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RESEARCH ARTICLE

Pharmacological inhibition of the primary endocannabinoid producing enzyme, DGL-α, induces autism spectrum disorder-like and co-morbid phenotypes in adult C57BL/J mice

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Abstract

Accumulating evidence links dysfunction in the endocannabinoid system (ECS) with the pathology of neurodevelopmental disorders, particularly autism spectrum disorder (ASD). Variants in ECS genes CNR1 and DAGLA are associated with neurological phenotypes in humans. The endocannabinoids (eCBs), 2-AG and AEA, which act at the primary cannabinoid receptor (CB1), mediate behaviors relevant to neurodevelopmental disorders. The overlap between these eCBs is poorly understood. Most ECS studies have focused on stress responses, anxiety, and epilepsy, however, its role in social behavior and communication has only recently come under investigation. This represents a critical gap in our understanding of the ECS and its relationship to ASD. Furthermore, the increasing prevalence of ASD and a lack of therapeutics emphasize a crucial need for novel therapeutic targets. To this aim, we used an inhibitor of the eCB producing enzyme DGL-α, DO34, and the CB1 inverse agonist, rimonabant, to evaluate the role of the primary eCB, 2-AG, in ASD. Adult male C57BL/6J mice were used in a series of behavioral paradigms which assessed social behavior, social communication, repetitive behaviors, anxiety and locomotor activity. DO34 and rimonabant increased anxiety-like behavior, while only DO34 induced hyperactivity, social deficits, and repetitive self-grooming behavior. These data indicate that reduced 2-AG bioavailability, or CB1 inhibition, each induce unique respective behavioral phenotypes relevant to neurodevelopmental disorders, particularly ASD. This suggests fundamental differences in CB1 signaling via 2-AG and the CB1 receptor itself, particularly for social behaviors, and that 2-AG signaling may represent a target for the development of novel therapeutics.

Lay Summary: Endocannabinoids play a critical role in the developing nervous system. Alterations in the endocannabinoid system are linked to neurodevelopmental disorders. Studies suggest these variants may play a critical role in the core symptoms of autism spectrum disorder. In this study, pharmacological inhibition of the primary endocannabinoid producing enzyme, DGL-α, induced a constellation of deficits in behavioral domains associated with autism.

KEYWORDS
autism spectrum disorders, DGL-α, endocannabinoids, mouse models, neurodevelopmental disorders

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by persistent deficits in social behavior and communication which co-occur with restricted, repetitive patterns of behavior, interests or activities (American Psychiatric Association, 2013). Epidemiological data indicates a notable increase in the prevalence of ASD from 1 in 150 children between 2002 and 2010 to 1 in 59 children in 2014 (Baio et al., 2018). This

dramatic increase has placed a large clinical and financial demand on the public healthcare system (Leigh & Du, 2015). This underscores that identification of causes and therapeutic targets are a public health priority.

Recent studies have revealed a relationship between alterations in the endocannabinoid system (ECS) and patients with non-syndromic ASD or syndromic ASD, such as Fragile X Syndrome (FXS; Aran et al., 2019; Karhson et al., 2018; Smith et al., 2017). Clinical studies have found lower levels of circulating endocannabinoids in ASD patients and post-mortem studies found lower expression levels of the primary cannabinoid receptor (CB1) in the brain (Aran et al., 2019; Karhson et al., 2018). Genetic studies detected associations between neurodevelopmental disorders and genetic variants in the genes for CB1 (*CNR1*) and the primary endocannabinoid (eCB) producing enzyme diacylglycerol lipase alpha (*DAGLA*: DGL-α; Miller et al., 2010; Prasad et al., 2012; Smith et al., 2017). Furthermore, variants in genes which are critical for intact eCB signaling, but not considered part of the ECS itself, such as Fragile X Mental Retardation Protein (*FMR1*), metabotropic glutamate receptors (*GRM5*), Neuroligin (*NLGN3, NLGN4*), Shank (*SHANK3*), and Homer (*H1a, H2a*) are associated with ASD (Foldy et al., 2013; Laumonnier et al., 2004; Wang et al., 2016; Wenger et al., 2016; Yan et al., 2005).

Preclinical studies with mouse models of ASD have partially identified the role ECS dysfunction may play in ASD pathology. Studies of mutations in *NLGN3* revealed alteration in eCB activity and phenotypes which, in some cases, are sensitive to the genetic background (Chadman et al., 2008; Jaramillo et al., 2014; Rothwell et al., 2014). Chadman et al. (2008) detected delayed growth and neurological reflex development in NLGN3R451C mice on a C57BL/6J background, while Jaramillo et al. (2014) found deficits in social, cognitive, and locomotor domains with this mutation against a 129S2/SvPasCrl background. Hosie et al. (2018) found that treatment with a CB1 agonist decreased aggressive behaviors in the NLGN3R451C model, and phenotyping by Rothwell et al. (2014) found the *NGLN3*KO mutation and NLGN3R451C caused unique and shared ASD-like behaviors on the C57BL/6J background. Studies with the FXS mouse model (*fmr1*-KO) revealed that FMRP, the protein lost due to the fragile X mutation, regulates the translation and localization of DGL-α at the post synaptic density (Jung et al., 2012; Maccarrone et al., 2010). Loss of FMRP resulted in delocalization of DGL-α and 2-AG, the eCB produced by DGL-α (Jung et al., 2012). This study found that increasing the bioavailability of 2-arachidonoyglycerol (2-AG) with an inhibitor of the 2-AG deactivating enzyme, monoacylglycerol lipase (MAGL), rescued the hyperactivity and reduced anxiety-like behavior seen in the *Fmr1*-KO mouse. Subsequent studies demonstrated that the scaffolding protein Homer, a protein correlated with ASD, is integral for appropriate 2-AG production (Ronesi et al., 2012; Ronesi & Huber, 2008).

DGL-α forms 2-AG through the hydrolysis of 1,2-diacylglycerol (DAG). 2-AG production occurs on demand through two mechanisms: activation of group I metabotropic glutamate receptors (mGluR5) (eCB_{mGluR}) or via *N*-methyl-D-aspartate (NMDA) receptors (eCB_{NMDA}) (Figure 1). 2-AG, produced at post-synaptic neurons, is a retrograde messenger which acts on presynaptic CB1 and CB2 receptors to suppress neurotransmitter release via P/Q and N-type Ca^{2+} channel inhibition (Sugiura et al., 1995; Sugiura et al., 2002; Suhara et al., 2000; Tanimura et al., 2010). Studies with $DAGL\alpha^{-/-}$ mice or the DGL-α inhibitor DO34, showed that interfering with DGL-α activity eliminated the two major forms of eCB mediated synaptic plasticity, depolarization induced suppression of excitation (DSE) and inhibition (DSI), in prefrontal cortex, hippocampus, striatum, and cerebellum (Gao et al., 2010; Ogasawara et al., 2016; Tanimura et al., 2010; Yoshino et al., 2011).

In regard to behavior, studies which interfered with 2-AG production detected phenotypes relevant to neurodevelopmental disorders. Schurman et al. (2019) compared $DAGL\alpha^{-/-}$ mice to DO34 in C57BL/6J mice in assays of learning and memory. DO34 treatment induced deficits in acquisition and reversal learning without deficits in expression, extinction, forgetting, perseveration or object location. In contrast $DAGL\alpha^{-/-}$ mice displayed profound deficits in all of these domains. Highly specific genetic deletion of DGL-α in direct pathway striatal medium spiny neurons (dMSNs) induced deficits in synaptic plasticity (DSE and DSI), social behavior, and increased repetitive self-grooming behavior (Shonesy et al., 2013; Shonesy et al., 2018). Studies that used either genetic or pharmacological disruption of DGL-α induced anxiety-like behavior (Bedse et al., 2017; Shonesy et al., 2014). These studies highlight the important contribution of 2-AG to behavioral domains impaired in neurodevelopmental disorders.

