Motor-like Tics are Mediated by CB₂ Cannabinoid Receptor-dependent and Independent Mechanisms Associated with Age and Sex

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Abstract
Δ⁹-Tetrahydrocannabinol (Δ⁹-THC) inhibits tics in individuals with Tourette syndrome (TS). Δ⁹-THC has similar affinities for CB₁/CB₂ cannabinoid receptors. However, the effect of HU-308, a selective CB₂ receptor agonist, on repetitive behaviors has not been investigated. The effects of 2,5-dimethoxy-4-iodoamphetamine (DOI)-induced motor-like tics and Δ⁹-THC were studied with gene analysis. The effects of HU-308 on head twitch response (HTR), ear scratch response (ESR), and grooming behavior were compared between wildtype and CB₂ receptor knockout (CB₂−/−) mice, and in the presence/absence of DOI or SR141716A, a CB₁ receptor antagonist/inverse agonist. The frequency of DOI-induced repetitive behaviors was higher in CB₂−/− than in wildtype mice. HU-308 increased DOI-induced ESR and grooming behavior in adult CB₂−/− mice. In juveniles, HU-308 inhibited HTR and ESR in the presence of DOI and SR141716A. HU-308 and beta-caryophyllene significantly increased HTR. In the left prefrontal cortex, DOI increased transcript expression of the CB₂ receptor and GPR55, but reduced fatty acid amide hydrolase (FAAH) and α/β-hydrolase domain-containing 6 (ABHD6) expression levels. CB₂ receptors are required to reduce 5-HT₂A/2C-induced tics in adults. HU-308 has an off-target effect which increases 5-HT₂A/2C-induced motor-like tics in adult female mice. The increased HTR in juveniles induced by selective CB₂ receptor agonists suggests that stimulation of the CB₂ receptor may generate motor tics in children. Sex differences suggest that the CB₂ receptor may contribute to the prevalence of TS in boys. The 5-HT₂A/2C-induced reduction in endocannabinoid catabolic enzyme expression level may explain the increased endocannabinoids’ levels in patients with TS.

Keywords Tic disorder · Premonitory urges · Anandamide · GPR55 · Tetrahydrocannabinvarin (THCV) · Cannabidivarin (CBDV) · α/β-Hydrolase domain-containing 6 (ABHD6)

Abbreviations
Δ⁹-THC Δ⁹-Tetrahydrocannabinol
CBD Cannabidiol
THCV Δ⁹-Tetrahydrocannabivarin
CBDV Cannabidivarin
2-AG 2-Arachidonoylglycerol
DOI 2,5-Dimethoxy-4-iodoamphetamine
HTR Head twitch response
ESR Ear scratch response
OCD Obsessive-compulsive disorder
CNS Central nervous system
PNS Peripheral nervous system
VTA Ventral tegmental area
ΔΔCt Delta-delta Ct
MAGL Monoacylglycerol lipase
FAAH Fatty acid amide hydrolase
ABHD6 α/β-Hydrolase domain-containing 6
GPR55 G protein-coupled receptor 55
MSNs Medium spiny neurons
GWAS Genome-Wide Association Study

Introduction
Δ⁹-Tetrahydrocannabinol (Δ⁹-THC) inhibits motor tics in adolescent and adult individuals with Tourette syndrome (TS), with onset around age 6 years and with a 3:1 boy:girl ratio [1–4]. In rodents, Δ⁹-THC dose-dependently reverses motor-like tics (sudden, repetitive twitches or movements that may represent Tourette syndrome motor tics), head
twitch response (HTR), ear scratch response (ESR), and grooming behavior, after induction of tic-like behavior with 2,5-dimethoxy-4-iodoamphetamine (DOI), a highly potent agonist of the serotonin 5-HT2A/2C receptors [5, 6]. ∆9-THC is a partial agonist of the cannabinoid CB1 and CB2 receptors, but can also act on other receptors, e.g., GPR55 [7, 8]. While the CB1 receptor is highly expressed on the surface of central and peripheral neurons, the cannabinoid CB2 receptor is highly expressed on cells of the immune system and activated microglia, but low expression levels of the CB2 receptor have been reported in the adult CNS under healthy physiological conditions [9, 10].

Evidence exists for the expression of functional CB2 receptors on neurons in different brain regions, including the striatum and brainstem, where it regulates dopamine release, while CB2 receptor expression levels in the brain can be significantly upregulated during CNS pathologies [9, 11–16]. For example, in adult male mice, exposure to JWH-133 (10, 20 mg/kg, intraperitoneally (i.p.)), a selective CB2 receptor agonist, reduces adult locomotor activity [14]. Similarly, JWH-133 reduces locomotor activity induced by cocaine [17]. In adult male mice, HU-308 (2.5, 5 mg/kg, i.p.), another selective CB2 receptor agonist, reduces dyskinesia-like behavior in a model of Parkinson’s disease [13]. However, HU-308 (40 mg/kg, i.p.) has no effect on the locomotor activity of adult Sabra female mice [18].

