A restricted population of CB_1 cannabinoid receptors with neuroprotective activity

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The CB_1 cannabinoid receptor, the main molecular target of endocannabinoids and cannabis active components, is the most abundant G protein-coupled receptor in the mammalian brain. Of note, CB_1 receptors are expressed at the synapses of two opposing (i.e., GABAergic/inhibitory and glutamatergic/excitatory) neuronal populations, so the activation of one and/or another receptor population may conceivably evoke different effects. Despite the widely reported neuroprotective activity of the CB_1 receptor in animal models, the precise pathophysiological relevance of those two CB_1 receptor pools in neurodegenerative processes is unknown. Here, we first induced excitotoxic damage in the mouse brain by (*i*) administering quinolinic acid to conditional mutant animals lacking CB_1 receptors selectively in GABAergic or glutamatergic neurons, and (*ii*) manipulating corticostriatal glutamatergic projections remotely with a *designer receptor exclusively activated by designer drug* pharmacogenetic approach. We next examined the alterations that occur in the R6/2 mouse, a well-established model of Huntington disease, upon (*i*) fully knocking out CB_1 receptors, and (*ii*) deleting CB_1 receptors selectively in corticostriatal glutamatergic or striatal GABAergic neurons. The data unequivocally identify the restricted population of CB_1 receptors located on glutamatergic terminals as an indispensable player in the neuroprotective activity of (endo)cannabinoids, therefore suggesting that this precise receptor pool constitutes a promising target for neuroprotective therapeutic strategies.

neuroprotection | neuromodulation | excitotoxicity

Endocannabinoids are a family of neuron-communication messengers that act by engaging CB_1 cannabinoid receptors, which are also targeted by Δ^9-tetrahydrocannabinol (THC), the main bioactive component of cannabis. Endocannabinoid signaling serves as a pivotal feedback mechanism to prevent excessive presynaptic activity, thereby tuning the functionality and plasticity of many synapses (1, 2). The CB_1 receptor is the most abundant G protein-coupled receptor in the brain, and is highly expressed in GABAergic terminals of the forebrain (particularly in cholecystokinin-positive and parvalbumin-negative interneurons) (3), where it inhibits GABA release. Functional CB_1 receptors reside as well on terminals of glutamatergic neurons in several brain regions, where they inhibit glutamate release (4). In concert with this well-established neuromodulatory function, the CB_1 receptor protects neurons in many different animal models of acute brain damage and chronic neurodegeneration, which, during recent years, has raised hope about the possible clinical use of cannabinoids as neuroprotective drugs, especially in still unexplored conditions such as Alzheimer's disease, Huntington disease (HD), amyotrophic lateral sclerosis, and stroke (5–7). However, the assessment of the physiological relevance and therapeutic potential of the CB_1 receptor in neurological diseases is hampered, at least in part, by the lack of knowledge of the neuron-population specificity of CB_1 receptor action. Here, by using various genetic models of CB_1 receptor loss of function, together with pharmacological and pharmacogenetic tools, we show that a unique population of CB_1 receptors, namely that located on glutamatergic terminals, plays an indispensable role in the neuroprotective activity of the endocannabinoid system in the mouse brain. This finding opens a new conceptual view on how the CB_1 receptor evokes neuroprotection, and provides preclinical support for improving the development of cannabinoid-based neuroprotective therapies.

Results

CB_1 Cannabinoid Receptors Located on Glutamatergic but Not GABAergic Neurons Protect Against Excitotoxic Damage. To evaluate the neuroprotective role of CB_1 receptors located on glutamatergic (excitatory) or GABAergic (inhibitory) terminals, we first used conditional mutant mice lacking CB_1 in glutamatergic neurons (Glu-$CB_1^{-/-}$ mice) or GABAergic neurons (GABA-$CB_1^{-/-}$ mice). These animals were injected in the striatum

Significance

Cannabinoids and their endogenous counterparts, the so-called endocannabinoids, promote neuroprotection in laboratory animals by engaging CB_1 cannabinoid receptors, one of the most abundant types of receptors in the brain. However, the assessment of the physiological relevance and therapeutic potential of the CB_1 receptor in neurological diseases is hampered, at least in part, by the lack of knowledge of the neuron-population specificity of CB_1 receptor action. This study shows that a unique and well-defined population of CB_1 receptors, namely that located on glutamatergic terminals, plays a key neuroprotective role in the mouse brain. This finding opens a new conceptual view on how the CB_1 receptor evokes neuroprotection, and provides preclinical support for improving the development of cannabinoid-based neuroprotective therapies.

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with quinolinic acid (QA), a widely used agonist of ionotropic NMDA-type glutamate receptors, at a dose (50 nmol in 1 μL PBS solution, unilaterally) that, in our hands, does not exert overt deficits in WT mice (Fig. 1*A* and Fig. S1). Glu-$CB_1^{-/-}$ mice were sensitive to excitotoxic damage, as determined by (*i*) the loss of dopamine- and cAMP-regulated phosphoprotein of 32 kDa [DARPP-32; a paradigmatic marker of medium-sized spiny neurons (MSNs), the cells that constitute ∼90% of total striatal neurons], and (*ii*) the decline in RotaRod performance (a well-established motor paradigm that relies, at least in part, on striatal function; Fig. 1*A*). In contrast, no significant neurotoxicity was observed in QA-treated GABA-$CB_1^{-/-}$ animals (Fig. 1*A*).

