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The role of the endocannabinoid system as a therapeutic target for autism spectrum disorder: Lessons from behavioral studies on mouse models

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ABSTRACT

Recent years have seen an impressive amount of research devoted to understanding the etiopathology of Autism Spectrum Disorder (ASD) and developing therapies for this syndrome. Because of the lack of biomarkers of ASD, this work has been largely based on the behavioral characterization of rodent models, based on a multitude of genetic and environmental manipulations. Here we highlight how the endocannabinoid system (ECS) has recently emerged within this context of mouse behavioral studies as an etiopathological factor in ASD and a valid potential therapeutic target. We summarize the most recent results showing alterations of the ECS in rodent models of ASD, and demonstrating ASD-like behaviors in mice with altered ECS, induced either by genetic or pharmacological manipulations. We also give a critical overview of the most relevant advances in designing treatments and novel mouse models for ASD targeting the ECS, highlighting the relevance of thorough and innovative behavioral approaches to investigate the mechanisms acting underneath the complex features of ASD.

1. Autism Spectrum Disorder (ASD) and its animal models: a matter of complexity

1.1. The complex nature and definition of ASD

Autism Spectrum disorder (ASD) is probably among the most complex pathologies of the central nervous system, starting from its diagnostic definition (Dover and Le Couteur, 2007; Klin et al., 2020). The criteria for diagnosing autism, as the disorder was originally called, were firstly proposed by Kanner (Kanner, 1943), subsequently refined by Rutter (Rutter, 1968) and then reformulated several times in the Diagnostic and Statistical Manual of Mental Disorders (American psychiatric association, 2013). According to the original definitions, a child can be ascertained for autism when the onset of the disturbance, characterised by pervasive social impairment and deficits in language and/or communication, occurred before 30 months, in the absence of the delusions and hallucinations typically found in schizophrenic patients (American psychiatric association, 1980). The subsequent revisions of the DSM tended to isolate pervasive developmental disorders, highlighting that autistic symptomatology is instead not confined to early childhood, but persists afterwards (American psychiatric association, 1987). Overall, the basic diagnostic criteria of autism include the following triad: social dysfunction, qualitative deficits in verbal/nonverbal communication and a restricted range of interests associated with the occurrence of repetitive behaviors. These criteria were substantially maintained in the more recent editions of DSM, although the first and second domains tended to be merged into a more general field of social/communication deficits, thus eliminating the triad concept (American psychiatric association, 2013).

Introducing the concept of “spectrum” (American psychiatric association, 2000) has drastically changed our view of autistic pathology: ASD is now defined as a single disorder that includes pathologies that were previously considered separate, i.e., autism, Asperger's syndrome, childhood disintegrative disorder and pervasive developmental disorder not otherwise specified (American psychiatric association, 2013). The term "spectrum" also refers to the wide range of symptoms and degrees of severity characterizing ASD patients, underlining the huge variability affecting the expression of both core (i.e., the ancient autistic “triad”) and additional symptoms (Rapin, 1991). This impressively high heterogeneity of ASD is therefore related to the fact that it has been somehow artificially defined as a single disorder, but it is in reality a combination of divergent disorders, in turn all having variable symptoms.

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1.2. The complexity of modelling ASD in laboratory animals: a challenge for behavioral testing

The complexity of the nature of ASD and of its definition obviously complicates the designing of animal models of this disorder. The latter task is already made difficult by the unknown aetiology of the disorder, including complex multigenic and environmental factors, with specific contributions and underlying mechanisms that still remain undefined. Furthermore, the lack of biomarkers of autistic pathology directs most of the possible research towards the behavioral validation of its animal models. Assessing the face validity of animal models of ASD is also a challenging and complex task, because of the large variety of behavioral symptoms. While additional ASD-relevant symptoms such as anxiety, sensory abnormalities, hyper-activity, and cognitive alterations can be tested in mice with well-established and standardized behavioral protocols, most of the core autistic symptoms are instead not easily modelled in rodents (Servadio et al., 2015). The core autistic symptom of the occurrence of repetitive behaviors and insistence on the sameness has been evaluated in laboratory mice through indirect measures of behavioral flexibility in different types of mazes (i.e, deficits in spontaneous alternation in Y- or T- mazes, or in reversal learning or working memory in the water maze), or through the assessment of home-cage or novelty-induced behavioral stereotypies [e.g. excessive self-grooming or marble burying (Moy et al., 2008)]. Novel protocols testing habit formation and insistence on sameness have been also developed in order to better model this behavioral ASD-like abnormality (Amodeo et al., 2012; Cupolillo et al., 2016; Roullet and Crawley, 2011).

Modelling the ASD-like core social deficits in laboratory rodents is probably the most challenging task, since autism seems intrinsically related to social dysfunction. Furthermore, proposing relevant parallelisms between human and mouse social behavioral test represents quite a challenge, because of the longer preferential use of rats in studies on social behaviors (Kondrakiewicz et al., 2019). Hence, a large body of recent research has focused on developing more appropriate methods to assess social ASD-like behaviors in mice. Social interest for example has been assessed in juvenile and adult mice using social preference tasks in the three chamber test (Nadler et al., 2004), or direct interaction tests, also evaluating juvenile play, affiliative behaviors, or aggression. Furthermore, communication has also been assessed in laboratory mice, as they emit ultrasonic vocalizations in social contexts at multiple stages of their life (Wohr, 2014; Wohr and Scattoni, 2013), i.e., during isolation on the first post-natal days (Branchi et al., 2001; D'Amato et al., 2005) and during adult social interactions (Holy and Guo, 2005; Moles et al., 2007). The analysis of all these mouse social behaviors has impressively evolved, leading to advanced tools for data analysis and to novel experimental protocols to assess complex social behaviors.

An example of a major effort to refine the analysis of mouse social behaviors comes from recent research proposing the automatized evaluation of social interactions in animal dyads and groups, using machine learning strategies (de Chaumont et al., 2019; Mathis et al., 2018; Nath et al., 2019). These recent approaches try to eliminate the major limiting factors affecting the analysis of mouse social behaviors, i.e., its time-consuming and observer-dependent characteristics, often pushing away researchers from including the evaluation of social phenotypes in their batteries of tests for genetic mouse models. Because of the necessary expertise in programming and informatics, the extensive application of these advanced technological tools to the analysis of mouse social behavior still requires some time, but it will probably become accessible to a larger audience very soon. Obviously, their application could reveal interesting outcomes and general advantages in rats too, to be adopted in future studies on social behaviors.

Among the most impressive advances in data analysis are probably those addressing mouse ultrasonic communication, since the phenomenon started being studied with basic bat-detectors and simple quantification of the calls and then moved to sensitive microphones and sophisticated qualitative assessment of ultrasonic spectrograms (Ferhat et al., 2016). These advances allowed the researchers to identify different type of calls and syllables within the repertoire of an adult mouse, and to study their modulation by the social context and other parameters (Hanson and Hurley, 2012; Kikusui et al., 2021; Matsumoto and Okanoya, 2016, 2018). These advances have so far concerned mostly the analysis of ultrasonic communication in mice, where they may specifically compensate the absence of frequency-specific calls with different affective value that are instead present in rats (as described in Section 1.3). This detailed qualitative analysis can also be applied during the first post-natal days, and it has allowed identifying alterations in the developmental ultrasonic profile of genetic models of ASD (Agarwalla et al., 2020; Fyke et al., 2021; Lai et al., 2014; Roy et al., 2012). The application of these novel analytical tools to genetic mouse models of ASD has indeed dramatically improved our ability to assess their face validity, often allowing subtle communicative alterations to emerge at different ages.

Finally, the evaluation of complex social behaviors in laboratory mice have benefitted from the recent designing of sophisticated experimental protocols e.g., targeting the social transmission of emotions and empathy (Jeon and Shin, 2011; Jordan and Mogil, 2006; Lecker et al., 2020). Knowing the affective state of others is a critical factor that can drive social interactions, and it is directly linked to ASD since an inability to decipher another's affective state is indeed a core autistic feature. Recent elegant studies have demonstrated the social transmission of affective states in laboratory mice by direct social interaction between an observer with no aversive experience and a demonstrator having experienced electric shocks: the enhanced stress levels and fear responses detected in the observer represent an index of the social transmission of a negative affective state (Sterley et al., 2018; Sterley and Bains, 2021). The ability of transmitting to cagemates a painful experience has also been evaluated in mice, through the development of a formalin test for emotional contagion (Laviola et al., 2017). Other authors have instead used the restrain stress protocol to show that sharing the stress with cage mates can reduced corticosterone levels of laboratory mice, while experiencing the stress alone among cage mates has the opposite effect, suggesting evidence of empathy and reversed empathy in laboratory mice (Watanabe, 2011).