Thus, it appears that there is a complex mechanism for the control of motor activity by the CB2 receptor and sex may contribute to these differences. Different selective CB2 receptor agonists (e.g., HU-308, JWH-133, HU-910) have been shown to modulate distinct signaling pathways [19]. Questioning their specificity, CB2 receptor agonists can also modulate other targets, including receptors other than the cannabinoid receptors, as well as transporters and enzymes [19]. Despite these considerations, HU-308 was selected as one of the best three selective CB2 receptor agonists to study the role of the CB2 receptor in diseases [20–23].

Similar to its effects on DOI-induced repetitive behaviors, ∆9-THC dose-dependently reduces HTR and ESR after the administration of SR141716A, a selective CB1 receptor antagonist/inverse agonist, to juvenile male albino ICR mice [24]. Like DOI, SR141716A administration has been proposed as a model for tic-like behavior, but similar model limitations as described before are applied to the SR141716A-induced repetitive behaviors model system [6]. The administration of SR141716A (rimonabant, Acomplia®, Zimulti®) to humans produces psychiatric and neurologic adverse effects such as suicidality, depressed mood, anxiety, insomnia, stress, and seizures. However, motor tics and premonitory urges were not observed in humans after taking rimonabant. In mice, SR141716A dose-dependently induces motor-like tics and premonitory urge-like behavior, effects which are reversed by the 5-HT2A/2C antagonist SR46349B [25]. However, in contrast to DOI, SR141716A does not increase grooming behavior in juvenile ICR mice [25], though it increases serotonin and dopamine release [26]. However, this appears to be species-dependent, as in rats, SR141716A increases grooming behavior [27].

The CB2 receptor makes a significant contribution to the control of locomotor activity [13, 14]. Despite the large body of work pointing to the role of the CB2 receptor in different diseases, the effect of CB2 selective agonists on stereotypical, repetitive behaviors has not been studied. As CB2 receptor expression is developmentally regulated, with the expression level being high after birth and very low in the adult brain [9, 28–30], it was important to study the effects of selective CB2 receptor ligands at different ages. The possible contribution of the CB2 receptor to the skewed ratio between boys and girls in TS was studied by testing motor-like tics in juvenile males and females.

Materials Methods

Animals

All experiments were approved by the Institutional Animal Use and Care Committees of Tel-Aviv University and Ariel University and were in accordance with the UK Home Office, EU directive 63/2010E, and the Animal (Scientific Procedures) Act 1986.

The specificity of HU-308 was tested in CB2 receptor knockout (CB2−/−) mice (JAX #005786), purchased from Jackson Laboratory, USA, and genotyped according to the instructions provided by the company. The experiments were performed as indicated in >7.5-week-old (7 males and 7 females, adult) CB2−/− mice.

Screening of the effects of HU-308 at different ages was conducted in C57BL/6 J (OlaHsd sub-strain). This strain was used in our previous study to screen the effects of ∆9-THC and CBD [6]. C57BL/6 J (OlaHsd sub-strain) male and female mice were purchased from Envigo, Israel or UK. The experiments were performed as indicated in 3-week-old (201 males and 66 females, unweaned, juvenile), 6-week-old (63 males and 28 females, pubertal, young adult), and >7.5-week-old (11 males and 7 females, adult) mice.

Drugs

SR141716A was synthesized by IRG, University of Aberdeen (according to US Patent 5,462,960). (R)(−)-DOI hydrochloride (CAS 82864-02-6), DMSO, and Kolliphor® EL were from Sigma-Aldrich (Rehovot, Israel). Ethanol was from Merck, Germany. HU-308 was from Tocris, UK. E-BCP was from Kanata Enterprises, India (99%). ∆9-THC (98%) was kindly provided by Prof. Mechoulam (The
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and latency to center, and body weight were analyzed by total distance, duration in the center of the cage, frequency and latency to center, and body weight were analyzed by
dissolved in vehicle made of 0.6:1:1.84 ethanol: Kolliphor® EL:saline. SR141716A (5 mg/kg, 10 mg/kg, 20 mg/kg) was dissolved in vehicles 0.6:1:1.84 DMSO: Kolliphor® EL:saline. SR141716A (5 mg/kg, 10 mg/kg, 20 mg/kg) was dissolved in vehicle made of 0.6:1:1.84 ethanol: Kolliphor® EL:saline. The drugs were freshly prepared, aliquoted, and stored for up to 3 months. Each aliquot was discarded after one use. Drugs were injected intraperitoneally (i.p.).

