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The Melanoma-Linked “Redhead” *MC1R* Influences Dopaminergic Neuron Survival

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Abstract

Objective—Individuals with Parkinson disease are more likely to develop melanoma, and melanoma patients are reciprocally at higher risk of developing Parkinson disease. Melanoma is strongly tied to red hair/fair skin, a phenotype of loss-of-function polymorphisms in the *MC1R* (melanocortin 1 receptor) gene. Loss-of-function variants of *MC1R* have also been linked to increased risk of Parkinson disease. The present study is to investigate the role of *MC1R* in dopaminergic neurons in vivo.

Methods—Genetic and pharmacological approaches were employed to manipulate *MC1R*, and nigrostriatal dopaminergic integrity was determined by comprehensive behavioral, neurochemical, and neuropathological measures.

Results—*MC1R*^{e/e} mice, which carry an inactivating mutation of *MC1R* and mimic the human redhead phenotype, have compromised nigrostriatal dopaminergic neuronal integrity, and they are more susceptible to dopaminergic neuron toxins 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Furthermore, a selective *MC1R* agonist protects against MPTP-induced dopaminergic neurotoxicity.

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Author Contributions

X.C., H.C., D.E.F., and M.A.S. contributed to conception and design of the study; X.C., H.C., W.C., M.M., B.Y., F.Z., R.L., H.L., K. R., C.R.V., Y.Y., and Y.W. contributed to acquisition and analysis of data; X.C. wrote the article.

Potential Conflicts of Interest

Nothing to report.

Interpretation—Our findings reveal a protective role of MC1R in the nigrostriatal dopaminergic system, and they provide a rationale for MC1R as a potential therapeutic target for Parkinson disease. Together with its established role in melanoma, MC1R may represent a common pathogenic pathway for melanoma and Parkinson disease.

Patients with Parkinson disease (PD), one of the most common neurodegenerative diseases, generally have reduced risk of developing almost all types of cancer, with one notable exception—melanoma, a malignant tumor of melanin-producing cells in skin.^{1,2} A large number of epidemiological studies have reported that occurrence of melanoma is higher than expected among subjects with PD, and melanoma patients are reciprocally more likely to develop PD.^{1,2} Although well documented since the 1970s, mechanisms underlying this association between PD and melanoma remain largely unknown. Recent epidemiological studies further demonstrated the positive, bidirectional link not only in the patients themselves but also in their relatives,^{3,4} suggesting a possible genetic basis for the association between the two seemingly distinct conditions.

A cyclic adenosine monophosphate–stimulating G-protein–coupled receptor (GPCR), melanocortin 1 receptor (MC1R) contributes to the regulation of skin physiology through the melanin synthetic pathway as well as pigmentation-independent mechanisms.^{5,6} Loss-of-function variants of *MC1R* in humans are associated with red hair and fair skin and increased risk of developing melanoma.^{7,8} In mice, an inactivating mutation of *MC1R* (*MC1R* extension, *e/e*) with a phenotype analogous to red hair/fair skin in humans,⁹ results in impaired skin protection and is sufficient to enhance melanoma formation.^{10,11} Furthermore, *MC1R^{e/e}* mice display greater oxidative damage in skin, suggesting an oxidative stress–mediated mechanism in carcinogenesis.¹¹ In addition to skin melanocytes, other tissue and cell types that express MC1R include adrenal tissue, immunocytes, endothelial cells,¹² and possibly human astrocytes¹³ and periaqueductal gray neurons,¹⁴ suggesting functions of MC1R extending beyond those in skin. In an analysis of 2 large prospective cohorts led by our epidemiologist collaborators, we found that red hair individuals and individuals homozygous for the red hair–associated Arg151Cys allele of Cys were associated with an increased risk for PD.¹⁵ The association was substantiated by another study linking PD with an alternative red hair *MC1R* variant in an independent cohort.¹⁶ Although other studies^{17–20} did not replicate a significant *MC1R*–PD association, which may be attributable to technical or population differences across studies,²¹ MC1R has nevertheless emerged as having a potential role at the interface of melanoma and PD.