To prove the direct antiexcitotoxic activity of CB_1 receptors located on glutamatergic terminals, we prepared organotypic cultures of corticostriatal slices from WT mice, and found that the loss of DARPP-32 immunoreactivity produced by incubation with QA (50 μM) was prevented by the cannabinoid receptor agonist THC (1 μM; Fig. 1*B*). This neuroprotective effect of THC was (*i*) impaired by the CB_1 receptor-selective antagonist rimonabant (5 μM) and (*ii*) absent in slices from Glu-$CB_1^{-/-}$ mice (Fig. 1*B*).

Cannabinoid Receptor Agonist Prevents Excitotoxic Damage Induced by Selective Activation of Corticostriatal Glutamatergic Neurons. To further support the antiexcitotoxic activity of glutamatergic-terminal CB_1 receptors, we selectively manipulated corticostriatal glutamatergic terminals in vivo by the *designer receptor exclusively activated by designer drug* (DREADD) pharmacogenetic technique. This is a newly developed tool based on the molecular evolution of muscarinic acetylcholine receptors, leading to a G_q protein-coupled receptor with negligible affinity for the native agonist (acetylcholine) but to which the orally bioavailable, pharmacologically inert agonist clozapine-*N*-oxide (CNO) binds with high potency and efficacy (8). Importantly, DREADDs lack detectable constitutive activity, thus allowing the remote control of neuronal activity in specific cell populations in vivo (9). Here, we injected stereotactically WT mice with a recombinant adenoassociated viral vector encoding an engineered G_q protein-coupled DREADD fused to mCherry (or only mCherry as control) into the motor cortex, where the somata of the glutamatergic afferents projecting onto the dorsolateral (motor) striatum reside. The expression of the transgene was driven by the calcium/calmodulin-dependent protein kinase II-α (CaMKIIα) promoter to confine it to principal (glutamatergic) neurons and to avoid other neuronal populations such as GABAergic interneurons. Animals were subsequently treated with CNO (or vehicle as control) in conditions that are known to evoke sustained neuronal activation (10 mg/kg body weight per day for 4 wk) (10). This procedure triggered excitotoxic damage in the striatum by enhancing glutamatergic transmission, as evidenced by (*i*) the CNO-induced reduction of DARPP-32 immunoreactivity and RotaRod performance and (*ii*) the abrogation of CNO action by the NMDA receptor-selective antagonist MK-801 (0.03 mg/kg body weight per day; Fig. 2 *A* and *B*). Of note, treatment with THC (2 mg/kg body weight per day) prevented the striatal damage evoked by DREADD-G_q-mediated activation of corticostriatal projections (Fig. 2 *A* and *B*).

Genetic Deletion of CB_1 Cannabinoid Receptors Aggravates HD-Like Striatal Neurodegeneration by Altering Glutamatergic but Not GABAergic Transmission. To assess the functional impact of the CB_1 receptor on glutamatergic and GABAergic signaling in a neurodegenerative-disease context, we conducted experiments in the R6/2 mouse, a well-established model of HD. This devastating disease constitutes so far the best paradigm to study the specific role of CB_1 receptors located on glutamatergic or GABAergic terminals because CB_1 receptors are expressed in the striatum at synapses established by neurons containing GABA (especially MSNs, the cells that primarily degenerate in HD) or glutamate (especially corticostriatal projecting neurons, which critically control MSN function) as transmitters, and play a key role in the control of motor behavior, one of the processes that is most typically affected in HD (11, 12). Moreover, a remarkable down-regulation of CB_1 receptors has been documented

