopez et al. (2020) and Luján et al. (2019)
CBD	3–20 mg/kg, i.p., repeated administration	Cocaine-induced ISA Cocaine-induced BSR	Long-evans rats *N* = 75 (M)	↓ cocaine self-administration with low but not high cocaine doses ↓ cocaine-enhanced BSR	Galaj et al. (2020)
CBD	15 mg/kg/day, t.d. 7 days	Cocaine-induced ESA	Wistar rats *N* = 52 (M)	↓ context-induced and stress-induced reinstatement	Gonzalez-Cuevas et al. (2018)
CBD + caffeine	20 mg/kg, i.p. Repeated administration	Cocaine-induced locomotor sensitization	Wistar rats (M)	↓ cocaine-induced hyperlocomotion	Prieto et al. (2020)
CBD	10, 20, 40 mg/kg, i.p.Cocaine-induced BSR Acute treatment	Spontaneous cocaine withdrawal	CD1 mice *N* = 100 (M)	↓ anxiety level ↓ hyperactivity ↓ withdrawal somatic signs	Gasparyan et al. (2020)
CBD	5 mg/kg, i.p. Acute treatment	Cocaine-induced ICSS	Sprague-dawley rats (M)	= reward-facilitating effect of cocaine	Katsidoni et al. (2013)
CBD	5 and 10 mg/kg, i.p. Chronic and acute treatment	Cocaine-induced ISA	Long-evans rats *N* = 40 (M)	= Cocaine self-administration = Cocaine seeking after withdrawal	Mahmud et al. (2017)
CBD	400 or 800 mg/day	Double-blind RCT	Cocaine-dependent individuals *N* = 79 (M/F)	Drug-cue induced craving Number of days to relapse (no results posted yet)	ClinicalTrials.gov ID: NCT02559167

CBD, cannabidiol; AMPH, amphetamine; METH, methamphetamine; CPP, conditioned place preference; ICSS, intracranial self-stimulation; ISA, intravenous self-administration; BSR, brain stimulation reward; NOR, novel object recognition; REM, rapid eye movement; RCT, randomized clinical trial; M, male; F, female; p.o., per os (oral administration); i.p., intraperitoneal injection; ICV, intracerebroventricular; ↑, increase; ↓, decrease; = , no effect.

treat the different phases of dependence to psychostimulants. As reviewed below, published reports focused mainly on evaluating the effects of CBD on the reinforcing and motivational actions of amphetamine, methamphetamine, and cocaine in different animal models (**Table 4**).

Amphetamine-type Substance Use Disorder

The potential of CBD to modulate amphetamine-induced rewarding properties was first reported in 2004. In this study, the administration of a low dose of CBD (5 mg/kg, i.p.) potentiated the extinction of amphetamine-induced CPP without affecting the learning process of place conditioning (Parker et al., 2004). Years later, another group showed that intracerebroventricular (ICV) injection of CBD (10 μg/5 μl) suppressed the methamphetamine-induced reinstatement in the CPP paradigm, even under stressed conditions (Karimi-Haghighi and Haghparast, 2018). Interestingly, these authors suggested later that the effect of CBD was associated with the normalization of methamphetamine-induced increase of gene expression of cytokines (interleukin-1β, interleukin-6, interleukin-10, and tumor necrosis factor α (TNF-α)) in the prefrontal cortex (PFC) and HIPP. However, in REM sleep-deprived rats CBD produced opposite effects (Karimi-Haghighi et al., 2020). Recently, CBD-mediated regulation of methamphetamine-induced CPP was further confirmed. Treatment with CBD (10, 20, 40 and 80 mg/kg, i.p.) 1 h prior to the administration of methamphetamine during conditioning sessions significantly and dose dependently attenuated CPP. Importantly, these effects were related with the regulation of the SigmaR1/AKT/GSK-3β/CREB signaling pathway that was up-regulated in the VTA, NAcc, HIPP, and PFC of methamphetamine-treated male Sprague-Dawley rats (Yang et al., 2020). Apart from the effects of CBD on CPP induced by amphetamine and methamphetamine, Hay et al. explored whether CBD modulates the motivation to obtain methamphetamine as well as the relapse into methamphetamine consumption using an intravenous SA paradigm. After a training phase, the administration of CBD (80 mg/kg, i.p.) significantly reduced active lever pressing and consequently the number of methamphetamine infusions, as well as methamphetamine-primed relapse to active lever pressing (Hay et al., 2018).

Chronic exposure to amphetamine-type derivatives could lead to neurodegeneration and neuro-inflammation phenomena with associated cognitive impairments. Therefore, in addition to the interest of modulating rewarding and motivational properties, it is also important to provide a neuroprotective effect to attenuate these alterations. In this sense, a recent report revealed that the ICV administration of CBD during the abstinence period after chronic exposure to methamphetamine (10 days) significantly reverses long-term memory in the novel object recognition test (Razavi et al., 2020). However, more studies are needed to further explore the therapeutic potential of CBD and to elucidate the neurobiological mechanisms involved.

Cocaine Use Disorder

One of the first reports suggesting the therapeutic potential of CBD for the modulation of cocaine rewarding properties employed the CPP paradigm. In Sprague-Dawley rats, CBD (5 mg/kg, i.p.) did not change the conditioning score but enhanced CPP extinction (Parker et al., 2004). Also, CBD (10 mg/kg, i.p.) disrupted the reconsolidation of place preference in rats and this effect was present for 2 weeks (de Carvalho and Takahashi, 2017). Very recently, Chesworth and Karl exhaustively explored CBD actions (10 mg/kg, i.p.) on the acquisition, consolidation, reconsolidation, extinction, and drug-primed reinstatement of cocaine (15 mg/kg) in the CPP paradigm. CBD significantly reduced the preference for the cocaine-context and the consolidation of cocaine memory. CBD had no effects on cocaine-induced CPP, the rate of extinction of cocaine memory, or the drug-primed reinstatement (Chesworth and Karl, 2020). However, a recent report of our group demonstrated that CBD (30 and 60 mg/kg, i.p.) significantly reduced cocaine priming- and social defeat-induced reinstatement of CPP (Calpe-Lopez et al., 2020). Likewise, Lujan et al. demonstrated that CBD (10 and 20 mg/kg, i.p.) significantly attenuated cocaine-induced CPP. Furthermore, they employed an intravenous SA paradigm and showed that CBD (20 mg/kg, i.p.) reduced the motivation to self-administer cocaine in a fixed ratio 1 schedule, as well as the breaking point during the progressive ratio stage. Interestingly, CBD effects on cocaine-induced reward and motivation could be related with an increase of CB1R and brain-derived neurotrophic factor (BDNF) expression, MAPK/CREB pathway phosphorylation and neural progenitor proliferation in the HIPP whereas a reduction of GluA1/2 AMPA subunit receptor ratio was found in the striatum of male CD1 mice that underwent cocaine SA (Luján et al., 2018). Also, it is relevant to point out that the effects of CBD on hippocampal neurogenesis plays a pivotal role in the reduction of cocaine SA (Luján et al., 2019). Recently, attenuating effects of CBD on the motivational properties of cocaine were also revealed by Galaj et al.. In this study CBD inhibited cocaine SA maintained by low, but not high, doses of cocaine, and dose-dependently lowered cocaine-enhanced brain-stimulation reward. Importantly, these effects were abolished by the blockade of CB2R, 5HT1a and TRPV1 suggesting their functional implication. Furthermore, *in vivo* microdialysis revealed a CBD-mediated reduction of cocaine-induced increases in extracellular dopamine in the NAcc (Galaj et al., 2020).

In addition to these previous findings, it was also explored whether CBD could be effective to prevent relapse. Gonzalez-Cuevas et al. revealed that the transdermal administration of CBD attenuated context-induced and stress-induced drug-seeking in an intravenous cocaine SA paradigm. Interestingly, CBD-mediated anti-relapsing effects were maintained up to 5 months after the end of the treatment although plasma and brain CBD levels were undetectable at this time (Gonzalez-Cuevas et al., 2018). Furthermore, the effects of CBD on cocaine plus caffeine-induced locomotor sensitization were investigated. Repeated treatment with CBD (20 mg/kg, i.p.) blunted the motor behavioral response induced by a challenge dose of cocaine plus caffeine (Prieto et al., 2020).

Another crucial aspect in the cocaine use disorder is the successful management of cocaine-induced withdrawal syndrome to maintain the abstinence and to prevent relapse.

Recently, our group evaluated the role of CBD to regulate behavioral and neurobiological alterations induced by cocaine in a new animal model of spontaneous withdrawal. The results of this study revealed that CBD (10, 20, and 40 mg/kg, i.p.) normalized motor and somatic signs disturbances and completely regulated anxiety-like behaviors induced by spontaneous cocaine withdrawal (progressive increasing doses of cocaine for 12 days, 15–60 mg/kg/day, i.p.). Furthermore, the administration of CBD blocked the increase of dopamine transporter (DAT) and TH gene expressions in the VTA of mice exposed to the cocaine withdrawal (Gasparyan et al., 2020).

On the contrary to the positive findings supporting the therapeutic potential of CBD in the regulation of the reinforcing and motivational actions of cocaine, one study found that CBD (5 mg/kg, i.p.) did not modify the reward-facilitating effect of cocaine in the ICSS paradigm (Katsidoni et al., 2013). Also, another publication showed that CBD (5 and 10 mg/kg, i.p.) did not attenuate the motivation to self-administer cocaine (breaking point) nor the cue-induced cocaine seeking in rats after a withdrawal period (Mahmud et al., 2017). These apparently contradictory results could be related, at least in part, with differences in the experimental design or in the administered doses of cocaine and CBD. However, the available information suggests that CBD could be a useful tool for the treatment of cocaine use disorder although additional studies are warranted.

Finally, a double-blind, randomized and placebo-controlled clinical trial was carried out in 79 patients with cocaine use disorder. The main goal was to evaluate the effects of CBD (400 or 800 mg/day) on cocaine-cue induced craving and the number of days to relapse. Although the results have not yet been published, the performance of this study points out the interest of the therapeutic potential of CBD for cocaine use disorder (NCT02559167).

CBD and Nicotine

Tobacco use is the cause of over 8 million deaths per year globally, resulting one of the biggest public health threats worldwide (World Health Organization (WHO), 2020). Nicotine is the main addictive substance responsible for cigarette smoking and withdrawal symptoms occurring upon smoking cessation. Nowadays, nicotine replacement therapy together with varenicline, a nicotinic receptor partial agonist, is the most effective smoking cessation drug. However, a significant proportion or smokers still fail to maintain long-term abstinence. Here we reviewed the scarce but recent results pointing out CBD as a candidate to be considered for modulating nicotine-induced reinforcing and withdrawal symptoms.

The first pilot clinical study evaluated the effects of CBD in smokers trying to achieve cessation. Inhaled CBD (400 μg/inhalation) was effective to reduce the number of cigarettes smoked after one week of treatment. Nevertheless, CBD treatment did not attenuate nicotine craving and showed only a slight, non-significant reduction in anxiety after the 7 days treatment (Morgan et al., 2013). A few years later, the administration of a single dose of CBD (800 mg) in non-treatment seeking, dependent, cigarette smokers after overnight abstinence did not improve verbal or spatial working memory, or impulsivity (Hindocha et al., 2018). However, the same group demonstrated that CBD (single 800 mg dose) reduced attentional bias after a period of tobacco abstinence without improving craving or withdrawal (Hindocha et al., 2018). Recently, a preclinical study was conducted to analyze the effects of CBD (10 and 30 mg/kg) in mice exposed to an animal model of pharmacologically precipitated nicotine withdrawal. Interestingly, CBD abolishes memory impairment and microglial reactivity induced by nicotine withdrawal (Saravia et al., 2019).

In summary, although the information on this issue is very limited, it appears that CBD may result an interesting therapeutic alternative for tobacco dishabituation (**Table 5**). However, further studies should be conducted to improve our knowledge of its usefulness and to increase our understanding of the possible mechanisms involved.

NEUROBIOLOGICAL MECHANISMS INVOLVED IN CBD-MEDIATED REGULATION OF ADDICTION

This section is aimed to analyze in an integrated way the mechanisms that could be underlying the "anti-addictive" actions of CBD. For that purpose, the most representative functional brain systems have been selected to dissect which targets and regulatory mechanisms may be modulated by CBD.

CBD and Dopaminergic System

The scientific community has long accepted the dopaminergic theory of addiction. The hedonic effects of different drugs of abuse are mediated mainly, at least initially, by the release of DA in the mesocorticolimbic system that comprises dopaminergic neurons projecting from the VTA to the NAcc. Released DA in the NAcc acts on high affinity D2 receptors and determines drug rewarding effects (Trifilieff et al., 2013). Also, DA stimulates the low-affinity D1 receptors associated with the consolidation of recent memory engrams (Wise, 2004). However, increased DA levels are not always present after the exposure to a drug of abuse since addiction encompasses a complex functional regulation including the interaction between different neurotransmission systems (Nutt et al., 2015). Despite this, the dopaminergic system plays a central role in addictive disorders.

Little is known about the effects of CBD on the mesolimbic system. One of the first reports revealed that systemically administered CBD had neither excitatory nor inhibitory effects on spontaneously recorded VTA dopaminergic neuronal activity levels (French et al., 1997). In accordance with this finding, systemic injections of CBD alone (10 and 20 mg/kg, i.p.) failed to significantly alter extracellular DA level in the NAcc (Galaj et al., 2020). However, intra-hypothalamic administration of CBD was reported to increase the release of dopamine extracellular levels collected from the NAcc (Murillo-Rodríguez et al., 2011). Due to the antipsychotic actions of CBD, along with the absence of extrapyramidal effects, numerous studies have been conducted to investigate the

TABLE 5 | Main findings from clinical and animal studies aimed to evaluate the therapeutic potential of CBD for the treatment of tobacco use disorder.

