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Comparison of the D2 Receptor Regulation and Neurotoxicant Susceptibility of Nigrostriatal Dopamine Neurons in Wild-Type and CB1/CB2 Receptor Knockout Mice

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Abstract

Motor dysfunctions of Parkinson Disease (PD) are due to the progressive loss of midbrain nigrostriatal dopamine (NSDA) neurons. Evidence suggests a role for cannabinoid receptors in the neurodegeneration of these neurons following neurotoxicant-induced injury. This work evaluates NSDA neurons in CB1/CB2 knockout (KO) mice and tests the hypothesis that CB1/CB2 KO mice are more susceptible to neurotoxicant exposure. NSDA neuronal indices were assessed using unbiased stereological cell counting, high pressure liquid chromatography coupled with electrochemical detection or mass spectrometry, and Western blot. Results reveal that CB1 and CB2 cannabinoid receptor signaling is not necessary for the maintenance of a normally functioning NSDA neuronal system. Mice lacking CB1 and CB2 receptors were found to be equally susceptible to the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). These studies support the use of CB1/CB2 KO mice for investigating the cannabinoid receptor-mediated regulation of the NSDA neuronal system in models of PD.

Keywords

MPTP; CB1 receptor; CB2 receptor; nigrostriatal

INTRODUCTION

The nigrostriatal dopamine (NSDA) neuronal system consists of densely clustered neurons originating in the midbrain substantia nigra and projecting axons to the striatum. These neurons function primarily to initiate and coordinate muscle movement (Dauer and

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Przedborski 2003). Progressive loss of these neurons causes motor dysfunctions seen in Parkinson Disease (PD) (Dauer and Przedborski 2003).

Cannabinoids are a class of lipophilic compounds that are neuroprotective against excitotoxicity and oxidative damage following neuronal injury (Fowler et al. 2010). Exogenous cannabinoids protect against progressive NSDA neuronal degeneration in toxicant-based models. Non-selective cannabinoid receptor agonists decrease NSDA degeneration caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice (Chung et al. 2011; Price et al. 2009). The non-selective CB1/CB2 cannabinoid receptor agonist WIN55, 212-2 is neuroprotective during a MPTP insult in CB1 knockout mice (Price et al. 2009), suggesting that CB2 receptor signaling mediates cannabinoid-mediated neuroprotection. However, CB1 receptor signaling also contributes to neuroprotection and inhibition of microglial activation following MPTP exposure (Chung et al. 2011). Thus, both CB1 and CB2 receptors likely mediate the neuroprotective effects of cannabinoid agonists in MPTP toxicity of NSDA neurons.

The role of endocannabinoid signaling via the CB1 and CB2 receptors in NSDA neurodegeneration is not known. Endocannabinoid signaling via the CB1 and/or CB2 receptors may be part of the normal function of NSDA neurons and participate in the protective mechanisms that mitigate neurotoxicant-induced lesions, such as inhibiting the release of glutamate (Fowler et al. 2010). The lack of these receptors might disrupt the normal function of the NSDA system and its response to MPTP-induced injury. The current work was performed to evaluate the integrity and D2 receptor mediated regulation of NSDA neurons in CB1/CB2 knockout (KO) mice. It was hypothesized that CB1/CB2 KO mice would be more susceptible to MPTP due to a lack of the protective effects of signaling through CB1 and CB2 receptors. Results reveal that cannabinoid receptors are not necessary for the development of a normally functioning NSDA neuronal system. Mice lacking the CB1 and CB2 receptors demonstrate a normal susceptibility to MPTP. This strain of mice represents a useful tool for assessing the function of cannabinoid receptors in the NSDA system under basal conditions as well as in models of PD.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (Jackson Labs, Bar Harbor, ME) age 8–12 weeks were used as wild-type (WT) controls for mice lacking both CB1 and CB2 receptors on a C57BL/6 background. Male CB1/CB2 KO mice, provided by Dr. Andreas Zimmer (Jarai et al. 1999), were obtained from Drs. Norbert Kaminski and Barbara Kaplan who maintain a colony at Michigan State University. Polymerase chain reaction (PCR) was used to confirm knockout of CB1 and CB2 receptor genes as described previously (Kaplan et al. 2010).

All animals were provided with food and water *ad libitum*, and housed at 25 C with a 12 h light/dark cycle. All experiments used the minimal number of animals required for statistical analyses and followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Michigan State Institutional Animal Care and Use Committee approved all experimental treatments.