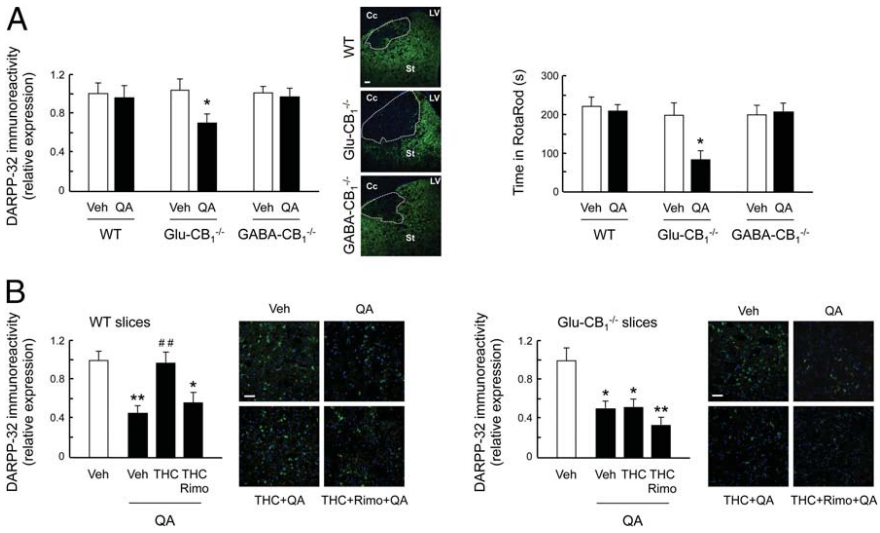

Fig. 1. CB_1 cannabinoid receptors located on glutamatergic but not GABAergic neurons protect against excitotoxic damage. (*A*) Glu-$CB_1^{-/-}$ and GABA-$CB_1^{-/-}$ mice, as well as WT ($CB_1^{floxed/floxed}$) littermates, were injected intrastriatally with vehicle (Veh) or QA (50 nmol in 1 μL PBS solution, unilaterally; n = 6–8 animals per group). RotaRod performance was evaluated during the following 3 d, and animals were killed the day after for determination of DARPP-32 immunoreactivity in the dorsolateral striatum (data expressed as relative values from the vehicle-treated WT group). (*B*) Corticostriatal slices from WT ($CB_1^{floxed/floxed}$) mice and Glu-$CB_1^{-/-}$ littermates were incubated for 24 h with vehicle or QA (50 μM) alone or with vehicle, THC (1 μM), and/or rimonabant (Rimo; 5 μM), and DARPP-32 immunoreactivity in the dorsolateral striatum was determined (data expressed as relative values vs. corresponding vehicle-treated group; n = 4–6 preparations per condition). Representative images of DARPP-32 staining (DARPP-32 in green; DAPI in blue) are shown in *A* (the area of apparent DARPP-32 loss is outlined; Cc, corpus callosum; LV, lateral ventricle; St, striatum) and *B*. (Scale bars: *A*, 100 μm; *B*, 50 μm.) (*$P < 0.05$ and **$P < 0.01$ vs. corresponding vehicle-treated group; ##$P < 0.01$ vs. QA vehicle-treated group.)

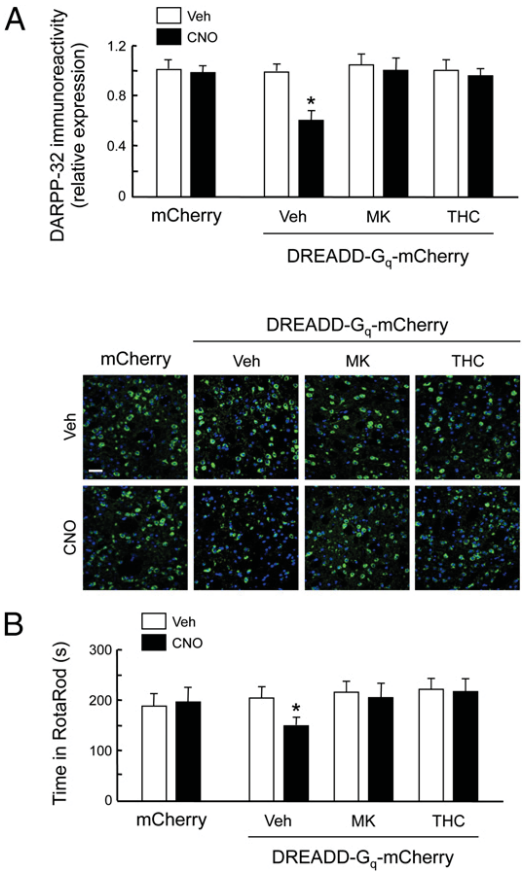

Fig. 2. Cannabinoid receptor agonist prevents excitotoxic damage induced by selective activation of corticostriatal glutamatergic neurons. (*A* and *B*) WT (C57BL/6N) mice were injected stereotactically into the motor cortex with a recombinant adenoassociated virus encoding DREADD-G_q-mCherry (or mCherry) under the control of the CaMKIIα promoter (n = 8–10 animals per group). Six weeks later, mice received daily i.p. injections of vehicle (Veh) or CNO (10 mg/kg body weight) alone or in combination with vehicle, MK-801 (0.03 mg/kg body weight), or THC (2 mg/kg body weight) for 4 wk. RotaRod performance was evaluated during the last 3 d of treatment, and animals were killed the day after for histological analyses. (*A*) DARPP-32 immunoreactivity in the dorsolateral striatum (data expressed as relative values vs. vehicle-treated mCherry group). Representative images of DARPP-32 staining are shown (DARPP-32 in green; DAPI in blue). (Scale bar, 50 μm.) (*B*) RotaRod performance (i.e., time to fall). (*$P < 0.05$ vs. corresponding vehicle-treated group.)

as one of the earliest and most characteristic neurochemical alterations found in the MSNs of HD animal models (13, 14) and patients with HD (15, 16). In striking contrast, CB_1 receptors located on glutamatergic terminals are fully preserved in (*i*) the striatum of symptomatic R6/2 mice (17) (Fig. S2) and (*ii*) the striatum (caudate-putamen) of patients with HD (Fig. S3).