CBD and nicotine					
Treatment	**Doses, route of administration, and treatment duration**	**Study design/model**	**Subjects, samples, and gender**	**Main outcomes**	**References**
Clinical studies					
CBD	400 µg/inhalation solution erosol, inh. 7 days	Double-blind placebo-controlled trial	Smokers *N* = 24 (12 M and 12 F)	↓ number of cigarettes smoked	Morgan et al. (2013)
CBD	800 mg, p.o. Acute treatment	Double-blind placebo-controlled trial	Non-treatment seeking dependent smokers *N* = 30 (15 M and 15 F)	= Verbal or spatial working memory = withdrawal-induced impulsivity	Hindocha et al. (2018)
CBD	800 mg, p.o. Acute treatment	Double-blind placebo-controlled trial	Non-treatment seeking dependent smokers *N* = 30 (16 M and 14 F)	↓ attentional bias ↓ pleasantness of cigarette images = Tobacco craving = Withdrawal symptoms	Hindocha et al. (2018)
Animal studies					
CBD	3, 10 and 30 mg/kg, s.c. Repeated treatment	Precipitated nicotine withdrawal	C57BL/6J mice (M)	↑ NOR discrimination index during nicotine withdrawal	Saravia et al. (2019)

CBD, cannabidiol; NOR, novel object recognition; M, male; F, female; inh., inhaled; p.o., per os (oral administration); s.c., subcutaneous injection; ↑, increase; ↓: decrease, = ; no effect.

interaction between CBD and the mesolimbic dopaminergic system employing animal models of schizophrenia. It has been proposed that CBD could act as a partial agonist of D2 receptors (Seeman, 2016) and normalize D3 receptor gene expression in several brain regions (PFC, HIPP, and NAcc) (Stark et al., 2020).

Considering the "anti-addictive" properties of CBD, previously mentioned in this review, it is important to determine how CBD modulates drug-induced alterations in the mesolimbic dopaminergic system. One of the first evidence was published by Renard et al., in an animal model of amphetamine-induced locomotor sensitization. They demonstrated that direct administration of CBD into the shell region of the NAcc completely abolished VTA dopaminergic neuronal activity sensitization induced by amphetamine (Renard et al., 2016). Interestingly, *in vivo* microdialysis studies revealed that systemic administration of CBD (10 and 20 mg/kg, i.p.) dose-dependently attenuated cocaine-induced DA release in the NAcc (Galaj et al., 2020). This effect could be explained by the hypothetical modulation of DA synthesis in the VTA. Indeed, our group has extensively explored the effects of CBD on drug-induced gene expression changes of TH, the rate limiting enzyme for dopamine synthesis in the VTA. In different animal models of ethanol consumption (voluntary consumption, SA, and binge-drinking) the administration of CBD significantly reduced ethanol rewarding and the motivational actions that were associated with a reduction in the gene expression of TH in the VTA (Viudez-Martínez et al., 2018a; Viudez-Martinez et al., 2018b; Viudez-Martínez et al., 2020). Similarly, CBD significantly decreases TH in mice exposed to spontaneous cocaine withdrawal (Gasparyan et al., 2020). Nevertheless, in an animal model of spontaneous cannabinoid withdrawal CBD enhanced TH gene expression in the VTA (Navarrete et al., 2018). These apparent discrepancies could be explained by two main facts. First, DA synthesis and release vary throughout the different phases of the addictive process, depending on whether consumption or withdrawal stages are present. Second, DA release in the NAcc depends on the mechanism of action of each drug of abuse. Accordingly, cannabis, unlike alcohol and psychostimulants would present a minimal effect that could account for these opposite regulations (Nutt et al., 2015).

Thus, available evidence suggests that CBD may functionally regulate the activity of the mesolimbic DA system and counteract the effects of dysregulated dopaminergic transmission induced by drugs such as amphetamine, cocaine, alcohol, or cannabis. These findings could be related, at least in part, to the reduction of the reinforcing and motivational effects of these drugs, as well as to the regulation of the withdrawal syndrome. Nevertheless, more studies are needed to precisely explore CBD-mediated regulation of dopaminergic mechanisms involved in drug addiction.

CBD and Opioidergic System

The endogenous opioid system is closely involved in the regulation of addictive behaviors. Opioid peptides do not directly affect dopaminergic neurons function in the VTA but inhibit gamma-aminobutyric acid (GABAergic) interneurons that innervate VTA dopaminergic neurons in the mesolimbic system (Johnson and North, 1992). The activation of MOR in the VTA through its endogenous ligands, β-endorphin and enkephalin, disinhibits the inhibition produced by the GABAergic interneurons and increases DA release in the NAcc whereas the selective blockade of these receptors significantly decreases basal DA release (Spanagel et al., 1992). Some drugs of abuse (e.g., alcohol, cannabis) stimulate the release of endogenous opioids leading to a MOR-mediated increase of DA release in the NAcc (Tanda and Di Chiara, 1998).

The interaction between CBD and opioidergic system components has been barely explored. A few studies evaluated changes in the main targets of the opioidergic system after CBD administration. The first reference was published in 2006 by Kathmann et al. They described the CBD-mediated allosteric

modulation of mu- and delta-opioid receptor by means of kinetic binding studies with ^{3}H-DAMGO (D-Ala^{2}, NMePhe^{4}, Gly-ol) in the cerebral cortex membrane of male Wistar rats. These effects only occur at very high concentrations and cannot be expected to contribute to the *in vivo* action (Kathmann et al., 2006). Recently, our group analyzed MOR gene expression changes after CBD administration in animal models of alcohol addiction. Interestingly, CBD-induced reduction of voluntary ethanol consumption, ethanol SA and binge-drinking was associated with a down-regulation of MOR in the NAcc (Viudez-Martínez et al., 2018a; Viudez-Martinez et al., 2018b; Viudez-Martínez et al., 2020). Similarly, the administration of CBD normalized increased MOR gene expression in the NAcc in mice exposed to an animal model of spontaneous cannabinoid withdrawal (Navarrete et al., 2018). Therefore, independently of the experimental paradigm employed, the phase of addiction assessed and the drug, the effect of CBD was in the same direction. Thus, it is possible to speculate that CBD negatively modulates MOR; however, more studies should be carried out to further explore the specific interaction between CBD and MOR receptors, as well as with other components of the opioidergic system.

In summary, available evidence suggests that CBD-induced modulation of drug reinforcing and motivational properties could be mediated, at least in part, by the functional regulation of the opioidergic system. However, it remains to elucidate the precise mechanisms involved.

CBD and Endogenous Cannabinoid System

ECS is a ubiquitous lipid signaling system distributed throughout the organism that participates in multiple intracellular signaling pathways (Piomelli, 2003; Zou and Kumar, 2018). ECS regulates several physiological functions and mediates the crosstalk between different neurotransmitter systems, therefore, representing a key player in the control of behavioral responses (Katona and Freund, 2012; Atkinson and Abbott, 2018). CB1R and CB2R, endogenous ligands or endocannabinoids (AEA and 2-arachidonoylglycerol (2-AG)), and their synthesizing (N-acylphosphatidylethanolamine specific phospholipase D (NAPE-PLD) and diacylglycerol lipases (DAGL-α and DAGL-β)) and degrading (FAAH and monoacylglycerol lipase (MAGL)) enzymes are the main components of the ECS, present in the central and peripheral nervous system (Mackie, 2005; Katona and Freund, 2012). As recently and extensively reviewed by our group and other authors, ECS is critically involved in the neurobiological substrate underlying drug addiction. Importantly, the functional localization of cannabinoid receptors in the mesocorticolimbic circuit participating in the modulation of the synthesis and release of dopamine is widely accepted (Maldonado et al., 2006; Parsons and Hurd, 2015; Sloan et al., 2017; Trigo and Le Foll, 2017; Manzanares et al., 2018).

Numerous studies were carried out to elucidate the interactive mechanisms between CBD and ECS components. One of the mechanisms is the inhibition of AEA hydrolysis and reuptake by blocking its catabolic enzyme (FAAH) and the corresponding membrane transporter, respectively (Bisogno et al., 2001; Laprairie et al., 2015). Regarding the interaction with CB1R, CBD was first thought to be an antagonist (Thomas et al., 2007; Pertwee, 2008), but recent results suggested that CBD could act also as a non-competitive negative allosteric modulator of CB1R (Laprairie et al., 2015; Tham et al., 2019). Interestingly, a statistical meta-analysis of all present information describing direct effects of CBD at cannabinoid receptors concluded that there is no direct CBD–CB1R interaction that may account for the reported changes in endocannabinoid signaling (McPartland et al., 2015).

There is also controversy about the pharmacological effect of CBD on CB2R. It was proposed that CBD could act as a partial agonist (Tham et al., 2019), inverse agonist or even as an antagonist (Thomas et al., 2007). A recent report suggested that CBD might act as an allosteric modulator (Martinez-Pinilla et al., 2017). Finally, CBD presents recognized antagonistic properties on GPR55 receptor (Ryberg et al., 2007; Sharir and Abood, 2010; Ibeas Bih et al., 2015).

The findings published by our group demonstrated that CBD down-regulates the gene expression of CB1R and GPR55 whereas up-regulates CB2R in the NAcc of C57BL/6J mice exposed to models of cannabinoid withdrawal (Navarrete et al., 2018) and alcohol addiction (Viudez-Martínez et al., 2018a; Viudez-Martínez et al., 2020). These effects may be related, at least in part, with CBD-mediated improvement of withdrawal symptoms and the reduction of alcohol consumption, motivation, and relapse. Similarly, Ren et al. showed a reduction of CB1R gene and protein levels in the NAcc core and shell subregions of rats exposed to a cue-induced heroin seeking procedure. Interestingly, these authors suggested that the effects of CBD on CB1R expression would present a mesolimbic specificity (Ren et al., 2009). Furthermore, CBD increased CB1R protein expression in the HIPP of mice exposed to a cocaine SA paradigm (Luján et al., 2018). On the other hand, the antagonism of CB2R by the administration of AM630 completely blocked the reduction of cocaine SA by CBD, suggesting its critical involvement in CBD-mediated effects (Galaj et al., 2020).

Taken together, it is possible to argue that ECS components play a pivotal role in the actions of CBD on withdrawal-related, reinforcement, motivation or relapse induced by alcohol, cocaine, or heroin. Thus, a greater effort is essential to further characterize the mechanisms involving the ECS that underlies potential therapeutic effects of CBD in drug addiction.

CBD and Serotonergic System

The serotonergic system has a pivotal role in the modulation of motivational and reinforcement processes and is involved in the regulation of the rewarding effects of certain drugs of abuse. Mesolimbic dopaminergic neurons are critically regulated by serotonergic projections from the medial and dorsal raphe nuclei entailing an inhibitory control (Di Giovanni et al., 2010; Müller and Homberg, 2015). There are a high number of serotonergic receptors subtypes with different functional profiles, suggesting the complexity of serotonin-mediated regulation of drug reward. Among these, 5HT1a receptors

stand out due to the large number of reports supporting its crucial role in drug addiction (Pinto et al., 2002; Risinger and Boyce, 2002; Müller et al., 2007; Kelaï et al., 2008; You et al., 2016). Importantly, CBD is known to act as a positive allosteric modulator at 5HT1a receptors (Russo et al., 2005; Campos and Guimarães, 2008) and this mechanism is closely involved in its anxiolytic and antidepressant actions (Fogaça et al., 2014; Linge et al., 2016; Sartim et al., 2016). Furthermore, the modulation of dopamine release in the NAcc by CBD was described and it appears to occur through a mechanism involving the activation of 5HT1a receptors (Norris et al., 2016).

To investigate the role of 5HT1a receptors in the CBD-mediated regulation of drug reward, our group analyzed the gene expression in the dorsal raphe (DR) of C57BL/6J mice that underwent an ethanol SA paradigm. Interestingly, the reduction in ethanol consumption and motivation induced by CBD was accompanied by a reduction of 5HT1a gene expression (Viudez-Martínez et al., 2018b). Pharmacological approaches employing the 5HT1a antagonist WAY-100635 confirmed the involvement of this receptor in the effects of CBD on drug-induced reward. First, intra-dorsal raphe injection of WAY-100635 abolished the CBD-mediated inhibition of the reward-facilitating effect of morphine measured in the ICSS paradigm (Katsidoni et al., 2013). Second, the administration of the selective 5HT1a antagonist completely blocked CBD plus naltrexone effects on ethanol SA (Viudez-Martínez et al., 2018b). Third, blockade of 5HT1a receptors attenuated CBD-mediated reduction of cocaine SA (Galaj et al., 2020). Therefore, all these results suggest that the effects of CBD on drug reward and motivation are mediated, at least in part, by 5HT1a receptors. It is tempting to hypothesize that the activation of these receptors by CBD in brain areas of the mesocorticolimbic circuit may play a critical role. A great effort is necessary to further elucidate and understand the interaction of CBD with the serotonergic system and its involvement in drug addiction.

CBD and Glutamatergic System

Glutamate (Glu) is the main excitatory neurotransmitter of the central nervous system. Glutamatergic synaptic plasticity in the mesocorticolimbic dopaminergic circuit is a key neuronal process in appetitive learning and significantly contributes to the development and maintenance of drug addiction (Yamaguchi et al., 2011; van Huijstee and Mansvelder, 2014). Drugs of abuse trigger critical adaptive changes in the reward system by inducing widespread modifications of glutamatergic synapses. The NAcc receives glutamatergic projections from the VTA (Yamaguchi et al., 2011) and other regions involved in the addictive process such as PFC, amygdala, and HIPP (Koob and Volkow, 2010; Floresco, 2015; Heinsbroek et al., 2020). The acquisition of drug reward associations depends on the convergence of dopaminergic and glutamatergic signaling in the NAcc (Neuhofer et al., 2019). Thus, glutamatergic neurotransmission plays an important role in the functional regulation of relevant brain structures involved in the neurocircuitry of drug addiction.

Few studies evaluated the effects of CBD on the different components of glutamatergic signaling in drug reward in animal models of addiction. The administration of CBD inhibited cue-induced heroin seeking in an intravenous SA paradigm and increased AMPA GluR1 protein levels in the NAcc core and shell subregions, achieving a normalization effect. However, mGluR5 protein levels were not modified by CBD (Ren et al., 2009). Also, CBD significantly reduced AMPA GluR1/2 protein levels in the striatum of mice self-administering cocaine (Luján et al., 2018). Finally, in an animal model of cocaine-induced intoxication, the administration of CBD reduced cocaine-induced seizures and this effect was associated with the activation of the mTor pathway with a subsequent significant reduction on Glu release in hippocampal synaptosomes (Gobira et al., 2015).

Therefore, although the available information is very limited, it is reasonable to suggest that CBD-mediated regulation of glutamatergic neurotransmission plays a crucial role in the modulation not only of drug reward but also of drug-induced neuroadaptive changes. However, more studies are needed to confirm this notion and to explore the effects of CBD in other targets of the glutamatergic system.