To our knowledge, no study has directly tested the effect of pharmacological interference with DGL-α activity on measures of social behavior or communication. Accumulating preclinical and clinical evidence shows that pharmacotherapeutics which target ECS dysfunction are promising treatments for neurodevelopmental disorders (Bar-Lev Schleider et al., 2019; Jung et al., 2012; Pretzsch et al., 2019; Wei et al., 2016). Therefore, this represents a critical gap in our knowledge regarding the ECS and neurodevelopmental disorders, particularly with respect to DGL-α and 2-AG.

To address this need, we used the DGL-α inhibitor DO34, and an inverse agonist of CB1, rimonabant, to evaluate pharmacological 2-AG depletion and CB1 inhibition on measures of social behavior and communication. Due to their relevance to ASD and neurodevelopmental disorders, assays of anxiety-like behavior and locomotor activity were also included.

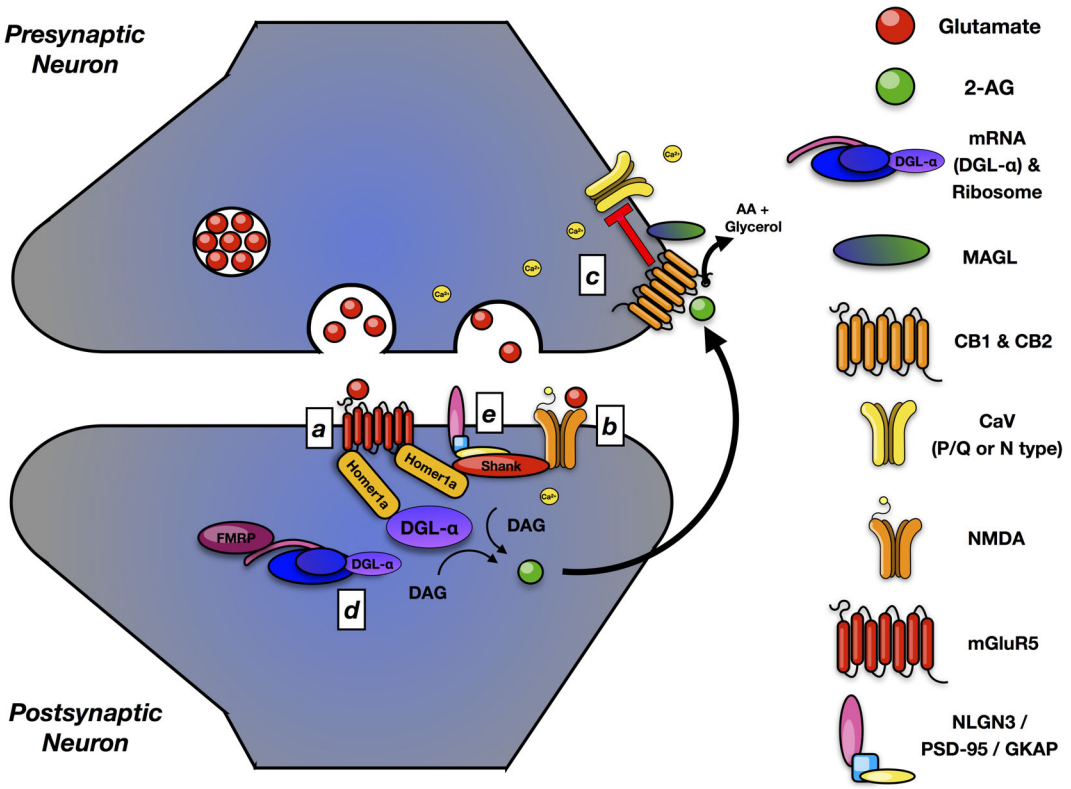

FIGURE 1 2-AG synthesis by DGL-α. Glutamate released from presynaptic vesicles can stimulate DGL-α to produce synthesize 2-AG from DAG via (*a*) mGluR5 activation or (*b*) NMDA mediated Ca^{2+} entry into the post-synaptic neuron. Newly synthesized 2-AG moves retrosynaptically to pre-synaptic CB1 and CB2 receptors which subsequently inhibit P/Q or N type Ca_V channels to suppress Ca^{2+} entry (*c*) and neurotransmitter release. FMRP, the protein lost due to the fragile X mutation, controls the appropriate (*d*) translation and localization of DGL-α within post-synaptic density. (*e*) NGLN3 binds to Shank proteins indirectly via post synaptic density protein 95 (PSD-95) and a guanylate kinase-associated protein (GKAP). Mutations in NLGN3 and Shank proteins are associated with ASD and altered ECS function

MATERIAL AND METHODS

Animals

Adult male C57BL6/J (B6) mice ($n = 51$) treated with DO34 50 mg/kg ($n = 14$), DO34 10 mg/kg ($n = 13$), rimonabant 2 mg/kg ($n = 13$); or vehicle ([Ethanol: Kolliphor:Saline]; $n = 14$), aged 3–6 months were bred in house (from breeders obtained from Jackson Labs) and used for all experiments. 129S1/SvImJ mice ($n = 6$) were used as the stranger mice because they have very low levels of activity so that all interactions were initiated by the subject mice (Moy et al., 2007). Adult female C57B6/L mice ($n = 6$) were used in the direct social interaction test. Because ASD affects a higher percentage of males than females, only male mice were used in the current study. Mice were housed 3–5 per cage with ad lib food and water and 12-h light/dark cycle. All experiments were conducted during the light phase between 9 am and 5 pm. All procedures were conducted in compliance with the National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and approved by the New York State Institute for Basic Research in Developmental Disabilities' Institutional Animal Care and Use Committee (IACUC).

Drug treatment

The drugs used were the DGL-α inhibitor DO34 (10 or 50 mg/kg) (AOBIOUS INC, Hopkinton, MA) and the CB1R antagonist rimonabant (2 mg/kg) (Sigma-Aldrich, St. Louis, MO). Animals were assigned to one of four experimental treatment conditions and administered DO34, rimonabant or vehicle by intraperitoneal (i.p.) injection at a volume of 10 ml/kg in a formulation containing ethanol:Kolliphor:saline (1:1:18; Sigma-Aldrich, St. Louis, MO). Drug pretreatment time was 2 h before behavioral testing. Two doses of DO34 were selected, one for full inhibition (50 mg/kg) and a second at the 50% inhibitory concentration (IC_{50}) (10 mg/kg) as established by Ogasawara et al. (2016) to evaluate the a possibility of dose dependent effects on behavior. Rimonabant dosage was selected based on dosage used by Bedse et al. (2017).

Behavioral testing

Order of testing

Subjects were run in five cohorts of nine mice, each with 2–3 mice per drug/dose. The order of tests was based on the need of performing first those tests that are more influenced by previous testing experience (such as the elevated plus maze), while leaving last tests involving a certain degree of stressful experience (such as those requiring social interactions). Therefore, tests were conducted in the following order: Day 1: EPM, Day 2: Open Field and Social Approach, Day 3: Direct Social Interaction. Tests were conducted >24 h apart to allow for drug washout. Mice received the same drug dose for each experiment.

Elevated plus maze (EPM)

Anxiety-like behavior was tested in the elevated plus maze as previously described (Chadman, 2011) The elevated (95 cm) plus maze consists of two open arms (30 × 5 cm) and two closed arms (30 × 5 × 15 cm) extending from a central (5 × 5 cm) area. A raised lip (0.25 cm) around the open arms minimized falling off the edges of the open arms. Mice were placed in the central area facing an open arm and allowed to traverse the maze freely for 5 min. Arm entries (70% of mouse in the arm) and time spent in the open and closed arms were tracked and scored using ANY-maze software (Stoelting, Inc., Wood Dale, IL). The center of the maze was lighted at 200 lx. This lighting condition was chosen based on Haller et al. (2004), where *CB1*-KO animals demonstrated anxiety-like behavior under high (198 lx), but not low (red) light conditions. Prior to each and all tests, behavioral equipment was cleaned using a 70% ethanol solution, followed by water, and dried.