We (18) and others (19) have recently reported that double-mutant mice expressing mutant huntingtin in a $CB_1^{-/-}$ background show an overt HD-like phenotype at earlier ages than their single-mutant littermates expressing mutant huntingtin in a normal $CB_1^{+/+}$ background. To test whether this detrimental consequence of knocking-out of CB_1 receptors is evoked by the deinhibition of glutamatergic and/or GABAergic transmission, we generated R6/2:$CB_1^{-/-}$ mice and their control littermates, and evaluated the effect of $GABA_A$ receptor or NMDA receptor-selective antagonists (picrotoxin and MK-801, respectively) at an early stage of the disease (4–8 wk) in which CB_1 receptor deletion is known to precipitate HD-like alterations (18). Picrotoxin administration (0.3 mg/kg body weight per day) to R6/2:$CB_1^{-/-}$ mice was unable to counteract the deleterious effect of CB_1 genetic ablation on striatal volume (Fig. 3*A*), striatal DARPP-32 expression (Fig. 3*B*) or RotaRod performance (Fig. 3*C*). Likewise, despite the remarkable loss of CB_1 receptors in the MSNs of R6/2 mice, picrotoxin did not prevent striatal neurodegeneration in our early-symptomatic (4–8 wk old; Fig. 3) or symptomatic (8–12 wk-old; Fig. S4) R6/2:$CB_1^{+/+}$ mice. In contrast, MK-801 administration (0.03 mg/kg body weight per day) rescued all these HD-like neuropathological and behavioral alterations of R6/2:$CB_1^{-/-}$ mice to the levels of their R6/2:$CB_1^{+/+}$ littermates (Fig. 3).

Cre Recombinase-Driven Deletion of CB_1 Cannabinoid Receptors in Corticostriatal but Not Striatal Neurons Aggravates HD-Like Neurodegeneration. To substantiate the selective neuroprotective activity of CB_1 receptors located on glutamatergic terminals in HD, we crossed R6/2 mice with CB_1 receptor-floxed mice, thus generating a HD-like mouse line that allows the spatiotemporally controlled excision of the *loxP*-flanked CB_1 receptor gene by Cre recombinase. Because this excision process can take several weeks in the mouse brain (20–22), we generated a new R6/2 mouse line (designated as R6/2L) that expresses a longer mutant tract (~250 CAG repeats) and has a longer survival (~30 wk) than "normal" R6/2 mice [similar to other CAG tract-expanded R6/2 mouse-derived lines previously reported (23, 24)]. We therefore injected stereotactically these R6/2L:$CB_1^{floxed/floxed}$ mice (and $CB_1^{floxed/floxed}$ control littermates) with a recombinant adenoassociated viral vector encoding Cre (or EGFP as control) into the

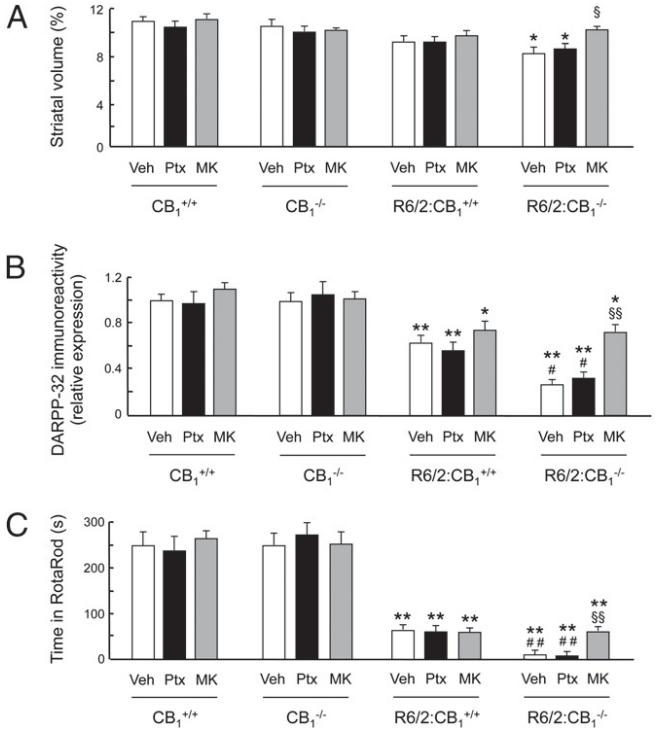

Fig. 3. Genetic deletion of CB_1 cannabinoid receptors aggravates HD-like striatal neurodegeneration by altering glutamatergic but not GABAergic transmission. (*A*–*C*) $CB_1^{+/+}$, $CB_1^{-/-}$, R6/2: $CB_1^{+/+}$, and R6/2:$CB_1^{-/-}$ mice were treated i.p. with vehicle (Veh), picrotoxin (Ptx; 0.3 mg/kg body weight per day), or MK-801 (MK; 0.03 mg/kg body weight per day) from week 4 to week 8 of age. (*A*) Striatal volume (percentage of total brain volume). (*B*) DARPP-32 immunoreactivity in the dorsolateral striatum (relative values vs. vehicle-treated $CB_1^{+/+}$ group). (*C*) RotaRod performance (i.e., time to fall). Data in *A*–*C* correspond to 8-wk-old mice at the end of the treatments (n = 8–12 animals per group). (*$P < 0.05$ and **$P < 0.01$ vs. corresponding $CB_1^{+/+}$ or $CB_1^{-/-}$ group; #$P < 0.05$ and ##$P < 0.01$ vs. corresponding R6/2:$CB_1^{+/+}$ group; §$P < 0.05$ and §§$P < 0.01$ vs. vehicle-treated R6/2:$CB_1^{-/-}$ group.)