CBD and Hippocampal Neurogenesis

In recent years, the major role of hippocampal neurogenesis in the addictive process has become increasingly established (Mandyam and Koob, 2012; Chambers, 2013; Deroche-Gamonet et al., 2019). A number of reports suggests that psychoactive substances with addictive potential modify neurogenesis in the adult HIPP (Castilla-Ortega et al., 2016). The subventricular zone (SVZ) and the subgranular layer of the hippocampal dentate gyrus (DG) are the brain regions where adult neurogenesis occurs. Drugs such as psychomotor stimulants, opioids or alcohol significantly impair several aspects of adult neurogenesis including the rate of progenitor proliferation, the survival of newly generated cells and the maturation and acquisition of cellular phenotype (García-Fuster et al., 2010; Taffe et al., 2010). These alterations may affect several drug-related psychological processes such as learning, memory and mood regulation (Canales, 2007).

One of the first reports showing the pro-neurogenic effect of CBD was published by Wolf et al.. The treatment with CBD (6 weeks) enhanced adult neurogenesis; however, this effect was not present in mice lacking CB1R suggesting the critical role of these receptors in the CBD-mediated actions on hippocampal neurogenesis (Wolf et al., 2010). The anxiolytic actions of CBD in mice exposed to chronic unpredictable stress were closely associated with the pro-neurogenic effect of CBD. The authors suggested that this phenomenon depends on the facilitation of the endocannabinoid-mediated signaling and subsequent cannabinoid receptors activation (Campos et al., 2013; Fogaça et al., 2018). Likewise, repeated administration of CBD at low doses (3 mg/kg, i.p.) increased cell proliferation and neurogenesis in the DG and SVZ (Schiavon et al., 2016). Interestingly, a recent critical review covers the potential therapeutic implications of the pro-neurogenic effects of CBD for the treatment of distinct psychiatric disorders, including drug addiction (Lujan and Valverde, 2020).

Recent advances focused on the study of the molecular basis that underlies the neurogenesis promoting actions of CBD in relation with the regulation or drug reward. Lujan et al. described

that CBD increased neural progenitor proliferation in the HIPP of cocaine self-administering animals. They explored the activation of MAPK pathway and its downstream pathways that regulate the expression of the transcriptional (CREB) and neurotrophic (BDNF) factors, responsible for the levels of neuronal hippocampal proliferation. Interestingly, the administration of CBD up-regulated ERK1/2 and CREB phosphorylation, as well as BDNF expression in the HIPP of mice that underwent cocaine SA. Furthermore, the number of BrdU/NeuN stained cells in the HIPP was significantly higher in CBD-treated animals (Luján et al., 2018). To further confirm the involvement of adult hippocampal neurogenesis in the CBD-mediated actions on cocaine reward, Lujan et al. carried out an elegant study administering temozolomide (25 mg/kg/day), a chemotherapy drug that blocks hippocampal neurogenesis. The results clearly demonstrated that in absence of the neurogenesis processes CBD does not modulate cocaine consumption and motivation (Luján et al., 2019). Thus, additional studies are warranted to further explore the therapeutic potential of CBD in addictive disorders regarding its pro-neurogenic as well as neuroprotective properties.

CONCLUDING REMARKS

The present review shows the current state of the art about the potential interest of CBD as a new pharmacological avenue for SUD. According to the findings from preclinical and clinical studies, CBD alone or in combination with commonly employed treatment strategies in drug addiction may configure a potential therapeutic option for improving the dishabituation process of addicted patients.

The great interest in the promising profile of CBD for the management of SUD was revealed by the significant number of clinical studies published or currently underway. One of the most representative examples is CUD for which numerous clinical trials evaluated the effects of CBD, mostly in combination with THC, on withdrawal symptoms, craving, and cannabis use. The information with CBD alone is still insufficient due to the small number of patients in the studies that were carried out to date. Additional clinical trials with more patients and longer treatment periods are warranted to further explore the efficacy and safety of CBD for the treatment of CUD. Interestingly, the results reported by our group in an animal model of spontaneous cannabinoid withdrawal support the implementation of randomized controlled trials (RCT) using only CBD. In addition, variables like motivation, reinforcement, withdrawal, relapse, and retention in treatment should be considered for a global overview during treatment for CUD. Smoking is another SUD in which clinical studies were predominantly conducted to evaluate CBD actions. Nevertheless, more information is required to accurately assess the therapeutic role that CBD could have in smoking cessation. Importantly, one of the main current limitations is the low oral bioavailability of CBD that requires the joint effort to develop new oral formulations to ensure adequate plasma levels and consequently reduce pharmacokinetic variability (Millar et al., 2018; Izgelov et al., 2020; Perucca and Bialer, 2020).

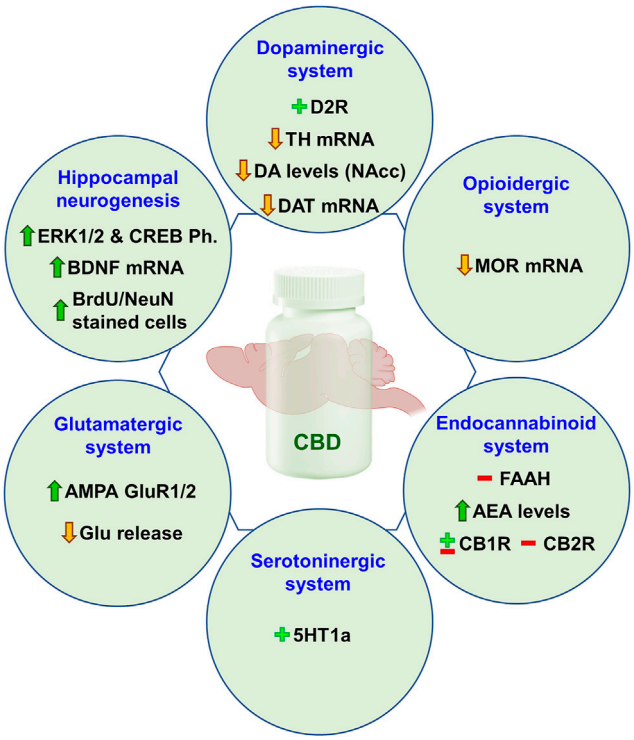

FIGURE 1 | Main preclinical findings regarding the neurobiological mechanisms underlying the "anti-addictive" potential of CBD in relation with dopaminergic, opioidergic, endocannabinoid, serotonergic, and glutamatergic systems, as well as hippocampal neurogenesis. D2r, dopamine receptor 2; TH, tyrosine hydroxylase; DA, dopamine; NAcc, nucleus accumbens; MOR, mu-opioid receptor; FAAH, fatty acid amide hydrolase; AEA, anandamide; CB1R, cannabinoid receptor 1; CB2R, cannabinoid receptor 2; 5HT1a, serotonin receptor 1a; AMPA GluR1/2, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor 1/2; Glu, glutamate; CREB, cAMP response element-binding protein; Ph., phosphorylation; BDNF, brain-derived neurotrophic factor.

On the other hand, the role of CBD in alcohol, opioid and psychostimulant use disorders lies mainly in the studies carried out with different animal models, which in turn motivated the performance of several ongoing clinical trials. The findings included in this review suggest that CBD may reduce the consumption, motivation or relapse of alcohol, opioids (i.e., heroin, morphine) and psychostimulants (amphetamine, methamphetamine, and cocaine), as well as the withdrawal-related signs of morphine and cocaine. The clinical trials recently launched will provide relevant information to know the outcome of the translational approach to patients suffering from these addictive disorders. In addition, it is important to highlight the protective actions derived from CBD treatment not only to attenuate drug-induced damages in the CNS, but also in peripheral tissues such as alcohol-induced liver steatosis or cirrhosis.

A fundamental aspect to optimize the therapeutic potential of CBD in the treatment of SUD is to improve our knowledge about the mechanisms that are involved in its actions. For that reason, the present review dedicates a special section to the interaction between CBD and distinct neurotransmission or functional regulation systems (**Figure 1**). Taking into account all the information that has been collected in this respect, the following ideas can be highlighted: 1) CBD can modulate dopaminergic neurotransmission in the mesolimbic circuit through the direct regulation of dopamine synthesis, release or effects on dopamine receptors, or by indirect mechanisms as the modulation of MOR; 2) the ECS plays a pivotal role in CBD-mediated effects on drug reward, involving the regulation of endocannabinoid signaling through the alteration of AEA levels and CB1R or CB2R function; 3) 5HT1a receptors are critically involved in the effects of CBD on drug addiction; and 4) hippocampal neurogenesis appears to be essential for the regulation of cocaine consumption and motivation by CBD.

In summary, we have ahead of us an exciting race to discover how CBD could contribute to the area of drug addiction from a therapeutic point of view. More preclinical and clinical studies are necessary to further evaluate the role of CBD as a new therapeutic intervention for SUD. In this regard, it is relevant to emphasize that according to the multiple pharmacological profile of CBD accounting for the anxiolytic, antidepressant or antipsychotic properties, comorbid clinical entities such as anxiety, depression or psychotic disorders could be also successfully managed. Importantly, taking into consideration the sex biological differences in terms of brain function and connectivity and its relationship with distinct vulnerability to develop a substance use disorder (Becker et al., 2017), it could be argued that CBD may display differential effects depending on sex (Viudez-Martínez et al., 2020), an aspect that needs to be further explored. The clinical studies that are currently underway will provide relevant information to improve our knowledge about the efficacy and safety of CBD for the treatment of SUD.

AUTHOR CONTRIBUTIONS

FN and JM designed the sections and contents of the review article. FN oversaw the organization to distribute the writing tasks among the authors and participated in article writing. MSSG, AG, and AO perform the literature searches and participated in the article writing. All the authors critically reviewed and approved the final version of the article.

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Cannabidiol and Sertraline Regulate Behavioral and Brain Gene Expression Alterations in an Animal Model of PTSD

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This study evaluated the effects of cannabidiol (CBD) and/or sertraline (STR) on behavioral and gene expression alterations induced by a new chronic animal model of post-traumatic stress disorder (PTSD). C57BL/6J male mice were repeatedly exposed to physical and psychogenic alternate stressful stimuli. Fear-related memory and anxiety-like behaviors were evaluated. The effects of the administration of CBD (20 mg/kg, i.p.) and/or STR (10 mg/kg, p.o.) were analyzed on behavioral and gene expression changes induced by the model of PTSD. Gene expression alterations of targets related with stress regulation, endocannabinoid and serotonergic systems were analyzed by real-time PCR. The results revealed an increased and long-lasting fear-related memory and anxiety-like behaviors in mice exposed to the animal model of PTSD. Treatment with CBD improved these behaviors in PTSD animals, effects that were significantly potentiated when combined with STR. Gene expression analyses revealed a long-term increase of corticotropin releasing factor (*Crf*) that was significantly normalized with the combination CBD plus STR. Cannabinoid receptors (*Cnr1* and *Cnr2*) were up regulated in PTSD mice whereas the serotonin transporter (*Slc6a4*) was reduced. Interestingly, CBD and STR alone or combined induced a significant and marked increase of *Slc6a4* gene expression. These results point out the cooperative action of the combination CBD plus STR to enhance fear extinction and reduce anxiety-like behaviors, normalizing gene expression alterations in this animal model of PTSD and suggesting that the combination of CBD with STR deserves to be further explored for the treatment of patients with PTSD.

Keywords: PTSD, mice model, cannabidiol, sertraline, mRNA

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INTRODUCTION

Post-traumatic stress disorder (PTSD) is a disabling mental condition caused by the exposure to frightening or threatening life events (APA, 2013). Around a 70% of worldwide population experience one or more traumatic events in any moment of their lives, whereas 10–15% develop PTSD. Type, severity and number of traumatic events, associated with individual susceptibility or the stage of life in which the trauma occurs influences the likelihood of developing PTSD (Kessler et al., 2017).

It remains essential to identify new therapeutic targets that may improve PTSD treatment. From a translational point of view, it is crucial to identify animal models to recapitulate PTSD-related clinical

traits by the exposure to different kind of stressors, mainly psychogenic (e.g., predator threat), physic (e.g., electric shock), and psychosocial (e.g., disturbances in housing conditions). However, it is unlikely that a single animal model will reproduce the complexity of the human disorder only mimicking core aspects of human PTSD such as fear dysregulation and increased anxiety-like behavior.

There is a broad range of multi-disciplinary experimental approaches to induce a PTSD-like syndrome (Daskalakis et al., 2013; Singewald and Holmes, 2019; Zhang et al., 2019). However, there is a need of chronic animal models of PTSD to induce intense and long-lasting (several weeks) emotional disturbances. These prolonged alterations will simulate more closely the time course of PTSD-related behavioral and neurochemical changes and, therefore, would permit to study the effects of chronic pharmacological treatments (3–5 weeks).

Currently approved medications for the treatment of PTSD are the selective serotonin reuptake inhibitors (SSRIs) paroxetine and sertraline (STR). These drugs present important limitations regarding the response rate that rarely exceeds 60%, and only 30% corresponds to complete remission (Berger et al., 2009). In addition, the available treatments present relevant side effects that may limit tolerance or even decrease therapeutic adherence (Shin et al., 2014). Therefore, there is an increasing need to develop new pharmacological strategies to improve the complex management of PTSD symptomatology. Interestingly, recent research advances revealed the pivotal role of the endocannabinoid system in the regulation of fear memory and emotional behavior in PTSD (Berardi et al., 2016). In this sense, cannabidiol (CBD) has attracted growing attention due to its lack of abuse potential (Viudez-Martinez et al., 2019), its multimodal mechanism of action (Elsaid and Le Foll, 2019) and especially its effects on the regulation of fear-related memories (Song et al., 2016). Indeed, several animal studies showed that CBD facilitates extinction, decreases retrieval or acquisition, and blocks reconsolidation of contextual fear memory evaluated in a fear conditioning (FC) paradigm (Bitencourt and Takahashi, 2018). Furthermore, human studies also suggested the therapeutic potential of CBD for the treatment of PTSD symptoms related with fear extinction, anxiety and sleep disturbances (Das et al., 2013; Shannon and Opila-Lehman, 2016; Elms et al., 2019). However, no previous studies have evaluated the effects of chronic CBD administration, alone or in combination with STR, on the behavioral and neurochemical impairments produced by an animal model of PTSD.