Open field

The open field test can be used to measure general exploration, anxiety, and locomotor activity in a novel environment. Mice were placed in a $40 \times 19 \times 22\ cm^3$ transparent plexiglass apparatus for 10 min. The center of the open field was defined as 7.5 × 7.5 cm square. Distance traveled, average speed, and center duration were scored using ANY-maze, while grooming and rearing were hand scored.

Social approach test

This experiment has two habituation phases (center and all three chambers) followed by two testing phases (sociability and novelty). The first test compares the preference for a social stimulus versus an inanimate object. The second test, or social novelty phase of the test, compares the preference for a now familiar social stimulus to a novel social stimulus. Social approach behaviors were tested in an apparatus with three chambers in a single 40-min session, divided into four phases. The subject mouse was acclimated to the apparatus for 10 min in the center chamber (Phase 1), and then for an additional 10 min with access to all three empty chambers (Phase 2). The subject was then confined to the middle chamber, while the novel object (an inverted wire cup, Galaxy Cup, Kitchen Plus, Streetsboro, OH) was placed into one of the side chambers, and the stranger mouse (Stranger 1), inside an identical inverted wire cup, was placed in the opposite side chamber. The location (left or right) of the novel object and stranger mouse counterbalanced across subjects. The chamber doors were opened simultaneously, and the subject had access to all three chambers for 10 min (Phase 3). After this, the fourth 10-min session provided a measure of preference for social novelty (Phase 4). The subject mouse was gently guided to the center chamber, the doors closed, and the novel object removed, and a second novel mouse (Stranger 2) was placed in the side chamber. The chamber doors were opened simultaneously, and the subject again had access to all three chambers for 10 min. The fourth 10-min phase provided a measure recognition and discrimination and is used to confirm olfactory abilities for detection and discrimination of social odors. Video tracking with ANY-maze (Stoelting, Inc.; Wood Dale, IL) automatically scored the time spent in each of the three chambers, time spent sniffing, and number of entries into each chamber during each 10-min phase of the test. Animals used as strangers were male 129S1/SvImJ mice habituated to the testing chamber for 30-min sessions on three consecutive days and were enclosed in the wire cup to ensure that all social approach was initiated by the subject mouse. Both end chambers maintained a lighting level of 26–27 lx with two desk lamps angled away from the maze.

Direct social interaction

Direct social interaction was assessed in 33 × 15 × 14 cm cage plastic cage with 3 cm of sawdust and a metal flat cage roof. Male test mice were isolated for 2 h in this cage prior to testing. An unfamiliar stimulus mouse (a 16-week year old C57BL/6 female) was then introduced into the testing cage and left there for 6 min. Videos were recorded by a camera mounted to face to the side of the cage. The ultrasonic microphone was mounted 2 cm above the top of the metal flat cage roof to record the session for subsequent scoring of USV parameters (see methods). Testing sessions were recorded, and videos were analyzed, with the ANY-maze software (Stoelting, Inc., Wood Dale, IL). One observer who was unaware of

the drug treatment of the animals scored the behavior of the test mice, quantifying the time spent performing each of the following behavioral categories and elements:

Affiliative behaviors
Sniffing the head and the snout of the partner, 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 (grooming the partner).

Nonsocial activities
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).

Assessment of estrus cycle

The estrous phase was assessed by analysis of vaginal smears performed on the day of the direct social interaction test in the female C57BL/6J stimulus mice. The evaluation of the test subjects was conducted after testing, in order to minimize the potential stress effects of the manipulation on direct social interaction. Cell types were identified in unstained wet preparations, and estrus stages categorized (Caligioni, 2009). Mounting behavior of C57Bl6/J male mice is significantly higher when females are in proestrus, estrus, and metestrus than in diestrus (Kim et al., 2016; Powers, 1970). All females were in proestrus or metestrus and this was not found to affect the data.

Ultrasonic vocalization analysis

Ultrasonic vocalizations were captured by a Noldus ultrasonic microphone (Noldus Information Technology Inc., Leesburg, VA) and rendered into audio files (.wav) by the UltraVox XT (3.0.80) software (Noldus Information Technology, The Netherlands). Wav files were converted and spectrograms were generated by DeepSqueak (Coffey et al., 2019). Sonogram parameters for short duration vocalization are: nfft = 0.0032 s, overlap = 0.0028 s, window = 0.0032 s. Sonogram parameters for long duration vocalizations are: nfft = 0.01 s, overlap = 0.005 s, window = 0.01 s.

Statistical analysis

EPM, open field, direct social interaction and USV data were analyzed using a one-way analysis of variance (ANOVA). To evaluate habituation to the three-chamber maze for chamber bias (left vs right) a repeated measures ANOVA was used. For the social approach task, a repeated measures ANOVA was used to compare time spent in the chamber and sniff time for Trial 3 (sociability) and Trial 4 (novelty). However, the time spent in each of the three chambers was not independent; for the analysis, only times spent in the side chambers (containing the stranger mouse and novel object) were compared. Time spent in the center chamber is shown in the graphs to illustrate where the subject mouse spent time during the entire 10-min phase. Chamber time, time spent sniffing the novel object versus the stranger mouse, and number of entries to the side chambers in the social approach test were analyzed. For USV data, the number of USV vocalizations failed the Shapiro-Wilks normality test, therefore outliers were removed using the ROUT method with Q = 10%, and data were analyzed using a one-way ANOVA. Statistical analyses were performed using IBM® SPSS Statistics Version 25 (IBM SPSS Japan, Tokyo, Japan) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA). Fisher's LSD post-hoc analysis was run when a main effect or when the repeated measure (stranger mouse or novel object) was significant to determine the group differences. Density plots were calculated and generated using IBM® SPSS Statistics Version 25 (IBM SPSS Japan, Tokyo, Japan) Sigma Plot 14 (Jandel Scientific).

RESULTS

Elevated plus maze

An effect of treatment on percentage of time spent in the open arms was detected ($F_{3,50} = 3.52$, $p < 0.05$). Animals treated with 50 mg/kg of DO34 or rimonabant spent less time in the open arms ($p < 0.01$ and $p < 0.05$ respectively; Figure 2(a)), with this effect being more pronounced in animals injected with high dose 50 mg/kg.

Total distance traveled was not affected, ($F_{3,50} = 0.93$, $p > 0.05$; Figure 2(b)), suggesting that locomotor activity in this test was not affected by inhibition of DGL-α or direct inhibition of CB1.

Three chambered maze

Open field (center habituation)

An effect of treatment on distance traveled during the center habituation trial was detected ($F_{3,50} = 4.50$, $p < 0.01$). DO34 (50 mg/kg) increased activity relative to all treatments ($p = 0.001$ vs. DO34 (10 mg/kg), $p < 0.05$ vs. rimonabant; $p < 0.01$ vs. vehicle; Figure 3(a)). DO34 (50 mg/kg) mice also demonstrated increased average speed during this trial ($F_{3,50} = 4.55$, $p < 0.01$; Figure 3(b)).