Experimental Procedures for Head Twitch Response (HTR), Ear Scratch Response (ESR), and Grooming Behavior Measurement

The experimental procedures for the DOI model system and for randomization have been previously described [6] and are detailed in the Supplementary Information.

Open Field Test

The test was performed similarly to the methods previously published [18]. The method is described in the Supplementary Information.

Marble Burying Test

The test was conducted similarly to methods previously published [31]. The method is described in the Supplementary Information.

Reverse Transcription and RT-PCR

In juvenile mice, the effects of DOI or 5 mg/kg ∆9-THC on genes of the endocannabinoid system were tested. The method is described in the Supplementary Information. The sequences of primers used in this study are detailed in the Supplementary Information.

Table 1 Sequences of primers used for mouse RT-PCR analyses

| Target        | Forward (F)/ reverse (R) | Sequence of primers   |
|---------------|--------------------------|-----------------------|
| GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CB1 receptor, cannabinoid CB1 receptor, CB2 receptor, cannabinoid CB2 receptor; GPR55, G protein-coupled receptor 55; MAGL, monoacylglycerol lipase; FAAH, fatty acid amide hydrolase; ABHD6, α/β-hydrolase domain-containing 6 |
| CB1 receptor  | F: TCTTAGACGGCCCTTGACAT R: AGGGACTACCCCTGAAGGAA | GAAACAGCCCGAGTCAGAGAG |
| CB2 receptor  | F: GAAAACAGGGCAGTCAGAGAG R: GAGGCTGCTATTCTACAGGG | |
| GPR55         | F: GTCCCATATCCCCACCTTCCT R: CATCTTGAATGGGAGGGA | |
| MAGL          | F: CAGAGAGGCGCACAACCTTTTC R: ATGCGCCCCAACGTCATATT | |
| FAAH          | F: GGAAGTGAACAAAGGGACCA R: TCCCCGCAGCTACAGTTACCT | |
| ABHD6         | F: CCTTGATCCCCATCACCCCGGA R: CCCGGACACATCAAGCATTGG | |

Results

Effects of HU-308 on DOI-induced Repetitive Behaviors in Adult CB2−/− Mice

In order to determine the on- versus off-target effects of HU-308 [19], the effects of DOI (1 mg/kg)-induced repetitive behaviors in the presence or absence of HU-308 (5 mg/kg) were tested in CB2−/− mice. HU-308 has neuroprotective effects [13, 21, 23], and activation of the CB2 receptor inhibits dopamine release [15]; therefore, we expected that HU-308 will reduce the DOI-induced motor-like tics. Surprisingly, the results show that in adult CB2−/− mice, HU-308 (5 mg/kg) had no effect on DOI-induced HTR and significantly increased DOI-induced ESR and grooming behavior in adult CB2−/− mice (Fig. 1a–c, respectively). These results show that the enhancing effects of HU-308 on DOI-induced repetitive behaviors in adult mice were not CB2 receptor-mediated. Sex comparison of the effect of HU-308 in adult CB2−/− mice suggests that females were more sensitive than males (Supplementary Figs. S1 and S2).
**Fig. 1** Effects of DOI in the presence or absence of HU-308 (5 mg/kg) on HTR (a, d), ESR (b, e), and grooming behavior (c, f) in adult wildtype (WT) and CB2−/− knockout mice (CB2−/− mice). In a–c, the effects of HU-308 on DOI in CB2−/− mice. In d–f, the effects of HU-308 on DOI in WT mice. Data represent mean ± SEM. n represents the number of animals in each group. The experiment was independently repeated a number of times according to the lowest n number. Two-way ANOVA analysis of variance followed by Bonferroni’s test for multiple comparisons was performed by GraphPad Prism 8. Asterisks aside from the graph are p value summary vs. vehicle + DOI group. Asterisks along the curve are p values of multiple comparisons (at a time point) of each dose vs. vehicle + DOI group. *P < 0.05; **P < 0.01; ***P < 0.001 significantly different.
Effects of HU-308 on DOI-induced Repetitive Behaviors in Adult Mice

To better understand if these enhancing effects of HU-308 on DOI-induced repetitive behaviors in adult mice were not dependent on the modulation of the CB2 receptor, we repeated this experiment in adult mice from another strain. We tested the effects of HU-308 on DOI-induced repetitive behaviors in a sub-strain of wildtype (WT) C57BL/6 J mice, which was used for subsequent experiments in juvenile mice. The results show that in adult WT mice, HU-308 (5 mg/kg) had no effect on DOI-induced HTR but significantly increased DOI-induced ESR and grooming behavior (Fig. 1d–f, respectively), replicating our results in CB2−/− mice. The effects of HU-308 on DOI-induced repetitive behaviors in young adult mice are detailed in the Supplementary Information (Supplementary Figs. S3, S4, and S5).