Drug Administration

γ -butyrolactone (GBL), quinelorane, raclopride, and MPTP (Sigma-Aldrich, St. Louis, MO) were dissolved in 0.9% saline. Vehicle (10 ml/kg) or quinelorane (0.1 mg/kg; i.p.) was given 1 min prior to either vehicle (10 ml/kg; i.p.) or GBL (750 mg/kg; i.p.) and mice were sacrificed by decapitation 1 h following treatment. Raclopride (1 mg/kg; i.p.) or vehicle (10 ml/kg) was given 1 h prior to decapitation. MPTP (10 or 20 mg/kg, s.c.), or vehicle (10 ml/

kg), was administered using an acute (single injection) or sub-chronic paradigm that involves daily injections for 5 days. Mice were decapitated 3 days following the last injection.

Striatal Neurochemistry and Western Blot Analyses

After euthanasia by decapitation, brains were immediately removed, frozen on dry ice, and stored at -80°C . Brains used for neurochemical and Western blot analyses were sectioned at 500 microns in a cryostat at -10°C (HM525 Microtome Cryostat, Microm International). For neurochemistry, the striatum was microdissected (Benskey et al. 2012), acidified in ice cold tissue buffer (0.05 M sodium phosphate, 0.03 M citrate, 15% methanol, pH 2.5), and sonicated using three 1 s bursts. DA and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured using HPLC-ED (Benskey et al. 2012). Peak heights of standards were used to determine content in samples. MPP⁺ was measured using HPLC-MS (Lehner et al. 2011). Protein content of samples was determined by a Lowry protein assay to normalize sample content.

For Western blot analysis, striatum and ventral midbrain were microdissected (Benskey et al. 2012), placed in either a sucrose buffer (0.25 M sucrose, 10 mM HEPES, 10 mM MgCl_2 , pH 7.4) or lysis buffer (1% sodium dodecyl sulfate (SDS), 20 mM Tris-HCl, pH 7.4), and homogenized by repeated 5 s bursts of sonication.

Cytosolic fraction isolation consisted of centrifugation for 10 min at 500 g at 4°C to pellet the sample. The supernatant was centrifuged at 18,000 RCF for 20 min at 4°C . Supernatants obtained from the second centrifugation were used to analyze cytosolic protein content. Membranes were isolated using ultracentrifugation for 10 min at 6,300 RCF at 4°C to pellet the sample. Supernatants were removed and centrifuged at 627,000 RCF for 30 min at 4°C using an Optima Max Ultracentrifuge (Beckman Coulter, Fullerton, CA). Pellets containing membrane fractions were re-suspended in PBS. Protein content was determined using a BCA protein assay.

The appropriate samples were probed by Western blot for tyrosine hydroxylase (TH), phosphorylated serine-40 TH (ser40TH), DA transporter (DAT), β -III tubulin, and synaptosome associated protein-25 (SNAP-25) using commercially available primary antibodies (1:2,000 AB152, 1:1,000 MAB369, 1:5,000 MAB1637, and 1:2,000 MAB331, Millipore, Billerica, MA or 1:1,000 #2791, Cell Signaling, Danvers, MA). Primary antibodies were reacted with secondary antibody (1:5,000 IRDye 800 goat anti-rabbit IgG or 1:10,000 IRDye 800 goat anti-rat IgG, Rockland, Gilbertsville, PA or 1:5,000 ALEXA Fluor 680 goat anti-mouse IgG, Molecular Probes, Eugene, OR). Antibody tagged proteins were visualized using an Odyssey infrared imaging system (LI-COR Biosciences). TH and ser40TH were normalized to β -III tubulin, and DAT normalized to SNAP-25 and expressed as relative density units (RDU).

TH Stereology

Mice were given a lethal dose of ketamine/xylazine (24.4 mg/kg; 3.6 mg/kg; i.p.) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. Brains were removed, post-fixed in 4% paraformaldehyde, and cryoprotected in 20% sucrose. Midbrain sections (60 μm) were prepared in a cryostat at -25°C . Every third tissue section was stained for TH (1:2,000 AB152, Millipore). Biotin conjugated goat anti-rabbit secondary antibody (1:500) (Vector Laboratories, Burlingame, CA) were reacted with an avidin-biotin complex using an ABC Vectastain kit (Vector Laboratories), followed by 3,3'-diaminobenzidine (Sigma-Aldrich). TH immunoreactive (TH-RI) cells were visualized using a 4X objective and the substantia nigra was delineated and counted using Stereo Investigator Software

version 6.55 (MicroBrightField, Inc., Williston, VT) as described previously (Behrouz et al., 2007).