Sociability trial

Figure 4 (a,b) illustrates social approach behaviors in B6 mice treated with DO34 (10 or 50 mg/kg), rimonabant,

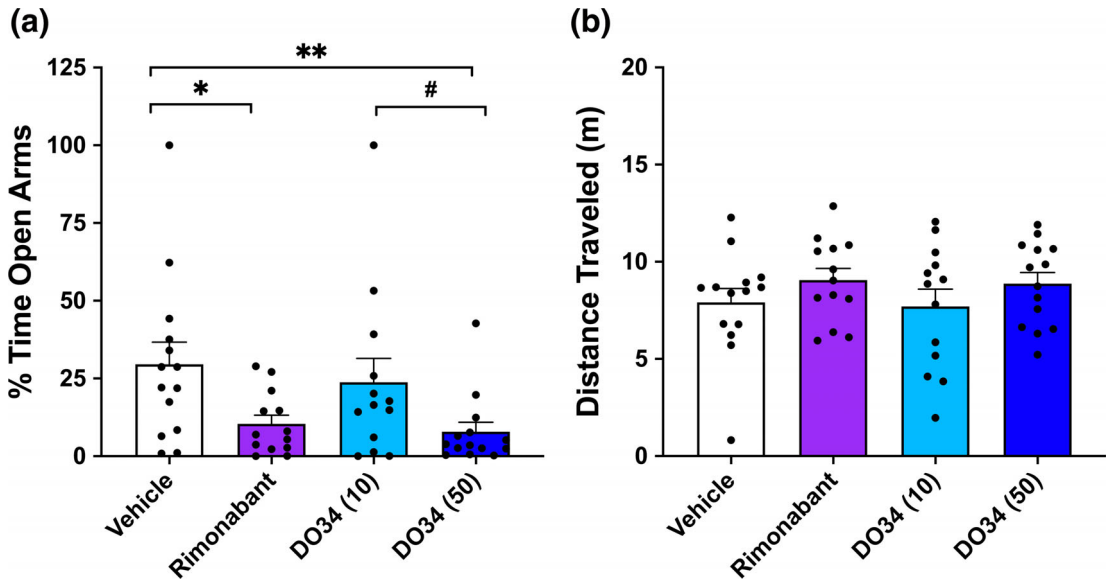

FIGURE 2 Elevated plus maze. DO34 (50 mg/kg) and rimonabant induced anxiety-like behavior in C57BL/6 mice. (a) DO34 (50 mg/kg) induced more anxiety-like behavior ($p < 0.01$) in comparison to vehicle-treated mice than rimonabant ($p < 0.05$ in comparison to vehicle). A trend toward significance was detected for DO34 (50 mg/kg) in comparison to DO34 (10 mg/kg) mice. (b) No significant difference was found for the distance traveled. $^{\#}p = 0.05$; * $p < 0.05$; ** $p < 0.01$. All data are presented as mean and $\pm SEM$. DO34 (50 mg/kg): $n = 13$; DO34 (10 mg/kg): $n = 14$; rimonabant $n = 13$; vehicle: $n = 14$

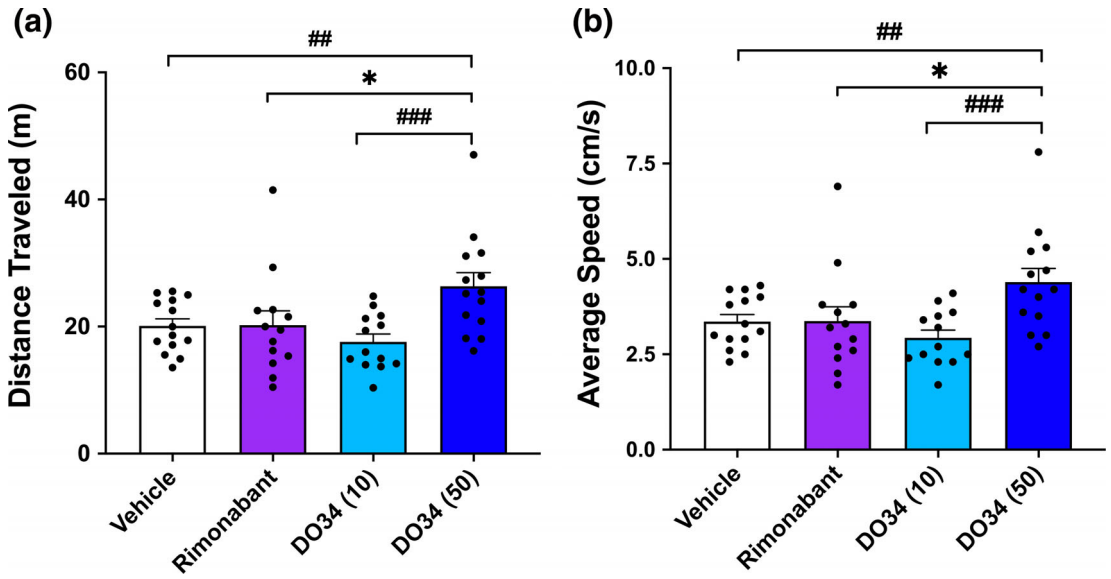

FIGURE 3 Open field. (a) DO34 (50 mg/kg) treatment increased locomotion relative to DO34 (10 mg/kg; $p = 0.001$), rimonabant ($p < 0.05$), and vehicle ($p = 0.01$). (b) DO34 (50 mg/kg) treatment increased the average speed (cm/s) relative to DO34 (10 mg/kg; p = 0.001), rimonabant (p < 0.05), and vehicle ($p < 0.05$). * $p < 0.05$; $^{\#\#}p = 0.01$; $^{\#\#\#}p = 0.001$. All data are presented as mean and $\pm SEM$. DO34 (50 mg/kg): $n = 13$; DO34 (10 mg/kg): $n = 14$; rimonabant $n = 13$; vehicle: $n = 14$

or vehicle. An effect of chamber was detected but not of treatment (chamber, $F_{1,50} = 33.16$, $p < 0.0001$; treatment x chamber, $F_{3,50} = 0.3587$, $p > 0.05$; Figure 4(a)). Mice treated with DO34, both 10 mg/kg ($p < 0.01$) and 50 mg/kg ($p < 0.0001$), or rimonabant ($p < 0.05$) showed a preference for the chamber containing the stranger mouse relative to the chamber with the novel object. Vehicle-treated mice showed a similar trend that did not achieve statistical significance (vehicle $p = 0.058$). When sniffing behavior was evaluated, each group, regardless of treatment spent more time sniffing the stranger mouse than the novel object (chamber, $F_{1,50} = 33.161$, $p < 0.0001$; treatment x chamber, $F_{1,50} = 0.359$, $p > 0.05$; Figure 4(b)) DO34 (50 mg/kg) $p < 0.0001$; DO34 (10 mg/kg) $p < 0.01$; rimonabant $p < 0.05$; vehicle $p < 0.01$).

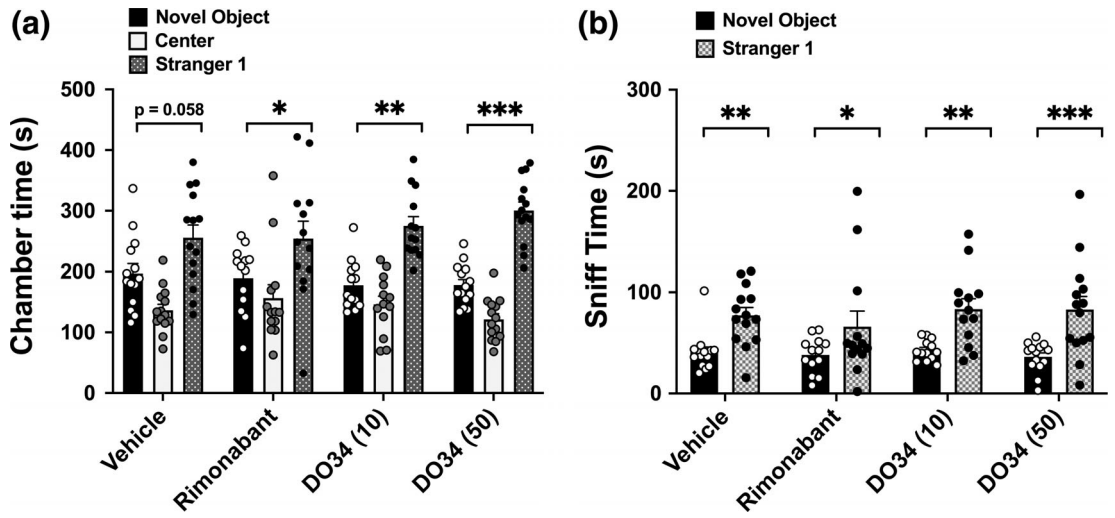

FIGURE 4 Sociability trial. (a) Chamber time: Mice treated with DO34 (50 or 10 mg/kg) or rimonabant demonstrated preference for the stranger mouse relative to the novel object. Vehicle-treated mice showed a similar trend that did not reach statistical significance. (b) Sniff time: All groups spent more time sniffing the stranger mouse relative to the novel object. (c) Distance traveled: Significant differences between groups for distance traveled during the sociability trial were not detected. * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$. All data are presented as mean and $\pm SEM$. DO34 (50 mg/kg): $n = 13$; DO34 (10 mg/kg): $n = 14$; rimonabant n = 13; vehicle: $n = 14$