1.3. Advantages and limitations of mouse versus rat studies on ASD

As mentioned above, social behavior has been by tradition studied preferentially in laboratory rats, somehow supporting the idea of the mouse as a "less social" species, mostly affected by territoriality and less involved in complex social interactions and behaviors. This ancient idea has been largely overcome in the recent years, mostly because of the clear invaluable advantages of studying social behavior and its dysfunction in laboratory mice. The major advantage of mouse *versus* rat studies on social behavior lies in the simplicity of manipulating the mouse genome, resulting in the availability of a myriad of inbred mouse strains and of genetically modified mouse lines, offering the unique possibility of understanding the genetic substrates of most basic aspects of social interactions.

Laboratory mice have become therefore an invaluable tool to investigate the molecular mechanisms underlying several social behaviors, also at early stages. Juvenile mice for instance are known to express a robust motivation to approach conspecifics of the same age, and play behavior is known to be not exclusive of young rats (Terranova et al., 1993). Genetic studies in juvenile mice [e.g., (Panksepp et al., 2007)] can therefore provide promising targets for intervention with high predictive validity, having an obvious special relevance for neurodevelopmental disorders in general and ASD in particular. The rapid advancing of gene targeting techniques also offers to the researchers novel unique tools to further dissect the molecular mechanisms underlying complex

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social behaviors. This is the case of social transmission of affective states, that has been evaluated in genetically modified mice (see also Section 1.2), allowing to shed light on the precise neuronal circuits involved in this complex social interaction (Sterley et al., 2018; Sterley and Bains, 2021). Recently, it has been shown in mice that the control of behavioral responses during social interaction essays can be disentangled in its finer components, i.e., in terms of the specificity of the cellular populations (e.g., astroglial versus neuronal cells) and subcellular components (e.g., mitochondrial versus plasmatic receptors) involved in brain metabolic functions (Jimenez-Blasco et al., 2020). Such a finely-tuned control of genetic manipulation of social functions in mice opens a brand new avenue of research perspectives that would be more difficult to be realized in laboratory rats, at least in terms of rapidity and precision of targeting.

Despite the clear advantages of using laboratory mice for studying social interactions and ASD, some aspects of social behaviors can still be investigated exclusively in rats. For example, although social play is studied in laboratory mice, rough-and-tumble play is uncommon in the mouse species (Panksepp and Lahvis, 2007), in contrast to rats where it constitutes a major behavioral component. Similarly, mice use ultrasonic communication during their social interactions, with levels of complexity that become increasingly evident, including the production of ultrasonic calls of different types according to the social context, as we described in Section 1.2. Nonetheless, the use of ultrasonic calls with different affective value has been so far demonstrated only in rats, since positive 50 kHz calls (also known as the rat laughter) and 22 kHz alarm calls have not been detected in mice so far (Knutson et al., 2002; Portfors, 2007; Wohr and Schwarting, 2013). This represents an unique advantage of the rat species to study affective communication of positive/reinforcing experiences, allowing to study the brain circuits and mechanisms underlying this important social function (Burgdorf et al., 2011, 2007), as well as the evolutionary role of communicative ways of sharing of positive versus negative feelings (Panksepp and Burgdorf, 2003).

1.4. How do we define behaviorally "valid" mouse models for ASD?

To face the challenges of basic research on ASD, a promising approach seems to consist in modeling single symptoms, rather than the whole disorder, giving up any attempt of a unitary explanation of ASD, as suggested by some authors (Happe et al., 2006). The definition of the precise criteria that a valid model for ASD should fulfill is still the subject of an intense debate (Chadman et al., 2019; Kazdoba et al., 2016; Servadio et al., 2015). An ideal animal model would be able to mimic all core behavioral autistic features in association with some of the secondary ones. Nonetheless, the behavioral validation of animal models for ASD is most often focused on the social domain only (Crawley, 2004, 2007; Moy et al., 2006; Silverman et al., 2010), as evident also from the data summaries illustrated here in Tables 2 and 3. Most reasonably, a rodent model should be considered as valid if it presents alterations in both core domains affected in ASD, i.e., social interaction/communication, and flexible/repetitive behaviors (Crawley, 2004, 2007; Wohr and Scattoni, 2013). The presence of additional behavioral abnormalities, such as hyper-activity, memory deficits or anxiety, may increase the validity of the model, but is not required (Kazdoba et al., 2016; Servadio et al., 2015).

As already reviewed in several articles [e.g., (Banerjee et al., 2014; Bey and Jiang, 2014; Ellegood and Crawley, 2015; Moy et al., 2006)], a large variety of mouse models for ASD have been proposed in the last decades, based on very different approaches. One strategy consists of identifying mouse strains that spontaneously present some major ASD-like symptoms, such as low sociability, poor communication abilities, and high levels of inflexible behaviors. The best-known example of this approach is the BTBR strain (McFarlane et al., 2008; Meyza et al., 2013), widely employed in ASD research and proposed as a tool to identify novel genes involved in the control of social behaviors. This model is characterized by agenesis of the corpus callosum (Wahlsten et al., 2003), as well as by several behavioral abnormalities, including alterations in ultrasonic communication both at early post-natal and adult ages, reduced social interest in the three chamber test, high levels of self-grooming and deficits in cognitive flexibility (Pearson et al., 2011; Scattoni et al., 2008, 2011; Yang et al., 2007). The main disadvantages of this model are (i) the problem of identifying an appropriate control strain and (ii) the possibility that strain-specific phenotypes of emotionality may explain the supposed ASD-like behavioral profiles (Oddi et al., 2013b) and play as major confounding factors on the social abnormalities. These problems are somehow intrinsic of the strain approach and indeed affect also the equivalent rat models based on strain differences [e.g., (Zhang-James et al., 2014)].

Other mouse models rely on the exposure (mostly prenatally) to adverse environmental factors (Dufour-Rainfray et al., 2011; Patterson, 2011), such as certain teratogens/drugs and viral/bacterial infections, suspected to contribute to the etiology of ASD on the basis of epidemiological data (American College of and Gynecologists' Committee on Obstetric, 2021; Atladottir et al., 2010; Davis et al., 1992; Engman et al., 2015; Fluegge, 2016; Mezzacappa et al., 2017; Zerbo et al., 2015). A well-known example of this approach is the valproic acid (VPA) model, developed mainly in rats (Ingram et al., 2000; Schneider and Przewlocki, 2005), and secondarily in mice (Chapman and Cutler, 1984; Roux et al., 2019). This model is based on VPA administration during the early phases of pregnancy in mice or rats and it is characterized by the early appearance of motor, sensory and social disturbances, as well as increased repetitive behaviors (Chapman and Cutler, 1984; Ingram et al., 2000; Roux et al., 2019; Schneider and Przewlocki, 2005). Similarly, the maternal immune activation model (MIA) induces motor, social and communication ASD-like deficits by injecting the viral mimic poly(I:C) (Malkova et al., 2012) or the bacterial Lipopolysaccharide (LPS) (Fernandez de Cossio et al., 2017) during pregnancy. The environmental approach has the advantage of having a sort of "construct validity", but has the main drawback of overlooking the major role of genetic factors in ASD etiopathology.

Indeed, another approach consists in manipulating the mouse genome to import into the animal model some of the genetic alterations associated with ASD, e.g., gene deletion/ mutations. The validity of this experimental strategy also has some problems, i.e., the heterogeneity of the genetic alterations known to be somehow involved in ASD, including basically almost all genes affecting neuronal function and development (Abrahams and Geschwind, 2008). These genes code for molecules involved in: i) chromatin remodelling and regulation of transcription (e.g., MECP2, FMR1), ii) actin cytoskeleton dynamics (TSC1, TSC2, NF1) iii) synaptic scaffolding proteins (e.g., SHANK3), iv) receptors and transporters (e.g., GRIN2A, GRIK2, GABAR, SLC6A4, SLC25A13, OXTR, AVPR1), v) second-messenger systems (PRKCB1, CACNA1C, NBEA), vi) cell adhesion (e.g.,neuroligin NLGN3, NLGN4), and vii) secreted proteins (e.g., RELN, LAMB1) [reviewed in (Persico and Bourgeron, 2006)]. Such list serves only to provide an indicative overview, as the number of implicated genes is supposed to be many-fold greater (Buxbaum et al., 2012).