Therefore, the main goals of this study were: 1) to characterize and validate a long-lasting animal model of PTSD by exposing C57BL/6J adolescent mice to alternating and unpredictable psychogenic (fox urine), physic (electric shock, movement restriction) and psychosocial stressors (wet bedding, tilted cage, food deprivation) during a 5-weeks period, including two intermediate resting weeks to add a pivotal re-exposure factor for modeling PTSD, and 2) to evaluate the effects of repeated administration of CBD, STR, and CBD plus STR combination on behavioral and neurochemical alterations induced by this animal model of PTSD. Fear-related memory and anxiety-like behaviors were evaluated by the FC paradigm, and by the novelty suppressed feeding test (NSFT), light-dark box (LDB) and elevated plus maze (EPM) tests, respectively. In addition, real-time quantitative polymerase chain reaction (qPCR) experiments were carried out to evaluate specific changes in the gene expression of targets involved in stress response [hypothalamus-pituitary-adrenal (HPA) axis] and pharmacological actions of CBD [cannabinoid receptors 1 (CB1r) and 2 (CB2r)], and STR [5-hydroxytryptamine transporter (5HTT)].

MATERIALS AND METHODS

Animals

A total of 94 C57BL/6J male 4-weeks old mice were purchased from Charles River laboratories (Lille, France). Mice, weighed 20–25 g, housed in groups of five per cage (40 × 25 × 22 cm) under controlled environmental conditions (temperature, 23 ± 2°C; relative humidity, 60 ± 10%, and 12 h light/dark cycle, lights on from 08:00 to 20:00 h), in an enriched environment with nesting material and *ad libitum* access to food (Teklad global 18% protein diet, Ref. 2014S, Envigo, Barcelona, Spain), and water except during behavioral evaluation. Experimental procedures were carried out in the animal facilities of Miguel Hernandez University located in San Juan de Alicante (Alicante. Spain). Behavioral evaluation was initiated during the adolescent period of mice (4 weeks old), after one-week acclimatization period to the animal housing room. Experiments were performed during the light cycle (from 16:00 to 18:00 h) placing home cages in the operant-task room 1 h before to start. All experimental procedures complied with the Spanish Royal Decree 53/2013, the Spanish Law 32/2007 and the European Union Directive of the 22nd of September 2010 (2010/63/UE) regulating the care of experimental animals and were approved by the Ethics Committee of Miguel Hernandez University. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath and Lilley, 2015).

Drugs

CBD was obtained from STI Pharmaceuticals (Essex, United Kingdom) and was dissolved in ethanol:cremophor: saline (1:1:18) to obtain the required dose of 20 mg/kg for its intraperitoneal administration (i.p.). STR was purchased from Pfizer laboratories (Madrid, Spain) and was dissolved in water to obtain the required dose of 10 mg/kg for its oral administration (p.o.). CBD and STR were freshly prepared every day immediately before its administration at a final volume of 10 ml/kg. Once-daily administration of CBD, STR, CBD plus STR or the corresponding vehicles (from 15:00 to 17:00 h) was carried out between weeks 11 and 14 of the model. A latency time of 90 (CBD) and/or 60 (STR) minutes was left before any behavioral evaluation according to previously published pharmacokinetics data (Deiana et al., 2012; Melis et al., 2012). Drug doses were selected according to prior literature (Wang et al., 2006; Blessing et al., 2015) and to preliminary results obtained with CBD in our laboratory (data not shown).

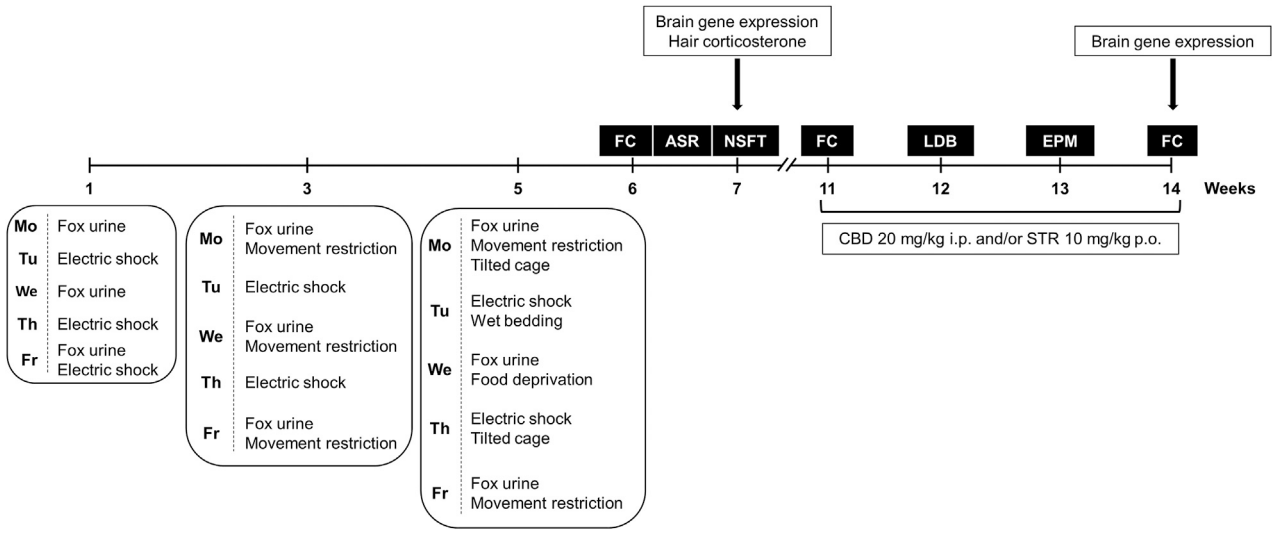

FIGURE 1 | Timeline diagram of the experimental procedure used for the development of the animal model of post-traumatic stress disorder (PTSD) (weeks 1–5) to evaluate PTSD-induced behavioral and neurobiological alterations under basal conditions (weeks 6 and 7), and to analyze the pharmacological actions of cannabidiol (CBD, 20 mg/kg, i.p.) and/or sertraline (STR, 10 mg/kg, p.o.) on long-lasting PTSD disturbances (weeks 11–14). FC: Fear Conditioning, NSFT: Novelty Suppressed Feeding Test, LDB: Light-Dark Box, EPM: Elevated Plus Maze.

Animal Model of Post-Traumatic Stress Disorder

The animal model of PTSD was induced by exposing mice to the following stressful stimuli at different time point for 5 weeks: 1) Fox urine: a perforated plastic tube (50 ml) containing a gauze impregnated in fox urine (Code blue, Fox Urine Cover Scent, Ref. OA1105, 3 ml) or saline (control mice) was placed in the central zone of each cage for 15 min, 2) Unescapable electric shock: animals were placed inside a 50 × 25 × 25 cm acrylic box with a floor consisting of a grid of parallel stainless steel bars (1 mm in diameter and 1 cm apart). Thirty seconds after animals were introduced in the box, they received a 1 mA scrambling shock or not (control mice) during 10 s, with an additional resting time of 20 s, 3) movement restriction: animals were introduced in perforated plastic falcon tubes (50 ml) for 15 min, or were left undisturbed in the home cage (control mice), 4) tilted cage: during dark cycle, home cages were tilted 30° for 12–14 h or not (control mice), 5) wet bedding: during dark cycle, mice were exposed to a cage with wet sawdust bedding for 12–14 h, or were left undisturbed (control mice); and 6) food restriction: during dark cycle, mice were food deprived for 12–14 h, or were left undisturbed (control mice).

Stressful stimuli were applied alternating 3 weeks of exposure (weeks 1, 3, and 5) with two intermediate weeks of resting (weeks 2 and 4), to avoid habituation and to add elements of unpredictability and re-exposure to the stressor. Importantly, the intensity of stress exposure was increased by adding new stressful stimuli from week to week as displayed in the **Figure 1**. Overall, these experimental aspects are especially relevant to induce long-lasting behavioral and neurochemical alterations in an animal model of PTSD.

Experimental Design

Procedure 1: Evaluation of Basal Behavioral and Neurobiological Alterations Induced by the Animal Model of Post-Traumatic Stress Disorder

This experimental phase was intended to evaluate basal behavioral and neurobiological disturbances induced by the animal model of PTSD (**Figure 1**). For that purpose, a total of 16 mice were used in this experiment, eight exposed to the animal model of PTSD and eight non-exposed. Fear-related memory and anxiety-like behavior were evaluated at weeks 6 and 7 by the fear conditioning (FC), the acoustic startle response (ASR), and the novelty-suppressed feeding test (NSFT) paradigms. Immediately after the last behavioral evaluation by NSFT mice were killed by cervical dislocation and brain and hair samples were obtained. Brain samples were used for relative gene expression analyses of targets of interest. Hair samples were used for hair accumulated corticosterone quantification as a peripheral biomarker of long-term HPA axis activity. All the behavioral paradigms of this procedure were made under blind conditions.

Procedure 2: Evaluation of the Effects of Cannabidiol and/or Sertraline Administration on Long-Lasting Behavioral and Gene Expression Alterations Induced by the Animal Model of Post-Traumatic Stress Disorder

This experimental phase evaluates the effects of CBD and/or STR administration on long-lasting behavioral and gene expression alterations induced by the animal model of PTSD (**Figure 1**). A total of 78 mice were used, 39 exposed to the animal model of PTSD and 39 non-exposed. After the model induction period and the subsequent basal behavioral evaluations at weeks 6 (FC) and 7 (NSFT), mice were left undisturbed for 3 weeks. After this period,

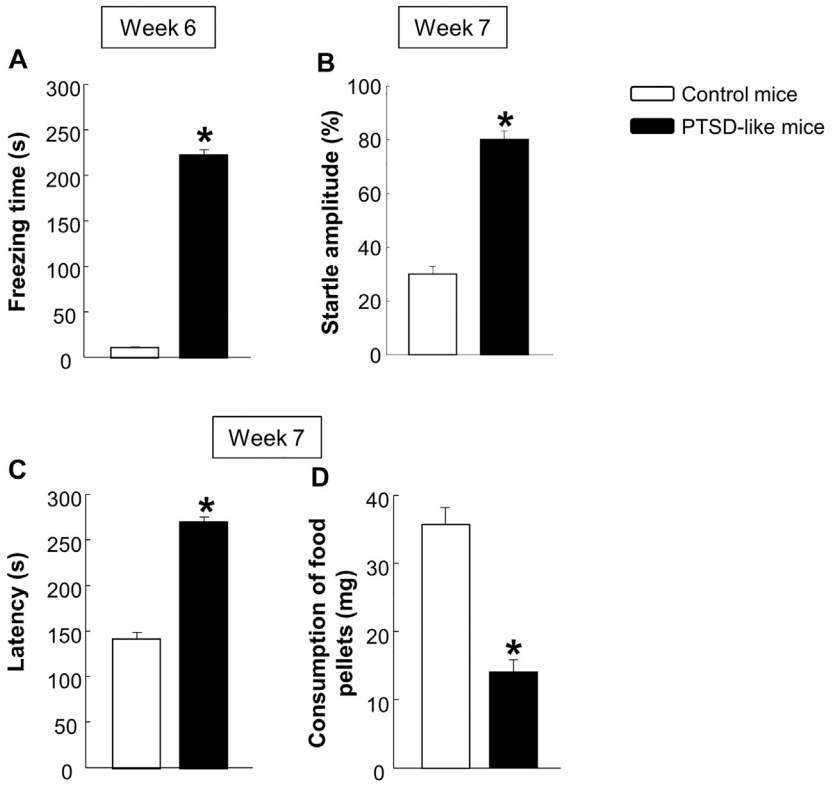

FIGURE 2 | Evaluation of the basal behavioral disturbances induced by the animal model of PTSD at weeks 6 and 7. Analysis of the freezing time (s) by the fear conditioning (FC) paradigm **(A)**, the startle amplitude **(B)** by the acoustic startle response (ASR), and of the latency time **(C)** and food pellets consumption **(D)** by the novelty suppressed feeding test (NSFT). Columns represent the mean and vertical lines ± SEM. *, Values from PTSD-like mice that are significantly different from control mice (Student's *t*-test, $p < 0.001$). Mice exposed to the PTSD-like model: N = 8; control mice: N = 8.

mice were randomly assigned to different treatment groups where CBD and/or STR effects on fear-related memory (weeks 11 and 14) and anxiety-like behavior (weeks 12 and 13) were analyzed. The first administration of CBD and/or STR was carried out 60 and/or 90 min before the FC at week 11, respectively, to evaluate the acute pharmacological effects. Subsequently, both drugs were administered once daily until week 14, evaluating its sub-chronic and chronic effects on different behavioral tests. CBD and/or STR actions on anxiety-like behavior were analyzed by the light-dark box (LDB; week 12) and the elevated plus maze (EPM; week 13) paradigms. At the end of the behavioral evaluation phase, mice were killed by cervical dislocation immediately after the last behavioral test (FC at week 14) and brain samples were removed. These samples were used to analyze relative gene expression of several targets of interest. All the behavioral paradigms of this procedure (FC, NSFT, LDB, and EPM) were made under blind conditions (for more detail see **Supplementary Material**).

Behavioral Analyses

Fear Conditioning Paradigm

Fear memory retention was evaluated using Pavlovian contextual fear conditioning protocol as described elsewhere (LeDoux, 2000). Briefly, in this behavioral paradigm mice were re-exposed to the same cage where they received electric shocks during the induction of the model of PTSD (or not in the case of control animals), without applying any shock in this evaluation phase. For a total of 5 min, freezing behavior was evaluated as the time of total absence of movements except those necessary to breathe.

Acoustic Startle Response

A previously described protocol was used to evaluate acoustic startle response of mice exposed to the animal model of PTSD and controls. Briefly, mice were placed in soundproof chambers equipped with loudspeakers controlled by STARTLE software (Panlab, Barcelona, Spain) (Ortega-Alvaro et al., 2011). Mice movement inside a Plexiglas cylinder was measured by a piezoelectric accelerometer and converted into a digital signal. Mice were acclimatized three days prior to test sessions by placing them each day in the apparatus for 5 min without background noise. The day of the evaluation, mice were exposed to 10 trials of 120 dB (40 ms, 8,000 Hz) acoustic startle stimulus applied every 44 s, recording the maximum of startle amplitude during a 100 ms sampling window.