Statistical Analyses

Data were analyzed using a Student's t-test or two-way ANOVA followed by Tukey's test for multiple comparisons. (Systat Software, Point Richmond, CA). An alpha value of p 0.05 was deemed statistically significant. A power analysis was performed to determine sample size based on estimated standard deviation, expected change, and $\alpha=0.05$. Eight and five mice per group provide sufficient power (80%) to detect meaningful differences between groups for neurochemistry and Western blot or stereology, respectively.

RESULTS

Basal levels of striatal DA (201 ± 18 vs 172 ± 12) and its metabolite DOPAC (41 ± 5 vs 50 ± 7) do not differ in WT versus CB1/CB2 KO mice. The basal activity of NSDA neurons as indicated by the ratio of DOPAC/DA (Lookingland and Moore 2005) was also equivalent in WT and CB1/CB2 KO mice (0.21 ± 0.03 vs 0.31 ± 0.06). The similarity in DA concentrations in the striatum of WT and CB1/CB2 KO mice was complementary to other axonal terminal markers of NSDA neurons in the striatum including striatal TH (12.1 ± 0.9 vs 12.2 ± 0.7), ser40TH (30 ± 2 vs 26 ± 2), DAT (29 ± 3 vs 38 ± 6), as well as substantia nigra TH-IR cells (12858 ± 288 vs 13909 ± 270) and ventral midbrain TH protein content (27 ± 2 vs 23 ± 3).

Treatment with GBL, a prodrug GABA_B receptor agonist, increased the amount of DA in the striatum of both WT and CB1/CB2 KO mice (Fig. 1a). This effect was attenuated by the pre-synaptic D2 receptor agonist quinlorane (Fig. 1a). Treatment with the D2 receptor antagonist raclopride decreased striatal DA concentrations (Fig. 1b), and increased DOPAC and the DOPAC/DA ratio (Figs. 1c & 1d) to a similar extent in both CB1/CB2 KO and WT mice.

Following a single injection of MPTP, CB1/CB2 KO mice have decreased concentrations of striatal MPP⁺ suggesting impaired conversion by glial MAO-B (Fig. 2a). Striatal TH content (Fig. 2b) was reduced following sub-chronic MPTP exposure in both genotypes, whereas neither MPTP nor genotype affected ser40TH (Fig. 2c). DA and DOPAC concentrations (Fig. 2d, e) were reduced following sub-chronic MPTP exposure in both genotypes. The DOPAC/DA ratio was significantly elevated in both WT and CB1/CB2 KO mice following MPTP (Fig. 2f).

DISCUSSION

Since mice exposed to cannabinoids perinatally show increases in their total striatal DA (Navarro et al. 1994), it was originally hypothesized that mice lacking CB1 and CB2 receptors from birth would show deficits in vesicular DA stores, which is indicative of impaired integrity of NSDA axon terminals (Drolet et al. 2004). However, the striatal neurochemical profile and NSDA cell body indexes are not significantly altered in CB1/CB2 KO mice. Therefore endocannabinoid activation of CB1/CB2 receptor signaling does not appear to be necessary for the normal development and neurochemical maintenance of NSDA neurons. It was also anticipated that the basal metabolic activity of NSDA neurons terminating in the striatum would be increased due to the lack of tonic inhibitory CB1 receptors, but there was no difference in the rate of DA metabolism or amount of phosphorylated TH between WT and CB1/CB2 KO mice. The results reveal that CB1 and CB2 receptors do not tonically inhibit the activity of NSDA neurons in adult mice.

Inhibitory D2 receptors regulate the activity of NSDA neurons through long-loop multisynaptic neuronal feedback and pre-synaptic autoreceptors (Lookingland and Moore 2005). Since cannabinoids have been shown to increase D2 receptors (Navarro et al. 1994) it was hypothesized that the lack of CB1 and/or CB2 receptors could disrupt D2 mediated regulation of NSDA neurons. Pharmacological blockade of D2 receptors stimulates NSDA neurons by removing both afferent neuronal and autoreceptor inhibitory mechanisms. Blockade of impulse flow and DA release in NSDA neurons following GBL administration disengages D2 autoreceptor-mediated inhibition of TH activity and increases *de novo* synthesis and accumulation of DA in axon terminals (Lookingland and Moore 2005). DA synthesis and accumulation is prevented by re-activation of autoreceptors with a D2 agonist. In the present study, there were no differences in D2 receptor regulation of NSDA neurons between WT and KO mice, revealing that lack of CB1/CB2 receptor signaling does not participate in DA autoregulatory and long loop multi-synaptic D2 receptor regulatory systems.