The role of all these genes in brain development is at the basis of a major hypothesis on autistic aetiology, i.e., the so-called "many genes, common pathways" (Geschwind, 2008), emphasizing the role of defective synaptic functioning and abnormal brain connectivity in ASD, both leading to altered information processing. One somewhat paradoxical finding about ASD is that, although there is proof that its aetiopathogenesis is the result of concomitant mutations in multiple loci, there is a growing list of genes whose disruption alone is sufficient for the whole autistic phenotype to occur. Based on such evidence, mouse models of single gene mutations may therefore be informative concerning the underlying mechanism(s) leading to ASD (Ey et al., 2011).

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The last approach used to design animal models of ASD indeed focused on other developmental pathologies featuring ASD-like traits, but with a defined, single genetic cause and therefore well-established mouse models. A widely used example of this "simplified" approach is provided by the Fmr1-KO mouse line (The Dutch-Belgian Fragile X Consortium, 1994), modelling the most common monogenic cause of ASD, that is Fragile X syndrome (FXS), a X-linked disorder due to the lack of FMRP protein coded by the FMR1 gene (Pieretti et al., 1991). Fmr1-KO mice display ASD-like deficits, i.e., reduced social interaction and social memory, deficits in reversal learning and ultrasonic communication alterations, as reviewed in (Pietropaolo and Subashi, 2014). An other example of this approach focuses on Rett syndrome that is a monogenic disorder caused by deficiency of methyl-CpG-binding protein-2 (Mecp2) and characterized by ASD-like symptoms. Mice carrying null mutation for Mecp2 (Chen et al., 2001), as well as various polymorphisms found in Rett patients (Shahbazian et al., 2002), display ASD-like phenotypes, including altered emission of ultrasonic vocalizations at infancy (Picker et al., 2006), and deficits in social and cognitive function in adulthood (Moretti et al., 2005). A criticism to this "monogenic" research strategy comes from the consideration that the genetic mutations induced in these models are not invariably present in ASD patients and may therefore be valid in modelling only a specific subset of patients (Budimirovic and Kaufmann, 2011). Nonetheless, recent research on pre-clinical models increasingly supports the view that investigating the consequences of single gene disruption may constitute a valuable tool for understanding ASD (Wohr, 2014; Wohr and Scattoni, 2013), a research approach that we have also followed in a major part of our research work based on the Fmr1-KO mouse line (Bernardet and Crusio, 2006; Oddi et al., 2013a), together with others' studies (Budimirovic and Kaufmann, 2011; Hulbert and Jiang, 2016; Verma et al., 2019).

In this complex and somehow undefined field of research, basically all these approaches to model ASD in laboratory mice have been employed to try to identify potential therapeutic targets to treat the disorder. Here, we will take advantage of examples of these different strategies to design mouse models of ASD to summarize the experimental evidence supporting the role of the endocannabinoid system (ECS) in the etiopathology of ASD. To this end, we will first review studies showing abnormalities in the functionality of the ECS in rodent models of ASD (Table 1), including several examples from the different approaches described above, e.g., based on strain differences (the BTBR mouse), prenatal exposure to environmental agents (the VPA or LPS models), candidate genes (NLGN-3 mutant mice), or monogenic developmental pathologies (the Fmr1-KO mouse). We will also summarize available data showing the correction of ASD-like behaviors in these models following pharmacological modulation of the ECS (Table 2), thus suggesting potential novel pharmacological approaches to ASD. Finally, we will discuss the possibility of inducing ASD-like behaviors in mice through pharmacological or genetic manipulations of the ECS (Table 3), thus discussing potential novel models for ASD. In line with the most prominent part of our experimental work on ECS and ASD, we will try as much as possible to keep our focus on the mouse species, though rat studies will be also included when necessary (e.g., for VPA and other environmental models). Our conclusive remarks will underline the relevance of extensive and refined behavioral analysis in assessing the role of the ECS in ASD.

2. Why should the endocannabinoid system play a role in ASD?

2.1. A converging etiopathological hypothesis for ASD: characteristics of the endocannabinoid system (ECS)

Recent studies have suggested that the multitude of possible causal factors involved in the pathogenic mechanisms of ASD may functionally converge into a relatively small subset of functional pathways that may represent an easier and more promising target for basic research on the disorder (Zoghbi and Bear, 2012). The functional convergence on particular signaling pathways and the shared synaptopathology of ASD raise the hope that similar therapeutic strategies may be effective for different forms of ASD which are related, but genetically distinct (Zoghbi and Bear, 2012). This "reductionist" approach is in line with the hypothesis of "many genes, common pathways" mentioned above (Geschwind, 2008).

In this context of searching for common causal pathways for ASD, the endocannabinoid system (ECS) seems a promising candidate to play a role in the etiopathology of this disorder and to provide a novel target of therapeutic interest. The ECS is an important modulator of neuronal functions, as demonstrated by the abundance of the cannabinoid CB1 receptors (CB1r) in the brain (Mackie, 2005). These are particularly enriched in cortex, hippocampus, amygdala, and cerebellum, i.e., brain areas that are strongly involved in the development of ASD. Furthermore, endocannabinoid signaling also regulates synaptogenesis and neuronal interconnectivity during development (Berghuis et al., 2007), processes whose defects are considered to be key mechanisms underlying ASD pathology (Pardo and Eberhart, 2007).

CB1 receptors are located in different cell types in the brain, including neurons and glial cells (Busquets-Garcia et al., 2018). As illustrated

Table 1
Evidence for ECS alterations in animal models of ASD.

Modelling approach	Model name	Description of ECS alteration	References
Monogenic associated syndromes	Fmr1-KO mice	↔ CB1r expression and 2-AG levels	(Zhang and Alger, 2010; Jung et al., 2012)
		↓ EC-mediated LTD at excitatory synapses in forebrain	(Jung et al., 2012)
		↑ MAGL activity in the striatum	(Maccarrone et al., 2010)
		↓ EC-mediated LTD at inhibitory synapses in hippocampus and striatum	(Maccarrone et al., 2010; Zhang and Alger, 2010)
Strain differences	BTBR mice	↑ CB1r binding and GTP-gamma activation	(Gould et al., 2012, 2014)
		↑CB2 gene expression in the cerebellum	(Liu et al., 2009)
Candidate gene	NLGN3-R451C knockin and NLGN3 knockout mice	↓ tonic EC signalling in the hippocampus, cortex, amygdala and dorsal striatum	(Foldy et al., 2013; Speed et al., 2015; Hosie et al., 2018; Martella et al., 2018)
		↔ tonic EC signalling and mobilization in the nucleus accumbens	(Rothwell et al., 2015)
Gestational exposure to environmental insults (e.g., teratogens, viral/bacterial infection)	VPA rats	↓ CB1r phosphorylation in the amygdala, hippocampus and dorsal striatum	(Servadio et al., 2016)
		↓ gene expression of DGL-α in the cerebellum	(Servadio et al., 2016)
		↓NAPE PLD and ↑FAAH in the whole brain	(Servadio et al., 2016)
	LPS rats	↓CB1, ↑ FAAH in the amygdala	(Doenni et al., 2016)

NLG: neurolegin. VPA: valproic acid. LPS: lipopolysaccharide. LTD: long-term depression. ECs: endocannabinoids. 2-AG: 2-arachidonoyl-glycerol. NAPE-PLD: N-acyl phosphatidylethanolamine-specific phospholipase D. DAGL-a: Diacylglycerol lipase alpha. FAAH: fatty acid amide hydrolase. MAGL: monoacylglycerol lipase. ↑increase. ↓decrease. ↔no change.

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Table 2
Pharmacological therapies targeting the ECS in animal models of ASD.