Effects of HU-308 on DOI-induced Repetitive Behaviors in Juvenile Mice

We expected to find similar results in juvenile mice. Surprisingly, in juvenile male mice, HU-308 (1 mg/kg, 5 mg/kg) reduced DOI-induced HTR, ESR, and grooming behavior (Fig. 2a–c). The DOI-induced HTR was significantly reduced by 21% and 13%, respectively (Fig. 2a, P < 0.05). The DOI-induced ESR was reduced by 64% (P < 0.05) and 50%, respectively (Fig. 2b). The DOI-induced grooming behavior was significantly reduced by 42% and 32%, respectively (Fig. 2c, P < 0.05). Compared with the results in adult mice, these results showed that in juvenile mice, HU-308 inhibits repetitive behaviors.

Age dependency was also demonstrated in female mice. In juvenile females, HU-308 (1 mg/kg, 5 mg/kg) significantly reduced DOI-induced HTR, resulting in 24% and 27% inhibition, respectively (Fig. 3a). HU-308 (0.2 mg/kg, 1 mg/kg, 5 mg/kg) had no significant effect on DOI-induced

Fig. 2 Effects of HU-308 (1 mg/kg, 5 mg/kg) on DOI (1 mg/kg)-induced HTR (a), ESR (b), and grooming behavior (c) in juvenile males. HU-308 (0.2 mg/kg) had no effects (Supplementary Fig. S6). Effects of HU-308 (0.2 mg/kg, 1 mg/kg, 5 mg/kg) on SR141716A (10 mg/kg)-induced HTR (d), ESR (e), and grooming behavior (f) in juvenile males. Data represent mean ± SEM. n represents the number of animals in each group. The experiment was independently repeated a number of times according to the lowest n number.
Effects of HU-308 on DOI-induced Repetitive Behaviors in Juveniles

In the presence of SR141716A, HU-308 (0.2 mg/kg, 1 mg/kg, 5 mg/kg) significantly decreased the frequency of HTR (Fig. 2d; \( P < 0.05 \)), resulting in an inhibition of 57%, 19%, and 58%, respectively. In the presence of SR141716A, HU-308 (1 mg/kg, 5 mg/kg) significantly decreased the frequency of ESR (Fig. 2b; \( P < 0.05 \)), resulting in an inhibition of 47%, and 29%, respectively. However, HU-308 (0.2 mg/kg, 1 mg/kg, 5 mg/kg) had no effect on grooming behavior in juvenile male mice (Fig. 2c). Average body weight was not different between groups (Supplementary Fig. S5). The effects of the vehicles (ethanol vs. DMSO) on SR141716A-induced repetitive behaviors are shown in the Supplementary Information (Supplementary Fig. S6).
S7a–c). SR141716A, dissolved in ethanol, dose-dependently increased HTR and ESR behaviors but not grooming behavior (Supplementary Fig. S7a–c). These results replicate another study [25].

Collectively, these results show that, in two model systems, HU-308 inhibits repetitive behaviors in juveniles. Therefore, we next studied its effects on basal repetitive behaviors, important to determine because this will impact its potential “therapeutic window.”

**Effect of HU-308 on Basal Repetitive Behaviors in Juvenile Mice**

In healthy juvenile females, compared with the basal HTR of the control group, HU-308 alone (0.2 mg/kg) significantly increased HTR (Fig. 3d). HU-308 (1 mg/kg, 5 mg/kg) had no effect on basal HTR (Fig. 3d). HU-308 (0.2 mg/kg, 1 mg/kg, 5 mg/kg) had no effect on basal ESR (Fig. 3e), while HU-308 (1 mg/kg, 5 mg/kg) significantly inhibited basal grooming behavior (Fig. 3f; $P < 0.05$).

In contrast, in healthy juvenile males, HU-308 alone significantly increased the frequency of HTR (Fig. 4a; $P < 0.05$). Compared with the basal HTR of the control group, HU-308 (1 mg/kg, 5 mg/kg) significantly increased HTR, resulting in an increase of 114% and 50% in basal HTR, respectively. Compared with the basal ESR of the control group, HU-308 (5 mg/kg) significantly increased ESR, resulting in an increase of 100% of basal ESR in juvenile male mice (Fig. 4b; $P < 0.05$). Compared with the basal grooming behavior of the control group, HU-308 alone had no effect on basal grooming behavior in juvenile male mice (Fig. 4c). Average body weight was not different between groups (Supplementary Fig. S8c). These results suggest that males are more sensitive than female...
mice to the effect of selective CB$_2$ receptor agonists on basal activity.

