RESEARCH ARTICLE

Communication and social interaction in the cannabinoid-type 1 receptor null mouse: Implications for autism spectrum disorder

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Abstract

Clinical and preclinical findings have suggested a role of the endocannabinoid system (ECS) in the etiopathology of autism spectrum disorder (ASD). Previous mouse studies have investigated the role of ECS in several behavioral domains; however, none of them has performed an extensive assessment of social and communication behaviors, that is, the main core features of ASD. This study employed a mouse line lacking the primary endocannabinoid receptor (CB1r) and characterized ultrasonic communication and social interaction in CB1$^{-/-}$, CB1$^{+/-}$, and CB1$^{+/+}$ males and females. Quantitative and qualitative alterations in ultrasonic vocalizations (USVs) were observed in CB1 null mice both during early development (i.e., between post-natal days 4 and 10), and at adulthood (i.e., at 3 months of age). Adult mutants also showed marked deficits in social interest in the three-chamber test and social investigation in the direct social interaction test. These behavioral alterations were mostly observed in both sexes and appeared more marked in CB1$^{-/-}$ than CB1$^{+/-}$ mutant mice. Importantly, the adult USV alterations could not be attributed to differences in anxiety or sensorimotor abilities, as assessed by the elevated plus maze and auditory startle tests. Our findings demonstrate the role of CB1r in social communication and behavior, supporting the use of the CB1 full knockout mouse in preclinical research on these ASD-relevant core domains.

Lay Summary

The endocannabinoid system (ECS) is important for brain development and neural function and is therefore likely to be involved in neurodevelopmental disorders such as Autism Spectrum Disorder (ASD). Here we investigated changes in social behavior and communication, which are core features of ASD, in male

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and female mice lacking the chief receptor of this system. Our results show that loss of this receptor results in several changes in social behavior and communication both during early development and in adulthood, thus supporting the role of the ECS in these ASD-core behavioral domains.

INTRODUCTION

Autism spectrum disorder (ASD) is a highly heterogeneous group of neurodevelopmental disorders characterized by a broad range of behavioral deficits (Harris et al., 2008; Shubrata et al., 2015); among these, alterations in social behaviors, especially in communication and social interest, are a major core component of ASD symptoms and provide a diagnostic criterion, together with the presence of repetitive/inflexible behaviors (Association, 2013). In the last years, an impressive impetus has been given to research on this neurodevelopmental disorder, with a special emphasis on preclinical animal models.

Despite the substantial and varied recent research efforts, the pathological mechanisms underlying ASD are far from being understood and therapeutic targets still need to be identified. It is known that ASD has a strong, though complex, genetic component, as it has been associated with a diverse array of copy number variants, chromosome duplications/deletions and point mutations (De Rubeis & Buxbaum, 2015; Folstein & Rutter, 1977; Sebat et al., 2007; Velinov, 2019). With regard to brain function, a view of ASD as a connectivity disorder has emerged (Keown et al., 2017), since patients often present with heterogeneous patterns of functional and structural connectivity between and within brain regions which differ significantly from neurotypical individuals (Assaf et al., 2010; Nair et al., 2013).

The endocannabinoid system (ECS) is a promising candidate to understand the etiopathology of ASD and to provide novel therapeutic targets. It is mainly a retrograde inhibitory signaling pathway that includes the primary cannabinoid type-1 receptor (*CB1*r) and the endogenous ligands, the endocannabinoids (Devane et al., 1992; Sugiura et al., 1995). It is a modulator of neuronal functions, as demonstrated by the abundance of *CB1* receptors in the brain (Mackie, 2005), and regulates synaptogenesis and neuronal interconnectivity during development (Berghuis et al., 2007; Mulder et al., 2008), all of them processes that are altered in ASD pathology (Pardo & Eberhart, 2007). In addition, brain expression levels of *CNR1*, the gene coding for *CB1*r, increase during the late embryonic stage and peak during postnatal development (Marsicano & Lutz, 1999).

Alterations in the expression of *CB1*r and other ECS components, as well as in their functionality, have been reported in ASD patients (Karhson et al., 2018; Smith et al., 2017) and in several animal models of ASD (reviewed by Zamberletti et al., 2017). Furthermore, recent clinical and preclinical studies support the efficacy of modulators of ECS in treating ASD symptoms (Bar-Lev Schleider et al., 2019; Jung et al., 2012; Pretzsch et al., 2019). Studies with null mice ($CB1^{-/-}$) have demonstrated that *CB1*r plays a key role in the regulation of several behavioral responses (Haller et al., 2004; Litvin et al., 2013; Shonesy et al., 2018), including ASD-relevant repetitive (Terzian et al., 2011; Varvel & Lichtman, 2002), and social behaviors (Haller et al., 2004; Haring et al., 2011; Litvin et al., 2013; Terzian et al., 2014). Mice lacking *CB1*r also have brain connectivity alterations, a neurological phenotype of ASD (Berghuis et al., 2007; Diaz-Alonso et al., 2012). However, to the best of our knowledge, no studies have been performed specifically on the role of *CB1*r and the ECS in modulating social communication, that is, one of the major domains altered in ASD.

The major form of social communication in mice is based on ultrasonic vocalizations (USVs), produced in the range above human hearing (> 20 kHz). USVs are produced throughout the life of the animal (Wohr & Scattoni, 2013). Shortly after birth, mouse pups vocalize in response to separation from the mother to induce maternal retrieval (Brudzynski, 2009; Scattoni et al., 2009). These isolation-induced USVs represent one of the earliest social behaviors that can be analyzed quantitatively (Ricceri et al., 2007), and have functional similarities to cries produced by human infants, namely the induction of maternal care. During the first postnatal week, mouse pups increase their number of vocalizations, while a subsequent decrease follows during the second postnatal week, that is, a typical inverted U-shaped developmental pattern (Branchi et al., 2001; Sungur et al., 2016). At adulthood, male mice emit USVs during courtship interactions with females (Hammerschmidt et al., 2009; White et al., 1998). USVs are also produced in female to female interactions in a resident-intruder setting, possibly as a strategy of the resident to reduce the aggressiveness of the intruder (Maggio & Whitney, 1985; Moles et al., 2007). Therefore, USVs may provide researchers with an accessible tool for modeling ASD-like communication deficits throughout development and adulthood (Crawley, 2004; Silverman et al., 2010), as demonstrated in several studies with mouse models of ASD (Belagodu et al., 2016; Gaudissard et al., 2017; Scattoni et al., 2008; Schmeisser et al., 2012; Spencer et al., 2011; Wohr et al., 2013; Wöhr et al., 2015).

Surprisingly, to our knowledge, ultrasonic communication in mice lacking *CB1*r has not been investigated yet, as only one USV study has been conducted in $CB1^{-/-}$ pups, but within a chronic stress paradigm (Fride et al., 2005). Also, most behavioral studies - including

those investigating social interest and interaction (Haller et al., 2004; Haring et al., 2011; Litvin et al., 2013; Terzian et al., 2014) - have so far focused exclusively on homozygous $CB1^{-/-}$ male mice. Hence, little is known about potential "dosage" and sex-dependent effects of the $CB1$ mutation on behavior, especially in relation to ASD-relevant phenotypes. Here we therefore examined ultrasonic communication in male and female $CB1$ null mutants, homo ($CB1^{-/-}$) or heterozygous ($CB1^{+/-}$) for the mutation, during development (i.e., between postnatal days 4 and 10) and at adulthood (3 months). Both quantitative and qualitative analyses of spectrographic measurements were performed in order to provide with an extensive characterization of USVs in $CB1$ null mutants and their WT littermates. To complete the assessment of ASD-relevant social phenotypes, social interest in the three-chamber test and social investigation towards a conspecific were also evaluated at adulthood. As confounding differences in anxiety (Fish et al., 2004; Simola & Granon, 2019; Veronesi et al., 2017; Vivian & Miczek, 1993) or sensori-motor abilities (Wada, 2017; Webber et al., 2013) may influence ultrasonic communication and social behavior, adult mice were also assessed in the elevated plus maze and auditory startle tests. The assessment of these behavioral confounders also contributed to the evaluation of the ASD-relevant phenotypes of the CB1 mouse line, since anxiety and enhanced auditory startle are recognized as additional ASD symptoms and are therefore often investigated in genetic mouse models of ASD (Crawley, 2007; Silverman & Crawley, 2014).

METHODS

Ethics approval

All experimental procedures were in accordance with the European Communities Council Directive 2010/63/EEC, and approved by local ethical committee ("Comité d'Ethique pour l'experimentation animale de Bordeaux", CE 50) and the French Ministry ("Ministere de l'enseignement superieur de la recherché et de l'innovation").

Animals

All experiments were performed in homozygous $CB1$ null mutant ($CB1^{-/-}$) mice with a targeted deletion of $CNR1$ gene and their heterozygous ($CB1^{+/-}$) and wildtype ($CB1^{+/+}$) littermates. Mice were obtained from breeders on a C57BL/6N congenic background, generated as previously reported (Marsicano et al., 2002). $CB1^{+/-}$ virgin males and females were paired for breeding in a temperature- ($21 \pm 1°C$) and humidity- (40%) controlled animal facility (lights on at 07:00 am); approximately two weeks afterwards, pregnant females were individually housed and left undisturbed. The day of birth was considered as postnatal day (PND) 0.