Novelty Suppressed Feeding Test

This behavioral test measures anxiety-induced hyponeophagia as the inhibition of food ingestion or approach to food in an anxiety-provoking environment (Bodnoff et al., 1988; Garcia-Gutierrez et al., 2010). After 24 h of food deprivation, mice were placed in a

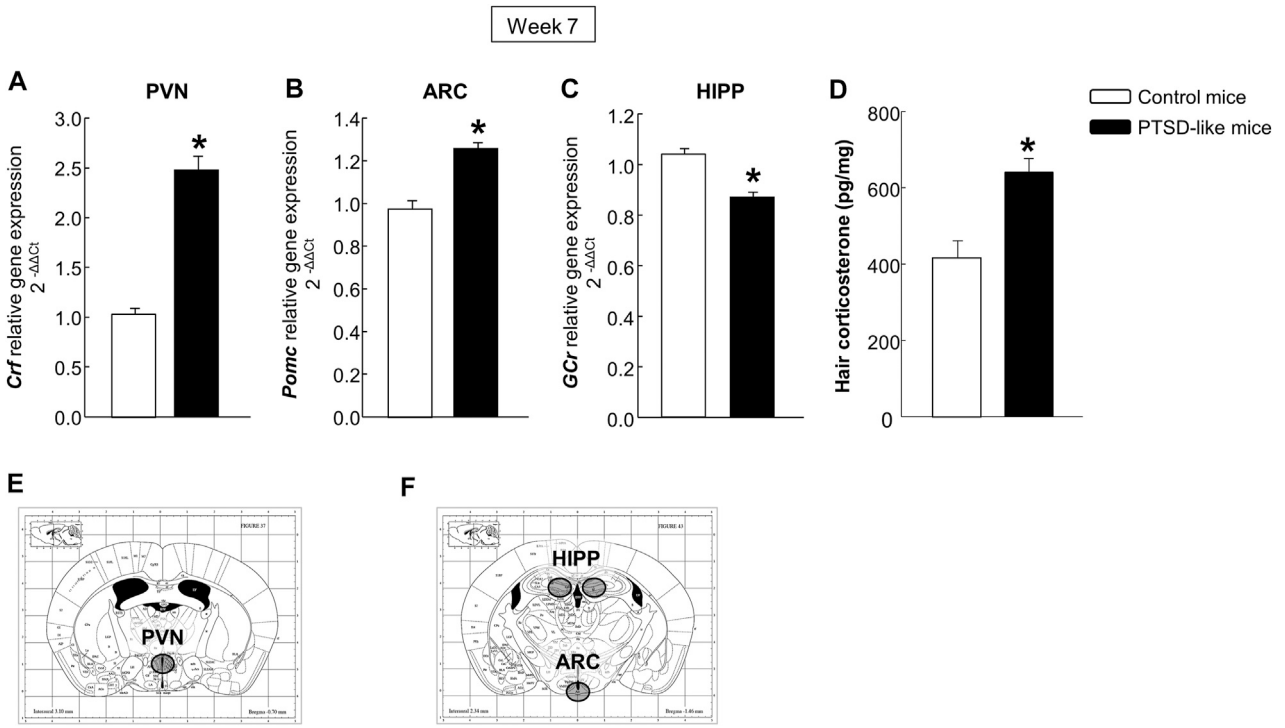

FIGURE 3 | Relative gene expression analyses of corticotropin releasing factor (*Crf*) in the paraventricular nucleus (PVN) **(A)**, proopiomelanocortin (*Pomc*) in the arcuate nucleus (ARC) **(B)**, and glucocorticoid receptor (*GCr*) in the hippocampus (HIPP) **(C)** by real time PCR. Quantification of hair corticosterone (pg/mg) **(D)** by Enzyme-Linked ImmunoSorbent Assay (ELISA). Columns represent the mean and vertical lines ± SEM of $2^{-\Delta\Delta C}$. *, Values from PTSD-like mice that are significantly different from control mice (Student's *t*-test, $p < 0.001$). Mice exposed to the PTSD-like model: N = 8; control mice: N = 8. **(E,F)** Representative images from *Paxinos and Franklin's mouse brain atlas* including the selected coronal sections to microdissect the regions of interest.

transparent square cage (40 × 40 × 50 cm) with a single pellet of food left on a white paper platform in the center of the cage. The latency time before the mouse started to eat was recorded up to a threshold period of 5 min. Once the mice started to eat, the total amount of food pellet consumption was measured during an additional 5-minutes time.

Light-Dark Box

Anxiety-like behavior was evaluated by the widely accepted LDB paradigm (Crawley and Goodwin, 1980; Garcia-Gutierrez et al., 2018). LDB was carried out in an apparatus with two methacrylate compartments (20 × 20 × 15 cm), one transparent and the other black and opaque, separated by an opaque tunnel (4 cm). Light compartment is illuminated with a lamp (60 W) that is placed 25 cm above it. At the beginning of the 5-min session, mice were placed in the light box facing the tunnel. The total time spent in the light box and the number of transitions between boxes were recorded. A mouse whose four paws were inside the new box was considered as having changed boxes.

Elevated Plus Maze

Another commonly used method for evaluating anxiety-like behavior in mice is the EPM (Lister, 1987; Garcia-Gutierrez et al., 2018). The apparatus consists of four arms (two open and two enclosed), that form a plus shape at 50 cm above the floor. The junction of the four arms is a central square platform (5 × 5 cm). At the beginning of the test, mice were placed in the central square, facing one of the enclosed arms. During a period of 5 min, the total time spent in the open arms (calculated as a percentage) and the number of transitions between open and enclosed arms were recorded. Animal arm entry was considered as the entry of its four paws into the arm.

Gene Expression Studies by Real Time PCR

Relative gene expression of corticotropin releasing factor (*Crf*) in the paraventricular nucleus (PVN), proopiomelanocortin (*Pomc*) in the arcuate nucleus (ARC), glucocorticoid receptor (*GCr*) in the hippocampus (HIPP), *Cnr1*, and *Cnr2* in the amygdala (AMY), and *Slc6a4* in the dorsal raphe nucleus (DR) were analyzed on brain samples obtained in Procedure 1 (week 7) and Procedure 2 (week 14). Briefly, mice were killed at the end of the experimental procedures by cervical dislocation and brains were removed from the skull and frozen at −80°C. Brain sections were cut (500 μm) in a cryostat (−10°C) containing the regions of interest according to Paxinos and Franklin atlas (Paxinos and Franklin, 2001), mounted in the slides and stored at −80°C. Sections were microdissected following the method described by Palkovits and previously performed by our group (Palkovits, 1983; Navarrete et al., 2012). Total RNA was extracted from brain micropunches with TRI Reagent extraction reagent (Applied Biosystem, Madrid,

Spain) and reverse transcription was carried out (Applied Biosystem, Madrid, Spain). Quantitative analyses of the relative expression of *Crf* (Mm01293920_s1), *Pomc* (Mm00435874_m1), *GCr* (Mm00433832_m1), *Cnr1* (Mm00432621_s1), *Cnr2* (Mm00438286_m1), and *Slc6a4* (Mm0043939_m1) genes was performed on the StepOne Sequence Detector System (Applied Biosystems, Madrid, Spain). All the reagents used in the study were obtained from Life Technologies, and the manufacturer's protocols were followed. The reference gene used was 18S rRNA (Mm03928990_g1). Data for each target gene were normalized to the endogenous reference gene, and the fold change in target gene expression was determined using the 2-ΔΔCt method (Livak and Schmittgen, 2001).

Hair Corticosterone Analysis

After cervical dislocation at week 7 (Procedure 1), mice hair of the dorsal zone was shaved using an electric razor. Hair samples were stored in 1.5 ml polypropylene tubes at -20°C. Extraction and analysis of corticosterone concentration were performed according to a previously described protocol (Erickson et al., 2017). Briefly, hair samples were washed with methanol (5 ml) twice rotating for 3 min. After methanol decantation, samples were placed on aluminum foil and dried in a protected hood for 3 days. Dried samples were weighed and transferred to 2 ml polypropylene tubes containing stainless steel grinding beads (2.8 mm Stainless Steel Grinding Balls Pre-Filled Tubes, OPS Diagnostics, Lebanon, NJ) that were placed in a bead beater (Mixermill MM300, Miguel Hernandez University, Alicante, Spain) to produce a powder. Powdered hair samples were incubated with 1.5 ml of methanol for 24 h on slow rotation to extract steroids. Tubes were centrifuged and steroid-containing supernatants were dried in a protected hood for 2–3 days to evaporate methanol. Dry extracts were analysed by a commercial competitive enzyme-linked immunosorbent assay (ELISA) kit (EIACOR, Invitrogen, Spain) following manufacturer instructions.

Statistical Analyses

Statistical analyses were performed using Student's *t*-test for comparing two groups, and two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post-hoc test for comparing four groups affected by two variables (treatment with CBD or STR). Differences were considered significant if the probability of error was less than 5%. SigmaPlot 11 software (Systat software Inc., Chicago, IL, United States) was used for all statistical analyses.

RESULTS

Procedure 1: Evaluation of Basal Behavioral and Neurobiological Alterations Induced by the Animal Model of Post-Traumatic Stress Disorder

Behavioral evaluation. Mice exposed to the new animal model of PTSD showed a significant increased freezing time in the FC (**Figure 2A**, Student's *t*-test, $t = -13.738$, $p < 0.001$, 14 d.f.), enhanced startle response in the ASR (**Figure 2B**, Student's *t*-test, $t = -3.002$, $p < 0.01$, 14 d.f.), and increased latency time (**Figure 2B**, Student's *t*-test, $t = -6.824$, $p < 0.001$, 14 d.f.) with decreased food consumption (**Figure 2C**, Student's *t*-test, $t = 2.202$, $p < 0.05$, 14 d.f.) in the NSFT, in comparison with control mice. According to these basal behavioral results, mice were randomly assigned to four experimental groups to be treated with CBD and/or STR or its corresponding vehicle in Procedure 2 (for more detail about mice assignment see **Supplementary Figure S1**).

Gene expression analyses. Statistical analyses indicated increased *Crf* (**Figure 3A**, Student's *t*-test, $t = -9.349$, $p < 0.001$, 14 d.f.) and *Pomc* (**Figure 3B**, Student's *t*-test, $t = -5.565$, $p < 0.001$, 14 d.f.) relative gene expression levels in the PVN and ARC, respectively, and decreased gene expression of *GCr* (**Figure 3C**, Student's *t*-test, $t = 5.734$, $p < 0.001$, 14 d.f.) in the HIPP of PTSD-like mice compared with control mice. These changes were accompanied by an increased corticosterone concentration in mice hair compared with controls (**Figure 3D**, Student's *t*-test, $t = -3.943$, $p < 0.01$, 14 d.f.).

In addition, mice exposed to the animal model of PTSD showed reduced *Cnr1* (**Figure 4A**, Student's *t*-test, $t = 5.647$, $p < 0.001$, 14 d.f.) and increased *Cnr2* (**Figure 4B**, Student's *t*-test, $t = -3.604$, $p = 0.003$, 14 d.f.) gene expression in the AMY, as well as enhanced gene expression of *Slc6a4* (**Figure 4C**, Student's *t*-test, $t = -3.337$, $p = 0.005$, 14 d.f.) in the DR compared with non-exposed mice.

Procedure 2: Evaluation of the Effects of Cannabidiol and/or Sertraline on Long-Lasting Behavioral and Gene Expression Alterations Induced by the Animal Model of Post-Traumatic Stress Disorder

Effects of Cannabidiol and/or Sertraline on Fear-Related Memory and Anxiety-Like Behavior Disturbances Induced by the Animal Model of Post-Traumatic Stress Disorder

Fear conditioning paradigm. Statistical analyses revealed a higher mean freezing time in PTSD-like mice compared with control mice at week 11 (**Figure 5A**, Student's *t*-test, $t = -14.178$, $p < 0.001$, 18 d.f.) and week 14 (**Figure 5D**, Student's *t*-test, $t = -21.269$, $p < 0.001$, 18 d.f.). Within control group, no significant differences were observed between CBD plus STR-treated animals compared to CBD and STR-treated mice at week 11 (**Figure 5B**, Two-way ANOVA, CBD: $F_{(1,37)} = 4.794$, $p < 0.05$; STR: $F_{(1,37)} = 4.712$, $p < 0.05$; CBD x STR: $F_{(1,37)} = 1.140$, $p = 0.293$), and at week 14 (**Figure 5E**, Two-way ANOVA, CBD: $F_{(1,37)} = 0.006$, $p = 0.940$; STR: $F_{(1,37)} = 0.201$, $p = 0.657$; CBD x STR: $F_{(1,37)} = 0.456$, $p = 0.504$). In PTSD-like mice, CBD and STR treatments significantly reduced the freezing time at week 11 (acute treatment), reaching a more pronounced reduction at week 14 (repeated treatment). Interestingly, pharmacological combination of CBD plus STR, compared with CBD or STR alone, achieved a superior effect in the reduction of the freezing

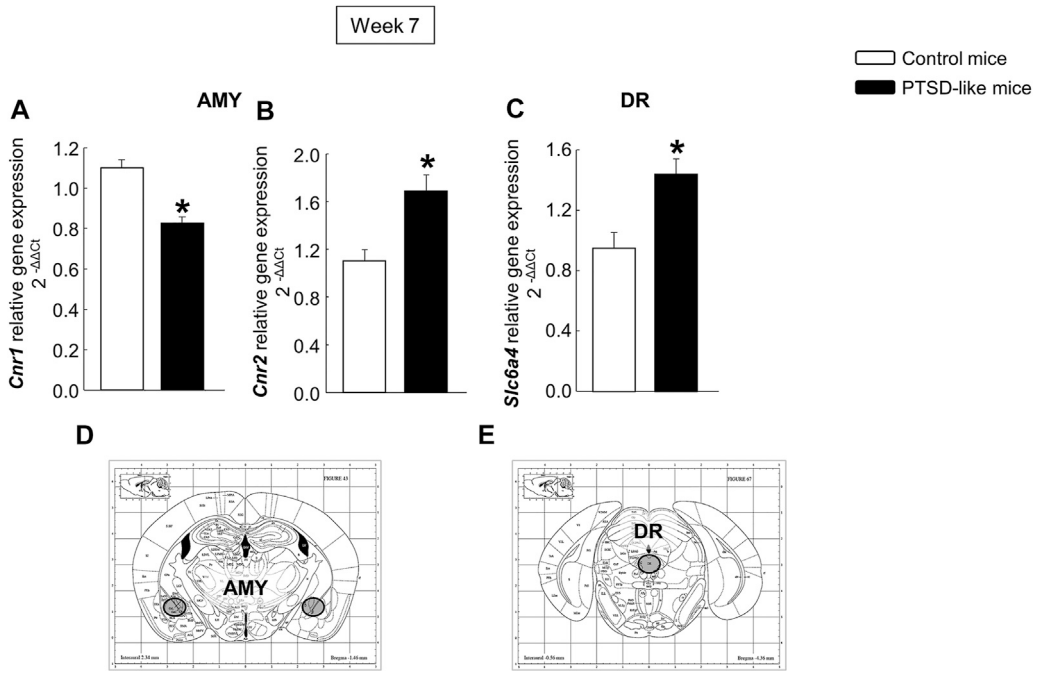

FIGURE 4 | Relative gene expression analyses of cannabinoid receptors 1 (*Cnr1*) **(A)** and 2 (*Cnr2*) **(B)** in the amygdala (AMY), and serotonin transporter (*Slc6a4*) in the dorsal raphe nucleus (DR) **(C)** by real time PCR. Columns represent the mean and vertical lines ± SEM of $2^{-\Delta\Delta C}$. *, Values from PTSD-like mice that are significantly different from control mice (Student's *t*-test, $p < 0.001$). Mice exposed to the PTSD-like model: N = 8; control mice: N = 8. **(D,E)** Representative images from *Paxinos and Franklin's mouse brain atlas* including the selected coronal sections to microdissect the regions of interest.

time in mice exposed to the PTSD model (without reaching statistical significance) at week 11 (**Figure 5C**, Two-way ANOVA, CBD: $F_{(1,38)} = 24.661$, $p < 0.001$; STR: $F_{(1,38)} = 19.226$, $p < 0.001$; CBD x STR: $F_{(1,38)} = 1.488$, $p = 0.231$) and at week 14 (**Figure 5F**, Two-way ANOVA, CBD: $F_{(1,38)} = 76.676$, $p < 0.001$; STR: $F_{(1,38)} = 86.029$, $p < 0.001$; CBD x STR: $F_{(1,38)} = 0.0823$, $p = 0.776$).