To explore such a role of MC1R in dopaminergic neurons and therefore a biological basis for the PD–melanoma link, we first assessed expression of MC1R in dopaminergic neurons of the substantia nigra (SN) in the mouse brain. Redhead *MC1R^{e/e}* mice were then employed to investigate effects of *MC1R* inactivation on the nigrostriatal dopaminergic system under basal conditions and in well-established 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models of PD.²² Furthermore, the neuroprotective potential of MC1R stimulation in an MPTP model was explored using a potent, specific MC1R agonist 1-(1-[3-methyl-L-histidyl-O-methyl-D-tyrosyl]-4-phenyl-4-piperidinyl)-1-butanone (BMS-470539).²³

Materials and Methods

Animals

First reported as an *extension* in 1993,⁹ *MC1R*^{e/e} mice carry a frameshift mutation due to a deletion of a single nucleotide at position 549. The mutation produces a prematurely terminated, nonfunctioning protein and results in a yellow/red coat in homozygous mice despite being on a C57BL/6J background.^{9–11} Heterozygous (*MC1R*^{e/+}) mice, conversely, do not display melanoma-related skin phenotypes. *MC1R*^{e/e} mice were backcrossed with C57BL/6J mice from the Jackson Laboratory (Bar Harbor, ME). Homozygous *MC1R*^{e/e} and littermate wildtype (WT) mice were used for experiments.

For effects of MC1R agonist BMS-470539, adult (3 months old) male C57Bl/6J mice were purchased from the Jackson Laboratory.

Animals were maintained in home cages at constant temperature with a 12-hour light/dark cycle and free access to food and water. All experiments were performed in accordance with a protocol approved by the Massachusetts General Hospital Animal Care and Use Committee.

Measurement of Locomotion

Locomotion was assessed using an automated open field recording system (San Diego Instruments, San Diego, CA). Horizontal locomotor activity during the first 60 minutes (7–8 PM) of the dark phase of the light cycle was recorded and analyzed as described.²⁴ Ambulation was quantified as sequential breaks in adjacent beams (55mm apart).

MC1R Immunohistochemistry

Naive adult male C57Bl/6J mice were perfused, and brains were postfixed. Following cryoprotection, brains were sectioned. The sections were heated for antigen retrieval²⁵ and then treated with H₂O₂ and blocking milk containing 0.3% Triton X-100. Primary anti-MC1R (Santa Cruz Biotechnology, Santa Cruz, CA; #SC-19485 at 1:50 dilution) was applied overnight at 4 °C. For a peptide blocking control, the primary antibody was preincubated with immunizing peptide (#SC-19485P, 1:25 dilution) overnight. For a control without primary antibody, it was replaced by phosphate-buffered saline only. Sections were Nissl counterstained.

Immunofluorescence Double Staining

For MC1R and tyrosine hydroxylase (TH) double labeling, mice were perfused and their brains were sectioned. Tyramide signal amplification (Life Technologies, Carlsbad, CA; #MP20911) was employed per the manufacturer's instructions. Briefly, sections were heated for antigen retrieval and incubated with the above primary MC1R antibody at 1:50 dilution and then stained with tyramide-labeled Alexa Fluor 488. Subsequent TH staining was performed by incubating the sections with anti-TH primary antibody (Sigma, St Louis, MO; #T1299, 1:500 dilution) and Alexa Fluor 546 donkey antimouse secondary antibody.

Immunohistochemistry for TH and Stereological Cell Counting

For TH immunohistochemistry in naive mice, the animals were perfused and the brains were postfixed for 48 hours. For TH immunohistochemistry with tissues from dopaminergic toxin experiments, mice were sacrificed by rapid cervical dislocation. The hinder brain block containing midbrain was immediately dissected and fixed for 48 hours. The brains were then cryoprotected, rapidly frozen by immersion in isopentane on dry ice, and sectioned at 30 μm . Sections were processed free-floating. Primary antibody was mouse monoclonal anti-TH (Sigma, #T1299) at 1:800.²⁶

For TH stereology, a complete set of serial sections were immunostained for TH and counterstained for Nissl. Sections were analyzed stereologically as described.^{26,27} The number of TH-positive (and TH-negative) neurons in the SN on both sides of each section was counted, and the total number of TH-positive (and TH-negative) neurons on each side (6- OHDA lesion) or both sides of the SN from individual animals was calculated.

Western Blotting

For Western blot analysis of MC1R in total protein, adrenal or brain tissues were obtained. Total protein was extracted and electrophoresed. After transferring, the membrane was blocked in 5% bovine serum albumin and then incubated with the above MC1R antibody at 1:500. β -Tubulin was probed using anti- β -tubulin (Cell Signaling Technology #2146) as loading control.