Three batches of mice were used, as described in detail in Table 1: one batch (36 males and 36 females) was tested for USVs during development between PND 4 and 10 (Experiment 1a); a subgroup of the same batch (24 males and 24 females) was tested again for USVs at adulthood (Experiment 1b). A second batch of adult mice (23 males and 26 females) underwent the tests of social interest in the three-chamber apparatus and of direct social interaction with an adult female. A third batch of mice (34 males and 27 females) was tested at adulthood in the elevated plus maze followed by the auditory startle test. Overall, our sample size (calculated according to the resource equation method Charan & Kantharia, 2013) combined with the statistical approach including parametric methods and repeated measures was in line with the "classical" (Still, 1982) and more recent (Charan & Kantharia, 2013; Festing & Altman, 2002) guidelines for

T A B L E 1 Experimental plan of the study

Experiment	Testing age/cohort	N (male)	N (female)	Behavioral test	Behaviors analyzed
1a	PND 4, 6,8,10/ cohort 1	9 WT	13 WT	Maternal separation	Ultrasonic vocalizations
		14 HET	17 HET		
		13 KO	6 KO		
1b	Adult (3 m)/cohort 1	6 WT	8 WT	Direct social interaction with an intact adult NMRI female	Ultrasonic vocalizations
		11 HET	11 HET		
		7 KO	5 KO		
2	Adult (3 m)/cohort 2	8 WT	12 WT	Three-chamber-test, direct social interaction with an ovx adult NMRI female	Social interest, social investigation
		10 HET	8 HET		
		5 KO	6 KO		
3	Adult (3 m)/cohort 3	9 WT	7 WT	Elevated plus maze, auditory startle	Anxiety-like behavior, startle reactivity
		16 HET	9 HET		
		9 KO	11 KO		

Note: A subgroup of the cohort used for Experiment 1a was re-assessed in Experiment 1b, while separate cohorts of adult mice underwent Experiments 2 and 3. Experiment 1a used male and female pups obtained from 11 litters, including all genotypes.
Abbreviations: Ovx, ovariectomized female; PND, postnatal day.

data analysis in animal studies. Nonetheless, we were partially limited in the sample size by the following factors; (a) litters including all three genotypes were preferentially selected in the study, (b) primiparous females had to be used for breeding in all experiments (as commonly done in studies including pup USVs, since previous maternal experience may affect USV's characteristics, e.g., Francia et al., 2006), and (c) breeding was based on heterozygous crossings as suggested by guidelines for mouse studies on behavioral genetics to control for maternal effects (Crusio, 1996; Crusio et al., 2009).

Experiment 1a used male and female pups obtained from 11 litters, including as much as possible all three genotypes ($CB1^{-/-}$, $CB1^{+/-}$, and $CB1^{+/+}$). The approach used here, including a high number of litters together with the use of WT and mutant littermates and testing all litter members without sampling, has been suggested as the most appropriate to protect from the risk of false positives related to litter effects, by past (Chiarotti et al., 1987; Zorrilla, 1997) and recent (Jimenez & Zylka, 2021) guidelines on mouse neurodevelopmental studies. On PND 4 pups were marked after testing by paw tattoo, using a non-toxic odor-less tattoo ink (Ketchum permanent Tattoo Inks green paste, Ketchum MFG. Co.), as previously described (Wohr et al., 2011; Yang et al., 2012). This procedure is routinely used for pups' marking and identification, as it is associated with minimal stress and pain. On the same day tail samples were collected for DNA extraction and subsequent PCR assessment of the genotypes as previously described (Marsicano et al., 2002). Mice were weaned at 3 weeks of age (PND 21), housed in same-sex cages in groups of 3–5 mice/cage in polycarbonate standard cages ($33 \times 15 \times 14$ cm in size; Tecniplast). Mice were left undisturbed until Experiment 1b began, that is, at 3 months of age. Animals for Experiments 2 and 3 were bred and housed as described from Experiment 1, but they were left undisturbed until PND 21, when they were weaned, identified, and genotyped and they were all tested at 3 months of age.

Stimulus mice used for the adult assessment of USVs (Experiment 1b) and of social interest and investigation (Experiment 2) were adult (10 weeks of age) female NMRI mice (Janvier, Le Genest-Saint-Isle), as this strain is commonly employed in social studies (Moles et al., 2000; Moles et al., 2007) since (a) it is characterized by high levels of sociability, (b) is an albino strain, so it facilitates the behavioral analysis during social encounters with B6 mutants, and (c) it has been used in several social tests with mouse models of ASD (Gaudissard et al., 2017; Gauducheau et al., 2017; Hébert et al., 2014; Oddi et al., 2015; Pietropaolo et al., 2014; Pietropaolo, Guilleminot, et al., 2011) and other neurodevelopmental and neurological disorders (Pietropaolo et al., 2015; Pietropaolo, Delage, et al., 2011; Pietropaolo et al., 2012), as well as in mouse social studies with CB1r antagonists (Pietropaolo et al., 2020). They were housed in groups of 3–4 per cage in the same conditions used for test subjects

and left undisturbed for 2 weeks before being used in behavioral tests. We employed intact and ovariectomized NMRI stimulus females, for the adult assessment of USVs (Experiment 1b) and social interest/investigation (Experiment 2), respectively. This allowed us to be in line with most ASD-relevant previous studies assessing USVs in adult male and female mice, including a recent mouse study on USVs and pharmacological inhibition of CB1r (Pietropaolo et al., 2020). These USV experiments mostly employed intact stimulus females (Caruso et al., 2020), concomitantly assessing their estrous phase, as controversial findings have been reported on the impact of estrous phase on USVs (Hanson & Hurley, 2012; Kim et al., 2016; Moles et al., 2007; Nyby et al., 1979; Yang et al., 2015). Hence, we have also used intact female stimuli for USV adult assessment, taking into account their estrous phase as well as that of the female resident in the statistical analysis of the data. The choice of an ovariectomized female for the three-chamber and direct social interaction tests allowed us to directly compare our findings with those from a previous study with CB1 male mutants (Terzian et al., 2014), concomitantly avoiding the potential impact of estrous phase on social interest/interaction and limiting the occurrence of sexual behaviors due to the longer duration of the testing session of direct social interaction. All animal cages were covered by a stainless metal wired lid, provided with sawdust (SAFE, Augy) and ad libitum food and water; they were provided with nesting material as environmental enrichment (Cotton Nestlets).

Behavioral testing

As mentioned above, three behavioral experiments were carried out, using three independent cohorts of mice (Table 1). First, ultrasonic communication was evaluated on PND 4, 6, 8, and 10 in response to maternal separation (Experiment 1a), and again at adulthood in response to an adult female intruder (Experiment 1b). Second, adult social interest and investigation were assessed respectively in the three-chamber and direct social interaction tests (Experiment 2). Finally, adult mice were tested for anxiety-like behavior in the elevated plus maze and for auditory startle response, that is, two behavioral confounding variables potentially acting on ultrasonic communication and social behavior (e.g., Simola & Granon, 2019; Webber et al., 2013). All behavioral procedures were based on experimental protocols used in our previous studies on genetic mouse models of ASD (Gaudissard et al., 2017; Gauducheau et al., 2017; Hébert et al., 2014; Oddi et al., 2015; Pietropaolo et al., 2014; Pietropaolo, Guilleminot, et al., 2011; Zhang et al., 2014). Behavioral tests were performed in adult mice with a 48 h-interval between subsequent tests, and they were carried out by experimenters blind to animals' genotypes. Except for pups' assessment, male and female mice were tested on

separate days, in order to avoid olfactory interference in the testing environment.

Experiment 1a: Assessment of isolation-induced USVs in pups

USVs of $CB1^{-/-}$, $CB1^{+/-}$, and $CB1^{+/+}$ littermates were repeatedly assessed on PND 4, 6, 8, and 10, during a 3-min daily session at room temperature (22°C–24°C). Pups were taken individually from the nest in a random sequence and placed into a glass container (10 × 8 × 7 cm; open surface), containing clean bedding material (3 cm). USVs were captured by an UltraSoundGate Condenser Microphone CM 16 (Avisoft Bioacoustics) placed 20 cm above the bedding. The microphone used is sensitive to frequencies of 15 to 180 kHz with a flat frequency response (± 6 dB) between 25 and 140 kHz. It was connected via an UltraSoundGate 116 USB audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were recorded with a sampling rate of 250 kHz in 16-bit format by Avisoft RECORDER (version 2.97; Avisoft Bioacoustics). At the end of the 3-min session, each pup was weighed and identified, while the container was cleansed with 70% ethanol solution and filled with clean bedding.

For acoustic analyses, recordings were transferred to Avisoft SASLab Pro (Version 5.20; Avisoft) and a Fast Fourier transformation was applied (512 FFT length, 100% frame, Hamming window, and 75% time window overlap). Call detection was provided by an automatic threshold-based algorithm and the accuracy of call detection by the software was verified manually by an experienced user. Based on previous studies (Wohr et al., 2011), the number of USVs was computed, as well as their mean duration, peak frequency and peak amplitude. In addition, call subtypes were determined by density plots depicting the distribution of total calls for each genotype at peak frequency versus peak amplitude, peak frequency versus duration, and peak amplitude versus duration, as described in details elsewhere (Mosienko et al., 2015; Wohr, 2014).

Experiment 1b: Assessment of interaction-induced USVs in adults

$CB1^{-/-}$, $CB1^{+/-}$, and $CB1^{+/+}$ male and female littermates were then tested at adulthood in a 33 × 15 × 14 cm plastic cage with 3 cm of sawdust and a metal flat cover. Male experimental subjects were habituated to this apparatus for 15 min prior to testing, while female subjects were isolated in the testing cage for 72 h, in order to induce a status of resident in adult females and therefore promote the emission of USVs toward an adult female intruder (Moles et al., 2007). An unfamiliar stimulus female mouse (an adult intact NMRI female) was then introduced into the testing cage of either male or female subjects and left

there for 3 min. Previous studies have shown that in these experimental settings USVs are mainly emitted by the male mouse in the male–female interaction (Wang et al., 2008; Warburton et al., 1989; Whitney et al., 1973), and by the female resident in the female–female interaction (Maggio & Whitney, 1985; Moles et al., 2007). The ultrasonic microphone previously described was mounted 2 cm above the cover of the testing cage; subsequent scoring of USV parameters was performed following the same procedures described for experiment 1a.