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dorsolateral striatum or the motor cortex. Cre expression was driven by the CaMKIIα promoter, and so it was confined to MSNs (injections into the striatum) or principal neurons (injections into the cortex). Cre-mediated excision of the *loxP*-flanked CB_1 receptor gene in dorsolateral MSNs of R6/2L:$CB_1^{floxed/floxed}$ mice (Fig. S5 *A* and *B*) had no significant effect on DARPP-32 expression (Fig. S5*C*) or RotaRod performance (Fig. S5*D*). In contrast, inactivation of the CB_1 receptor gene in the motor cortices of R6/2L:$CB_1^{floxed/floxed}$ mice (Fig. 4 *A* and *B*) worsened those two hallmarks of striatal integrity (Fig. 4 *C* and *D*).

Discussion

In this report, we show that a restricted population of CB_1 receptors, namely that located on glutamatergic terminals, plays an indispensable role in the neuroprotective activity of the endocannabinoid system. The size of this pool of glutamatergic-terminal CB_1 receptor molecules seems to be much smaller than that of GABAergic-terminal CB_1 receptors (4, 5). However, CB_1 receptors located on glutamatergic terminals are strongly coupled to heterotrimeric G protein signaling (25) and, in fact, participate in the control of important neurobiological processes such as neuronal excitability (22), motor activity (26), feeding behavior (27), and anxiety (28). Our present findings support that this specific pool of CB_1 receptors should be considered a new key player in the excitotoxicity hypothesis of neural disease (29, 30). On mechanistic grounds, it is very plausible that, upon intense activation of a glutamatergic projection, glutamate spillover out of the synapse would trigger in the target neuron the activation of the perisynaptic machinery of endocannabinoid generation (5), composed of type 1 metabotropic glutamate receptors (mostly mGluR5), $G_{q/11}$ proteins, phospholipase C-β, and diacylglycerol lipase-α, thus producing the endocannabinoid 2-arachidonoylglycerol, which would engage presynaptic CB_1 receptors located on the glutamatergic terminal, thereby inhibiting excess excitatory transmission (5) and buffering the potential neurotoxic effects of extrasynaptic NMDA receptors in the postsynaptic neuron (31, 32).

In the precise case of HD, it has been long suggested that the early and massive down-regulation of CB_1 receptors located on MSNs plays a pathogenic role in promoting disease onset and progression (12, 33, 34). Thus, as the CB_1 receptor couples to several cell-autonomous neuroprotective pathways (6, 35), one might suppose that its down-regulation in MSNs would render these cells more susceptible to damage. However, as it is well established that CB_1 receptors located on MSNs inhibit GABA release (4, 5), it would also be conceivable that their notable loss enhanced extracellular GABA availability, thereby constituting an adaptive mechanism aimed at attenuating excitatory transmission and, in turn, excitotoxicity of MSNs. These possibilities notwithstanding, here, by using various pharmacological and genetic approaches, we were unable to detect any overt effect of the MSN-CB_1 receptor pool on striatal damage. In contrast, impairing the function of the corticostriatal-terminal CB_1 receptor pool produced remarkable deleterious effects in the striatum. This strongly supports that (*i*) the detrimental effects elicited by the complete genetic elimination of CB_1 receptors in HD mouse models (18, 19) result from the inactivation of CB_1 receptors located on corticostriatal projections rather than on MSNs, and (*ii*) the beneficial effects exerted by pharmacological administration of THC on HD-like progression in symptomatic R6/2 mice (18) reflects the engagement of CB_1 receptors located on corticostriatal projections rather than those on MSNs. Hence, from a translational point of view, it is tempting to speculate that the glutamatergic-neuron CB_1 receptor pool may constitute a therapeutic target to attenuate neurodegeneration in patients with HD. THC and other cannabinoids have a favorable drug safety profile and are already used in the clinic as antiemetic, anticachectic, antispastic, and analgesic compounds (36). Although