For Western blot analysis of MC1R in membrane and cytosolic fractions, membrane and cytosolic fractions were obtained with a Membrane Protein Extraction Kit (Thermo Scientific, Waltham, MA; #89842). Na^+/K^+ -adenosine triphosphatase (Cell Signaling Technology #3010) and β -tubulin (Cell Signaling Technology #2146) were probed as loading controls for membrane and cytosolic fractions, respectively.

To validate specificity of the MC1R primary antibody in vitro, B16 mouse melanoma cells were treated with short hairpin RNA (shRNA) construct targeting mouse MC1R (target sequence: 5'-AATGGAGATCAGGAAGGGATG-3') according to published methods.²⁸ Cells and reagents were kind gifts from Dr Rutao Cui, Boston University School of Medicine. Primary MC1R antibody was diluted at 1:1,000.

Laser Capture Microdissection and Reverse Transcriptase Polymerase Chain Reaction

To detect MC1R mRNA in dopaminergic neurons in the mouse brain, approximately 2,000 nigral TH-positive neurons were laser-captured. RNA was extracted, and reverse transcriptase polymerase chain reaction (RT-PCR) was performed.²⁹ Primer sequences were: forward TGGTAAGTGTCAGCATCGTG and reverse TGATAACGCAGCGCATAGAA.

Determination of Protein Carbonyls

Protein carbonyls in brain tissues were detected using the Oxyblot Protein Oxidation Detection Kit (Millipore, Billerica, MA).²⁶ Band density was analyzed and normalized with Ponceau staining using the ImageJ system.

Determination of Oxidative DNA Damage

Nuclear DNA was digested enzymatically, and N-labeled standard lesions were added. Lesions were separated by high-performance liquid chromatography (HPLC) and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS/MS) as previously described.^{11,30}

6-OHDA Lesion

Mice were pretreated with desipramine, and a solution of 6-OHDA (5.0 µg/µl) was infused into the left striatum as previously described.²⁶

Rotational Behavior Assessmen

Spontaneous and amphetamine (5mg/kg)-induced rotational behavior was tested at 3 weeks after the 6-OHDA lesion using an automated rotometry system (San Diego Instruments) for 60 minutes as previously described.²⁶

MPTP Treatment Regimens

For MPTP toxicity in *MC1R^{e/e}* mice, a subacute regimen was employed.^{31,32} Mice were administered intraperitoneally (i.p.) with MPTP-HCl 20mg/kg or saline once daily for 4 consecutive days and sacrificed 7 days after the last MPTP injection.

For effects of MC1R agonist BMS-470539 on MPTP toxicity, a single dose of MPTP-HCl (40mg/kg)^{31,32} or saline was injected i.p., and BMS-470539 dihydrochloride (Tocris Bioscience, Bristol, UK) or vehicle (water) was administered 10 minutes before and 60 minutes after MPTP. Animals were sacrificed 7 days after the injections. The same treatment paradigm was employed to examine whether BMS-470539 changes MPTP metabolism. Mice were sacrificed 90 minutes after MPTP administration.

MC1R Agonist BMS-470539 and Its Pharmacokinetics

Male C57Bl/6J mice were dosed with BMS-470539 (20mg/kg subcutaneously [s.c.])²³ and sacrificed at 5 time points (n=3) from 5 to 120 minutes. BMS-470539 was measured in plasma and brain by LC-MS/MS/MS by Cyprotex (Watertown, MA).

Measurement of MPTP Metabolite

Striatal 1-methyl-4-phenylpyridinium (MPP⁺) was measured using HPLC-ultraviolet (UV) as previously described.³³

Measurement of Dopamine and Metabolites

Dopamine (DA) and metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined by standard HPLC with electrochemical detection, as previously described.²⁶

Statistical Analysis

All values are expressed as mean±standard error of the mean. The difference between two groups was analyzed by Student *t* test. Multiple comparisons among groups were performed

by 1- way or 2-way analysis of variance (ANOVA), and Tukey post hoc test was performed when an overall significance was demonstrated with 1-way ANOVA or a main effect of genotype was demonstrated with 2-way ANOVA. $p < 0.05$ is considered statistically significant.