The estrus phase of adult females was assessed by analysis of vaginal smears (Caligioni, 2009) performed on the testing day in both the experimental subjects and NMRI stimulus mice. The evaluation of $CB1^{-/-}$, $CB1^{+/-}$, and $CB1^{+/+}$ females was conducted after their testing, in order to minimize the potential stress effects of the manipulation necessary for determining the estrous phase. Stimulus NMRI females were approximately half in diestrus and half in estrus phases, and their assignment to social encounters was equally distributed between genotypes and sexes. The estrus phase of experimental female subjects included proestrus, estrus and diestrus, following a distribution that was balanced across genotypes ($CB1^{-/-}$ females included 2 in proestrus, 2 in estrus and 1 in diestrus; $CB1^{+/-}$ were 4 in proestrus, 4 in estrus and 3 in diestrus; and $CB1^{+/+}$ included 3 in proestrus, 2 in estrus and 3 in diestrus).

Experiment 2: Assessment of social interest and social investigation in adult mice

Mice of a second cohort were assessed first in the three-chamber test for social interest and 48 h later in the direct social interaction; both tests used an ovariectomized NMRI adult female as the social stimulus, since the estrous phase of the stimulus animal is known to affect social interest and investigation (Baudoin et al., 1991; Liu et al., 2010). The estrus phase of experimental female subjects was assessed as described in Experiment 1b, and no differences in the distribution of estrous phases were found between genotypes.

Three-chamber test for social interest

The three-chamber apparatus was made of transparent Plexiglas (its detailed description was provided elsewhere Gauducheau et al., 2017). Each side compartment contained a perforated stimulus cage (8 × 8 × 15 cm) placed at a distance of 5.5 cm from the side walls.

Each experimental animal was placed in the middle of the central compartment and allowed to explore the whole apparatus for two trials of 5 min each (Pietropaolo, Guilleminot, et al., 2011). On the first trial the stimulus cages were empty and the experimental mouse was left undisturbed to explore the apparatus and habituate to the testing environment. At the end of this trial, the experimental mouse was confined in the central compartment using two transparent Plexiglas magnetic doors for 40 s.

On the second trial, a stimulus mouse (an adult ovariectomized NMRI female) was introduced in one of the stimulus cages, while a novel object (a glass red cylinder) was introduced in the other one. The position of the social stimulus and of the object was counterbalanced between genotypes. The apparatus and the stimulus cages were cleansed with 70% ethanol solution at the end of the second testing trial. The time spent in each of the side compartments containing the stimulus cages was computed from the video files obtained from a camera placed above the center of the apparatus. An experimenter blind to stimulus position and animals' sex and genotypes performed the analysis using Observer XT (version 7, Noldus).

Direct social interaction with an adult female
Each experimental animal was confined in one of the side compartments of the three-chamber apparatus and an unfamiliar stimulus NMRI female was introduced and left for 10 min. Testing sessions were recorded by a camera placed on the side of the compartment and videos analyzed with Observer XT. One observer who was unaware of the genotype and sex of the animals scored the behavior of the test mice, quantifying the time spent performing affiliative behaviors (Gaudissard et al., 2017; Gauducheau et al., 2017; Oddi et al., 2015; Pietropaolo et al., 2014; Pietropaolo, Guilleminot, et al., 2011), that is, sniffing the head and the snout of the partner, its anogenital region, or any other part of the body; contact with partner through traversing the partner's body by crawling over/under from one side to the other or allo-grooming. Nonsocial activities were also measured: rearing (standing on the hind limbs sometimes with the forelimbs against the walls of the cage); digging; self-grooming (the animal licks and mouths its own fur). The testing session was analyzed in 5-min bins, in order to assess social habituation, that is, the expected time-dependent decrease in affiliative behaviors that is known to occur after the initial minutes of a social encounter and is often altered in mouse models of ASD (Pietropaolo et al., 2014; Shah et al., 2013). Furthermore, the separate analysis of the first 5 min allowed a better comparison of our results with those obtained from other studies on social interaction/interest tests, since short testing session (typically between 3 and 5-min-duration) are most commonly employed in experiments on both male and female mice (e.g., Moles et al., 2007; Nadler et al., 2004; Ricceri et al., 2007).

Experiment 3: Assessment of potential confounding nonsocial behavioral alterations in adult mice

Mice of a third cohort were assessed first for anxiety-like behavior in the elevated plus maze and 48 h later in for auditory startle response. The estrus phase of female subjects was assessed as described in Experiment 1b at the end of each behavioral test, and no differences in the distribution of estrous phases were found between genotypes. Prior to each test, behavioral equipment was cleaned using a 70% ethanol solution, followed by water, and dried with paper towels.

Elevated plus maze
The maze described in details elsewhere (Pietropaolo & Crusio, 2009; Pietropaolo, Guilleminot, et al., 2011) was placed 55 cm above floor level, in a quiet testing room with diffuse dim lighting. A digital camera was mounted above the maze, and images were transmitted to a PC running the Ethovision (Version 11, Noldus Technology) tracking system. To begin a trial, the mouse was gently placed in the central square with its head facing one of the open arms and allowed to explore freely for 5 min. We measured the percent time in open arms as ($time_{open\ arms}$/$time_{(open\ +\ closed\ arms)}$) x 100. Total distance moved was also assessed.

Auditory startle response
The whole-body startle response to low intensity auditory stimuli was measured using startle response boxes (SR-LAB, San Diego Instruments), as described in details elsewhere (Gaudissard et al., 2017). Briefly, mice were habituated to the boxes for 24 h prior to testing for 5 min to reduce stress. On the days of testing, mice were presented with pulses of 20-ms duration and varying intensity: +6, +12, +18, and + 24 dB over a white background noise at 66 dB (namely 72, 78, 84, and 90 dB). Startle reactivity was assessed by the scores obtained for the mean of trials for each stimulus level presented.

Statistical analysis

Data from experiment 1a were analyzed using a $3 \times 2 \times 4$ parametric analysis of variance (ANOVA) with genotype and sex as between-subject factors, and day as within-subject factor. For all other experiments, data from males and females were analyzed separately using a one way ANOVA with only genotype as between-subject factor. These separate analyses were necessary as male and female mice had to be tested (a) on different days to avoid odor interference in all experiments, and (b) using a different experimental protocol to allow USV detection in Experiment 1b.

Within-subject factors, that is, stimulus compartment, 5-min-bins, stimulus intensities, were added to the ANOVAs of the data of social interest, social interaction, and auditory startle. Post hoc comparisons using Fisher's PLSD test were performed when appropriate. To better conform to the assumptions of parametric ANOVA, a natural logarithmic transformation was applied to the startle reactivity scores (Experiment 3). Data from the density plots did not undergo statistical analysis, but were used to obtain a qualitative three-dimensional evaluation of USV data, as in previous studies (Mosienko

et al., 2015; Wohr, 2014). All statistical analyses were performed using SPSS Statistics Version 25 and GraphPad Prism 8 (GraphPad Software).

RESULTS

Experiment 1a: Assessment of isolation-induced USVs in mouse pups

CB1 mutation affected the body weight of mouse pups and this effect was detected only in females, where it differed across postnatal days (interaction sex x genotype x day: $F_{6,198} = 3.58$, $p < 0.01$, and interaction genotype x day in females: $F_{6,33} = 3.596$, $p < 0.05$; Figure 1). On PND 4, both $CB1^{+/-}$ and $CB1^{-/-}$ females weighted less than their $CB1^{+/+}$ littermates, and this difference was still found on PND 10, but for $CB1^{-/-}$ pups only (post hoc, $p < 0.05$). No significant genotype difference in body weight gain emerged in male pups (genotype and interaction genotype x day, all n.s.).

All USV parameters followed a developmental pattern, with changes across PNDs. As expected, the number of USVs emitted by pups of both sexes showed a peak occurring on PND 4 and 6 followed by a decrease on PND 8 and PND 10 (day effect: $F_{3,198} = 44.272$, $p < 0.0001$; Figure 2a,b). This pattern was altered in *CB1* mutants, with slight differences between sexes (genotype x day: $F_{6,198} = 2.645$, $p < 0.05$; sex x genotype x day: $F_{6,198} = 2.309$, $p < 0.05$). The most prominent decrease was observed in males on PND 10, and it was less marked in $CB1^{-/-}$ littermates only (genotype x day in males $F_{6,99} = 2.674$, $p < 0.05$; post hoc: $p < 0.05$; Figure 2a), while in females it was observed on PND 8 and it was attenuated in both $CB1^{+/-}$ and $CB1^{-/-}$ animals (genotype x day in females $F_{6,99} = 2.95$, $p < 0.05$; post hoc: $p < 0.05$; Figure 2b).

A similar pattern was detected also on USV duration, with a peak occurring on PND 4–6 and a reduction afterwards (day effect: $F_{3,198} = 19.13$, $p < 0.0001$; Figure 2c, d). This pattern was more marked in male than in female mice, with a more dramatic decrease in call duration on

the last days in males (interaction sex x day: $F_{3,198} = 5.67$, $p < 0.01$), where it tended to be attenuated in $CB1^{-/-}$ mice (interaction genotype x day in males: $F_{6,99} = 2.75$, $p < 0.05$; post hoc: $p < 0.05$, Figure 2c; in females, genotype effects or its interactions: all n.s., Figure 2d).