Light-dark box. PTSD-like mice spent less time in the lighted box (**Figure 6A**, Student's *t*-test, $t = 4.190$, $p < 0.001$, 18 d.f.) than control mice. Additionally, the number of transitions was reduced in PTSD-like mice compared with control mice (**Figure 6D**, Student's *t*-test, $t = 2.535$, $p < 0.05$, 18 d.f.). Within control mice group, only CBD treatment significantly increased the time spent in the lighted box (**Figure 6B**, Two-way ANOVA, CBD: $F_{(1,37)} = 16.739$, $p < 0.001$; STR: $F_{(1,37)} = 1.400$, $p = 0.245$; CBD x STR: $F_{(1,37)} = 0.508$, $p = 0.481$). Within PTSD-like mice, both CBD and STR treatment increased the time spent in the lighted box. Interestingly, CBD plus STR combination increased the time of permanence in the lighted box compared with CBD or STR alone, without reaching statistical significance (**Figure 6C**, Two-way ANOVA, CBD: $F_{(1,38)} = 16.271$, $p < 0.001$; STR: $F_{(1,38)} = 22.939$, $p < 0.001$; CBD x STR: $F_{(1,38)} = 1.394$, $p = 0.246$). STR treatment increased the number of transitions in both control and PTSD-like mice (**Figure 6E**, Two-way ANOVA, CBD: $F_{(1,37)} = 0.593$, $p = 0.446$; STR: $F_{(1,37)} = 9.272$, $p < 0.01$; CBD x STR: $F_{(1,37)} = 0.212$, $p = 0.648$; and **Figure 6F**, Two-way ANOVA, CBD: $F_{(1,38)} = 0.774$, $p = 0.385$; STR: $F_{(1,38)} = 30.088$, $p < 0.001$; CBD x STR: $F_{(1,38)} = 0.00452$, $p = 0.947$).

Elevated plus maze. PTSD-like mice spent less time in the open arms than control mice (**Figure 7A**, Student's *t*-test, $t = 2.962$, $p < 0.01$, 18 d.f.), and no differences were observed in the number of transitions between opened and closed arms (**Figure 7D**, Student's *t*-test, $t = 0.750$, $p = 0.463$, 18 d.f.). Within control mice no differences were observed in the time spent in open arms (**Figure 6B**, Two-way ANOVA, CBD: $F_{(1,37)} = 1.564$, $p = 0.220$; STR: $F_{(1,37)} = 0.584$, $p = 0.450$; CBD x STR: $F_{(1,37)} = 1.202$, $p = 0.281$). However, within PTSD-like mice, CBD or STR treatment significantly increased the time spent in the open arms, effect that was more pronounced with the CBD plus STR combination without reaching statistical significance (**Figure 7C**, Two-way ANOVA, CBD: $F_{(1,38)} = 41.191$, $p < 0.001$; STR: $F_{(1,38)} = 18.328$, $p < 0.001$; CBD x STR: $F_{(1,38)} = 0.008$, $p = 0.927$). STR treatment increased the number of transitions in control mice (**Figure 7E**, Two-way ANOVA, CBD: $F_{(1,37)} = 0.923$, $p = 0.343$; STR: $F(1,37) = 10.553$, $p < 0.01$; CBD x STR: $F_{(1,37)} = 0.0317$, $p = 0.860$) and in PTSD-like mice (**Figure 7F**, Two-way ANOVA, CBD: $F_{(1,38)} = 0.000317$, $p = 0.986$; STR: $F_{(1,38)} = 4.869$, $p < 0.05$; CBD x STR: $F_{(1,38)} = 0.0203$, $p = 0.888$).

Effects of Cannabidiol and/or Sertraline on Relative Gene Expression Alterations Induced by the Animal Model of Post-Traumatic Stress Disorder

HPA axis. Crf gene expression increased in the PVN of PTSD-like mice compared with control mice (**Figure 8A**, Student's *t*-test, $t = -3.459$, $p < 0.01$, 18 d.f.). Within the control group, STR treatment induced an upregulation in *Crf* gene expression

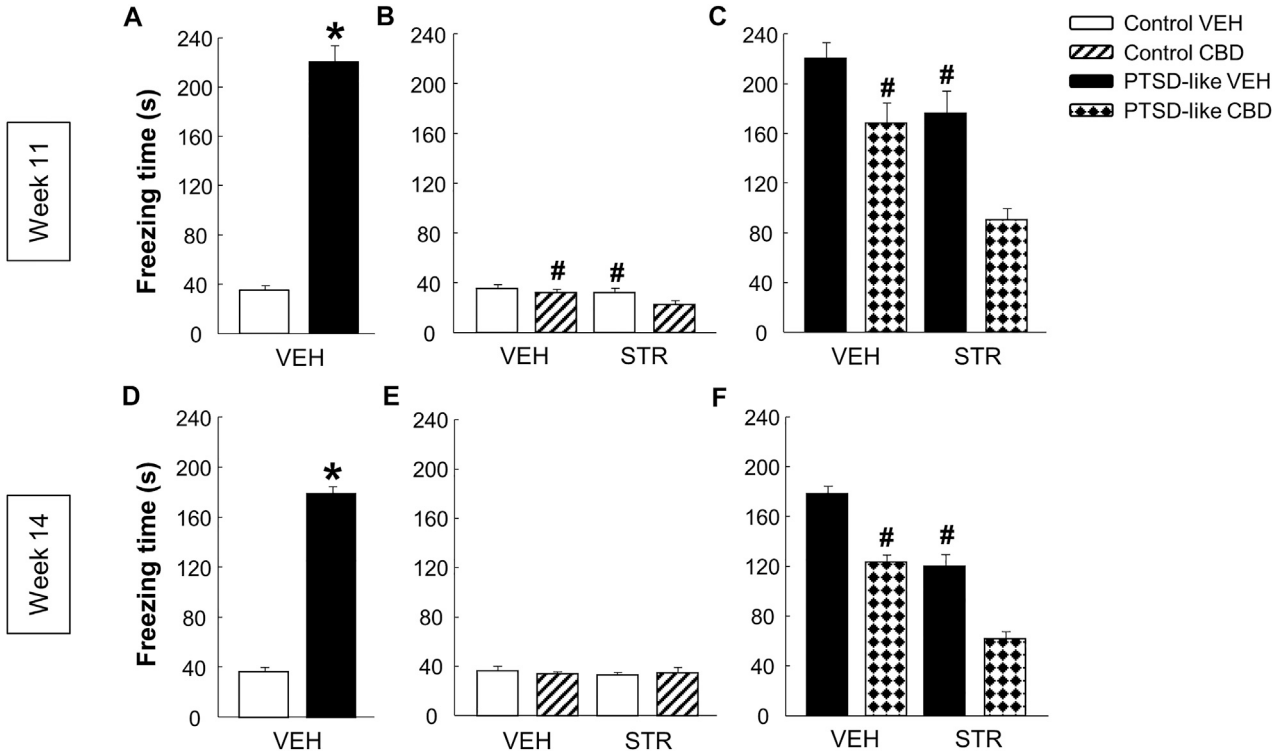

FIGURE 5 | Evaluation of fear-related memory at weeks 11 and 14. Analysis of the freezing time (s) by the fear conditioning paradigm between control and PTSD-like VEH-treated mice at week 11 **(A)** and week 14 **(D)**. Effects of acute (week 11) and chronic (week 14) administration of CBD (20 mg/kg, i.p.) and/or STR (10 mg/kg, p.o.), or its corresponding VEH, on the freezing time (s) of control mice **(B, E)** and PTSD-like mice **(C,F)**. Columns represent the means and vertical lines ± SEM. *, Values from PTSD-like VEH-treated mice that are significantly different from control VEH-treated mice (Student's *t*-test, $p < 0.001$). #, Values from CBD- or STR-treated groups that are significantly different from VEH-treated mice (Two-way ANOVA, $p < 0.05$). and, Values from CBD plus STR-treated mice that significantly different from CBD- and STR-treated control (Two-way ANOVA, $p < 0.05$) and PTSD-like mice (Two-way ANOVA, $p < 0.001$). Mice exposed to the PTSD-like model: N(VEH) = 10, N(CBD) = 10, N(STR) = 9, N(CBD plus STR) = 10; control mice: N(VEH) = 10, N(CBD) = 9, N(STR) = 9, N(CBD plus STR) = 10.

(**Figure 8B**, Two-way ANOVA; CBD: $F_{(1,37)} = 0.861$, $p = 0.360$; STR: $F_{(1,37)} = 6.702$, $p < 0.05$; CBD x STR: $F_{(1,37)} = 0.0597$, $p = 0.808$). In the PTSD-like mice group, Two-way ANOVA revealed that STR reduced the gene expression of *Crf*, achieving a more pronounced reduction when combined with CBD (**Figure 8C**, Two-way ANOVA, CBD: $F_{(1,38)} = 0.733$, $p = 0.398$; STR: $F_{(1,38)} = 8.885$, $p < 0.01$; CBD x STR: $F_{(1,38)} = 4.246$, $p < 0.05$). In addition, PTSD-exposed mice also showed decreased gene expression of *Pomc* in the ARC compared with control mice (**Figure 8D**, Student's *t*-test, $t = 3.416$, $p < 0.01$, 18 d.f.), but no differences were observed in both control (**Figure 8E**, Two-way ANOVA, CBD: $F_{(1,38)} = 0.561$, $p = 0.459$; STR: $F_{(1,38)} = 0.158$, $p = 0.693$; CBD x STR: $F_{(1,38)} = 2.859$, $p = 0.100$) and PTSD-like mice (**Figure 8F**, Two-way ANOVA; CBD: $F_{(1,37)} = 0.0340$, $p = 0.855$; STR: $F_{(1,37)} = 0.233$, $p = 0.632$; CBD x STR: $F_{(1,37)} = 0.0370$, $p = 0.849$) after CBD and/or STR administration. Finally, *GCr* gene expression in the HIPP increased in PTSD-like mice compared with controls (**Figure 8G**, Student's *t*-test, $t = -2.359$, $p < 0.05$, 18 d.f.) and no differences were observed among the four groups of control treated mice (**Figure 8H**, Two-way ANOVA, CBD: $F_{(1,37)} = 1.621$, $p = 0.212$; STR: $F_{(1,37)} = 0.00139$, $p = 0.970$; CBD x STR: $F_{(1,37)} = 0.0626$, $p = 0.804$). Within PTSD-like group, CBD decreased the *GCr* gene expression and STR increased it (**Figure 8I**, Two-way ANOVA, CBD: $F_{(1,38)} = 6.94$, $p < 0.05$; STR: $F_{(1,38)} = 6.022$, $p < 0.05$; CBD x STR: $F_{(1,38)} = 0.414$, $p = 0.524$).

Cannabinoid receptors. *Cnr1* gene expression was significantly increased in PTSD-like mice compared with controls (**Figure 9A**, Student's *t*-test, $t = -2.223$, $p < 0.05$, 18 d.f.). Within control group, no differences were observed in the *Cnr1* gene expression with drug administration (**Figure 9B**, Two-way ANOVA, CBD: $F_{(1,37)} = 1.421$, $p = 0.241$; STR: $F_{(1,37)} = 0.319$, $p = 0.576$; CBD x STR: $F_{(1,37)} = 0.105$, $p = 0.747$). Nevertheless, CBD and its combination with STR increased the *Cnr1* gene expression in PTSD-like mice (**Figure 9C**, Two-way ANOVA, CBD: $F_{(1,39)} = 18.716$, $p < 0.001$; STR: $F_{(1,39)} = 0.583$, $p = 0.450$; CBD x STR: $F_{(1,39)} = 9.955$, $p < 0.01$). In addition, *Cnr2* gene expression was significantly increased in PTSD-like mice (**Figure 9D**, Student's *t*-test, $t = -4.763$, $p < 0.001$, 18 d.f.). In the control group, CBD or STR increased *Cnr2* gene expression (**Figure 9E**, Two-way ANOVA, CBD: $F_{(1,39)} = 20.301$, $p < 0.001$; STR: $F_{(1,39)} = 27.577$, $p < 0.001$; CBD x STR: $F_{(1,39)} = 1.052$, $p = 0.312$). In addition, in the PTSD-like group CBD treatment decreased while STR increased *Cnr2* gene expression (**Figure 9F**, Two-way ANOVA, CBD: $F_{(1,39)} = 4.281$, $p < 0.05$; STR: $F_{(1,39)} = 17.287$, $p < 0.001$; CBD x STR: $F_{(1,39)} = 0.179$, $p = 0.675$).