Treatment action on ECS	Drug	Dose	ASD model	Therapeutic effects	Behavioral tests	References
CB2 antagonist	AM630	1 mg/kg; acute	Fmr1-KO mouse	rescue of anxiety alterations and audiogenic seizures	EPM	(Busquets-Garcia et al., 2013)
CB1 antagonist	SR141716A	1 mg/kg; acute	Fmr1-KO mouse	rescue of cognitive deficits, audiogenic seizures, altered nociception and abnormal mTOR signaling	NOR	(Busquets-Garcia et al., 2013)
		0,03-1 mg/kg; acute/chronic	Fmr1-KO mouse	rescue of cognitive deficits	NOR	(Busquets-Garcia et al., 2013; Gomis-Gonzalez et al., 2016)
	AM251	2 mg/Kg	BTBR mouse	no effect on sociability deficits	3 comp	(Wei et al., 2016)
		10μM, in bath application	NLGN-3 mouse	rescue of increased inhibitory transmission in somatosensory cortex	_	(Speed et al., 2015)
CB1-agonist	ACEA	20μM, perfusion	NLGN-3 mouse	rescue of alterations in tonic EC signalling in the dorsal striatum	_	(Martella et al., 2018)
	Win 55,212-2	0,3 or 1 mg/kg; acute	NLGN-3 mouse	Rescue of enhanced aggressiveness	resident-intruder	(Hosie et al., 2018)
FAAH-inhibitor	URB597	0,3 mg/kg	Fmr1-KO mouse	amelioration of cognitive deficits, no effect on anxiety	PA, EPM	(Qin et al., 2015)
		0,3 mg/kg	Fmr1-KO mouse	rescue of sociability deficits	3 comp	(Wei et al., 2016)
		0,3 mg/kg and 1 mg/Kg	BTBR mouse	rescue of sociability deficits	3 comp	(Wei et al., 2016)
		0.05 mg/Kg acute	VPA-rat	rescue of communication and social deficits at infancy, adolescence and adulthood. Elimination of anxiety and stereotypic behaviors	USVs, homing, hole board, social play, 3 comp, EPM	(Servadio et al., 2016)
	PF-3845	10 mg/Kg acute	VPA-rat	rescue of social deficits	3 comp	(Kerr et al., 2013)
	PF-04457845	1 mg/Kg acute	LPS-rat	rescue of social deficits	direct SI	(Doenni et al., 2016)
MAGL-inhibitor	JZL-184	16 mg/kg	Fmr1-KO mouse	no effect	PA	(Qin et al., 2015)
				normalization of hyperactivity and altered anxiety	EPM, OF	(Jung et al., 2012)

FAAH: fatty acid amide hydrolase. MAGL: monoacyl-glycerol lipase. NLG: neurolegin. VPA: valproic acid. LPS: lipopolysaccharide. EPM: elevated plus maze. OF: open field. 3-COMP: three compartment test. NOR: novel object recognition. PA: passive avoidance. USVs: ultrasonic vocalizations. SI: social interaction.

in Fig. 1, CB1r are mainly expressed pre-synaptically in both GABAergic and glutamatergic neurons where they are activated by their endogenous ligands, i.e., the endocannabinoids (ECs): anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG). These endogenous cannabinoids are produced at post-synaptic site by membrane depolarization or activation of metabotropic receptors (Piomelli, 2014; Piomelli et al., 1998). Indeed calcium elevation generated by these two type of events is able to trigger plasma membrane lipid remodeling which generates as final products AEA, by the biosynthetic enzyme N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD), and 2-AG, by the Diacylglycerol lipase alpha (DAGL-a) (Piomelli, 2003). The retrograde binding of AEA and 2-AG to pre-synaptic CB1r can trigger several intracellular pathways to reduce intracellular calcium and inhibit neurotransmitter release (Piomelli, 2003). Endocannabinoids are terminated by re-uptake mechanisms; AEA is degraded by fatty acid amide hydrolase (FAAH), and 2-AG by monoacyl-glycerol lipase (MAGL). The ECS differs from classical neurotransmitter systems for the fact that there is no constitutive tone, but on-demand production of ECs, CB1r activation followed by immediate ECs degradation (Piomelli, 2003).

2.2. Modulating the functionality of the ECS

The ECS is therefore a neuromodulatory network of lipid signaling pathways, including the endocannabinoids together with their receptors and the associated metabolic enzymes (Di Marzo and Piscitelli, 2015). Although endocannabinoids exert their central effects mainly through the cannabinoid type 1 receptor CB1r, they also interact with the other G-protein coupled cannabinoid type 2 receptor (CB2r), mostly involved in inflammatory processes and in the modulation of the immune system (Jordan and Xi, 2019; Turcotte et al., 2016). Additionally, they can also interact with non-CB1r/CB2r targets, e.g., the transient receptor potential vanilloid type 1 (TRPV1) channel, peroxisome proliferator-activated receptor (PPAR)-alpha, and PPAR-gamma as well as the G protein-coupled receptor GPR55 (Pistis and Melis, 2010).

Several pharmacological tools are available for studying the ECS (mostly mentioned in Tables 2 and 3), as extensively described elsewhere (An et al., 2020). Most commonly, the ECS is activated by CBr agonists, e.g., the natural cannabinoid Δ(9)-tetrahydrocannabinol (THC) and the synthetic WIN 55,212-2, ACEA, or CP-55,940 (Griffin et al., 1998), or indirectly by pharmacological inhibition of AEA and 2-AG reuptake and degradation (e.g., URB597, JZL-184, JZL-195). The latter approaches are useful for activity-dependent potentiation of endocannabinoid signals and would not have an effect in the absence of endocannabinoids production as normally happens with systemic CBr agonists administration (Monory and Lutz, 2009). The ECS can be blocked mainly by CBr antagonists (e.g., rimonabant or SR141716A, AM251) or inhibition of endocannabinoids biosynthesis [e.g., DO-34 for DAGL (Mitra et al., 2021) and LEI-401 for AEA (Mock et al., 2020)]. In addition, transgenic mouse models with constitutive and cell type specific

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Table 3
ASD-like phenotypes induced by pharmacological and genetic targeting of the ECS in rodents.

Action on ECS	GT	Drug	Dose	ASD-like behavioral phenotype	Behavioral test	References
CB1-antagonist	WT mice	AM251	2 mg/Kg, acute	↓ sociability, ↔ anxiety	3-comp, EPM	(Wei et al., 2016)
			5 mg/Kg, acute	↓ social interaction and social memory	SI	(Litvin et al., 2013; Umathe et al., 2009)
		SR141716A	3 or 10 mg/Kg, acute	↓ social interaction and USV emission,↑ repetitive behavior	SI	(Pietropaolo et al., 2020; Terzian et al., 2014)
CB2-antagonist	WT rats	AM630	1 mg/Kg, subchronic	↔ social interaction	SI	(Argue et al., 2017)
		SR144528	0.1 mg/Kg, acute	↔ social interaction	SI	(Trezza and Vandershuren 2009)
FAAH-inhibitor	WT mice	URB597	1 mg/Kg, acute	↔ sociability or anxiety	3 comp, EPM	(Wei et al., 2016)
	WT rats	URB597	0.01 mg/Kg, acute	↑ social interaction	SI	(Manduca et al., 2014)
			0.1 mg/Kg, acute	↑ social interaction	SI	(Trezza et al., 2012; Trezza and Vandershuren 2008)
MAGL-inhibitor	WT mice	JZL-184	8 mg/Kg, acute	↑ social interaction	SI	(Manduca et al., 2016)
	WT rats	JZL-184	1 mg/Kg, acute	↑ social interaction	SI	(Schiavi et al., 2019)
CB1-agonist	WT mice	Win 55,212-2	0.4 mg/Kg, acute; 0.1 mg/Kg, chronic	↓ social interaction	SI	(Pietropaolo et al., 2015)
			0.1 mg/Kg, acute	↓ social interaction	3-COMP	(Gould et al., 2012)
	WT rats	Win 55,212-2	0.1 -0.3 mg/Kg, acute	↑ social interaction	SI	(Almeida et al., 2014)
	WT mice	CP 55,940	0.1 mg/Kg, acute	↔ social interaction	SI	(Tambaro et al., 2013)
	WT rats	ACEA	0.1 -1 mg/Kg, acute	↔ social interaction	SI	(Almeida et al., 2014; Argue et al., 2017))
CB1-deletion	CB1-KO mice			↓ sociability and social exploration	SI, 3-COMP	(Haller et al., 2004; Haring et al., 2015, 2011; Fyke et al., 2021; Gould et al., 2012; Terzian et al., 2014; Uriguen et al., 2004)
				↓ social memory	SI	(Litvin et al., 2013)
				↓USV communication	USVs, SI	(Fyke et al., 2021)
				↑ repetitive behaviours	SI	(Litvin et al., 2013; Terzian et al., 2011)
↑ ECs	FAAH-KO			↑ sociability and social exploration	SI, 3-COMP	(Cassano et al., 2011; Wei et al., 2015)
↓ ECs	DAGLa-KO			↓ sociability and social exploration	3-COMP	(Shonesy et al., 2018)

GT: genotype. FAAH: fatty acid amide hydrolase. MAGL: monoacyl-glycerol lipase. ECs: endocannabinoids. DAGL-a: Diacylglycerol lipase alpha. EPM: elevated plus maze. 3-COMP: three compartment test. USVs: ultrasonic vocalizations. SI: social interaction. ↑increase. ↓decrease. ↔no change.