The peak frequency of the calls increased on PND 8 and 10, and this pattern differed between sexes and genotypes (genotype x sex: $F_{2,66} = 3.07$, $p = 0.05$; Figure 2e,f). The highest increase in peak frequencies was observed in males on PND10 and this was markedly reduced in $CB1^{-/-}$ pups only (interaction genotype x day in males: $F_{6,33} = 10.463$, $p < 0.0001$; Figure 2e), while in females it was detected already on PND 8, and it was almost absent in both $CB1^{-/-}$ and $CB1^{+/-}$ pups (interaction genotype x day in females: $F_{6,33} = 4.989$, $p < 0.05$; Figure 2f). The peak amplitude of USVs tended instead to decrease (softer calls) on PND8 and PND10 (day effect: $F_{3,198} = 155.959$, $p < 0.0001$; Figure 2g,h), with no differences between sexes and genotypes (all effects and interactions, n.s.).

In a subsequent detailed analysis based on 28,756 calls emitted by $CB1^{+/+}$ pups, 44,724 calls by $CB1^{+/-}$ pups, and 31,452 calls by $CB1^{-/-}$ pups, different clusters of isolation-induced USVs were revealed by density plots (Figure 3). In $CB1^{+/+}$ mice a single cluster was identified on PND 4, most USVs being characterized by peak frequencies between 60 and 70 kHz. On PND 6, a second cluster between 80 and 100 kHz appeared, became more prominent on PND 8, and included most USVs as a single cluster on PND10. A similar pattern was observed in $CB1^{+/-}$ and $CB1^{-/-}$ pups except on PND10, when both mutants continued to produce the majority of their USVs in two distinguishable clusters. This effect was found in both male and female mice.

Experiment 1b: Assessment of interaction-induced USVs in adult mice

The *CB1* mutation affected the number of USVs produced at adulthood by both males and females (genotype
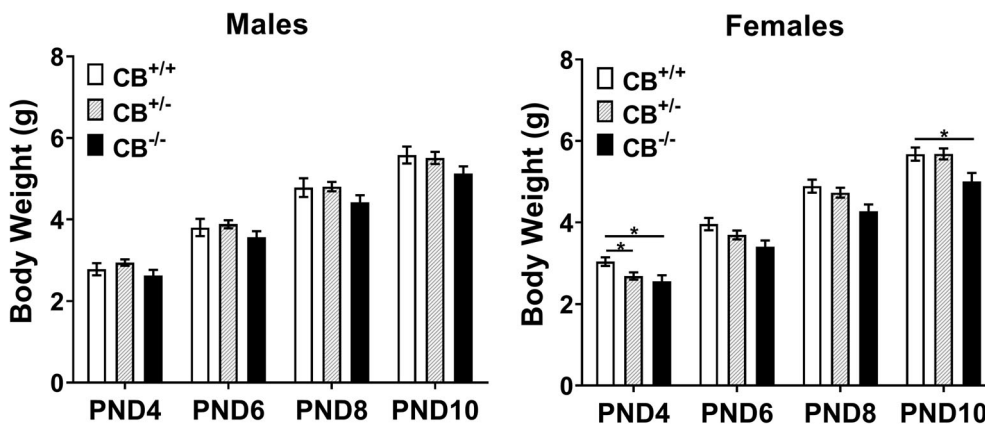

FIGURE 1 Effects of the *CB1* null mutation on body weight during development (Experiment 1a). * = $p < 0.05$. N (males) = 9 ($CB1^{+/+}$), 14 ($CB1^{+/-}$), 13 ($CB1^{-/-}$). N (females) = 13 ($CB1^{+/+}$), 17 ($CB1^{+/-}$), 6 ($CB1^{-/-}$). Male and female pups were obtained from 11 litters, including all three genotypes (CB1−/−, CB1+/−, and CB1+/+). Data are mean ± *SEM*

FIGURE 2 Effects of the *CB1* null mutation on ultrasonic vocalization (USV) during development in mouse pups. Isolation-induced USVs were analyzed in terms of quantitative (a, b) and qualitative (C to H) parameters. * = $p < 0.05$. N (males) = 9 ($CB1^{+/+}$), 14 ($CB1^{+/-}$), 13 ($CB1^{-/-}$). N (females) = 13 ($CB1^{+/+}$), 17 ($CB1^{+/-}$), 6 ($CB1^{-/-}$). All pups were obtained from 11 litters, including all genotypes. Data are mean $\pm$ SEM
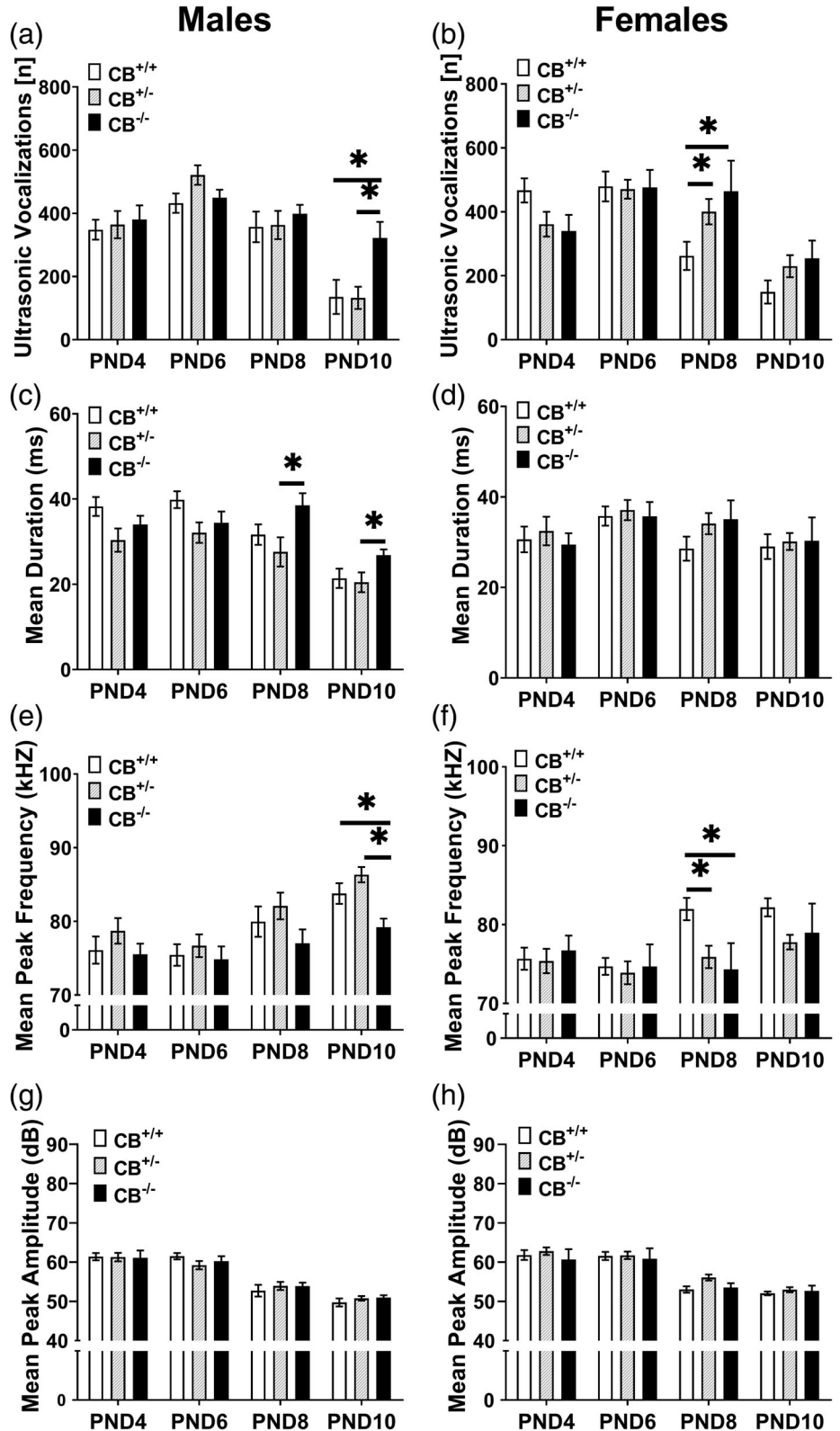
effect, respectively: $F_{2,21} = 15.89$, 4.06, and $p < 0.05$; Figure 4a,e), with $CB1^{-/-}$ mice emitting less USVs than their $CB1^{+/+}$ and $CB1^{+/-}$ littermates (post hoc: $p < 0.05$). No differences in other parameters, including duration, peak frequency and peak amplitude were detected in either sex (all genotype effects, n.s.; Figure 4b–d, f–h).