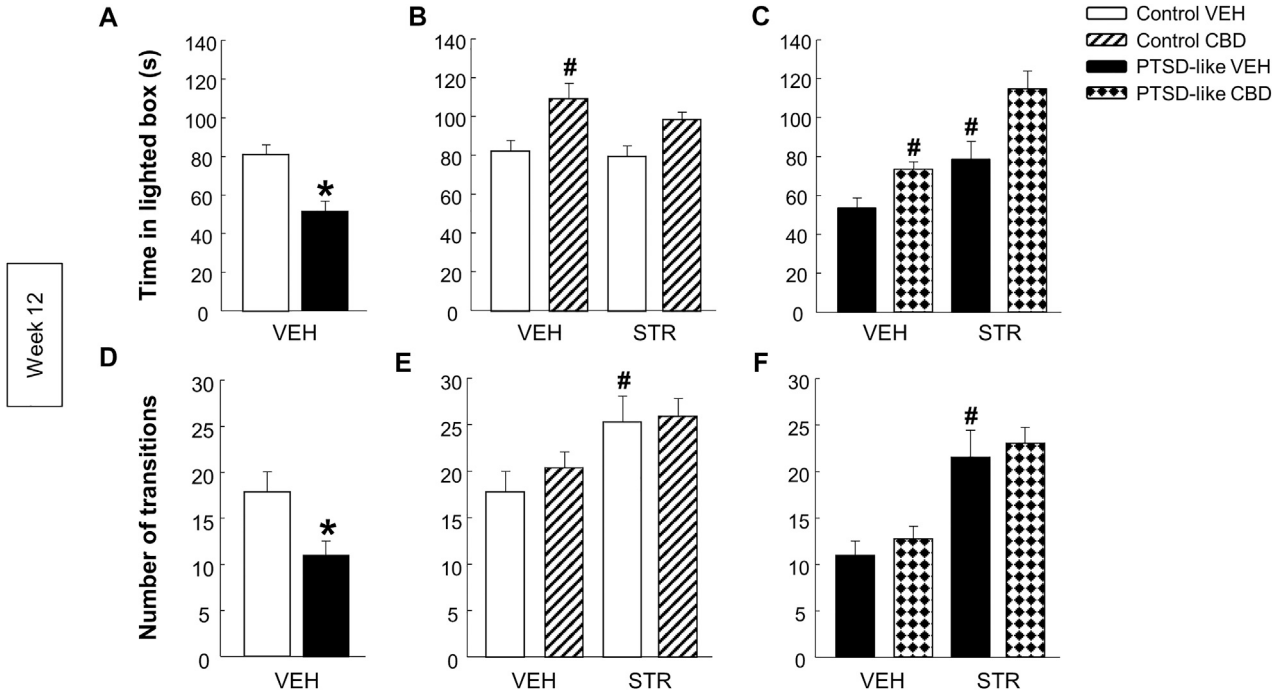

FIGURE 6 | Evaluation of anxiety-like behavior by the light-dark box (LDB) paradigm at week 12. Comparative analysis between control and PTSD-like VEH-treated mice of the time spent in the lighted box (s) **(A)** and the number of transitions **(D)**. Effects of CBD (20 mg/kg, i.p.) and/or STR (10 mg/kg, p.o.) administration, or its corresponding VEH, on the time spent in the lighted box (s) **(B,C)** and the number of transitions **(E,F)** of control and PTSD-like mice. Columns represent the mean and vertical lines ± SEM. *, Values from PTSD-like VEH-treated mice that are significantly different from control VEH-treated mice (Student's *t*-test, $p < 0.001$). #, Values from CBD- or STR-treated groups that are significantly different from VEH-treated mice (Two-way ANOVA, $p < 0.001$). and, Values from CBD plus STR-treated mice that are significantly different from CBD- and STR-treated mice (Two-way ANOVA, $p < 0.001$). Mice exposed to the PTSD-like model: N(VEH) = 10, N(CBD) = 10, N(STR) = 9, N(CBD plus STR) = 10; control mice: N(VEH) = 10, N(CBD) = 9, N(STR) = 9, N(CBD plus STR) = 10.

Serotonin transporter. *Slc6a4* gene expression was significantly decreased in PTSD-like mice (**Figure 10A**, Student's *t*-test, $t = 3.550$, $p < 0.01$, 18 d.f.). Within control group, only CBD enhanced *Slc6a4* gene expression (**Figure 10B**, Two-way ANOVA, CBD: $F_{(1,39)} = 25.440$, $p < 0.001$; STR: $F_{(1,39)} = 0.0931$, $p = 0.762$; CBD x STR: $F_{(1,39)} = 0.113$, $p = 0.739$). Within PTSD-like mice, CBD or STR significantly increased *Slc6a4* gene expression in comparison with VEH-treated group, and a similar effect was reached with CBD plus STR combination but without achieving statistical significance (**Figure 10C**, Two-way ANOVA, CBD: $F_{(1,38)} = 9.050$, $p < 0.01$; STR: $F_{(1,38)} = 10.984$, $p < 0.01$; CBD x STR: $F_{(1,38)} = 2.726$, $p = 0.108$).

DISCUSSION

The results of the present study reveal that the administration of CBD alone or in combination with STR significantly regulated the long-lasting behavioral and neurochemical disturbances in this animal model of PTSD. This statement is supported by the following observations: 1) Mice exposed to the animal model of PTSD showed a pronounced increase of fear-related memories, hyperarousal and anxiety-like behaviors together with gene expression alterations in the HPA-axis, *Cnr1*, *Cnr2* and *Slc6a4* genes, including higher hair accumulated corticosterone concentrations, 2) Exposure of mice to the animal model of PTSD produced a long-lasting enhancement of fear-related memories and anxiety-like behaviors, as well as gene expression changes in HPA-axis, *Cnr1*, *Cnr2* and *Slc6a4* genes, and 3) The administration of CBD (20 mg/kg, i.p.), STR (10 mg/kg, p.o.) and its combination significantly reduced fear-related memories, anxiety-like behaviors and long-term gene expression alterations of PTSD-like mice.

For improving the understanding of the pathophysiological hallmarks of PTSD, it is crucial the development of animal models to reproduce, at least in part, the intensity and the duration of PTSD symptoms. These are critical to identify therapeutic targets leading to safer and more effective pharmacological strategies. In the present study, a chronic animal model of PTSD was developed to induce intense and long-lasting emotional and brain gene expression disturbances. The development of the animal model of PTSD was carried out during mice adolescent period, being this fact critical to induce pronounced and long-lasting alterations related with the exposure to early traumatic experiences. Indeed, mice exposed to this model showed remarkable and enduring disturbances in fear extinction and anxiety-like behavior, that were notorious even 9 weeks after the end of the induction. In the FC, the small reduction of the freezing time observed at weeks 11 (1.38%) and 14 (20.18%) compared to the week 6 (baseline) highlights the fear extinction deficits in mice

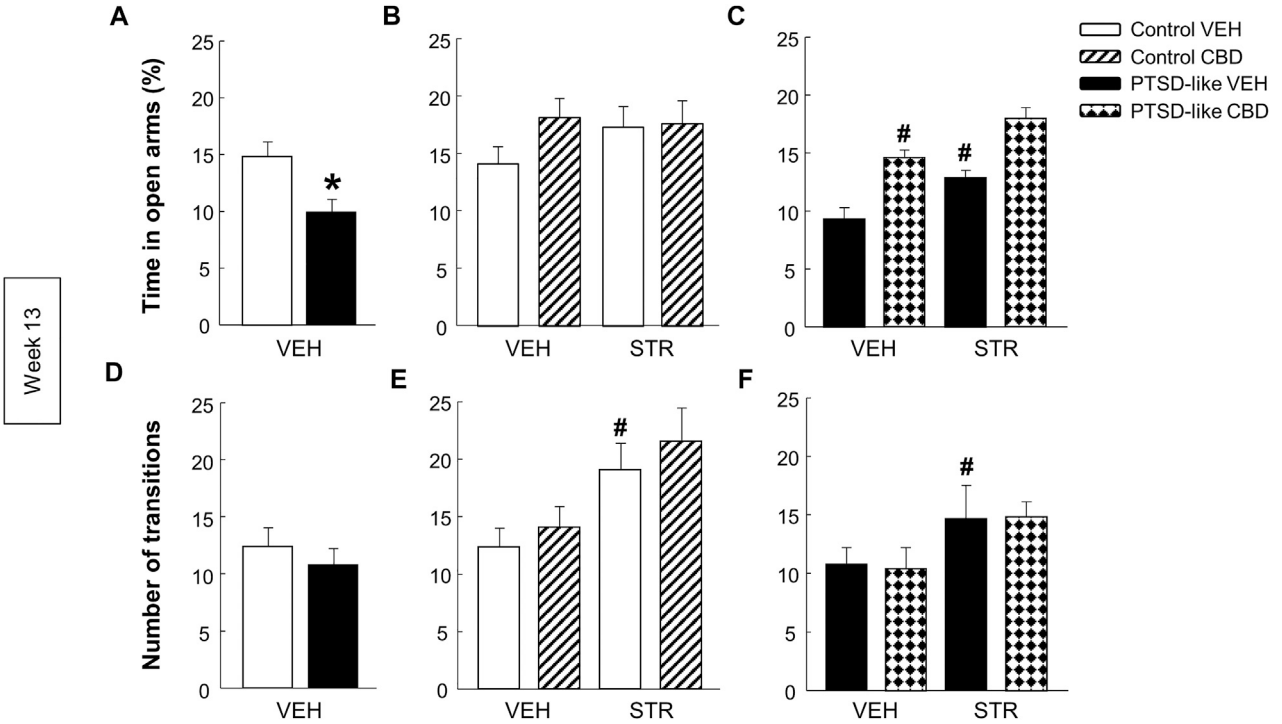

FIGURE 7 | Evaluation of anxiety-like behavior by the elevated plus maze (EPM) paradigm at week 13. Comparative analysis between control and PTSD-like VEH-treated mice of the time spent in the open arms (s) **(A)** and the number of transitions **(D)**. Effects of CBD (20 mg/kg, i.p.) and/or STR (10 mg/kg, p.o.) administration, or its corresponding VEH, on the time spent in the open arms (s) **(B,C)** and the number of transitions **(E,F)** of control and PTSD-like mice. Columns represent the mean and vertical lines ± SEM. *, Values from PTSD-like VEH-treated mice that are significantly different from control VEH-treated mice (Student's *t*-test, $p < 0.001$). #, Values from CBD- or STR-treated groups that are significantly different from VEH-treated mice (Two-way ANOVA, $p < 0.001$). and, Values from CBD plus STR-treated mice that are significantly different from CBD- and STR-treated mice (Two-way ANOVA, $p < 0.001$). Mice exposed to the PTSD-like model: N(VEH) = 10, N(CBD) = 10, N(STR) = 9, N(CBD plus STR) = 10; control mice: N(VEH) = 10, N(CBD) = 9, N(STR) = 9, N(CBD plus STR) = 10.

exposed to the animal model of PTSD. Therefore, it is worth to mention that the long-term PTSD-related impairments triggered by this model facilitate the simulation, at least in part, of chronic emotional disturbances in PTSD patients, and results ideal to evaluate the effects of chronic drug treatments (that usually require 3–5 weeks to result effective).

In the present study, 2-weeks after the end of the induction of the PTSD model, gene expression analyses revealed that *Crf* and *Pomc* were significantly increased in the PVN and ARC of PTSD animals, respectively. In addition, hair accumulated corticosterone was also elevated, confirming the maintained hyperactivity of the HPA axis during the PTSD model. Furthermore, *GCr* gene expression was downregulated in the HIPP. This effect may be related, at least in part, with the increase of corticosterone concentrations and the negative feed-back regulation. On week 14, we found a moderate increase of *Crf* gene expression in the PVN whereas *Pomc* gene expression was reduced in the ARC and *GCr* gene expression increased in the HIPP. It is tempting to hypothesize that a long-term reduction of HPA axis activity may underlie these alterations, in accordance with the clinical evidence pointing out that PTSD at advanced stages may be associated with hypocortisolism (Miller et al., 2007). However, future studies are needed to further characterize long-term disturbances in the regulation of the HPA axis.

The endogenous cannabinoid and serotonergic systems are strongly involved in the regulation of the emotional response. Mice exposed to the animal model showed reduced gene expression of *Cnr1* in the AMY and enhanced gene expression of *Cnr2* in the AMY and *Slc6a4* in the DR, suggesting the involvement of these targets in the behavioral changes observed in PTSD-like mice under basal conditions (weeks 6 and 7). Interestingly, up-regulation of *Cnr2* gene expression was maintained at week 14, whereas for *Cnr1* and *Slc6a4* an opposite effect was observed in the long-term.