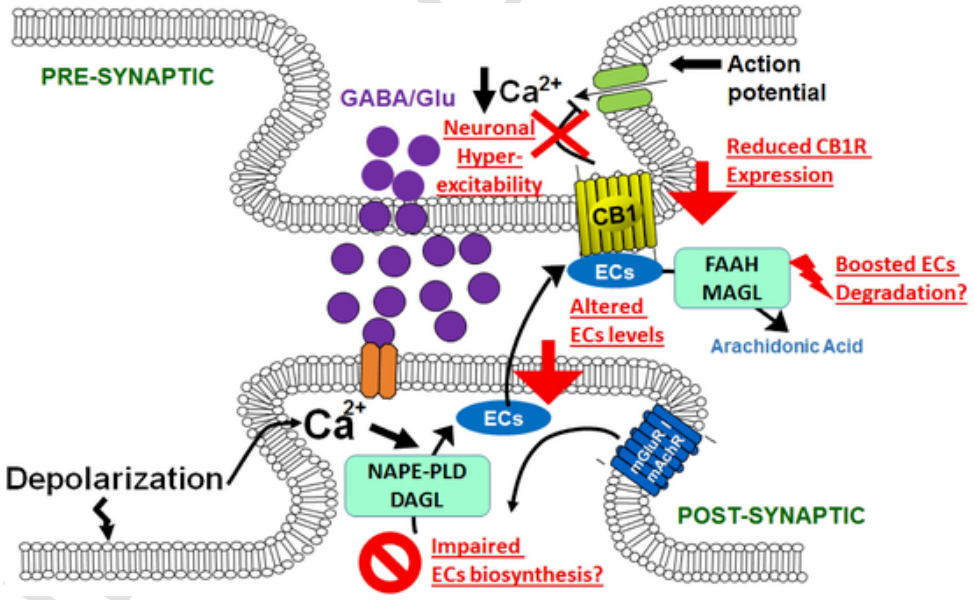

Fig. 1. Schematic representation of ECS functionality and its alterations in patients with ASD.
ECs: endocannabinoids. AEA: anandamide. 2-AG: 2-arachidonoyl-glycerol. NAPE-PLD: N-acyl phosphatidylethanolamine-specific phospholipase D. DAGL-a: Diacylglycerol lipase alpha. FAAH: fatty acid amide hydrolase. MAGL: monoacyl-glycerol lipase. The elements of the system that are known to be altered in ASD patients are marked in red color. Direction of the red arrows indicates increase or decrease.

deletion of the gene encoding the CB1r (Bellocchio et al., 2010, 2013; Monory et al., 2006), as well as the genes of associated metabolic proteins [NAPE-PLD, DAGL-a, FAAH and MAGL; reviewed in (Busquets-Garcia et al., 2018)] are available (see also Table 3). These genetically modified engineered mice have allowed identifying a plethora of ECS-dependent regulation of brain and peripheral functions (Cristino et al., 2020), involving different cell types and cellular processes (Busquets-Garcia et al., 2015).

3. Clinical and pre-clinical evidence of ECS alterations in ASD: identifying novel therapeutic strategies

3.1. Alterations in ECS functionality in ASD patients and related pharmacological approaches

An etiopathological role of alterations in ECS functionality has been already suggested for several psychiatric (Rubino et al., 2015) and neurological disorders (Iannotti et al., 2016), such as schizophrenia, anxiety, depression, neurodegenerative diseases, epilepsy and others (Cristino et al., 2020). This is not surprising, considering the major impact of the ECS in the modulation of neuronal and synaptic functions, as well as of brain development. Indeed, a clear contribution of the ECS has been demonstrated on behavioral functions that are altered in several neuropathologies, including ASD. The impairments span across cognitive and emotional abilities, circadian rhythms, epileptic seizures and social interaction [e.g., (Marco and Laviola, 2012; Monory et al., 2006; Trezza et al., 2012; Vaughn et al., 2010)]. Beside this indirect evidence, recent findings have provided a more direct link between ECS functionality and ASD. Human neuroimaging studies have for instance demonstrated that variations of the cannabinoid receptor gene are involved in the modulation of social reward responsivity, specifically in the striatal response to happy faces (Chakrabarti et al., 2006), a function which is specifically altered in ASD patients.

As illustrated in Fig. 1, several alterations in ECS functionality have been described in ASD patients. Post-mortem analyses showed reduced expression of CB1r in several areas of ASD brains (Purcell et al., 2001), while recent studies reported that AEA plasmatic concentrations were significantly lower in ASD children compared to controls (Karhson et al., 2018), in the absence of changes in blood levels of 2-AG (Aran et al., 2019b). It is still not clear whether the reduced AEA levels could be due to a reduced activity of NAPE-PLD, the enzyme that synthesizes AEA (Schultz et al., 2021), or increased activity of FAAH, the enzyme that degrades them. Nonetheless, it is increasingly evident that AEA circulating levels can represent a useful biomarker for the early identification of subpopulations of affected patients and individual at risk and to design specific therapeutic strategies. Circulating ECs could indeed contribute to predict autism risk prior to the onset of behavioral abnormalities and to initiate early interventions, since alterations in AEA levels appeared to be inversely correlated with age in ASD patients, as the most marked reductions were detected in ASD children (Aran et al., 2019b; Karhson et al., 2018). Also, it has been suggested that a decrease in the levels of circulating endocannabinoids may be a central mechanism linking certain environmental risk factors to ASD, e.g., early exposure to pharmacological agents (Schultz et al., 2021). Treatments compensating the reduced EC tone would then be a possible valuable strategy to rescue or prevent these environmentally-induced forms of ASD (Schultz et al., 2021).

Taking into account the relevance of the endocannabinoids in ASD, it is not surprising that some phytocannabinoids, such as Cannabidiol (CBD), have been suggested as therapeutic strategies for this disorder (Poleg et al., 2019). It has been indeed hypothesized that its therapeutic effects could be due to the inhibition of FAAH (Di Marzo and Piscitelli, 2015) and therefore a compensation of the low levels of AEA found in ASD patients (Aran et al., 2019b; Schultz et al., 2021). Once again, the assessment of EC levels could be a useful tool to screen sub-populations of ASD patients who would more markedly benefit from treatments with phytocannabinoids, as suggested by some authors (Schultz et al., 2021).

Several clinical studies demonstrated the therapeutic effects of CBD in pathological conditions associated with ASD, including social phobia (Bergamaschi et al., 2011) and epilepsy (Devinsky et al., 2016), as well as for the ASD and cognitive symptoms of Fragile X patients (Heussler et al., 2019; Tartaglia et al., 2019). Moreover, there are preliminary reports of beneficial effects of CBD in idiopathic ASD itself (Barchel et al., 2018). For instance, recent clinical studies demonstrated that CBD-rich cannabis (with a ratio of CBD:THC of 20:1) reduced behavioral outbreaks in children with ASD and severe behavioral problems (Aran et al., 2019a), and significantly reduced disruptive behavior and secondary autistic measures in ASD patients (Aran et al., 2021), such as alterations in Social Responsiveness Scale and Autism Parenting Stress Index (in the absence of treatment-related serious adverse events). Furthermore, a study using resting state fMRI demonstrated that the brain response to a single oral administration of CBD differed between individuals with and without ASD. CBD significantly altered the functional connectivity of the cerebellar vermis with several of its subcortical (striatal) and cortical targets (Pretzsch et al., 2019), but only in ASD patients, suggesting the efficacy of this phytocannabinoid in treating the abnormal levels of cerebellar excitation–inhibition levels, known to be correlated with abnormal cerebro-cerebellar functional connectivity in patients with ASD (Hegarty et al., 2018).