Stimulus NMRI females were approximately half in diestrus and half in estrus phases, and their assignment to social encounters was balanced between genotypes and sexes. In males, no significant main effect of the estrous phase of the stimulus females ($F_{1,18} = 1.24$, 0.51, 0.03, 1.17, for number, mean duration, peak amplitude and

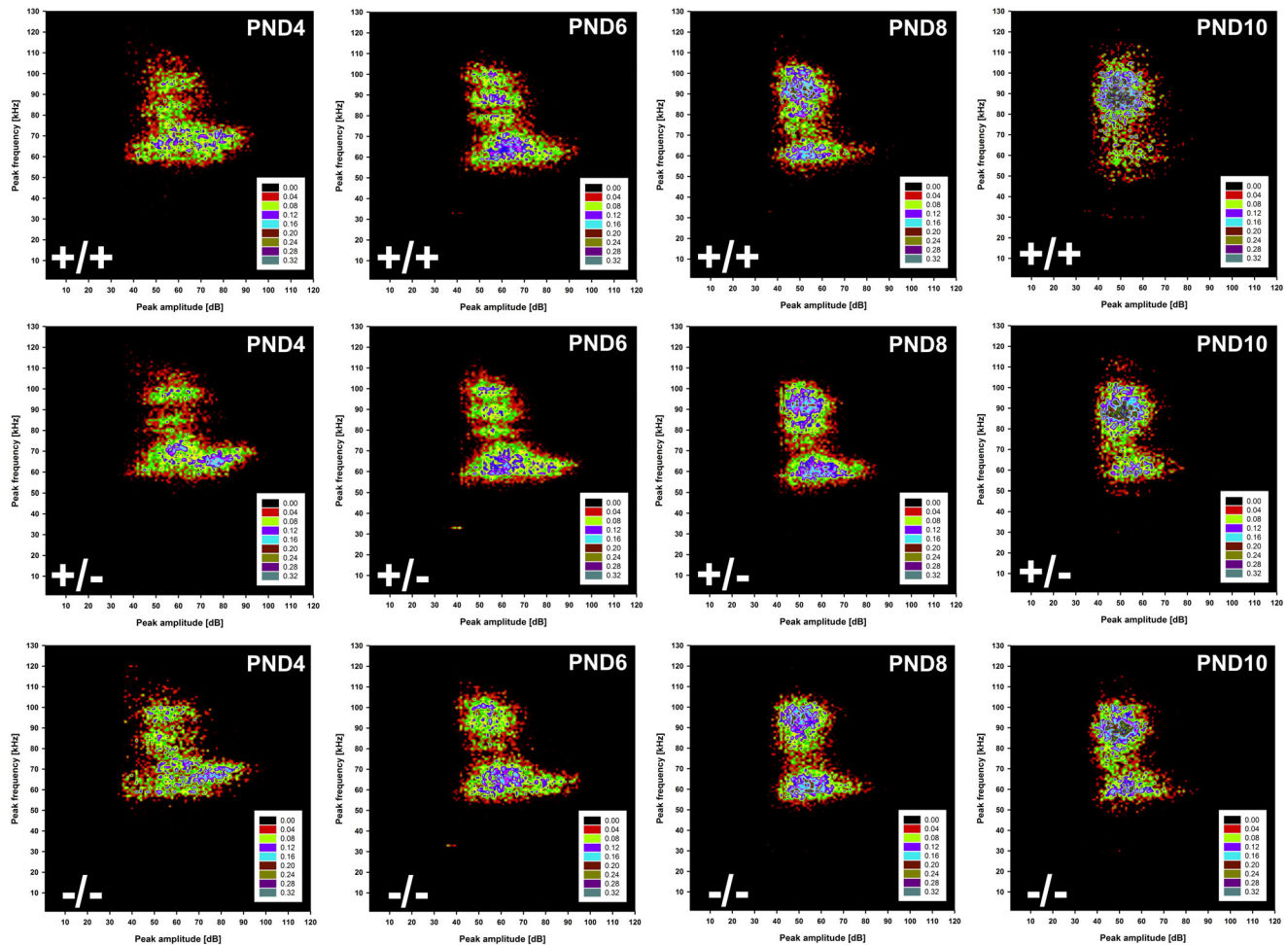**FIGURE 3** Density plots depicting the distribution of individual ultrasonic vocalizations by postnatal day (PND) in mouse pups. Distribution of individual USVs depending on peak frequency in kHz and amplitude in dB in $CB1^{+/+}$, $CB1^{+/-}$, and $CB1^{-/-}$ littermates. Color coding reflects frequency in percentages. Pooled data for both sexes are represented, as no difference between males and females was detected

peak frequency, all n.s.) or its interaction with genotype ($F_{2,18}$ = 0.09, 1.02, 0.79, 0.73, for number, mean duration, peak amplitude, and peak frequency, all n.s.) was detected on any USV parameter. In females, similar results on the impact of the estrous phase of the stimulus animals were obtained, with no main effect ($F_{1,18}$ = 0.03, 0.01, 0.62, 0.05, for number, mean duration, peak amplitude and peak frequency, all n.s.) or interaction with genotype ($F_{2,18}$ = 0.03, 1.13, 1.4, 0.83, for number, mean duration, peak amplitude and peak frequency, all n.s.). The estrus phase of experimental female subjects included proestrus, estrus, and diestrus, following a distribution that was mostly balanced across genotypes. The estrous phase of the experimental subjects did not induce any significant main effect ($F_{2,15}$ = 0.29, 0.94, 1.14, 1.12, for number, mean duration, peak amplitude and peak frequency, all n.s.) or interaction with genotype ($F_{4,15}$ = 0.07, 0.69, 0.93, 1.89, for number, mean duration, peak amplitude and peak frequency, all n. s.) on all considered USV parameters.

As in Experiment 1a, a detailed analysis (Figure 5) was performed in males based on 4106 calls for $CB1^{+/+}$, 11,289 calls for $CB1^{+/-}$, 1082 calls for $CB1^{-/-}$ mice, and in females based on 9237 calls for $CB1^{+/+}$, 8600 calls for $CB1^{+/-}$, and 1283 calls for $CB1^{-/-}$ mice. In $CB1^{+/+}$ males the majority of calls were clustered between 70 and 85 kHz for peak frequency and 5 to 25 ms for the mean duration; while $CB1^{+/-}$ littermates exhibited a similar pattern, $CB1^{-/-}$ males showed substantially more variation in their calls in both mean peak frequency and duration, with the majority of calls occurring in clusters between 65 and 90 kHz and durations between 5 and 50 ms. In $CB^{+/+}$ females most USVs were distributed in two clusters, one between 70 and 75 kHz and another between 80 and 85 kHz, both with durations between 5 and 40 ms. These two clusters were less distinguishable in $CB^{+/-}$ females, and tended to disappear in $CB1^{-/-}$ mice, emitting USVs with a wider variation in both mean peak frequency and duration.
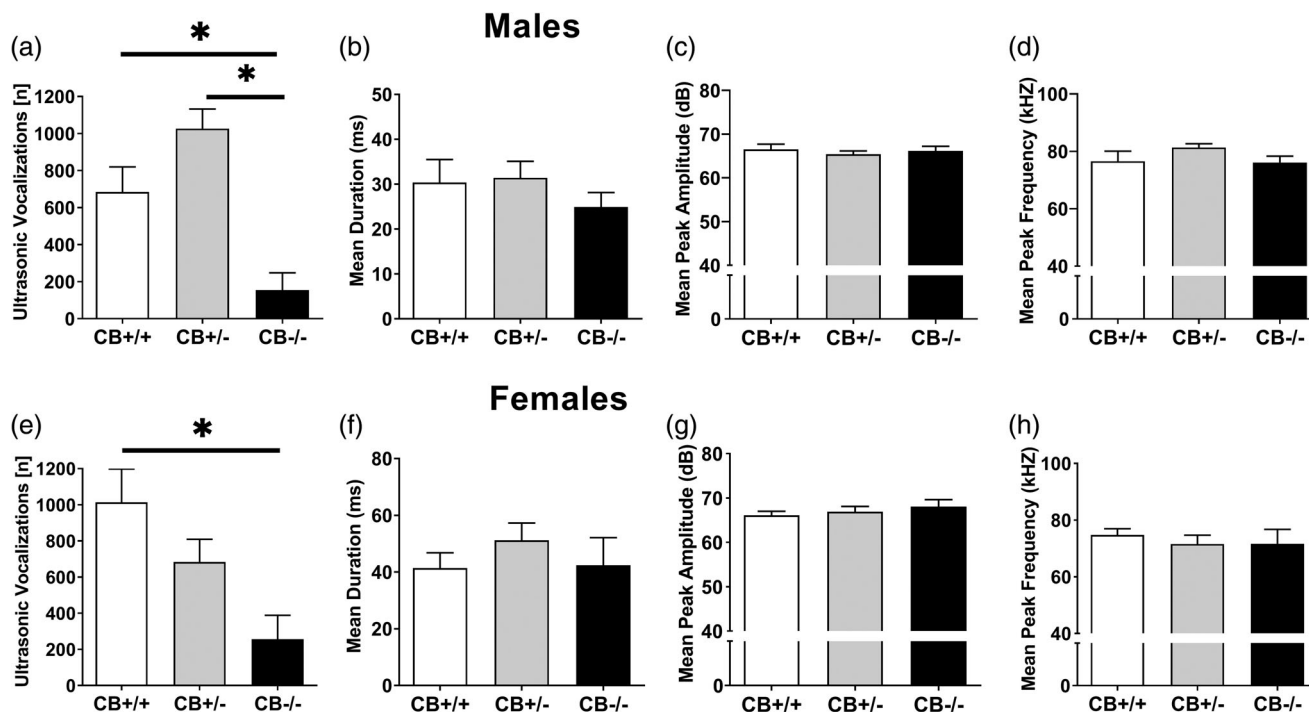
FIGURE 4 Effects of the *CB1* null mutation on interaction-induced ultrasonic vocalization (USV) in adult mice. USVs were analyzed in male and female mice during a 3-min session of direct social interaction with an intact NMRI stimulus female. USVs were analyzed in terms of quantitative (a, e) and qualitative (b, c, d, f, g, h) parameters. * = $p < 0.05$. N (males) = 6 ($CB1^{+/+}$), 11 ($CB1^{+/-}$), 7 ($CB1^{-/-}$). N (females) = 8 ($CB1^{+/+}$), 11 ($CB1^{+/-}$), 5 ($CB1^{-/-}$). Data are mean ± SEM

Experiment 2: Assessment of social interest and social investigation in adult mice

One $CB1^{+/-}$ and a $CB1^{+/+}$ mouse, both females, were excluded respectively from the analysis of the data of the three chamber and direct social interaction tests because of problems in video recording (the precise n for each test is described in the legend of Figure 6). In the three-chamber test, CB1 mutation affected social interest in both sexes (interaction genotype x compartment: $F_{2,20} = 5.32$ and $F_{2,22} = 12.04$, $p < 0.05$, respectively in males and females; Figure 6a–d). $CB1^{+/+}$ and $CB1^{+/-}$ mice showed a clear preference for the compartment containing the social stimulus the (compartment effect: $F_{1,7} = 46.91$ and $F_{1,9} = 10.97$, $p < 0.01$, respectively in $CB1^{+/+}$ and $CB1^{+/-}$ males, and $F_{1,11} = 43.66$ and $F_{1,6} = 16.73$, $p < 0.01$, respectively in $CB1^{+/+}$ and $CB1^{+/-}$ females), while social interest was absent in $CB1^{-/-}$ adult males and females (compartment effect: $F_{1,4} = 0.49$ and $F_{1,5} = 3.09$, n.s., respectively in male and female mutants).