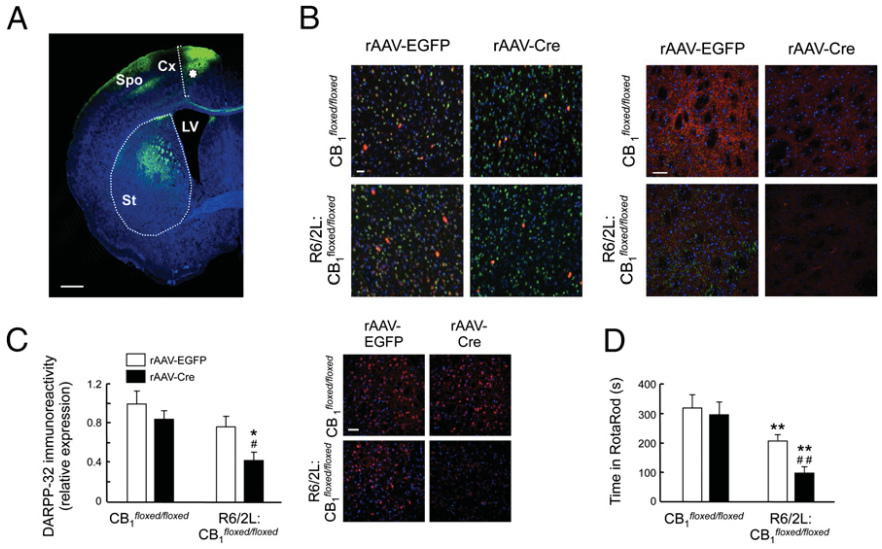

Fig. 4. Cre recombinase-driven deletion of CB_1 cannabinoid receptors in corticostriatal neurons aggravates HD-like neurodegeneration. (*A–D*) Four-week-old R6/2L:$CB_1^{floxed/floxed}$ mice and $CB_1^{floxed/floxed}$ littermates were injected stereotactically into the motor cortex with rAAV encoding Cre recombinase (or EGFP) under the control of the CaMKIIα promoter (n = 8–12 animals per group). At week 20 of age, RotaRod performance was evaluated, and animals were killed the day after for histological analyses. (*A*) Example of a brain hemisphere injected with rAAV-CaMKIIα promoter-EGFP (EGFP in green; DAPI in blue). Note the striatal EGFP labeling. Cx, cortex; LV, lateral ventricle; Spo, injection spillover; St, striatum (*approximate site of injection). (Scale bar, 500 μm.) (*B*) (*Left*) Representative images of CB_1 receptor and vGluT-1 mRNA in situ hybridization in the motor cortex (CB_1, red; vGluT-1, green; DAPI, blue). Note the Cre-mediated reduction of CB_1 mRNA expression. The few spotted CB_1 highly labeled cells are interneurons. (Scale bar, 100 μm.) (*Right*) Representative images of CB_1 receptor immunostaining in the dorsolateral striatum (CB_1, red; EGFP, green; DAPI, blue). Note the Cre-mediated reduction of CB_1 protein expression. (Scale bar, 50 μm.) (*C*) DARPP-32 immunoreactivity in the dorsolateral striatum (relative values vs. corresponding rAAV-EGFP–injected $CB_1^{floxed/floxed}$ group). Representative images of DARPP-32 staining are shown (DARPP-32, red; DAPI, blue). (Scale bar, 50 μm.) (*D*) RotaRod performance (i.e., time to fall; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. corresponding $CB_1^{floxed/floxed}$ group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. rAAV-EGFP–injected R6/2L:$CB_1^{floxed/floxed}$ group).

exhaustive clinical studies are indeed necessary to assess whether cannabinoid-based medicines could be used for the management of neurodegenerative diseases, the findings reported here, by providing a specific neurobiological substrate for cannabinoid-evoked neuroprotection in preclinically relevant models, may contribute to improving the development of therapeutic approaches aimed at targeting the glutamatergic-neuron CB_1 receptor population.

Materials and Methods

Animals. We used conditional mutant mice, generated by the Cre-lox technology, in which the CB_1 receptor gene is primarily absent from cortical glutamatergic neurons of the dorsal telencephalon ($CB_1^{floxed/floxed;Nex\text{-}Cre/+}$ mice; herein referred to as Glu-$CB_1^{-/-}$ mice) or from forebrain GABAergic neurons ($CB_1^{floxed/floxed;Dlx5/6\text{-}Cre/+}$ mice; herein referred to as GABA-$CB_1^{-/-}$ mice) (26, 27). Hemizygous mice transgenic for exon 1 of the human *huntingtin* gene with an expanded CAG tract (~160 CAG repeats; R6/2 mice) (37) were purchased from Jackson Laboratory [code B6CBA-Tg(HDexon1)62Gpb/1J]. Double-mutant R6/2:$CB_1^{-/-}$ mice were generated by crossing R6/2 mice with $CB_1^{-/-}$ mice as described previously (18). In some experiments, we used another HD-like mouse line, designated as R6/2L, which expresses a longer mutant tract (~250 CAG repeats) and has a longer survival (~30 wk) than the aforementioned normal R6/2 line. This new line was obtained by exploiting the fact that transmission of the CAG tract in R6/2 mice is unstable, with a tendency to expand through the male line. We crossed R6/2L mice with $CB_1^{floxed/floxed}$ mice to generate the double-mutant R6/2L:$CB_1^{floxed/floxed}$ line as follows: R6/2L males were first cross-mated with $CB_1^{floxed/floxed}$ females. The R6/2L:$CB_1^{floxed/+}$ F1 males were back-crossed with the aforementioned $CB_1^{floxed/floxed}$ females to obtain the R6/2L:$CB_1^{floxed/floxed}$ double-mutants and the respective $CB_1^{floxed/floxed}$ controls. The colony was maintained by back-crossing R6/2L:$CB_1^{floxed/floxed}$ males with $CB_1^{floxed/floxed}$ females. In all experiments, mutant mice were compared with their corresponding littermates. Animal housing, handling, and assignment to the different experimental groups were conducted as described previously (18). All experimental procedures were performed in accordance with the guidelines and with the approval of the animal welfare committee of Madrid Complutense University (DC 86/609/EU).