Novelty trial

Figure 5(a)-(d) illustrate the preference for social novelty in each of the groups when the novel object has been substituted with a second stranger mouse. An effect of chamber was detected but not of treatment (chamber, $F_{1,50} = 4.51$, $p < 0.05$; treatment x chamber, $F_{3,50} = 0.332$, $p > 0.05$). When sniffing time was analyzed, an effect of chamber but not treatment was found (chamber, $F_{1,50} = 5.364$, $p < 0.05$; treatment × chamber, $F_{3,50} = 1.144$, $p > 0.05$; Figure 5(b)). Treatment altered locomotor behavior during this trial ($F_{3,50} = 2.959$, $p < 0.05$). DO34 (50 mg/kg) and DO34 (10 mg/kg) decreased locomotor behavior ($p = 0.01$ and $p < 0.05$, respectively; Figure 5(c)). No significant effects for number of entries between chambers were detected (chamber, $F_{1,50} = 7.068$, $p = 0.01$; treatment × chamber, $F_{3,50} = 1.082$; $p > 0.05$; not shown). Treatment groups demonstrated differences in the time spent immobile during this trial ($F_{3,50} = 6.472$, $p < 0.001$; Figure 5(d)). Pairwise comparisons revealed that DO34 (50 mg/kg) treated mice spent more time immobile than DO34 (10 mg/kg; $p < 0.01$), rimonabant ($p < 0.05$), and vehicle-treated mice ($p = 0.0001$). Heat maps revealed that DO34 (50 mg/kg) treated mice spent increased amounts of time in one spot in the right chamber of the maze, regardless of whether this chamber contained the stranger mouse or the novel object (Figure 6). Evaluation of the habituation phase to the empty maze showed no preference for either the left or right side of the chamber, nor an effect of treatment (chamber, $F_{1,50} = 0.177$, $p > 0.05$; treatment × chamber, $F_{3,50} = 2.242$, $p > 0.05$).

Direct social interaction

An effect of treatment was detected for time engaged in affiliation behaviors during the direct social interaction ($F_{3,47} = 3.33$, $p < 0.05$). DO34 (50 mg/kg) treated mice engaged in less affiliative behaviors than DO34 (10 mg/kg; $p < 0.05$), rimonabant ($p < 0.01$) and vehicle injected mice ($p < 0.05$; Figure 7(a)). When individual affiliative behaviors (sniffing: anogenital or rest of body, mounting, allogrooming) were analyzed, an effect for anogenital sniffing was detected ($F_{3,47} = 3.47$, $p < 0.05$). DO34 (50 mg/kg) engaged in less anogenital sniffing than DO34 (10 mg/kg; $p < 0.01$), and vehicle injected mice ($p < 0.01$). DO34 (50 mg/kg) compared to rimonabant treatment trended toward but did not achieve significance ($p = 0.06$; Figure 7(b)).

An effect of treatment was detected for time engaged in non-social behaviors ($F_{3,47} = 3.72$, $p < 0.05$; Figure 8(a)). DO34 (50 mg/kg) treated mice engaged in more non-social behaviors than DO34 (10 mg/kg; $p < 0.05$), rimonabant ($p < 0.05$) and vehicle ($p < 0.01$) injected animals. When individual non-social behaviors were analyzed (e.g., digging, rearing, self-grooming), an effect of treatment was again detected for self-grooming behaviors ($F_{3,47} = 2.98$, $p < 0.05$; Figure 8(b)). DO34 (50 mg/kg) treated mice engaged in more self-grooming than rimonabant and vehicle-treated mice ($p < 0.05$ and $p < 0.01$, respectively) but not DO34 (10 mg/kg; $p > 0.05$).

Ultrasonic vocalizations (USVs)

USVs produced by the male during the 6-min direct social interaction with a novel female were recorded and