In the direct social interaction test, all mice displayed social habituation, as demonstrated by the reduced time spent in affiliation from the first to the last 5-min of the testing session (effect of 5-min bins, $F_{1,19} = 28.19$, $F_{1,22} = 4.51$, $p < 0.05$, respectively, in males and females; Figure 6b,e). CB1 mutation reduced social investigation, as demonstrated by the reduced time spent in affiliation by $CB1^{-/-}$ males and females compared to their littermates (genotype effect,

respectively: $F_{2,19} = 3.61$ and $F_{2,22} = 4.42$, $p < 0.05$; post hoc: $p < 0.05$; Figure 6b,e). In mice of both sexes, this effect was mainly due to a reduction in the time spent performing anogenital sniffing (Figure 6c–f); in males, this reduction was observed in both $CB1^{+/-}$ and $CB1^{-/-}$ mice during the entire duration of the test (genotype effect: $F_{2,19} = 11.62$, $p < 0.001$; post hoc: $p < 0.05$; Figure 6c). In females, reduced anogenital sniffing was detected only in $CB1^{-/-}$ mice and during the first 5 min of the test (genotype effect: $F_{2,22} = 4.45$, $p < 0.05$; interaction genotype x 5-min bin: $F_{2,22} = 4.39$, $p < 0.05$; post hoc: $p < 0.05$; Figure 6f). No difference among experimental groups was found on nonsocial behaviors (data not shown).

Experiment 3: Adult assessment of potentially confounding nonsocial alterations

In the elevated plus maze, the *CB1* mutation in males or females did not results in behavioral differences either in the percent time spent in the open arms (all genotype effects, n.s.; Figure 7a,b), or in the total distance traveled (data not shown). Similarly, no differences between genotypes were detected in the auditory startle response in both sexes, with only an overall expected effect of pulse intensity ($F_{3,72} = 12.529$, $F_{3,93} = 16.49$, $p < 0.0001$ in males and females, respectively; all genotype effects, n.s.; Figure 7c,d).
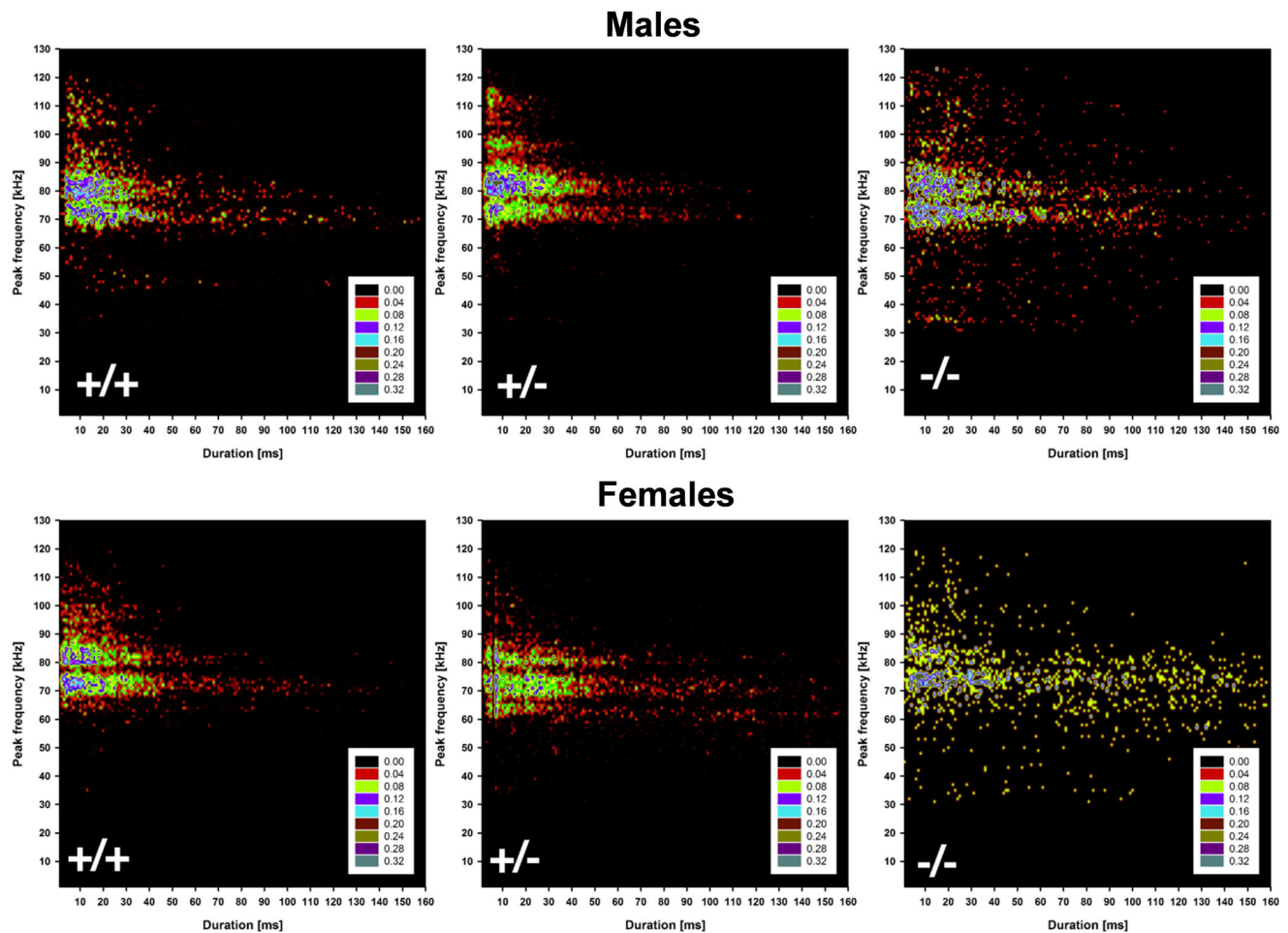
Males

Females

FIGURE 5 Distribution of individual ultrasonic vocalizations (USV) in adult males and females. Density plots depict the distribution of individual USV emitted during 3-min social interaction with an intact NMRI adult female, plotted by frequency in kHz and duration in ms. Color coding reflects frequency in percentages

DISCUSSION

Our data demonstrate that the *CB1* mutation affects ultrasonic communication, both during development and at adulthood, as well as social interest/investigation at adult age. These ASD-relevant behavioral alterations were observed in both male and female mice, and overall seemed more marked in $CB1^{-/-}$ than $CB1^{+/-}$ mutants. Importantly, the adult USV alterations were not confounded by differences in anxiety or sensori-motor abilities, as assessed by the elevated plus maze and auditory startle tests. These findings add to previous evidence showing that *CB1* inhibition in mice induces repetitive and inflexible behaviors (Gomes et al., 2011; Pietropaolo et al., 2020; Terzian et al., 2011; Varvel & Lichtman, 2002), that is, another core ASD-like symptom. Hence, our results further support the potential role of the ECS in the etiopathology of ASD and therefore its relevance as a therapeutic target for autistic pathologies. Our data also support the validity of the *CB1* null mouse line not only for preclinical studies on ASD, but also for studies on the neurobiological mechanisms

underlying the general control of social behaviors and communication.