Results

MC1R Is Expressed in Dopaminergic Neurons of the SN of Adult C57BL Mice

To probe a role of *MC1R* in dopaminergic neurons of the SN, we first assessed expression of MC1R in naive C57BL/6J mouse brain. Western blot analysis of MC1R immunoreactivity in the striatum and the ventral midbrain revealed a band corresponding to a molecular weight of ~35kDa, the known size of MC1R (Fig 1). Immunohistochemistry demonstrated, in a coronal section of the ventral midbrain, positive staining for MC1R in the SN pars compacta (SNpc), and the staining vanished when antibody peptide was applied or when primary antibody was omitted. Immunofluorescence double labeling indicated colocalization of MC1R and TH, a marker for dopaminergic neurons within SNpc. MC1R appeared to be located on cell surface as well as in cytoplasm, consistent with Western blot results showing the presence of a ~35kDa band in both membrane, although weak, and cytosolic fractions extracted from ventral midbrain and striatal tissues. Specificity of MC1R primary antibody was indirectly verified in vitro by shRNA knockdown of the mouse MC1R in mouse B16 melanoma cells.²⁸ MC1R is known to be expressed in B16 cells.²⁸ Reduced MC1R was demonstrated in cells treated with mouse MC1R shRNA but not scrambled construct or vehicle-treated control. shRNA treatment did not alter β -tubulin band density. The presence of MC1R protein is supported both technically and conceptually by its mRNA expression in the SN, more specifically, in dopaminergic neurons by laser capture microdissection of nigral TH-positive neurons and RT-PCR. A band corresponding to predicted size of MC1R was demonstrated. Despite lack of theoretically definite negative control (eg, MC1R knockout mice), our data using 3 different methods suggest the existence of MC1R in the SN in C57BL/6J mice.

Impaired Nigrostriatal Dopaminergic Neuronal Integrity in Redhead *MC1R^{e/e}* Mice

To investigate potential MC1R influence on the nigrostriatal dopaminergic system, we monitored locomotor activity in *MC1R^{e/e}* mice by open field testing for the first hour of the dark phase in a 12-hour-light/12-hour-dark cycle.²⁴ Despite being generally healthy and fertile, redhead *MC1R^{e/e}* mice displayed progressive decline in locomotor activity. Compared to littermate WT controls, there was a significant decrease in the number of adjacent photobeam breaks in 8-month-old ($p=0.05$) and 14-month-old *MC1R^{e/e}* mice ($p=0.01$). Young *MC1R^{e/e}* mice performed normally (Fig 2A).

We then assessed neurochemical and morphological measures of dopaminergic nigrostriatal neuron integrity^{26,27} in *MC1R^{e/e}* mice as they age. HPLC coupled with electrochemical detection revealed 16% and 26% reduction in DA content in the striatum of 8- and 14-month-old *MC1R^{e/e}* mice, respectively, significantly lower than their WT littermates (8 months old, $p<0.05$; 14 months old, $p<0.01$; see Fig 2). The reduced striatal DA in *MC1R^{e/e}* mice is likely caused by loss of dopaminergic neurons in the SN, as demonstrated by

immunohistochemistry and stereological counting of cells stained positive for TH. *MC1R^{e/e}* mice at 8 months of age had 24% fewer TH-positive neurons ($p<0.01$), and at 14 months of age there were 39% fewer nigral dopaminergic neurons compared to WT littermates ($p=0.01$). Nissl counterstaining and stereological counting of TH-negative neurons in the SNpc revealed no difference between *MC1R^{e/e}* and WT littermates at any of the ages. Total summed counts of TH-positive and TH-negative cells in the SNpc, which represent its total number of neurons, were significantly lower in *MC1R^{e/e}* mice at 8 ($p=0.01$) and 14 months ($p<0.01$) but not at 2 months ($p=0.20$) of age as compared to WT littermates. Representative images of TH staining in the SN showed loss of TH-positive cells in 8- and 14-month-old redhead *MC1R^{e/e}* mice. There is no significant change in the size of either the nigra or individual TH-positive neurons (Fig 2D and quantitative data not shown).

Increased Oxidative Damage in the Ventral Midbrain of Adult *MC1R^{e/e}* Mice

Our collaborative team previously reported an oxidative stress mechanism in *MC1R*-mediated, UV-independent carcinogenesis in skin using *MC1R^{e/e}* mice.¹¹ Oxidative stress has been implicated in neurodegenerative diseases including PD.³⁴ To determine whether disrupted *MC1R* is associated with changes in oxidative stress in the nigrostriatal system, we assessed protein carbonyls, a marker of oxidative protein damage, by Oxyblot.²⁶ Increased protein carbonyls were detected in the ventral midbrain ($p<0.05$; Fig 3A) but not the striatum (see Fig 3B) of adult (3–6 months old) *MC1R^{e/e}* mice compared to littermate WT controls.