Drug Treatments. THC (Health Concept) was stored in DMSO. Just before the experiments, solutions of vehicle [1% (vol/vol) DMSO in Tween-20/saline solution (1:18, vol/vol)] and THC (2 mg/kg body weight per day) were prepared for i.p. injections. CNO (Santa Cruz) was prepared fresh in saline solution just before the experiments and administered i.p. at 10 mg/kg body weight per day. Stock solutions of MK-801 (Sigma) and picrotoxin (Sigma) were prepared in ethanol and, just before the experiments, diluted into sterile distilled H_2O [final ethanol concentration 2% (vol/vol)]. Animals received i.p. injections of vehicle, MK-801 (0.03 mg/kg body weight per day), or picrotoxin (0.3 mg/kg body weight per day). These doses of MK-801 and picrotoxin were selected from our previous studies on feeding behavior (27) and memory (38), as well as from preliminary experiments on motor activity. Specifically, the drug doses used here were the highest ones that had no significant effect per se but were able to block CB_1 receptor-evoked effects on those parameters.

QA-Induced Excitotoxicity. Conditional mutant mice lacking CB_1 receptors in glutamatergic or GABAergic neurons, as well as WT mice [$CB_1^{floxed/floxed}$ littermates or C57BL6/N mice (Harlan), depending on the experiment], were injected stereotactically (unilaterally) with vehicle (1 μL PBS solution) or QA (30–150 nmol in 1 μL PBS solution, adjusted to pH 7.5) (39) at the following dorsolateral-striatum coordinates (to bregma): anteroposterior +0.6, lateral +2.0, dorsoventral −3.0. RotaRod performance was evaluated during the following 3 d. Mice were killed the day after by intracardial perfusion and their brains were excised for immunofluorescence analyses.

Viral Vectors. G_q-coupled human M3 muscarinic DREADD (hM3Dq) fused to mCherry (10) (provided by Brian L. Roth, University of North Carolina, Chapel Hill, NC) and HA-tagged Cre recombinase, or mCherry and EGFP as respective controls, were subcloned in a recombinant adenoassociated virus (rAAV) expression vector with a minimal CaMKIIα promoter (provided by Karl Deisseroth, Stanford University, Stanford, CA) by using standard molecular cloning techniques. All vectors used were of an AAV1/AAV2 mixed serotype, and were generated by calcium phosphate transfection of HEK293T cells and subsequent purification as described previously (22).

DREADD-Induced Excitotoxicity. Eight-week-old male C57BL6/N mice were injected stereotactically with CaMKIIα-hM3Dq-rAAV or CaMKIIα-mCherry-rAAV (in 1.5 μL PBS solution) aimed at targeting the motor cortex projecting onto the dorsolateral striatum. Each animal received two bilateral injections at the following coordinates (to bregma): anteroposterior +1.5, lateral ±1.2, dorsoventral −1.7; and anteroposterior −0.5, lateral ±1.2, dorsoventral −1.2. Six weeks after surgery, mice were assigned to different experimental groups and injected i.p. with vehicle or CNO (10 mg/kg body weight per day) (10), together with vehicle, MK-801 (0.03 mg/kg body weight per day), or THC (2 mg/kg body weight per day), for 4 wk. RotaRod performance was analyzed during the last 3 d of treatment. Mice were subsequently killed by intracardial perfusion, and their brains were excised for immunofluorescence analyses.

Cre Recombinase-Driven Deletion of CB_1 Receptors. Four-week-old R6/2L:$CB_1^{floxed/floxed}$ mice and their $CB_1^{floxed/floxed}$ littermates were injected stereotactically with CaMKIIα-Cre-rAAV or CaMKIIα-EGFP-rAAV (in 1.5 μL PBS solution) into the motor cortex projecting onto the dorsolateral striatum or into the dorsolateral striatum. In the case of the cortex, each animal received two bilateral injections at the following coordinates (to bregma): anteroposterior +1.5, lateral ±1.2, dorsoventral −1.7; and anteroposterior −0.5, lateral ±1.2, dorsoventral −1.2. In the case of the striatum, each animal received one bilateral injection at the following coordinates (to bregma): anteroposterior +0.6, lateral +2.0, dorsoventral −3.0. At age 20 wk, RotaRod performance was analyzed. Mice were subsequently killed by intracardial perfusion, and their brains were excised for immunofluorescence analyses and in situ hybridization.