We next tested E-BCP, another selective CB$_2$ receptor agonist [32]. In healthy juvenile male mice, E-BCP alone (1 mg/kg, 5 mg/kg, 10 mg/kg) dose-dependently increased HTR (Fig. 4d). Compared with basal HTR of the control group, E-BCP alone (5 mg/kg, 10 mg/kg) significantly increased HTR by 400% and 500%, respectively (Fig. 4d; $P < 0.05$). E-BCP alone (10 mg/kg) significantly increased basal ESR by 500% (Fig. 4e). E-BCP alone (5 mg/kg, 10 mg/kg) significantly increased basal grooming behavior by 33% and 73%, respectively (Fig. 4f; $P < 0.05$). The similarity of these results with that of HU-308 on basal repetitive behaviors in juveniles suggests that these effects are indeed CB$_2$ receptor-mediated.

In juvenile male mice, HU-308 (1 mg/kg, 5 mg/kg) significantly reduced the number of rears and ambulatory behavior (Supplementary Fig. S10a,b; $P < 0.05$) but not grooming behavior (Supplementary Fig. S10c). Average body weight was not different between groups (Supplementary Fig. S10d). These results are in line with the inhibitory effect of JWH-133 on locomotor activity [14]. In contrast, DOI (1 mg/kg) significantly increased ambulation and rearing behaviors in juveniles ($P < 0.05$; $n = 6$; VG results, not shown) and SR141716A at a dose of 10 mg/kg, but not at a lower dose, increases ambulation behavior and travel distance in adolescent male rodents [33, 34].

Collectively, these results show that HU-308 reduces locomotor activity but significantly increases repetitive behaviors, inducing a phenotype of motor-like tics without hyperactivity in juvenile males, while DOI and SR141716A induce a phenotype of motor-like tics with hyperactivity in juvenile males.

**DOI and ∆$_9$-THC Induce Left Lateralization in the Endocannabinoid System**

Further support for the involvement of the CB$_2$ receptor in juvenile males comes from an RT-PCR study, which focused on the dorsolateral prefrontal cortex (PFC), because in a Genome-Wide Association Study (GWAS), significant genetic mutations in patients with TS found in the PFC and have raised interest in this region [35]. In our study, the CB$_2$ receptor expression level was significantly increased by DOI in the left but not in the right PFC (Fig. 5a, d $P < 0.05$).

DOI significantly altered the mRNA expression level of elements of the endocannabinoid system in the left but not in the right PFC (Fig. 5). In addition to the increased expression level of the Cnr2 gene (encoding the CB$_2$ receptor), the expression level of Gpr55 (encoding gene of GPR55) was significantly increased by DOI in the left, but not in the right, PFC (Fig. 5h, k). However, Cnr1 (encoding gene of CB$_1$ receptor) expression levels were not affected by DOI (Fig. 5g, j). In line with these results, genetic variations of the CNRI gene in patients were not correlated with TS [36], further supporting that DOI-induced motor-like tics may closely model TS.

In contrast, Abhd6 (encoding gene of ABHD6) and Faah (encoding gene of FAAH) expression levels were significantly decreased by DOI in the left, but not in the right, PFC (Fig. 5b, i vs. Fig. 5e, l). In the left PFC, DOI reduced the expression level of Mgll (encoding gene of MAGL) ($P = 0.09$; Fig. 5c, f).

Similar effects to those of DOI on gene expression were found with ∆$_9$-THC alone. This may explain why (1) ∆$_9$-THC induces psychosis, similarly to DOI, apart from the effect on CB$_2$ receptor expression (Fig. 6a–i), and (2) treatment with ∆$_9$-THC only temporarily alleviates the symptoms of TS.