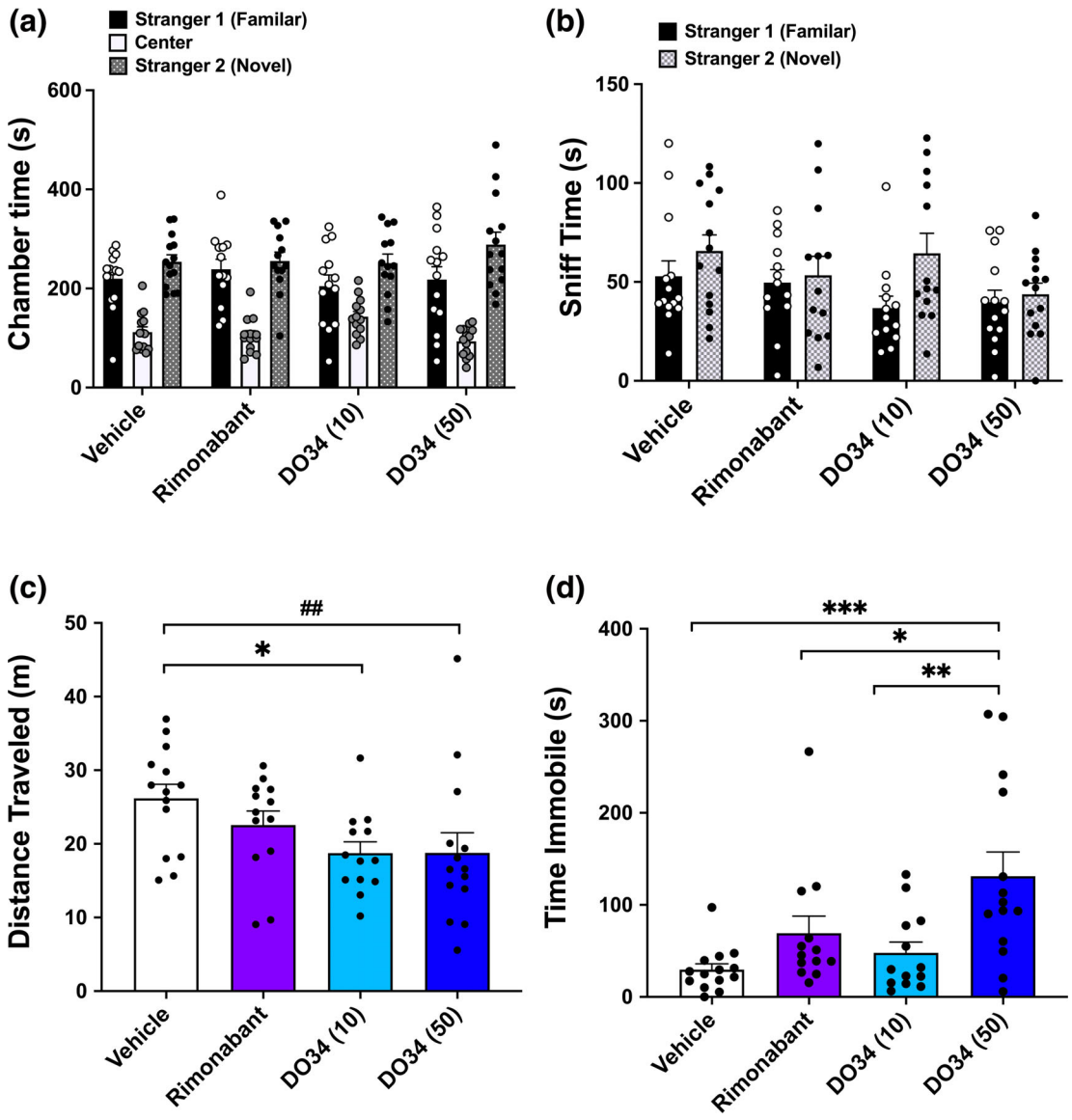

FIGURE 5 Novelty trial. (a) Chamber time: DO34 (50 mg/kg) treated mice spent more time in the side chamber containing stranger mouse 2 (novel) than the chamber containing stranger mouse 1 (familiar). (b) Sniff time: DO34 (50 mg/kg) and rimonabant-treated mice appeared to have similar average times spent sniffing the stranger and novel object in comparison to DO34 (10 mg/kg) and vehicle-treated mice, however, no statistically significant differences were detected. (c) Distance traveled: DO34 (50 mg/kg) and DO34 (10 mg/kg) demonstrated increased levels of locomotor behavior during the novelty trial ($p = 0.01$ and $p < 0.05$, respectively). (d) DO34 (50 mg/kg) treatment increased the time spent immobile relative or all other treatment groups ($p < 0.01$ vs. DO34 (10 mg/kg); $p < 0.05$ vs. rimonabant; $p < 0.0001$ vs. vehicle). * $p < 0.05$; $^{\#\#}p = 0.01$; ** $p < 0.01$; *** $p < 0.001$. All data are presented as mean and $\pm SEM$. DO34 (50 mg/kg): $n = 13$; DO34 (10 mg/kg): $n = 14$; rimonabant $n = 13$; vehicle: $n = 14$

analyzed. A significant effect of treatment was found for the number of USVs produced (treatment, $F_{3,45} = 3.114$, $p < 0.05$). DO34 (50 mg/kg) treated mice produced significantly fewer vocalizations than DO34 (10 mg/kg) and vehicle-treated mice (both, $p = 0.01$), but not rimonabant-treated mice ($p > 0.05$; Figure 9).

DISCUSSION

To investigate the contribution of 2-AG-CB1 signaling in behaviors relevant in ASD, we utilized two pharmacological approaches. First, a reduction in DGL-α synthesis of the primary CB1 ligand, 2-AG (Ogasawara et al., 2016), and second, antagonism of CB1 in with the inverse agonist, rimonabant (Rinaldi-Carmona et al., 1995). Our main findings are as follows: (1) inhibition of DGL-α with DO34 induced anxiety-like behavior, hyperactivity, social behavior deficits, communication deficits, and increased self-grooming behavior; and (2) CB1 inverse agonism induced anxiety-like behavior and communication deficits but not hyperlocomotion, increased self-grooming behaviors, and only partially influenced social behavior.

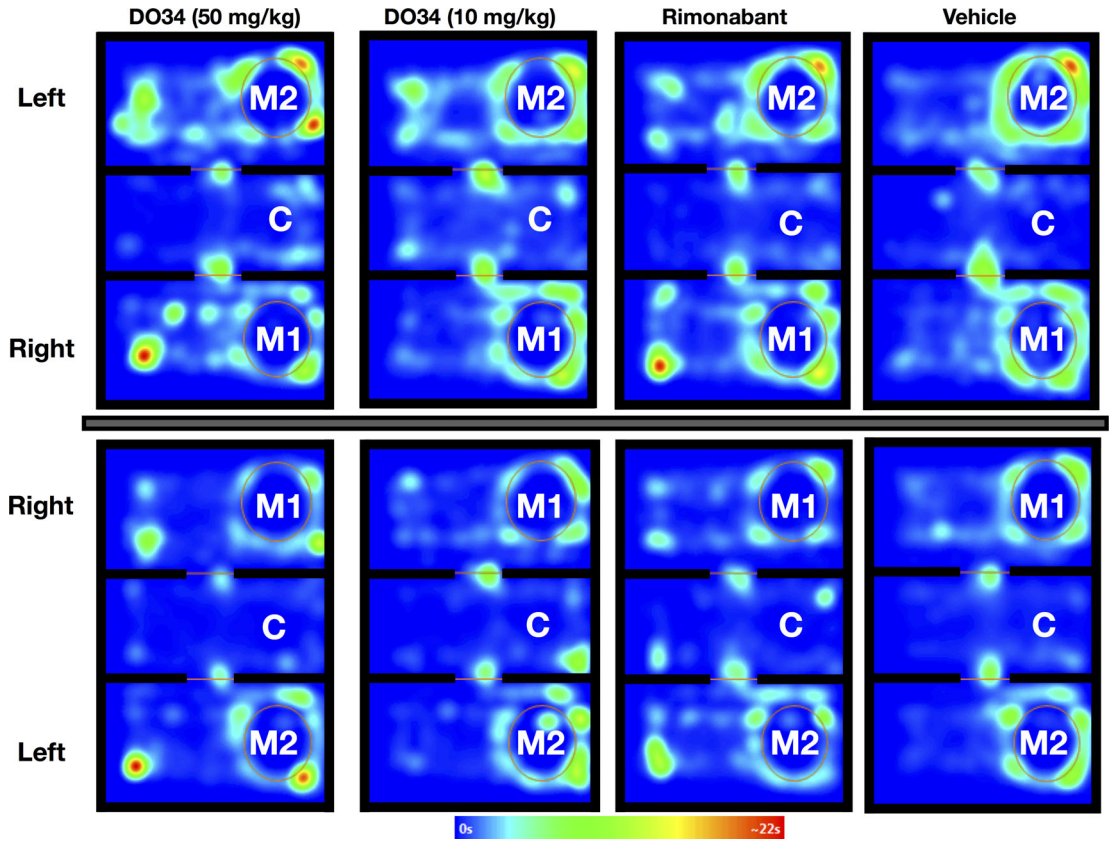

FIGURE 6 Time immobile during the novelty trial. Groups were counterbalanced so that an equal number of mice from each treatment group were presented with either the familiar mouse (M1) in the right chamber and the novel mouse (M2) in the left chamber *(top panel)* or the familiar mouse (M1) in the left chamber and the novel mouse (M2) in the right chamber *(bottom panel)*. Heat maps illustrate that DO34 (50 mg/kg) treated mice remained immobile in the right chamber of the maze regardless of whether the chamber contained the stranger mouse or novel object. M1 = familiar mouse, M2 = novel mouse, C = center chamber of maze