The present study performed for the first time a comprehensive analysis of ultrasonic communication in *CB1* mutants during early postnatal development (between PND 4 and 10) and at adulthood (at 3 months of age). The alterations in USVs observed in *CB1* mutants were both quantitative and qualitative at both time points. During the postnatal period both male and female $CB1^{-/-}$ pups showed altered day-dependent patterns of expression of multiple USV parameters. These patterns included a developmental profile (Branchi et al., 2001; Sungur et al., 2016) characterizing the number and mean duration of calls produced by $CB1^{+/+}$ mouse pups, with a peak around PND 4–6, followed by a reduction on PNDs 8 and 10 (Figure 2a–d). We observed here a slight divergence from the typical inverted-U shaped developmental USV patterns described in mouse pup studies (e.g., Branchi et al., 2001; Sungur et al., 2016) that could be due to the specific genetic background of our *CB1* mice, that is, the C57Bl6/N. B6/N mice differ from other
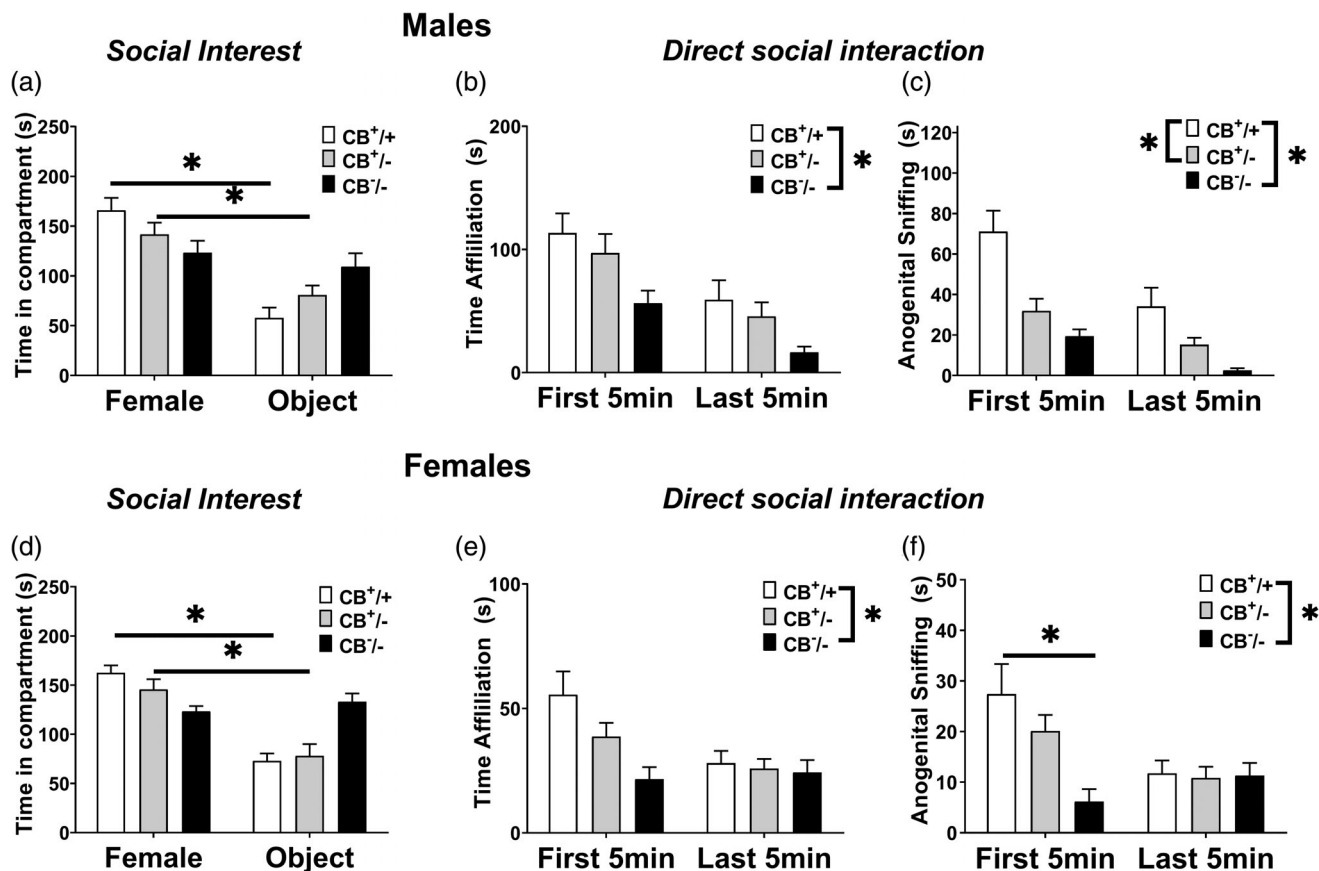
Males

(a) Social Interest

(b) Direct social interaction

(c)

Females

(d) Social Interest

(e) Direct social interaction

(f)

FIGURE 6 Effects of the *CB1* null mutation on social interest and investigation in adult mice. Social interest (a, d) was assessed in the three-chamber test. Social investigation was evaluated 48 h afterwards during a 10-min session of direct social interaction (b,c,e,f), based on the time spent performing affiliative behaviors (Gaudissard et al., 2017; Gauducheau et al., 2017; Oddi et al., 2015; Pietropaolo et al., 2014; Pietropaolo, Guilleminot, et al., 2011), that is, sniffing the head and the snout of the partner, its anogenital region, or any other part of the body; contact with partner through traversing the partner's body by crawling over/under from one side to the other or allogrooming. Both tests used an unfamiliar ovariectomized NMRI stimulus female. The testing session was analyzed in 5-min bins in order to assess social habituation. * = $p < 0.05$ (in b, c, e, and F, it refers to the main effect of genotype on the entire 10-min testing session). N (males) for both tests = 8 ($CB1^{+/+}$),10 ($CB1^{+/-}$), 5 ($CB1^{-/-}$). N (females) for the three-chamber test = 12 ($CB1^{+/+}$), 7 ($CB1^{+/-}$), 6 ($CB1^{-/-}$). N (females) for the direct social interaction test = 11 ($CB1^{+/+}$), 8 ($CB1^{+/-}$), 6 ($CB1^{-/-}$). Data are mean ± SEM

B6 sub-strains in the number, peak amplitude and peak frequency of their USVs during infancy (Wohr et al., 2008), while at adulthood show slightly higher anxiety levels and reduced auditory startle than B6/J, without differing in their social behaviors (Matsuo et al., 2010).

While $CB1^{-/-}$ pups demonstrated a similar peak in USV rate and duration, they did not show a comparable reduction on the following days. This finding suggests a delay in the development of communication abilities in $CB1^{-/-}$ pups, a finding supported by the analysis of other parameters of pups' USVs. Indeed, USV mean peak frequencies also followed a clear developmental pattern, increasing from PNDs 4 to 10 (Figure 2e,f), but this linear increase was markedly reduced in $CB1^{-/-}$ male and female pups. Furthermore, density plots revealed in $CB1^{+/+}$ pups the presence at PND 4 of a single cluster of lower mean peak frequency calls (50–70 kHz), associated at PND 6 and 8 with a second cluster of higher frequency calls (80–100 kHz), and disappearing on PND 10, when

only the higher frequency cluster remained (Figure 3). In $CB1^{+/-}$ and $CB1^{-/-}$ pups both the high and low frequency clusters were instead still evident at PND 10; this finding resembles the pattern observed in the *Shank1* mouse model of ASD (Sungur et al., 2016), and further supports a delay in the communication abilities of *CB1* mutants.

The hypothesis of a general developmental delay in *CB1* mutants is further supported by their reduced body weight gain during the first 10 postnatal days (Figure 1); this reduced body growth was previously described in mouse pups following not only genetic (Fride et al., 2005), but also pharmacological inhibition of *CB1*r (Fride et al., 2001; Schechter et al., 2012), and it has been linked to reduced suckling and milk intake (Fride et al., 2001; Schechter et al., 2012). Nonetheless, body weight differences cannot directly explain the alterations in USV emission rates of *CB1* mutant pups, since they appear much earlier during postnatal development (i.e.,. at PND 4, while
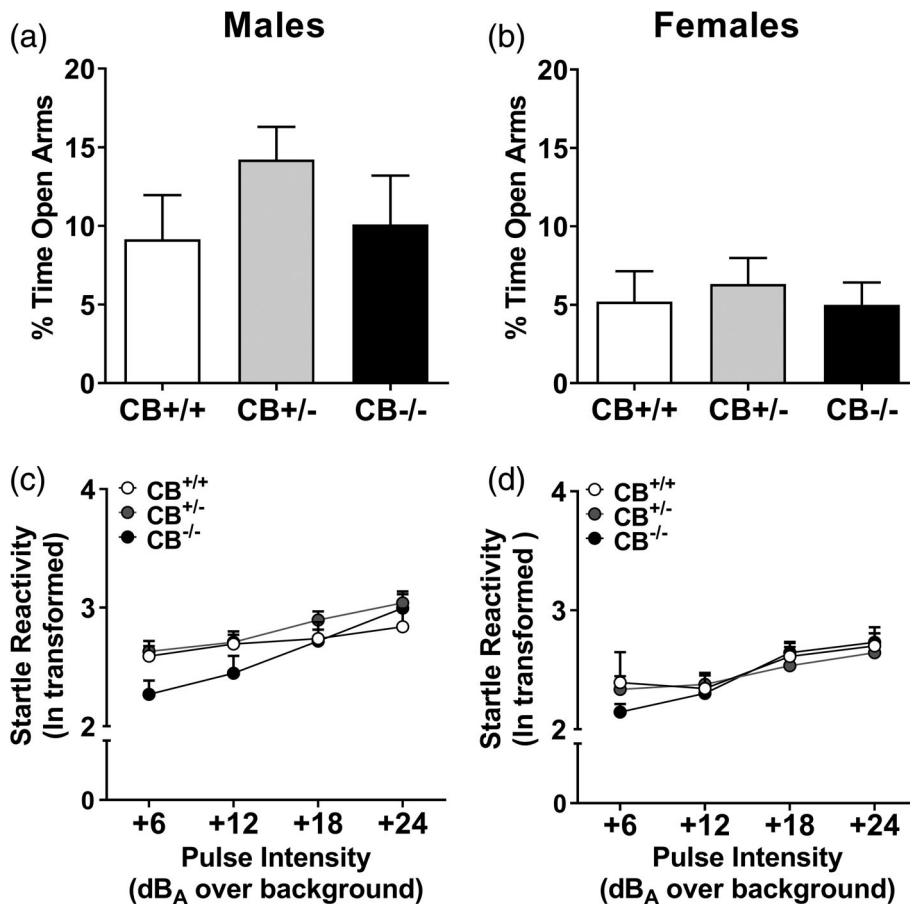
FIGURE 7 Effects of the *CB1* null mutation on confounding nonsocial behaviors in adult mice. Anxiety-like behavior (a, b) in the elevated plus maze and auditory startle response (c, d) were assessed in adult (3-months old) mice. N (males) = 9 ($CB1^{+/+}$), 16 ($CB1^{+/-}$), 9 ($CB1^{-/-}$). N (females) = 7 ($CB1^{+/+}$), 9 ($CB1^{+/-}$), 11 ($CB1^{-/-}$). Data are mean $\pm$ SEM

USV alterations were first detected at PND 8). Furthermore, reductions in body weight are thought to lead to reduced emission rates of USVs because of the decreased pulmonary-thoracic size (Scattoni et al., 2008), while here an increase in USV number was observed on the last postnatal days (Figure 2a,b). Interestingly, an increase in USV rate was also previously described on PNDs 11–13 in rats following systemic administration of the *CB1* inverse agonist/antagonist rimonabant (McGregor et al., 1996). It is instead possible that an overall developmental delay in terms of reflexes and neuro-physiological development may be associated with the USV quantitative and qualitative alterations found in *CB1* mutant pups; future studies evaluating in depth developmental milestones (Branchi et al., 2004) are needed to investigate this issue that is, to our knowledge, still unknown in these mouse mutants.