MPTP is used widely as a toxicant-based model of neurodegeneration that causes predictable injuries to NSDA neurons associated with displacement and metabolism of vesicular DA and metabolic stress secondary to inhibition of mitochondrial Complex I activity (Benskey et al. 2012; Behrouz et al. 2007; Petroske et al. 2001; Anderson et al. 2006). Despite CB1/CB2 KO mice having impaired striatal glial conversion of MPTP to MPP⁺ following a single injection, they do not differ from WT in the neurochemical profile following repeated MPTP administration. Decreased striatal DA, TH, and DOPAC in mice treated sub-chronically reflects axon terminal degeneration, impaired synthesis and metabolism of DA (Anderson et al. 2006; Petroske et al. 2001), and is paralleled by other markers of axon terminal degeneration (Behrouz et al. 2007; Jackson-Lewis et al. 1995). These data suggest that lack of CB1 and/or CB2 signaling is neither protective nor detrimental to the susceptibility of NSDA neurons to MPTP toxicity. Together, these data demonstrate that CB1/CB2 KO mice represent a useful tool for assessing the function of cannabinoid receptors in the NSDA system under basal conditions as well as in models of PD.

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Fig. 1.

D2 autoreceptor regulation of NSDA neurons terminating in the striatum of WT and CB1/CB2 KO mice. **a)** Concentrations of DA in the striatum of WT and CB1/CB2 KO mice given either saline & vehicle, GBL (750 mg/kg) & vehicle, or GBL & quinelorane (0.1 mg/kg) 1 h prior to decapitation. Columns represent means of DA concentrations (ng/mg protein) as determined by HPLC-ED + 1 SEM, $n=7-8$. * Indicates values in GBL-treated mice significantly different from values in saline vehicle treated mice of the same genotype ($p < 0.05$). # Indicates values in GBL + quinelorane treated mice significantly different from GBL treated mice of the same genotype ($p < 0.05$). **b c & d)** Concentrations of DA (**b**), DOPAC (**c**), and DOPAC/DA (**d**) in the striatum of WT and CB1/CB2 KO mice given saline or raclopride (1 mg/kg) 1 h prior to decapitation. Columns represent means (ng/mg protein) as determined by HPLC-ED + 1 SEM, $n=7-8$. * Indicates values in raclopride treated mice significantly different from saline treated controls with the same genotype ($p < 0.05$).

Fig. 2.

Effects of MPTP treatment in the striatum of WT and CB1/CB2 KO mice. **a**) Mice were injected with MPTP (10 mg/kg) and sacrificed 1, 2, 4, or 8 h later. Zero time mice were treated with saline and sacrificed 1 h later. MPP⁺ concentrations were measured in the striatum using HPLC-MS. Data points represent mean MPP⁺ (ng/mg protein) $\pm$ 1 SEM, $n=5$. Filled symbols indicate values in MPTP treated mice significantly different from zero time controls of the same genotype ($p < 0.05$). * Indicates values in WT mice significantly different from CB1/CB2 KO mice at the corresponding time point ($p < 0.05$). **b & c**) Mice were treated with saline or MPTP (10 mg/kg) daily for 5 days and decapitated 3 days after the final injection. TH and ser40TH protein were normalized to β -III tubulin. TH protein (**b**) and ser40TH (**c**) data are expressed as mean normalized RDU $\pm$ 1 SEM, $n=6-10$. * Indicates values in MPTP treated mice significantly different from saline controls of the same genotype ($p < 0.05$). **d e & f**) Mice were treated with either saline or MPTP (20 mg/kg) daily for 5 days and decapitated 3 days after the final injection. Concentrations of DA (**d**), DOPAC (**e**), and DOPAC/DA (**f**) were measured in the striatum. Columns represent means (ng/mg protein) as determined by HPLC-ED $\pm$ 1 SEM, $n=9-11$. * Indicates values in MPTP treated mice significantly different from saline controls of the same genotype ($p < 0.05$).