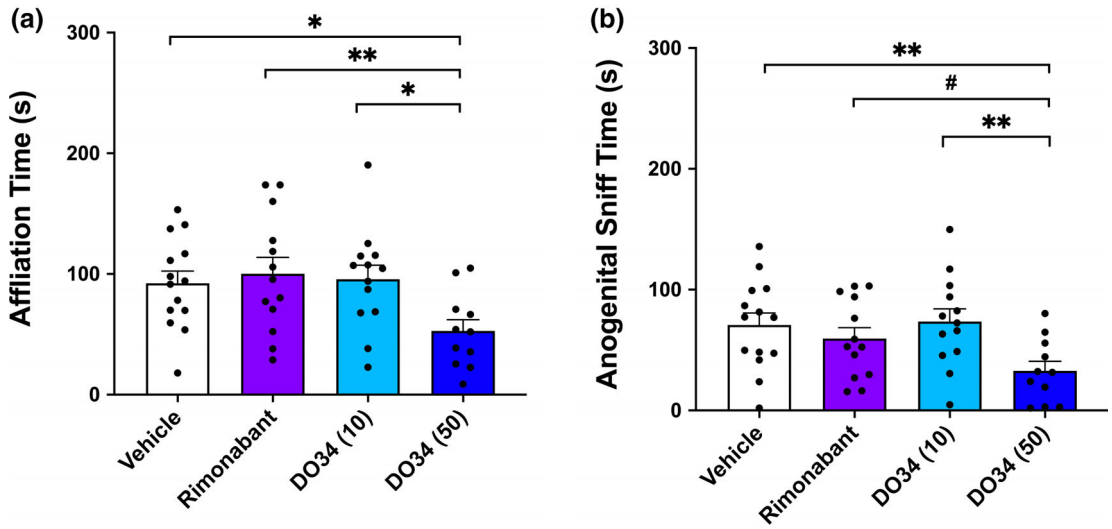

FIGURE 7 Affiliation behaviors during a direct social interaction with a novel female. (a) DO34 (50 mg/kg) treatment decreased affiliative behaviors in comparison to DO34 (10 mg/kg; $p < 0.05$), rimonabant ($p < 0.01$), and vehicle ($p < 0.05$) treated mice. (b) Of the affiliation behaviors analyzed, DO34 (50 mg/kg) treatment reduced anogenital sniffing in comparison to DO34 (10 mg/kg) treatment and vehicle ($p < 0.01$); when compared to rimonabant treatment a trend was detected that failed to achieve statistical significance ($p = 0.06$). All data are presented as mean and ±*SEM*. $^{\#}p = 0.06$, * $p < 0.05$, ** $p < 0.01$. DO34 (50 mg/kg): $n = 11$; DO34 (10 mg/kg): $n = 13$; rimonabant $n = 13$; vehicle: $n = 14$

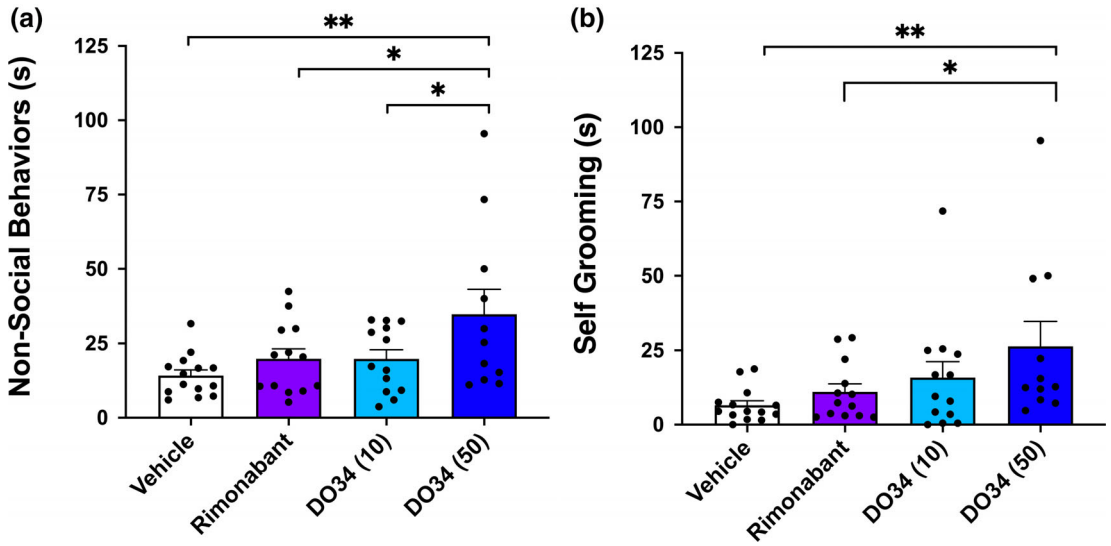

FIGURE 8 Non-social behaviors during a direct social interaction with a novel female. (a) DO34 (50 mg/kg) treatment increased non-social behaviors in comparison to DO34 (10 mg/kg; $p < 0.05$), rimonabant ($p < 0.05$), and vehicle ($p < 0.01$) treated mice. (b) DO34 (50 mg/kg) treatment increased self-grooming behavior in comparison to rimonabant ($p < 0.05$), and vehicle ($p < 0.01$) treated mice, but not DO34 (10 mg/kg; $p < 0.05$) mice. All data are presented as mean and $\pm SEM$. * $p < 0.05$, ** $p < 0.01$ DO34 (50 mg/kg): $n = 11$; DO34 (10 mg/kg): $n = 13$; rimonabant $n = 13$; vehicle: $n = 14$

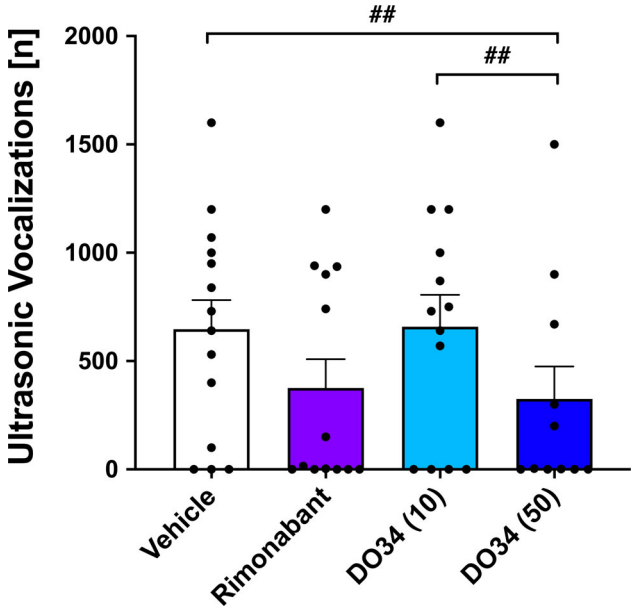

FIGURE 9 Number of ultrasonic vocalizations (USVs) produced during an interaction with a novel female. DO34 (50 mg/kg) reduced the number of USVs relative to DO34 (10 mg/kg) and vehicle-treated animals but not rimonabant-treated animals. $^{\#\#}p = 0.01$. $n = 11$; DO34 (10 mg/kg): $n = 13$; rimonabant $n = 13$; vehicle: $n = 14$

Consistent with previous reports, inhibition of DGL-α or antagonism of CB1 induced anxiety-like behavior in C57BL/6 mice (Bedse et al., 2017; Bluett et al., 2017). In agreement with these findings, mice that are null for CB1 ($CB1^{-/-}$) show anxiety-like phenotypes in the elevated plus maze, light-dark box, and open field (Haller et al., 2002; Haller et al., 2004; Uriguen et al., 2004). Our findings with rimonabant are consistent with those reported clinically. Rimonabant was approved for the treatment of obesity in the European Union (E.U.), however, reports of neuropsychiatric side effects, such as anxiety, depression, and suicidal ideation, resulted in its removal from the market (Christensen et al., 2007; Moreira & Crippa, 2009). Interestingly, acute inhibition of 2-AG had a more pronounced effect for anxiety-like behavior (% time in the open arms) on the elevated plus maze than CB1 inhibition by rimonabant. The results we obtained may be due to contributions of 2-AG signaling at cannabinoid receptor 2 (CB2). 2-AG acts as a full agonist at both CB1 and CB2 (Sugiura et al., 2002). Therefore, reduction in 2-AG production would reduce signaling at both CB1 and CB2, whereas rimonabant, which is highly specific for CB1, does not affect 2-AG-CB2 activity (Ogasawara et al., 2016). Our studies are consistent with previous pharmacological studies which indicate that 2-AG-CB2 signaling contributes to anxiolysis (Almeida-Santos et al., 2013; Busquets-Garcia et al., 2011).

Previous studies have found that inhibition of CB1 with rimonabant does not induce a hyperactive phenotype (Long, Li, et al., 2009; Long, Nomura, et al., 2009; Marinho et al., 2015). Consistent with these studies CB1 inhibition with rimonabant did not induce a hyperactive phenotype, in the open field test but reduction of 2-AG did. Interestingly, hyperactivity did not contribute to our findings on the EPM or on the social approach test. This strongly suggests that effects on locomotor behavior due to a reduction in 2-AG are highly context dependent.