Oxidative DNA damage markers 8,5'-cyclo-2'-deoxyadenosine (cdA) and 8,5'-cyclo-2'-deoxyguanosine (cdG) were evaluated in *MC1R^{e/e}* mice using established LC-MS/MS/MS.^{11,30} Increased levels of *S*-cdA and *S*-cdG, which lead to A to T and G to A mutations, respectively, were demonstrated in ventral midbrain in adult *MC1R^{e/e}* mice (5–8 months old) as compared to WT littermates ($p=0.036$, *S*-cdA; $p=0.046$, *S*-cdG; see Fig 3C). *R*-cdA did not show a significant difference between the 2 genotypes (13.6 ± 0.8 and 12.4 ± 1.0 lesions per 10^7 nucleosides, WT and *MC1R^{e/e}*; $p>0.05$). There were no significant changes in *S*-cdA and *R*-cdA levels in the striatum in adult redhead *MC1R^{e/e}* mice compared to WT controls (see Fig 3D).

MC1R^{e/e} Mice Are More Susceptible to Dopaminergic Neurotoxin 6-OHDA

In humans, loss-of-function variants of *MC1R* produce red hair/fair skin phenotype and are linked to higher risk of both melanoma^{7,8} and PD.^{15,16} Our collaborative team previously reported the lower threshold for melanoma induction in redhead *MC1R^{e/e}* mice.¹¹ To investigate whether the *MC1R* disruption may contribute to greater susceptibility to PD, we assessed dopaminergic phenotype of redhead *MC1R^{e/e}* mice in a well-established 6-OHDA model of PD.^{22,26} Adult (6–8 months old) *MC1R^{e/e}* and >littermate WT mice received unilateral intrastratial infusion of 10 μ g 6-OHDA. This model induces progressive and retrograde degeneration of the nigrostriatal system by a combined effect of reactive oxygen species and quinones.²² The animals were then assessed for behavioral, neurochemical, and anatomical measures of dopaminergic deficits (Fig 4A).²⁶ Comparing to WT littermates, *MC1R^{e/e}* mice displayed a statistically significant increase in ipsilateral net rotations after

stimulation with a DA-releasing agent amphetamine ($p < 0.05$) despite no difference in spontaneous rotations ipsilateral to the lesion (see Fig 4B).

The greater DA imbalance between ipsilateral and contralateral sides in $MC1R^{e/e}$ mice was confirmed by HPLC analysis of DA content in the striatum. 6-OHDA induced greater depletion of DA and a further increase in ratio of HVA, a DA metabolite, to DA in the striatum in $MC1R^{e/e}$ mice than WT littermates on the lesioned side ($p < 0.01$, striatal DA; $p < 0.01$, HVA/DA; see Fig 4C). After normalization for the significant difference in DA on the unlesioned side between the 2 groups of animals ($p < 0.01$) by expressing it as percentage of contralateral control value, $MC1R^{e/e}$ mice had $12.8 \pm 2.1\%$ residual DA on the lesioned side, significantly lower than the residual in WT mice ($31.3 \pm 3.0\%$; $p < 0.01$).

A more substantial loss of nigral dopaminergic neurons induced by 6-OHDA infusion was demonstrated in $MC1R^{e/e}$ mice compared to their WT littermates (see Fig 4D). Stereological cell counts revealed fewer remaining TH-positive neurons on the lesioned side ($p < 0.01$). The loss of dopaminergic neurons was still greater as a proportion of the unlesioned contralateral side in $MC1R^{e/e}$ mice ($32.4 \pm 4.6\%$) than in WT ($49.8 \pm 1.6\%$; $p < 0.01$; see Fig 4E), despite reduced numbers on the unlesioned side ($p < 0.01$), which confirms the compromised dopaminergic integrity of intact $MC1R^{e/e}$ mice as shown in Figure 2. These results demonstrate higher susceptibility of redhead $MC1R^{e/e}$ mice to the dopaminergic toxin 6-OHDA.