Furthermore, rare genetic variants in the core endocannabinoid system genes (e.g., coding for CB1r, CB2r, DAGL-α, FAAH and MAGL) were identified in molecular testing data from a large sample of patients with a broad spectrum of neurological disorders, showing that heterozygous rare variants in DAGL-α were significantly associated with seizures and ASD (Smith et al., 2017). In addition, increased expression of CB2 receptors has been described in blood monocytes of ASD patients (Siniscalco et al., 2013), suggesting a role of the ECS in mediating the immune alterations of ASD pathology. CB2r is indeed able to modulate development, migration, proliferation, and effector functions of immune cells (Basu and Dittel, 2011), and alterations in immune system have been reported in ASD patients (Ormstad et al., 2018; Siniscalco et al., 2018). Overall, the existing clinical data on ECS alterations point towards a reduced endocannabinoid tone which might then be the leading cause for the neuronal hyper-excitability often associated to ASD (Fig. 1) (Bozzi et al., 2018; Sohal and Rubenstein, 2019). Based on this large body of evidence from ASD patients, several studies have employed animal models in the last years to better evaluate the role of the ECS in the etiopathology of ASD.

3.2. Alterations of ECS in ASD animal models

Alterations in ECS functionality have been described in several animal models of ASD [reviewed also in (Zamberletti et al., 2017)], based on the approaches described in Section 1.4 of the present review (Table 1).

The ASD model that has been the most extensively investigated in relation to the ECS is the Fmr1-KO mouse, since this is also a model of another major (but monogenic) developmental disorder associated with ASD, that is Fragile X syndrome (reviewed in (Pietropaolo and Subashi, 2014). As Fragile X syndrome is also a developmental pathology lacking therapeutic targets, a large amount of research has been devoted on the role of the ECS in Fragile X syndrome on its own. Although the validity of the Fmr1-KO mouse to model ASD is still debatable, because the construct validity of this animal model is limited to Fragile X syndrome (i.e., mutation in the X-linked Fmr1 gene and lack of FMRP protein), its relevance to study ASD is nowadays widely recognized, since Fragile X syndrome represents the most common monogenic cause of ASD (Chaste et al., 2012; Clifford et al., 2007; Harris et al., 2008; Kielinen et al., 2004; Losh et al., 2012; Reddy, 2005; Toriello, 2012). Nonetheless, caution should be taken in the strict application of the findings from the Fmr1-KO model to ASD, since it is possible that they better suit a distinct clinical subpopulation of ASD patients with Fragile X syndrome. Alterations in the ECS functionality have been demonstrated in Fmr1-KO mice by several studies, highlighting a complex imbalance in EC signaling. It was demonstrated that the coupling between mGluR activation and ECs mobilization was altered in Fmr1-KO mice in several brain areas (Jung et al., 2012; Maccarrone et al., 2010; Zhang and Alger, 2010), in the absence of alterations in the expression of

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cannabinoid CB1r (Zhang and Alger, 2010) and baseline levels of 2-AG (Jung et al., 2012). Enhanced activity of diacylglycerol lipase (DAGL) was also reported in the dorsal striatum of these mutants (Maccarrone et al., 2010). This might provide a probable substrate for the enhanced glutamatergic activity often observed in the Fmr1-KO mouse model.

We also obtained results supporting altered ECS functionality in Fmr1-KO mice, as demonstrated by the analysis of the typical behavioral effects of acute pharmacological challenge of the ECS in domains unrelated to ASD, using either the CB1r antagonist rimonabant, or an agonist, WIN 55,212-2 (Pietropaolo, 2020). On one hand, Fmr1-KOs showed a similar reduction to WT littermates in fasting-induced food intake, i.e., a classical behavioral effect of acute rimonabant administration (3 mg/Kg, intraperitoneal) which has been shown to depend on peripheral CB1r (Bellocchio et al., 2013; Gomez et al., 2002). On the other hand, mutant mice showed a marked reduced classical behavioral (motor and sensory) response to the acute injection of WIN 55,212-2 (Pietropaolo, 2020), as assessed by the tetrad experiment (i.e., assessing the hypolocomotion, hypothermia, analgesia and catalepsy typically induced by a 3 mg/Kg dose of this CB1r agonist). These results suggest a hypo-responsiveness of central, but not peripheral CB1r in Fmr1-KO mice, that may explain the reduced activation by the exogenous agonists.

A similar result was obtained in the BTBR mouse model for ASD, i.e., showing a reduced sensitivity to the sedative effects of high doses of CBr agonists, in this case, THC (Onaivi et al., 2011). The same authors showed upregulation of gene expression of CB2r in the cerebellum of untreated BTBR mice (Liu et al., 2009), an effect usually observed in C57BL6/mice following acute administration of sedative doses of WIN 55,212-2 (Liu et al., 2009). BTBR mice also showed an enhancement of CB1r functionality in cortical (Gould et al., 2012) and hippocampal areas (Gould et al., 2014) without any reported impact on physiological ECS-mediated processes. Some authors suggested that CB1r upregulation could be related to the hyper-responsiveness of the HPA axis characterizing these mice (Gould et al., 2014). The role of oxytocin was instead underlined by others (Wei et al., 2016), as this neuropeptide drives anandamide mobilization in some brain areas in the mouse, e.g., the nucleus accumbens (NAc), a brain structure that regulates motivated behavior (Wei et al., 2015). Interestingly, AEA mobilization in this brain area is potentiated by social contact and inhibited by social isolation in mice (Wei et al., 2015).

Alterations in ECS functionality were also detected in mouse models based on the “candidate gene” approach. This is the case for instance of mouse models based on mutations in the cell-adhesion molecule neuroligin NLG3 that are implicated in monogenic heritable ASD. These mutant mice displayed at adulthood reduced ultrasound vocalization and a lack of social novelty preference, with variable cognitive deficits (Radyushkin et al., 2009), together with increased occurrence of repetitive behaviors (Rothwell et al., 2014). NLG3 mutants showed reduced CB1r-dependent modulation of GABAergic transmission in several brain areas, including the hippocampus (Foldy et al., 2013), cortex (Speed et al., 2015), dorsal striatum (Martella et al., 2018) and amygdala (Hosie et al., 2018), but not in the nucleus accumbens (Rothwell et al., 2014). These findings suggest the presence of region-specific alterations in the balance of excitatory and inhibitory synaptic transmission (E/I balance), a mechanism known to play a central role in the etiopathology of ASD.

Finally, evidence for the involvement of the ECS in ASD was obtained from environmental-based models, i.e. the VPA and LPS models, although to our knowledge all studies on this issue were conducted in rats. Rats prenatally exposed to VPA displayed altered expression of phosphorylated CB1r in the amygdala, hippocampus and dorsal striatum (Kerr et al., 2013). Since alterations in CB1r phosphorylation may reflect abnormalities in CB1r-activation (Daigle et al., 2008), it has been suggested that the changes observed in VPA rats may reveal a compensatory response aimed at normalizing CB1-mediated signaling and the relative imbalance of the EC system (Garcia et al., 1998), as suggested by the reduced expression of DAGL-α, and NAPE-PLD and FAAH in VPA brains (Kerr et al., 2013; Servadio et al., 2016). In LPS-treated rats, alterations similar to those observed in ASD patients were found in the amygdala, including reduced CB1r expression and increased FAAH levels (Doenni et al., 2016).

3.3. Pharmacological targeting of the ECS in ASD models

The data from mouse models of ASD support the presence of alterations in the functionality of the ECS, involving multiple components of the systems, sometimes with different modalities according to the considered model. Nonetheless, a common aspect of multiple animal models relies in an imbalance in EC signaling, suggesting the therapeutic relevance of its correction by targeting different components of the ECS, including the functionality of CB receptors as well as the synthesis/degradation of the 2-AG and AEA (as illustrated in Table 2).