Organotypic Cultures. Corticostriatal slices (300 μm thick) were obtained from adult WT ($CB_1^{floxed/floxed}$) and Glu-$CB_1^{-/-}$ ($CB_1^{floxed/floxed;Nex\text{-}Cre/+}$) littermates, and cultured under semidry conditions in Neurobasal medium supplemented with B27 (1%), N2 (1%), glutamine (1%), penicillin/streptomycin (1%), Fungizone (1%), and ciprofloxacin (5 μg/mL), as described previously (40). Slices were incubated for 24 h with vehicle (PBS solution) or QA (50 μM), alone or in combination with vehicle (0.1% DMSO), THC (1 μM), and/or rimonabant (5 μM). Slices were subsequently fixed with formalin and processed in 15-μm sections, which were analyzed at equivalent regions of the rostral to caudal axis. Counting of DARPP-32 immunoreactivity was conducted in the dorsolateral striatum in a one-in-six series per slice.

Synaptosomes. Synaptosomes were obtained from mouse striata as described previously (41) and used for immunomicroscopy analyses (as detailed later). Glutamate release was assayed in synaptosomal preparations from the P2 fraction with glutamate dehydrogenase, and the fluorescence of NADPH was followed by online fluorimetry (LS-50 luminescence spectrometer; PerkinElmer) (41). Stock solutions of WIN-55,212-2 were prepared in DMSO (final concentration in the assay, 0.1%).

In Situ Hybridization. Cryosections (14 μm thick) were incubated with digoxigenin-labeled riboprobes against mouse CB_1 receptor and/or FITC-labeled riboprobes against mouse vesicular glutamate transporter-1 (vGluT-1) or GAD-67 as described previously (27). For signal amplification, we used the TSA Plus Cyanine 3 and Fluorescein System (PerkinElmer). Cell nuclei were visualized with DAPI. Preparations were analyzed in an Axioplan 2 microscope (Carl Zeiss). Coexpression data were obtained with ImageJ software (National Institutes of Health) by counting fluorescence in a one-in-six series per animal in the deep motor cortex (layers 5/6) and the dorsolateral striatum, ranging from bregma +1.5 to −0.5 coronal coordinates.

Immunomicroscopy (Mouse Samples). Coronal free-floating sections (30 μm thick) were obtained from paraformaldehyde-perfused mouse brains. Synaptosomes were seeded onto polylysine-coated cover glasses. Samples were incubated with antibodies against DARPP-32 (1:1,000; BD), CB_1 cannabinoid receptor (1:500; provided by Ken Mackie, Indiana University, Bloomington, IN), vGluT-1 (1:500; Synaptic Systems), and/or Bassoon protein (1:500; Synaptic Systems), followed by staining with the corresponding Alexa Fluor 488, 594, or 647 antibodies (1:1,000; Life Technologies) (18). Nuclei were visualized with Hoechst 33342 or DAPI. Counting of DARPP-32 immunoreactivity in the dorsolateral striatum was conducted in a 1-in-10 series per animal (from bregma +1.5 to −0.5 coronal coordinates), and data were calculated as immunoreactive area per total cell nuclei, except for the QA-induced in vivo excitotoxicity experiments (Fig. 1*A*), in which data of immunoreactive area were referred to total counted area. Confocal fluorescence images were acquired by using TCS-SP2 software and a SP2 AOBS microscope (Leica). Pixel quantification and colocalization were analyzed with ImageJ software.

Immunomicroscopy (Human Samples). Paraffin-embedded postmortem 4-μm-thick brain sections containing caudate putamen were provided by Jean-Paul Vonsattel (New York Brain Bank at Columbia University, New York, NY), and were obtained and handled following the ethical guidelines of the provider's institution. Samples ($n = 4$ sections per individual) were obtained from donors with HD [grades 3–4; $n = 7$; a 54-year-old male, 56-year-old male, 56-year-old male, 58-year-old female, 59-year-old female, 61-year-old female, and 72-year-old female] and control subjects with no background of neuropsychiatric disease [n = 5; a 49-year-old male, 57-year-old male, 57-year-old male, 68-year-old female, and 74-year-old male]. Immunohistochemical analysis (42) was performed with anti-CB_1 cannabinoid receptor antibody (1:100; Thermo Scientific). Sections were further incubated with biotinylated goat anti-rabbit antibody (1:200), avidin–biotin complex (Vector Laboratories), and a diaminobenzidine substrate-chromogen system (Dako) to give a visible reaction product. For immunofluorescence analysis (42), sections were sequentially incubated with anti–vGluT-1 (1:250; Synaptic Systems), Alexa Fluor 488 (Life Technologies), anti-CB_1 cannabinoid receptor (1:50; Thermo Scientific), and Alexa Fluor 546 (Life Technologies) antibodies. Sections were treated with 1% Sudan black in 70% ethanol to quench autofluorescence.

Behavior. Motor coordination (i.e., RotaRod performance) was evaluated as described previously (18). All assays were conducted before drug injections.

MRI. Striatal volume was measured by MRI in a BIOSPEC BMT 47/40 device (Bruker) operating at 4.7 T as described previously (18).

Statistics. Data are presented as mean ± SEM. Statistical comparisons were made by ANOVA with post hoc Student–Newman–Keuls test or by unpaired Student *t* test. A *P* value of less than 0.05 was considered significant.

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