Our findings on neonatal USVs point out to a developmental delay in CB1$^{-/-}$ pups, since the day-dependent reduction of USV rate is supposed to correspond to increased pup maturity and reduced dependence from the mother (Caruso et al., 2020). Furthermore, previous studies have shown that USV rate in rodent pups reflect their affective state (Branchi et al., 2001; Zimmerberg et al., 1994), with an increase corresponding to an adverse emotional state (Caruso et al., 2020). An increase in pups' USV rate has been described in several mouse models of ASD [reviewed in (Caruso et al., 2020)] and it has been often

interpreted as a sign of enhanced emotional distress; when accompanied by qualitative alterations in spectrographic parameters, such as mean duration or peak frequency, it has also been interpreted as a marker of altered communication abilities, within the context of mother-infant interactions. Interestingly, infants with ASD or at risk for developing ASD, when compared to typically developing infants, emit more cries with certain specific spectrographic characteristics (e.g., higher fundamental frequencies), that have been linked to more aversive conditions and negatively affect parental responses (Esposito et al., 2017; Esposito & Venuti, 2010). Other studies have interpreted the enhanced rate of USVs described in mouse models of ASD as similar to the excessive talking and repetitive speech often found in autistic patients (e.g., Gauducheau et al., 2017, Wheeler et al., 2014). Interestingly, an enhanced mean duration of USVs was also described in the Fmr1-KO mouse model for ASD, both in pups (Gaudissard et al., 2017) and juveniles (Gauducheau et al., 2017), thus suggesting that longer calls may represent a consistent ASD-like phenotype, at least in young mice.

An alternative explanation for USV alterations in *CB1* pups may involve their altered response to stress, as USVs were assessed following a short maternal separation; several studies demonstrated an abnormal behavioral response of *CB1* null mice to stressors in general (Busquets-Garcia et al., 2016; Miller et al., 2008), often

accompanied by enhanced neuro-endocrine reactivity (Barna et al., 2004). Furthermore, previous data demonstrated specifically an altered ultrasonic response to acute stress in $CB1^{-/-}$ mouse pups (Fride et al., 2005), similar to that previously described in rats following pharmacological $CB1$ modulation (Myose et al., 2019; Varga et al., 2012; Varga et al., 2017). Moreover, the persistence of USV alterations into adulthood in our $CB1$ mutants strongly supports the presence of a genuine deficit in communication abilities in these mutants, which was indeed confirmed in the non-stressful context of direct social interaction with an adult female.

Male and female $CB1^{-/-}$ mice showed in fact again at adulthood quantitative and qualitative USV alterations, including a reduction in the call rate (Figure 4a–e), and higher variations in the peak frequency and mean duration of the calls (i.e., reduced clustering), as revealed by the analysis of the density plots (Figure 5). The reduced USV rate is in line with what is observed in other ASD mouse models (Silverman et al., 2010), for example, the BTBR (McFarlane et al., 2008; Scattoni et al., 2011), *Shank* (Ey et al., 2011; Schmeisser et al., 2012) or *Fmr1*-KO mouse lines (Rotschafer et al., 2012), thus supporting the relevance of this quantitative USV alteration as an ASD-like phenotype.

Interestingly, the USV alterations of $CB1$ mutants were not accompanied by altered anxiety or reduced startle response, as found in other mouse models for ASD (Pietropaolo & Subashi, 2014; Yang et al., 2015). In fact here $CB1$ mutants did not differ from their WT littermates in the elevated plus maze and auditory startle tests (Figure 7), in line with previous studies showing that behaviors in these tests were not consistently and robustly affected by $CB1$ homozygous deletion (Haller et al., 2002; Haller et al., 2004; Marongiu et al., 2012). While the USV alterations observed in $CB1$ male and female mutants at adulthood were not linked to emotional or sensori-motor abnormalities, they were instead associated with deficits in social interest in the three-chamber test and in social investigation (Figure 6); interestingly, the genotype differences were more marked for anogenital sniffing, a behavior that has been shown to positively correlate with USVs rate in adult mice (Moles et al., 2007; Nyby, 1983). This finding, together with the presence of social and USV alterations in both sexes, suggests that $CB1$ mutation may affect social interactions and communication by acting on the general sociability of mice. It is possible that other confounding factors may influence the genotype effects observed here on adult social behaviors, such as deficits in olfactory abilities, as well as general reduced explorative behavior and altered locomotor activity. Nonetheless, these behavioral confounders were ruled out by previous studies describing no abnormalities in olfactory discrimination tests (Hutch et al., 2015), and inconsistent alterations in object exploration and locomotion (e.g., Dubreucq et al., 2010; Haller et al., 2002; Haring et al., 2011; Lafenetre et al., 2009).

Our data from the total distance moved in the elevated plus maze and nonsocial exploration in the direct social interaction test further support the lack of confounding differences in locomotion/exploration between WT and $CB1$ mutants.

The deficits showed in social interest and behavior in adult $CB1$ mutants are in agreement with previous reports on $CB1^{-/-}$ male mice (Haller et al., 2004; Haring et al., 2011; Litvin et al., 2013; Terzian et al., 2014), even with stimuli of different sex (Haller et al., 2004; Haring et al., 2011; Litvin et al., 2013; Terzian et al., 2014) and genetic background (Terzian et al., 2014), thus confirming these as a robust behavioral phenotype of $CB1$ null mice. Reduced social investigation and social interest in CB1 $^{-/-}$ male mice was previously described toward an ovariectomised female in hormonally-induced estrous phase (Terzian et al., 2014), thus suggesting the limited impact of the estrous phase of the female stimulus on the social deficit of CB1 mutants. Also, similar deficits in USV rate and social investigation (again, especially on anogenital sniffing) were observed following pharmacological $CB1$ inhibition by rimonabant in male mice, in a dose-dependent manner (Pietropaolo et al., 2020). In agreement with our results, stimulating the endocannabinoid tone by the administration of anandamide hydrolysis inhibitor URB597 induced an increase in social investigation, as well as in the emission rate of USVs (although mostly those at 50 kHz) in adult rats (Manduca et al., 2014). To our knowledge, this is the first time that USV and social alterations are described also in $CB1^{+/-}$ mice, also including female subjects; here, females seem more sensitive to the early effects of $CB1$ mutation, since during development $CB1^{+/-}$ females (but not males) differed in body weight and USVs from WT littermates similarly to $CB1^{-/-}$. At adulthood, "a dosage" effect of $CB1$ mutation seemed evident in males and females, with one allele somehow protecting from the effects of $CB1$ deletion, and C$B1^{+/-}$ positioning between $CB1^{-/-}$ and $CB1^{+/+}$. Despite a traditional focus on the male sex, ASD-research is indeed increasingly interested in evaluating pathological behavioral phenotypes also in females subjects, as ASD female patients may have unique clinical presentations relative to their male counterparts, a factor that may have led to under diagnosis of ASD in the female sex (Loomes et al., 2017). Hence, the presence of communication and social phenotypes in $CB1$ mutant females add to the value of the $CB1$ null mouse to study ASD, an issue that is receiving increasing attention in preclinical research on this pathology (e.g., studies in female *Fmr1*-KO mice modeling ASD, (Gauducheau et al., 2017).

In conclusion, our data support the use of the $CB1$ null mouse in preclinical research on ASD. The lack of nonsocial alterations, that is, emotional or sensori-motor abnormalities, does not undermine the validity of $CB1$ mutants to study ASD, although they may be considered ASD-like phenotypes; first, because recapitulating the

full ASD-like phenotypes is increasingly considered an unrealistic and unnecessary goal of mouse models (Crawley, 2004, 2007; Moy et al., 2006; Silverman et al., 2010), second because it allows to rule out important confounds potentially acting on social and communication behaviors. Thus, the *CB1* null mouse may be instrumental in specifically investigating the neurobiology of social behaviors and communication, that is, the core ASD symptoms, without including other nonsocial symptoms. This approach is particularly suitable to the *CB1* null model, because of the availability of mutant *CB1* mouse lines with region- and cell-specific deletions (Bellocchio et al., 2010; Busquets-Garcia et al., 2016; Hebert-Chatelain et al., 2014; Marsicano & Lutz, 1999; Oliveira da Cruz et al., 2016; Robin et al., 2018), allowing dissecting the behavioral role of *CB1* according to its expression site (e.g., glutamatergic, gabaergic, dopaminergic neurons, in the whole cell or mitochondria only). Hence, future studies combining region-and cell-specific deletions of *CB1* will be able to identify the structures and circuits responsible for the social and communication deficits, thus providing novel avenues for research on ASD.

CONFLICT OF INTEREST
The authors have no conflict of interest to report in relation to the work described.

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