When the impact of pharmacological manipulations of CB receptors was studied, the effects seem to critically depend on the type of animal models and of the behavioral alterations analyzed. In Fmr1-KO mice, CB1Rr blockade (also through genetic approaches, i.e., generation of double knock-outs for CB1r and Fmr1) eliminated the cognitive and nociceptive alterations together with the enhanced susceptibility to audiogenic seizures, while the pharmacological blockade of CB2R rescued anxiety abnormalities (Busquets-Garcia et al., 2013, 2014). As some of these beneficial effects were accompanied by normalization of overactivated mTOR signalling and were observed also following pharmacological inhibition of mTOR or mGluR5 in the same mutant mice, some authors have suggested that mTOR pathway -regulating cell growth, proliferation/survival, and metabolism (Lipton and Sahin, 2014; Wipperman et al., 2019)- may represent a key mechanism to explain the role of ECS in ASD (Busquets-Garcia et al., 2014). The same authors have supported the potential therapeutic applications of rimonabant for FXS and ASD, although beneficial effects were described only on the cognitive ASD-relevant deficits of Fmr1-KO mice (Gomis-Gonzalez et al., 2016). Given that the same authors failed to detect alterations in LTD in the hippocampus of Fmr1-KO mice (Busquets-Garcia et al., 2013, 2014), it is possible that ECS blockade might impact on FMR1-KO cognitive deficits not by acting on CB1-dependent synaptic plasticity alterations, but merely on intracellular signaling mechanisms. This hypothesis may explain the discrepancies obtained from other ASD mouse models, e.g., the BTBR (Wei et al., 2016) and NLG-3 (Speed et al., 2015) lines, where no behavioral rescue or region-specific brain effects were induced by CBr-inhibitors (Table 2).

The use of direct or indirect CBr agonists seems instead a more convincing pharmacological approach, in line with the evidence of an overall reduced functionality of the ECS in ASD, supported by clinical and pre-clinical studies (see Fig. 1 and Table 1). In NL-3 mice, the administration of CB1r synthetic agonists induced a partial rescue of striatal LTD (Martella et al., 2018), as well as beneficial behavioral effects, i.e., a significant reduction in aggressive behavior (Hosie et al., 2018). Nonetheless, despite the efforts of considerably lowering the doses (Gomis-Gonzalez et al., 2016), it is unlikely that therapeutic strategies directly targeting central CB receptors may still be actively investigated, due to the risk of severe adverse psychiatric effects, e.g., appearance of anxiety, depression and suicidal thoughts (Akbas et al., 2009). Nevertheless, the neurosteroid pregnenolone was recently shown to be produced upon excessive CB1 receptor activation and to act as a signal-specific allosteric endogenous inhibitor of the receptor itself, thereby providing protection against ECs overload (Vallee et al., 2014). Thus, pregnenolone-derivatives exploiting this mechanism of action are currently being tested in clinical settings and might provide a much safer way to inhibit CB1 receptor activity than orthosteric synthetic antagonists.

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A stronger interest has been instead devoted to target the endocannabinoids, AEA in particular, since they emerge as a common altered element across multiple models (including ASD patients) and therefore a potential central factor in the etiopathology of ASD (Fig. 1). Administration of inhibitors of FAAH (e.g., URB597), thus increasing AEA levels, has shown to eliminates the cognitive (Qin et al., 2015) and social deficits of Fmr1-KO mice (Wei et al., 2016). Also, enhancing 2-AG signaling through administration of JZL184, a pharmacological inhibitor of the 2-AG degrading enzyme MAGL, corrected some of the Fmr1 mutants' behavioral alterations, e.g. hyperactivity and anxiety abnormalities (Jung et al., 2012), without effects on the cognitive alterations (Qin et al., 2015), that once again seem to have an independent modulation in this model. The therapeutic value of FAAH inhibition was observed also in BTBR mice, where it rescued their social impairments. Interestingly, this effect was independent from changes in anxiety-levels and was prevented by CB1r blockade (Wei et al., 2016). Enhancing AEA signaling through inhibition of its degradation was also effective in mitigating the behavioral phenotypes induced by prenatal VPA (Kerr et al., 2016; Servadio et al., 2016) or LPS (Doenni et al., 2016) exposure in environmental rat models of ASD.

In line with the relevance of the endocannabinoids as a therapeutic target in ASD, some phytocannabinoids, such as CBD have been suggested as treatments for this syndrome (Poleg et al., 2019), as previously mentioned. The high interest in CBD and its derived compounds, e.g., cannabidivarin (CBDV), stems from the fact that these molecules do not induce the noxious psychotropic effects typical of directly targeting central CB receptors, as for example THC (Agarwal et al., 2019). Furthermore, CBDV has powerful anti-inflammatory properties (Burstein, 2015), probably due to its action as agonist of transient receptor potential channels (De Petrocellis et al., 2011). Hence, several preclinical studies have been conducted to evaluate the therapeutic efficacy of CBDV in animal models of ASD. For instance, chronic systemic administration of CBDV rescued the social deficits as well as the brain alterations of MeCP2-308 mice (Vigli et al., 2018), a validated model of Rett syndrome. CBDV also eliminated the cognitive deficits of Mecp2 mice, concomitantly normalizing the upregulation of brain CB1r and CB2r, thus further supporting the role of the ECS in this ASD-related developmental disorder (Zamberletti et al., 2019a).

CBDV also rescued the cognitive, social and repetitive alterations shown by VPA rats, restoring hippocampal endocannabinoid signaling and neuroinflammation induced by prenatal VPA exposure (Zamberletti et al., 2019b). Our unpublished results from Fmr1-KO mice also supported the efficacy of CBDV chronic treatments for ASD-like behavioral alterations, although the early timing of intervention appeared to be crucial (Pietropaolo, 2020). The efficacy of CBDV was in fact markedly higher when the treatment was administered starting at weaning than at adulthood in our Fmr1-KO mice. Hence, at least some behavioral alterations may be irreversible by CBDV beyond certain age of development. It remains to be tested if the therapeutic window we highlighted, including the adolescent age (4-7 weeks of age in rodents), may be generalized to other modalities targeting the ECS. Adolescence is an early life phase that is indeed characterized by high levels of neurobehavioral plasticity in rodents as well as in humans (Spear, 2000), and is easier to target with pharmacological interventions compared to earlier developmental phases (where the mother-pup interaction has a major confounding impact).

4. ASD-like phenotypes induced by manipulations of the endocannabinoid system: designing novel animal models for ASD

4.1. Behavioral effects of pharmacological targeting the ECS on ASD-relevant domains in WT animals

The recent predominant research focus on genetic mouse models of ASD has somehow attenuated the interest in pharmacological approaches, though they may provide complementary precious information on the etiopathology of this disorder. Evaluating the ASD-like behavioral effects of pharmacological manipulations of the ECS in wild-type rodents can in fact contribute to shed light on the involvement of this complex neuromodulatory system in this developmental disease. Cannabis intoxication has been shown in humans and laboratory animals to share several symptoms with ASD, such as cognitive and somatosensory impairment, social withdrawal and repetitive behavior (Zehra et al., 2018). However little is still known on the effects of recreational, non-intoxicating doses of cannabinoids on ASD-relevant behaviors, i.e., social behavior. In humans, recreational doses of THC have been shown to modulate the perception of social threats in a recent functional magnetic resonance imaging study (Phan et al., 2008), by significantly reducing amygdala reactivity without affecting primary visual and motor cortical areas. A recent study has suggested an association between maternal cannabis use in pregnancy and the incidence of autism spectrum disorder in the offspring (Corsi et al., 2020).

To date, the evaluation of the behavioral effects of administration of CBr agonists, e.g., THC or synthetic cannabinoids suspensions yielded to contradictory results in wild-type mice [e.g., (Almeida et al., 2014; Argue et al., 2017; Gould et al., 2012; Pietropaolo et al., 2015; Tambaro et al., 2013)], displaying either pro or antisocial effects, according to the dose and the type of tests employed (Table 3). One possible reason for this discrepancy might be the differential expression and activation of CB1r in different neuronal populations. Indeed an elegant series of studies shed light on the biphasic effects of cannabinoids on both feeding and anxiety (Bellocchio et al., 2010; Rey et al., 2012; Ruehle et al., 2012) showing that opposite effects of different doses were mediated by activation of CB1r on different neuronal populations, either glutamatergic or GABAergic (Bellocchio et al., 2010; Ruehle et al., 2012). A similar scenario may apply also to the effects of cannabinoids on sociability, as suggested by recent studies on conditional knockout mice for CB1r in different neuronal types (Haring et al., 2015, 2011; Terzian et al., 2014). Recently, it has emerged a novel link between THC and social interest, involving CB1r in non-neuronal populations, i.e., in astrocytes (Jimenez-Blasco et al., 2020). This innovative work has demonstrated that 24 h after THC administration, the activation of CB1r located in association with mitochondrial membranes in astrocytes induces a down-regulation of glucose metabolism, thereby decreasing the amount of lactate that is shuttled to neurons. This in turn causes oxidative and bioenergetic stress in the neurons and induces the appearance of social withdrawal in mice (Jimenez-Blasco et al., 2020). It will be interesting to investigate if similar mechanisms are involved in the pathophysiological mechanisms underlying social alterations typical of ASD.