Recently, some preclinical and clinical reports suggested the usefulness of CBD as a new alternative for the treatment of PTSD (Schier et al., 2012; Jurkus et al., 2016; Shannon and Opila-Lehman, 2016; Bitencourt and Takahashi, 2018; Crippa et al., 2018; Bonaccorso et al., 2019; Elms et al., 2019; Lisboa et al., 2019). Despite the available, although scarce, reports in rodents regarding the effects of CBD on fear extinction or anxiety-like behavior in animal models of PTSD (Campos et al., 2012; Shallcross et al., 2019), no previous studies evaluated the effects of the repeated administration of CBD, alone or in combination with STR, on long-term behavioral and neurochemical alterations produced by a chronic animal model of PTSD. The results demonstrate that CBD significantly attenuated freezing time under acute and chronic administration and produced an anxiolytic action on

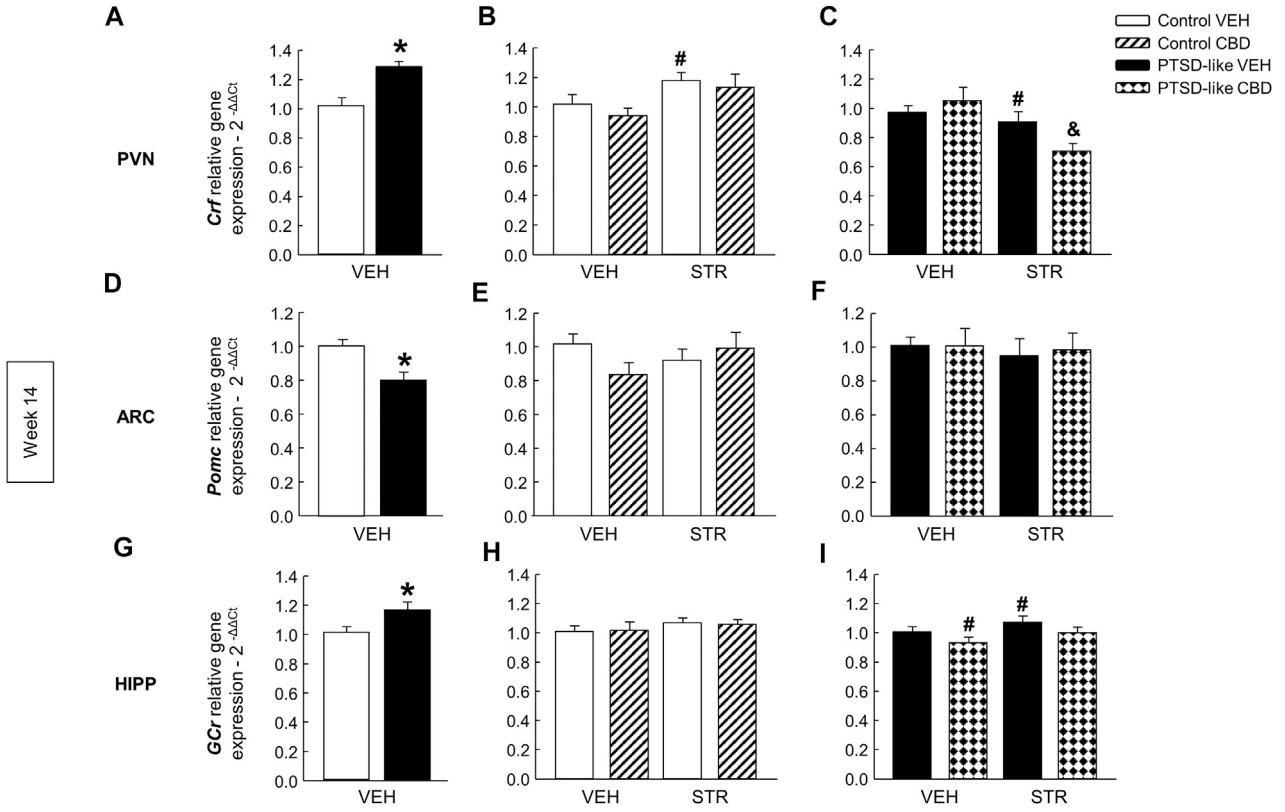

FIGURE 8 | Relative gene expression analyses of Hypothalamus-Pituitary-Adrenal (HPA) axis markers by real time PCR at week 14. Comparative analysis between control and PTSD-like VEH-treated mice of the relative gene expression of corticotropin releasing factor (*Crf*) in the paraventricular nucleus (PVN) **(A)**, proopiomelanocortin (*Pomc*) in the arcuate nucleus (ARC) **(D)**, and glucocorticoid receptor (*GCr*) in the hippocampus **(G)**. Effects of CBD (20 mg/kg, i.p.) and/or STR (10 mg/kg, p.o.) administration, or its corresponding VEH, on the relative gene expression of *Crf* in the PVN **(B,C)**, *Pomc* in the ARC **(E,F)**, and *GCr* in the HIPP **(H,I)** of control and PTSD-like mice. Columns represent the means and vertical lines ± SEM of $2^{-\Delta\Delta Ct}$. *, Values from PTSD-like mice that are significantly different from control mice (Student's *t*-test, $p < 0.05$). #, Values from CBD- or STR-treated mice that are significantly different from VEH-treated mice (Two-way ANOVA, $p < 0.05$). and, Values from CBD plus STR-treated mice that are significantly different from CBD- and STR-treated mice (Two-way ANOVA, $p < 0.05$). Mice exposed to the PTSD-like model: N(VEH) = 10, N(CBD) = 9, N(STR) = 9, N(CBD plus STR) = 10; control mice: N(VEH) = 10, N(CBD) = 9, N(STR) = 9, N(CBD plus STR) = 10.

the LDB and EPM paradigms, effects that were similar to those produced by STR. Interestingly, the combination of CBD plus STR induced more pronounced effects, reducing the freezing time approximately by half, and producing a higher increase of the latency time in the lighted box or open arms. Therefore, these results provide novel and relevant information regarding the therapeutic potential of CBD but especially of CBD plus STR combination for attenuating trauma-related memories and anxiety-like behaviors in PTSD.

Real time PCR analyses revealed that the long-lasting increase of *Crf* in the PVN of PTSD-like animals was significantly normalized by CBD plus STR combination whereas no changes were observed in control mice. Furthermore, upregulation of *GCr* in the HIPP of PTSD-like mice was only normalized by CBD while no changes were observed in control animals. These results are related with previous reports by our group showing that low to moderate doses of CBD did not change *Crf*, *Pomc* and *GCr* gene expressions in non-stressed mice whereas an intermediate CBD dose (15 mg/kg) induced a normalization effect in mice exposed to acute restraint stress (Viudez-Martinez et al., 2018).

Furthermore, gene expression analyses of *Cnr1* and *Cnr2* in the AMY, and *Slc6a4* in the DR were also performed as these targets are closely involved with the mechanisms of action of CBD and STR, respectively, and in PTSD-induced emotional alterations. *Cnr1* relative gene expression was increased in the AMY of PTSD-like mice, and CBD, STR or its combination produced an up-regulation. It has been accepted that CBD act as an indirect agonist of CB1r by increasing anandamide (AEA) levels through the blockade of its degradation and its reuptake (Bisogno et al., 2001). In addition, in the last years some authors suggested that CBD could act also as a negative allosteric modulator of CB1r (Tham et al., 2019). On the other hand, CBD reduced the increased gene expression of *Cnr2* in the AMY of PTSD animals presenting the opposite effect in control mice, especially in combination with STR. According to the idea that CBD is an inverse agonist or an antagonist at the CB2r (Thomas et al., 2007), it is plausible that the up regulation found in control animals may be related with this mechanism. Thus, significant differences in CBD-mediated regulation of *Cnr2* between control and PTSD-like animals may depend on a differential modulation

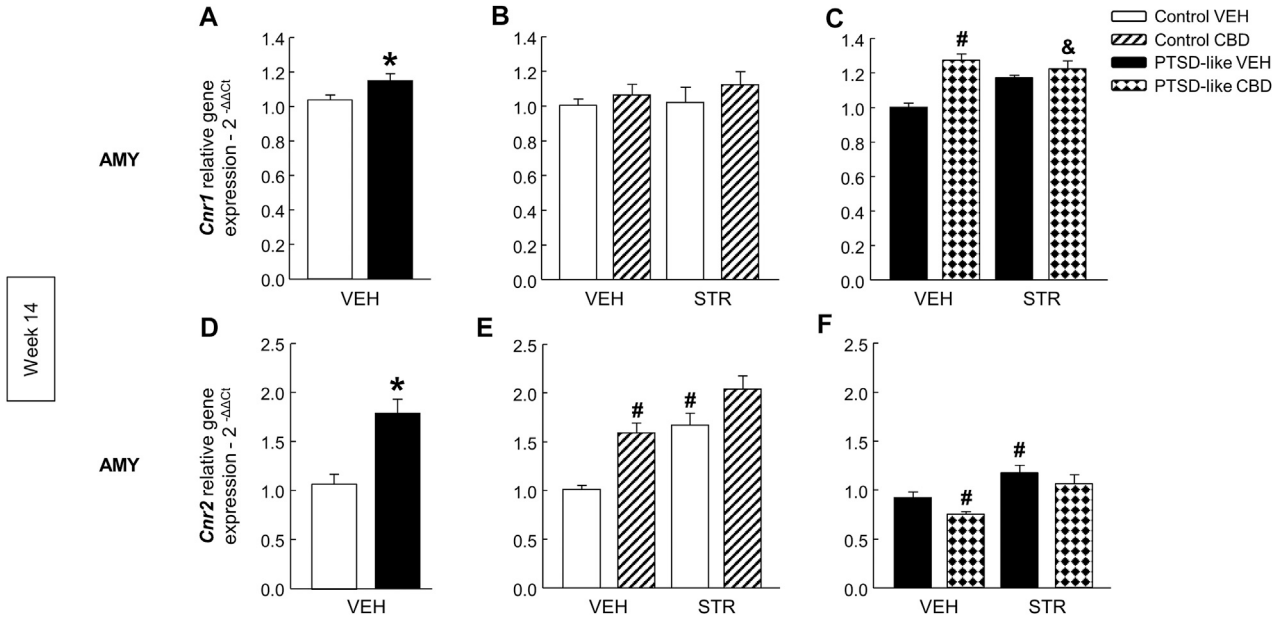

FIGURE 9 | Relative gene expression analyses of cannabinoid receptor 1 (*Cnr1*) and 2 (*Cnr2*) in the amygdala (AMY) by real time PCR at week 14. Comparative analysis between control and PTSD-like vehicle (VEH)-treated mice of the relative gene expression of *Cnr1* **(A)** and *Cnr2* **(D)**. Effects of CBD (20 mg/kg) and/or STR (10 mg/kg) administration, or its corresponding VEH, on the relative gene expression of *Cnr1* **(B,C)** and *Cnr2* **(E,F)** of control and PTSD-like mice. Columns represent the means and vertical lines ± SEM of $2^{-\Delta\Delta Ct}$. *, Values from PTSD-like mice that are significantly different from control mice (Student's *t*-test, *Cnr1*: $p < 0.05$, *Cnr2*: $p < 0.001$). #, Values from CBD- or STR-treated mice that are significantly different from VEH-treated mice (Two-way ANOVA, $p < 0.05$). and, Values from CBD plus STR-treated mice that are significantly different from CBD- and STR-treated mice (Two-way ANOVA, $p < 0.05$). Mice exposed to the PTSD-like model: N(VEH) = 10, N(CBD) = 9, N(STR) = 9, N(CBD plus STR) = 10; control mice: N(VEH) = 10, N(CBD) = 9, N(STR) = 9, N(CBD plus STR) = 10.

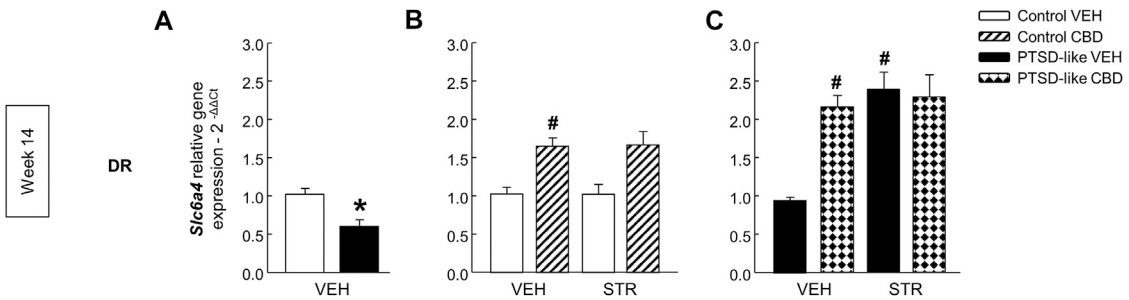

FIGURE 10 | Relative gene expression analyses of serotonin transporter (*Slc6a4*) in the dorsal raphe nucleus (DR) by real time PCR at week 14. Comparative analysis between control and PTSD-like vehicle (VEH)-treated mice of the relative gene expression of *Slc6a4* **(A)** in the DR. Effects of CBD (20 mg/kg) and/or STR (10 mg/kg) administration, or its corresponding VEH, on the relative gene expression of *Slc6a4* in the DR of control **(B)** and PTSD-like **(C)** mice. Columns represent the means and vertical lines ± SEM of $2^{-\Delta\Delta Ct}$. *, Values from PTSD-like mice that are significantly different from control mice (Student's *t*-test, *Slc6a4*: $p < 0.001$). #, Values from CBD- or STR-treated mice that are significantly different from VEH-treated mice (Two-way ANOVA, $p < 0.05$). Mice exposed to the PTSD-like model: N(VEH) = 10, N(CBD) = 9, N(STR) = 9, N(CBD plus STR) = 10; control mice: N(VEH) = 10, N(CBD) = 9, N(STR) = 9, N(CBD plus STR) = 10.

of the endocannabinoid system tone depending on the exposure or not to the animal model of PTSD.

Interestingly, CBD, STR or the combination of both drugs significantly upregulated *Slc6a4* gene expression. In control mice, only CBD or CBD plus STR treatments significantly increased *Slc6a4*. The 1A serotonin receptor (5HT1A) is one of the main targets of the actions mediated by CBD (Campos et al., 2012), mainly due to 5HT1A receptor activation (Russo et al., 2005) or allosteric modulation. Interestingly, it has been recently reported that 5HT1A receptors are involved in the induction of cortical serotonin release by CBD treatment (Linge et al., 2016). Therefore, it is possible to hypothesize that CBD reduces serotonin concentrations in the DR by upregulation of *Slc6a4* gene expression (Norris et al., 2016). Importantly, considering that CBD can interact with more than 65 different targets (Elsaid and Le Foll, 2019), additional studies are needed to further assess the underlying mechanisms involved in the effects of CBD on PTSD-related behavioral disturbances.

In conclusion, these results provide unequivocal evidence for the efficacy of CBD alone, and particularly in combination with STR, to significantly promote fear extinction and reduce anxiety-like behavior in animals exposed to an intense and long-lasting animal model of PTSD. Moreover, gene expression analyses also provide important clues regarding the short- and long-term neurobiological basis of this model of PTSD, and the mechanisms that could be underlying the pharmacological effects of CBD and STR. Some of the limitations that should be highlighted are the lack of female mice to evaluate gender-dependent effects, or the performance of a dose-response curve to have a more complete pharmacological profile of CBD and/or STR in this long-lasting animal model of PTSD. Future studies are warranted to explore the therapeutic potential of CBD for the treatment of PTSD, especially considering the increased effect when combined with STR.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Commettee of Miguel Hernandez University.

AUTHOR CONTRIBUTIONS

All named authors made an active contribution to the conception, design, performance and statistical analysis of the results. AG carried out the behavioral and real time-PCR analyses. AG and FN carried out ELISA immunohistochemical analyses and wrote the first draft of the manuscript. FN and JM critically reviewed the content, validated the accuracy of the data and approved the final version for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.694510/full#supplementary-material

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