**Discussion**

This study demonstrates that the CB$_2$ receptor has a role in the control of repetitive behaviors. In support of the contribution of CB$_2$ receptors to the control of motor movements are previous studies showing that (1) in rodents, the CB$_2$ receptor is expressed on the soma and nerve terminals of dopaminergic neurons projecting from the substantia nigra to the striatum in the nigrostriatal pathway [12, 37, 38] and from the ventral tegmental area (VTA) to the nucleus accumbens in the mesocortical pathway [15]; (2) the CB$_2$ receptor controls the release of dopamine in the dorsal striatum (caudate nucleus and putamen) and nucleus accumbens [12, 15]; (3) in healthy animals, the CB$_2$ receptor mediates M$_4$ muscarinic acetylcholine receptor-induced inhibition of dopamine release [11]; (4) in non-human primates, the CB$_2$ receptor is expressed on globus pallidus (internal and external) output neurons of the basal ganglia [39]; (5) in humans, the CB$_2$ receptor is expressed by dopaminergic neurons of the substantia nigra pars compacta (SNc) [40], Purkinje neurons as well as neurons of the dentate nucleus, and in the white matter of the cerebellum in patients with loss of motor coordination [41]. Most of these studies have focused on neuronal cells; however, in some of these studies, CB$_2$ receptors have been localized on glial cells as well [15, 37, 38, 41], suggesting that the CB$_2$ receptor is expressed by neuronal and glial cells in brain areas that control motor function.

In this study, several limitations in the models employed need to be taken into account: (1) DOI and SR141716A are administered systemically, thus affecting multiple brain regions including those that do not cause tics [42–44]; (2) systemic administration of DOI or rimonabant to humans does not lead to the appearance of tics; (3) the tested drugs are used as pre-treatments prior to the administration of...
DOI or SR141716A. This is not the case in humans, who are treated only after the appearance of symptoms; (4) CB2 expression in the CNS changes in pathological diseases but our models use only healthy mice; (5) Tourette syndrome consists of both motor and vocal tics and while DOI induces motor-like tics it does not induce vocal tics [6]. Similarly, administration of SR141716A to juvenile and adult mice does not induce vocalizations; (6) in mice, under the experimental conditions employed, SR141716A does not induce peripheral motor-like tics, making it only a partial model for motor-like tics.

**A Role for CB2 Receptor in Movement Disorders**

Following activation of 5-HT2A/2C receptors by DOI, repetitive behaviors were higher in adult CB2−/− than in wildtype mice, and the deletion of CB2 receptor reveals its contribution to 5-HT2A/2C receptor-induced repetitive behaviors. Interestingly, CB2−/− mice with deleted CB2 receptor on dopamine neurons show increased hyperactivity [45]. Previous studies showed that in healthy animals CB2 receptor inhibits the release of dopamine [11, 12, 15]. Collectively these results suggest that (1) during healthy brain development, Gαi protein-coupled CB2 receptors are required to reduce the magnitude of dopamine release, including when stimulated by activation of 5-HT2A/2C receptors, and (2) this mechanism, in turn, reduces the frequency of repetitive behaviors in healthy animals.

Our results further suggest that losing expression of functional brain CB2 receptors will contribute to a robust motor tic phenotype. These results imply that a sudden and profound drop in the cerebral expression level of Gαi protein-coupled CB2 receptor during adulthood may possibly contribute to the appearance of adult-onset tic disorders [46]. Vice versa, the severity of motor tics gradually declines through adolescence, and by adulthood, most patients experience a significant reduction in the number of tics [1]. One possible explanation for this
is that the cerebral expression level of the Gαi protein-coupled CB2 receptor is gradually re-stabilized in adult TS patients with reduced tics.

**HU-308 Increases DOI-induced Motor-like Tics but has a Novel Target in Adult Mice**

In the presence or absence of CB2 receptor expression, HU-308 significantly increased DOI-induced ESR and grooming behavior, implying that another target mediates the effect of HU-308 on motor-like tics in adult mice. Indeed, HU-308 has a number of off-target receptors including 5-HT2A, cholecystokinin 1 (CCK-1), tachykinin 2 (NK2), and angiotensin 1 (AT1) receptors, and the dopamine and norepinephrine transporters [19]. Identification of the off-target receptor(s)/transporter(s) of HU-308 in this model may lead to the discovery of a new pathway that regulates motor tics.

**HU-308 Increase of Motor-like Tics in Juveniles is CB2 Receptor-mediated**

Surprisingly, in juveniles, HU-308 alone significantly increased basal repetitive behaviors. The stimulatory effects of HU-308 on basal motor-like tics were mimicked by E-BCP, another CB2 receptor-selective agonist. These results suggest a possible role for stimulation of the CB2 receptor in the development of motor tics in children. Thus, it is possible that in juveniles, in the presence of basal activity of D2 autoreceptors (i.e., lack of dopamine), the CB2 receptor will favor the coupling to Gαs protein [12] (extended in the Supplementary Information). This may mean that selective CB2 receptor agonists, such as HU-308 and E-BCP, and endogenous CB2 receptor agonists, such as 2-arachidonoylglycerol (2-AG), will possibly enhance the release of dopamine in children, resulting in increased frequency of motor tics.