An alternative pharmacological approach to activate the ECS rather than administration of CB1r agonists consists of promoting endogenous endocannabinoids' signalling (Table 3), for instance by enhancing AEA brain levels (as already mentioned above). The administration of AEA hydrolysis inhibitors (e.g.,URB597) either systemically or directly into brain limbic areas (e.g., amygdala and nucleus accumbens), has been reported for instance to increase social activity in rats (Trezza et al., 2012) and reduced the anxiety induced by social defeat in mice (Rossi et al., 2010). Prosocial effects were also detected following administration of JZL-84 in WT rats and mice (Manduca et al., 2016; Schiavi et al., 2019). Hence, activating the physiological functions of the ECS seems to induce promoting effects on social behaviors.

In line with the these results, the blockade of CB1r signaling is almost universally recognized as an « anti-social » treatment in animal research (Table 3), and could therefore be instrumental in inducing ASD-like phenotypes in WT animals with unaltered endocannabinoid tone (in contrast to genetic mouse models of ASD, e.g., the Fmr1-KO and BTBR lines, where normalization of ECS alterations through CB1 inhibition resulted in rescuing of certain ASD-like phenotypes, as described before). Systemic administration of the CB1r blocker AM251 in adult male capuchin monkeys induced a decrease in social and an increase in

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self-directed behaviors, as well as resistance to aversive memory extinction, without concomitant changes in the subjects' locomotor activity (Gonczarowska et al., 2019). AM251 was shown to decrease social interest also in rats (Seillier et al., 2013) and mice (Umathe et al., 2009; Wei et al., 2015), where it also altered social memory (Litvin et al., 2013). In line with these studies, we have investigated the ability of the CB1r antagonist rimonabant of inducing ASD-like behaviors in wild type B6 mice, i.e., the background that is mostly used to engineer ASD models, including the Fmr1-KO mouse. We demonstrated that an acute injection of increasing doses of rimonabant reduced the levels of affiliative behaviors towards a female, in particular of anogenital sniffings, and the number of ultrasonic vocalizations, while it increased those of self-grooming, i.e., it induced an ASD-like phenotype similar to that observed in Fmr1-KO mice (Pietropaolo et al., 2020). Interestingly, no effect on social behaviors was detected following administration of selective CB2 inhibitors (Argue et al., 2017; Trezza and Vanderschuren, 2009), suggesting an exclusive role of CB1 receptor in modulating social interactions.

4.2. Behavioral effects of genetic targeting the ECS in WT mice

Interestingly, ASD-like behaviors were also described following the genetic deletion of CB1r, thus suggesting the possible relevance of CB1r-KO mice to model ASD (Table 3). Impaired sociability (Haller et al., 2004) and social exploration (Haring et al., 2015, 2011) was observed in these KOs [although the effects vary according with the testing context (Gould et al., 2012) and the type of social stimulus (Terzian et al., 2014)], as well as altered social memory (Litvin et al., 2013). While repetitive behaviors, such as enhanced levels of self-grooming, were observed in CB1r-KOs (Litvin et al., 2013; Terzian et al., 2011), we have recently described qualitative and quantitative alterations in ultrasonic vocalizations in CB1r-KO mice, both during development (i.e., in the first post-natal week) and at adulthood (Fyke et al., 2021). These abnormalities were detected through an extensive spectrographic analysis of the calls, including multiple testing days and qualitative parameters. CB1r-KO mice showed also a lack of social interest in the 3-chamber test at adulthood, as well as reduced social investigation in the direct interaction test (Fyke et al., 2021). The emerging importance of CB1r-KO mice to study the role of the ECS in ASD is remarkable, especially because of the availability of KOs in specific neuronal populations, helping to disentangle the impact of glutamatergic, gabaergic, serotonergic, dopaminergic or glial pools of these receptors in the modulation of social and ASD-relevant behaviors (Haring et al., 2015, 2011; Litvin et al., 2013; Terzian et al., 2011, 2014).

In line with the pharmacological findings summarized before, the genetic deletion of FAAH or DAGL induced respectively pro-social or ASD-like behavioral phenotypes (Cassano et al., 2011; Shonesy et al., 2018; Wei et al., 2015), thus confirming the critical role of the ECs. Interestingly, the pro-social phenotype of FAAH-knock-out mice was accompanied by increased serotonergic tone in cortical areas and both elements were eliminated by the acute administration of rimonabant, thus suggesting the critical relevance of anandamide-mediated hyperactivation of CB1r (Cassano et al., 2011).

5. Conclusions and perspectives

Clinical and pre-clinical data strongly support the involvement of the ECS in the etiopathology of ASD, and its relevance as a therapeutic target to design novel pharmacological treatments. Evidence from mouse models of ASD and pharmacological manipulations of the ECS in wild type animals highlight the importance of an imbalance in ECS signaling as a common potential etiopathological mechanism of this complex disorder.

The ECS could therefore be considered as a good candidate to fit into the theory of ASD of "multiple genes, common pathways", also because of its major modulatory role of neuronal functions and brain development. In this latter respect, some authors have recently suggested that the impact of the ECS in ASD could be specifically related to its modulation of brain developmental processes (Yeh and Levine, 2017), an issue that has been poorly elucidated in the context of ASD models. The use of induced pluripotent stem cell derived neurons has already provided valuable insight into the pathogenesis of neurological disorders such as ASDs (Wen et al., 2014), and should therefore be applied to future studies to try to differentiate the relevance of the ECS in abnormal brain development from mature synaptic functionality (Yeh and Levine, 2017).

The complexity characterizing ASD etiopathology reflects somehow the complexity of the ECS itself. On one hand, the discovery of the relevance of endocannabinoids in ASD raised new possibilities for safe targeting the ECS to treat this disorder, as demonstrated by the evidence reviewed here supporting the therapeutic use of phytocannabinoids, such as CBD and CBDV. These compounds are promising because of their anti-inflammatory properties, and because they can enhance endocannabinoid levels without the adverse effects of CB1r direct modulation. On the other hand, our knowledge of the complexity of the ECS has further increased, complicating clinical approaches. The discovery that endocannabinoids can activate different novel receptors and that their metabolic pathways are often shared with other mediators has led to the inclusion of the ECS into an expanded signaling system, known as the endocannabinoidome (Cristino et al., 2020). This enlarged ECS, including several mediators that are biochemically related to the endocannabinoids, together with their receptors and metabolic enzymes, is likely to provide novel molecular targets of therapeutic relevance to be tested in future studies on ASD (Cristino et al., 2020).

Finally, the studies reviewed here support the central relevance of behavioral analysis in modern neuroscientific research. Indeed, the investigation of the role of the ECS as in ASD pathology may be considered itself as a powerful example of the reasons why behavior matters to brain science. This is evident from the need of a thorough behavioral characterization of mouse models of ASD both to demonstrate the therapeutic effects of pharmacological treatments targeting the ECS and to evaluate the ability of manipulations of the ECS to induce ASD-like phenotypes. This need is due to the lack of biomarkers for ASD other than behavioral alterations, and to the complexity of the behavioral ASD-relevant phenotypes. Furthermore, research on the ECS and ASD can provide with an opportunity to further develop behavioral science itself, as demonstrated by the advanced behavioral approaches used in some studies reviewed here to investigate the role of the ECS in modulating complex social behaviors in mice, e.g., ultrasonic communication. The strong complexity of ASD symptomatology affecting highly developed social domains represents indeed a challenge for behavioral neuroscience, thus contributing to assign a centrality to behavioral analysis in the present and future neuroscientific research.

Data availability

No data was used for the research described in the article.

Declaration of Competing Interest

The authors report no declarations of interest.

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