Our results on the social approach test indicate that DGL-α inhibition did not affect social behavior or

locomotor activity when one stranger mouse was present in the apparatus (sociability), however when a second stranger mouse was introduced (novelty trial) DO34 treatment increased immobility time. These results are intriguing, since DO34 treatment increased locomotor activity in the open field test. It is possible that our findings on the social approach test reflect a context specific social anxiety phenotype and that the presence of two stimulus mice in the apparatus is necessary to elicit this phenotype. This effect was only found with DO34 and not with rimonabant. These data suggest the possibility that this behavior is mediated by 2-AG signaling at CB1 and CB2. Indeed, CB2 signaling contributes to social behavior and anxiety in mice (Almeida-Santos et al., 2013; Argue et al., 2017; Rodríguez-Arias et al., 2015).

Our results share similarities with those of Shonesy et al. (2014) who showed that genetic deletion of DGL-α induces social deficits and increases self-grooming behavior. Interestingly, FXS and prenatal exposure to valproate are linked to DGL-α dysfunction (Jung et al., 2012; Kerr et al., 2013; Tang & Alger, 2015). Persons with these syndromes have unique social phenotypes, one of which is social anxiety (Budimirovic et al., 2006; Cassidy & Allanson, 2010; Christensen et al., 2013; Harris et al., 2008; Hong et al., 2019; Kaufmann et al., 2004). Our results here suggest the connection between DGL-α activity and social anxiety needs to be investigated further.

We employed a second test of social behavior, direct social interaction with a novel female, to examine the effects of our manipulations under a different context. Inhibition of DGL- α at 50 mg/kg reduced affiliation behaviors and increased non-social behaviors, specifically self-grooming behavior. These effects were not found with CB1 antagonism.

Recent studies using genetic methods to knock out DGL-α function in dorsal striatum detected reduced social interest and increased repetitive self-grooming behavior (Shonesy et al., 2013; Shonesy et al., 2018). Folkes et al. (2020) demonstrated that 2-AG and CB1 function to inhibit glutamatergic drive in the basolateral amygdala–nucleus accumbens (BLA-NA) circuit. They found that enhancement of glutamatergic activity in the BLA-NA induced social deficits, and increased immobility time in C57BL/6J mice, while pharmacological augmentation of 2-AG activity in this circuit rescued social behavior in *SHANK3B*$^{-/-}$ mice. Our manipulations appeared to show dose dependent effects with regard to self-grooming. Rimonabant produced a non-significant increase in self-grooming behavior while DO34 10 mg/kg, and 50 mg/kg produced larger increases. These findings with regard to rimonabant are consistent with those found by Terzian et al. (2014) in a similar direct social interaction paradigm with wild type C57BL/6N mice. Overall our results suggest that reduction in 2-AG production results in larger insults to social behavior and repetitive self-grooming than inverse agonism at CB1 with rimonabant.

When we analyzed the USVs produced during this interaction, a different pattern was detected. Rimonabant treatment reduced the number of vocalizations males produced, however, this effect was more pronounced in the case of selective 2-AG reduction.

These data suggest that 2-AG and CB1 each make contributions to communication behavior. It is possible that 2-AG signaling (at CB1, CB2, or both), and CB1 activity independent from 2-AG-CB1 signaling (e.g. AEA-CB1, constitutive receptor activity) each contribute to USV production. This may represent functional redundancy of the ECS with regard to communication, similar to that which is detected with anxiety-like and social behavior (Bedse et al., 2017; Wei et al., 2016). Future studies should be aimed at further elucidating the role of these ECS components in communication behavior.

DO34 does not augment levels of the second eCB, AEA, and thus it unlikely that changes in AEA signaling occurred in these manipulations. (Ogasawara et al., 2016). It is important to note that the relationship between AEA signaling and social behavior is largely unexplored. A single study with Male Sprague–Dawley rats showed that 2-AG and AEA have overlapping developmental roles in social play behavior (Manduca et al., 2015). In regard to neurodevelopmental disorders, a single study found that increasing AEA levels rescued social deficits in two mouse models of ASD, namely *Fmr1*-KO and BTBR mice (Wei et al., 2016). It is possible that 2-AG and AEA exhibit functional redundancy with social behavior, as is the case with anxiety behavior (Bedse et al., 2017). Therefore, we cannot exclude the possibility that inhibition of AEA-CB1 signaling plays a role in our results. Additionally, it must be considered that the effects obtained here could have been influenced by an off-target effect. While DO34 is highly selectivity for DGL-α, it does cause a reduction in the levels of free arachidonic acid and the prostaglandins, PGD_2 and PGE_2 (Ogasawara et al., 2016). Therefore the possibility of this influence cannot be ruled out, however in consideration of the overall agreement our results share with studies that have used highly specific genetic methods, it is likely these effects are due to DO34's action at DGL-α (Shonesy et al., 2014; Shonesy et al., 2018). Additionally, it should be noted that dysfunction of DGL-α has been most closely studied in the *fmr1*-KO mouse model of the X-linked syndrome, FXS, therefore, we chose male mice for this study. Future studies would do well to explore the role of DGL-α in female mice, as this could provide valuable insight into phenotypic sex differences of autism behaviors.

The bimodal nature of CB1 signaling may explain some of the results obtained here. With respect to anxiety-like behavior, CB1 signaling exhibits anxiolytic properties at moderate levels of stimulation and anxiogenic effects at high levels of stimulation (Bhattacharyya et al., 2017; Ruehle et al., 2012). Whether or not CB1 exhibits bimodal

properties with regard to social behavior has not been directly investigated to the best of our knowledge.

On the surface our data appear somewhat at odds with $CB1^{-/-}$ mice studies which indicate a strong role for CB1 in social behavior (Haller et al., 2004; Litvin et al., 2013; Terzian et al., 2014). We do not view the results obtained in our study as contradictory, but complementary. The CB1 mutation used in those studies were bred on a CD1 background, an outbred strain with greater background genetic variability. This likely contributed to the phenotypic differences we detected. Indeed, studies with the *fmr1*-KO mouse model have demonstrated the influence that background can have on the expression ASD-like phenotypes (Dobkin et al., 2000; Pietropaolo et al., 2011).

Furthermore, $CB1^{-/-}$ mice, relative to wild-type mice, have an altered developmental trajectory, and therefore phenotypic differences compared to pharmacological studies are expected. The $CB1^{-/-}$ mouse continues to help unravel the importance of the ECS in ASD relevant behaviors, however a complete genetic knockout of CB1 does not closely recapitulate human pathology, as genetic variants for *CNR1* and *DAGLA*, not complete loss of the gene, are associated with ASDs. Indeed, ASD is a uniquely human disorder. Mouse models are only rough approximations which provide insight, by proxy, into selective pathology related to ASD. Therefore, the development of novel mouse models is critical for improving our understanding of this complex and diverse disorder.

Our manipulations of the ECS illustrate a crucial point: neurodevelopmental disorders are a complex mix of alterations due to different mechanisms, these data, and those from previous studies, suggest that the ECS supports a cluster of mechanisms responsible for certain wavelength ranges of behavior. This is particularly evident in regard to social behavior and communication. Here we supply a novel pharmacological mouse model for exploring behaviors which are relevant to neurodevelopmental disorders, particularly ASD. Our findings illustrate the need for exhaustive studies regarding imbalances in 2-AG signaling and neuropathology. Furthermore, these findings support and extend the accumulating body of evidence that the ECS, particularly with respect to 2-AG, is a target of therapeutic interest for neurodevelopmental disorders.

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AUTHOR CONTRIBUTIONS

William Fyke performed the data collection under the supervision of Kathryn K. Chadman. William Fyke performed the analysis and interpretation under the supervision of Juan Marcos Alarcon, Milen Velinov, and Kathryn K. Chadman. William Fyke drafted the manuscript and Juan Marcos Alarcon, Milen Velinov, and Kathryn K. Chadman provided critical revisions. All authors approved the final version of the manuscript for submission.

CONFLICT OF INTERESTS

The author(s) have no potential conflicts of interest to declare with respect to the research, authorship, and/or publication of this article.

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