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**Fig. 6** Effects of Δ⁹-THC (5 mg/kg) on the mRNA expression level of elements of the endocannabinoid system and GPR55 in the left (a–c, g–i) and right (d–f, j–l) prefrontal cortex of juvenile male mice. The experiment was independently repeated 5 times. Expression level was normalized to GAPDH and expressed relative to the control group (vehicle + saline). Expression level was compared with this of the control group (vehicle + saline) and analyzed with Student’s t-test, unpaired, two tails (or one tail as indicated), followed by Welch’s correction. *P < 0.05 significantly different.
Behavioral Response to HU-308 is Dependent on Age and Sex

In contrast to the enhancing effect by HU-308 of DOI-induced motor-like tics in adult mice, in juvenile mice, HU-308 inhibited DOI-induced HTR, ESR, and grooming behavior. These inhibitory effects of HU-308 were mimicked in another model system of SR141716A-induced motor-like tics, where HU-308 inhibited SR141716A-induced HTR and ESR. These results suggest that the effect of selective CB2 receptor agonists on motor-like tics and urge-like responses is dependent on age. The implications for drug development are that selective CB2 receptor ligands should be tested at different developmental stages within the same model.

Our study found that in juvenile mice, (1) HU-308 and E-BCP, selective CB2 receptor agonists, enhanced basal repetitive behaviors in juvenile males, suggesting these effects were CB2-mediated; (2) the intensity of the effects of HU-308 in females was lower than in males, e.g., HU-308 had a lower or no effect on HTR in juvenile females; (3) HU-308 significantly decreased basal grooming behavior in juvenile females but not in males, suggesting that CB2 receptor stimulation may possibly reduce the frequency of caudally located motor tics in juvenile females; (4) HU-308 (0.2 mg/kg) significantly inhibited DOI-induced HTR and grooming behavior in females but not in males.

Collectively, these results suggest that the CB2 receptor contributes to the skewed ratio between juvenile males and females with TS, reducing the prevalence of TS in juvenile females. Possible explanations for these results are related to common pathways between sex hormones and cannabinoids [47]. In specific brain areas, estrogen modulates the inhibitory effect of cannabinoids on GABAergic and glutamatergic transmission [48]. In addition, 17-beta-oestradiol increases CB2 receptor expression on osteoclast [49]. Thus, it may be possible that in juvenile females, estrogen modulates GABA release while increased estradiol level may contribute to the increased expression level of the CB2 receptor, which in turn may reduce the release of dopamine [14] in the basal ganglia. Revealing the mechanism may explain why juvenile females are, relatively to males, more protected from the generation of motor tics.

Activation of 5-HT2A/2C Receptors Induces Lateralization in the Endocannabinoid System

Activation of 5-HT2A/2C receptors reduced the expression level of transcripts encoding ABHD6 and MAGL enzymes, which hydrolyze the endocannabinoid 2-AG, and FAAH which hydrolyses anandamide. As there can be differences between gene and protein expression, we discuss below the different possible scenarios. In the first scenario, gene and protein expressions are in opposite directions. RNA-binding proteins that regulate translational processes are crucial for proper neuronal function though the control of post-transcriptional events [50]. In our model system, this may result in no change in the protein expression level of the above enzymes or may lead to an actual increase in the expression level of these enzymes, independent of a change of gene transcript. Such an increased enzymatic activity will reduce the level of the above endocannabinoids, damaging neuronal and glia functioning. According to this scenario, small molecules that inhibit these enzymes may lead to the development of new therapeutics for the treatment of motor tics. Such a candidate is ABX-1431, which inhibits MAGL; however, a clinical trial with ABX-1431 in adult patients with Tourette syndrome did not show significant results [51].

In the second scenario, gene and protein expressions are in the same direction. This may result in an increase in 2-AG and anandamide levels. These results suggest the existence of a mechanism for a “sustained” increase of 2-AG and anandamide levels in TS. This is important as a clinical study found increased 2-AG and anandamide levels in the CSF of patients with TS [52]. Another mechanism has been proposed for “acute” increase of 2-AG level, where activation of M4 muscarinic acetylcholine receptors expressed on a population of striatal D1-expressing medium spiny neurons (MSNs) increases the synthesis of 2-AG, which is then retrogradely released to stimulate presynaptic CB2 receptors on dopaminergic terminals [11]. Indeed, activation of 5-HT2A/2C receptors by DOI induces the release of acetylcholine in the prefrontal cortex [53]. Therefore, it is possible that both mechanisms exist in the prefrontal cortex leading to increased 2-AG level. However, while the “acute” mechanism has been associated with the initial response to stress, a fight-or-flight survival mechanism, the “sustained” mechanism has been associated with long-term effects of stress, leading, for example, to memory impairment [54].

Our results imply that this increased 2-AG level may possibly be a result of 5-HT2A/2C receptor stimulation and can start as early as childhood, leading to left prefrontal cortex lateralization in the expression levels of components of the endocannabinoid system. Interestingly, the left dorsolateral prefrontal cortex controls error-related processes, while the left dorsolateral premotor cortex controls accurate movement timing of either hand [55, 56]. Indeed, lateralization in single-hand finger movements, with longer touch duration, shorter movement time, and more errors, has been presented by children with TS and can persist into adulthood [57, 58]. Correlating 2-AG levels in the brain with those of the CSF levels from treated animals and from patients with errors in sequential finger tasks may help to diagnose patients with TS.
GPR55 Inhibitors as Novel Drugs for TS

Our results suggest that activation of 5-HT$_{2A/C}$ receptors will increase the expression of both CB$_2$ receptor and GPR55. Interestingly, 2-AG is more potent at GPR55 than at CB$_1$ and CB$_2$ receptors but has a similar efficacy at these receptors [59]. In the periphery, CB$_2$ receptors heterodimerize with GPR55 to inhibit GPR55 activity [60]. This suggests that an increase in the number of both receptors may increase the number of heterodimers to reduce GPR55 activity, which in turn may impair movement coordination [61]. The potential increased GPR55 expression supports the development of GPR55 inhibitors to treat TS and suggests that a drug combination of Δ$_9$-THC with potent GPR55 inhibitors such as tetrahydrocannabinvarin (THCV) and cannabidivarin (CBDV) [7, 31, 62] may provide a more efficacious combination of cannabinoids to treat motor tics and to improve motor coordination in patients with TS.

In another system, similar opposing effects of the CB$_1$ receptor (as a tumor suppressor) to GPR55 (as an oncogene) have been documented, in which DNA methylation of the CNR1 and GPR55 genes were also differentially regulated in samples from patients with colorectal cancer compared to control samples [63]. Further application of bioinformatics will be important to direct future studies in the field of Tourette syndrome.

Summary

This study discovered that (1) the deletion of CB$_2$ receptor expression enhances repetitive behaviors in adult mice; (2) HU-308 modulates a novel target that increases 5-HT$_{2A/C}$ receptor-induced repetitive behaviors in adult mice; and (3) stimulation of the CB$_2$ receptor by selective agonists enhances repetitive behaviors in juvenile mice. This study suggests that stimulation of the CB$_2$ receptor in children may contribute to the appearance of motor tics and to the prevalence of motor tics in boys. The results support the development of CB$_2$ receptor and GPR55 inhibitors (i.e., antagonists, inverse-agonists, negative allosteric modulators), but also suggest that development of enzyme enhancers (enzyme potentiators) of ABHD6, MAQL, FAAH enzymes, and possibly their combination with or without a CB$_2$ receptor inhibitor and a GPR55 inhibitor will provide alternative approaches to treat patients with TS that are diagnosed with increased 2-AG level.

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Author Contribution SAG conceived and designed the research. IRG synthesized SR141716A. RGP contributed to the pharmacological experiments. SAG and PM were the PIs and co-mentored VG. VG (Ph.D. student) was the main contributor to this research, performed experiments, analyzed, and graphed data. SAG mentored VB (M.Sc. student). VB performed the dissections, qPCR experiments and analysis, and genotyped the CB$_2^{-/-}$ mice. SAG, VG, VB, and PM wrote the manuscript. RGP contributed with critical comments.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Declarations

Ethics approval All experiments were approved by the Institutional Animal Use and Care Committees of Tel-Aviv University and Ariel University and were in accordance with the UK Home Office, EU directive 63/2010E, and the Animal (Scientific Procedures) Act 1986.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests SAG is a member of the Clinical Advisory Committee for Tourette syndrome, Tourette Syndrome Association of Israel (TSAI), and a member of the International Consortium For Medical Cannabis and Related Drugs For Tic Disorders, Tourette Association of America (TAA). SAG is Section Editor for the Endocannabinoid system of the Journal of Cannabis Research and is the founder of Fridel Pharma. RGP is a member of the Board of Directors of the Internation Cannabinoid Research Society and of the International Association for Cannabinoid Medicines. RGP receives royalties for his published books “Handbook of Cannabis” and “Endocannabinoids.” SAG, IG, and RGP have filed patent applications related to cannabinoids. The authors VG, VB, and PM have no financial/non-financial interests.

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