$MC1R^{e/e}$ Mice Are More Susceptible to Dopaminergic Neurotoxin MPTP

Exacerbated dopaminergic neurotoxicity was demonstrated in $MC1R^{e/e}$ mice in another well-established, complementary toxin model of PD.^{22,31} After systemic administration, MPTP enters the brain and is locally converted to its active metabolite MPP^+ , which in turn is selectively transported into dopaminergic neurons, where it exerts toxicity by inhibiting complex I of the mitochondrial electron transport chain.^{22,32} A subacute regimen (20mg/kg MPTP-HCl i.p. once daily for 4 days) induced greater reduction of striatal DA in adult (6–8 months old) $MC1R^{e/e}$ mice than in WT littermates 7 days after the last MPTP administration ($p < 0.01$; Fig 5A). Even with normalization to saline-treated control mice to account for basal differences between the 2 control groups ($p = 0.01$), a greater percentage loss of striatal DA in $MC1R^{e/e}$ mice treated with MPTP ($82.2 \pm 2.5\%$) persisted compared to WT MPTP-treated mice ($59.6 \pm 3.1\%$; $p = 0.05$). $MC1R^{e/e}$ mice also displayed a 350% increase in DA turnover rate (HVA/DA ratio) after MPTP administration in comparison to a 180% increase in WT littermates ($p < 0.05$).

Anatomically, TH immunohistochemistry (see Fig 5B) and quantitative stereological analysis indicated an extensive loss of dopaminergic neurons in MPTP-treated $MC1R^{e/e}$ mice, with residual TH-positive SN neuron counts that were $35.5 \pm 5.5\%$ of those in saline-treated $MC1R^{e/e}$ littermates, whereas the WT MPTP group had $55.1 \pm 5.4\%$ of the nigral TH-positive neurons compared to their saline-treated WT littermates, with the $MC1R^{e/e}$ mice showing significantly greater losses of dopaminergic nigral neurons compared to their controls, either by absolute cell counts ($p < 0.01$) or as a proportion of their vehicle-treated controls' counts ($p < 0.05$; see Fig 5C).

To evaluate whether exacerbated MPTP toxicity in *MC1R^{e/e}* mice may be due to greater conversion of MPTP to its toxic metabolite MPP⁺ (or to slower rates of its clearance), we measured levels of MPP⁺ in the striatum³³ of *MC1R^{e/e}* and littermate WT mice 90 minutes and 6 hours after a single i.p. injection of MPTP-HCl 20mg/kg. The 2 time points represent the peak and late phase, respectively, of MPP⁺ accumulation in brain after i.p. MPTP administration in naive WT mice.³² There was no statistically significant difference in striatal MPP⁺ levels between *MC1R^{e/e}* and WT controls at either time point, suggesting *MC1R* disruption does not alter metabolism of the dopaminergic toxin, and therefore that the exacerbated toxicity in *MC1R^{e/e}* mice is not due to increased local toxic metabolite after systemic injection (see Fig 5D).

MC1R Agonist Protects against MPTP-Induced Dopaminergic Neurotoxicity

The compromised nigrostriatal integrity in *MC1R^{e/e}* mice and their high susceptibility to both 6-OHDA and MPTP suggest a protective role of MC1R signaling in the nigrostriatal dopaminergic system. To further probe this role, we investigated whether pharmacological activation of MC1R protects against MPTP toxicity using a commercially available MC1R agonist BMS-470539. BMS-470539 is a selective, potent MC1R agonist used in the laboratory to understand the role of MC1R in immunomodulation and inflammatory responses.^{23,35} We first determined the pharmacokinetic profile of BMS-470539 in blood and brain in naive C57Bl/6J mice. After systemic injection (20mg/kg s.c.), the concentration of BMS-470539 in plasma peaked at 15 minutes (1,318±189ng/ml); in brain it peaked at 30 minutes (18±2ng/ml) and remained detectable until at least 120 minutes at ~10ng/ml (n=3 each time point), corresponding to the agonist's reported median effective concentration of ~10ng/ml.^{23,35}

Based on the pharmacokinetics and metabolism of MPTP,³² we injected i.p. adult (3 months old) C57Bl/6J mice with a single dose of MPTP-HCl at 40mg/kg, and BMS-470539 (100mg/kg) was injected s.c. 10 minutes before and 60 minutes after MPTP. Mice were sacrificed 7 days later. The single dose regimen induced 66% reduction in DA in the striatum. BMS-470539 attenuated MPTP-induced DA depletion to 49% of that of the control group, resulting in significantly greater residual DA content compared to MPTP mice treated with vehicle ($p<0.01$; Fig 6). Measurement of DOPAC showed similar preservation in the DA metabolite in MPTP mice treated with BMS-470539. TH immunohistochemistry and stereological cell counting revealed 65% loss of dopaminergic neurons in the SN in the MPTP group, and treatment with BMS-470539 improved cell survival to 53% of that of the control group ($p<0.05$). BMS-470539 itself did not alter striatal DA content or the number of TH-positive neurons. A separate experiment using the same treatment regimen showed BMS-470539 did not change MPTP metabolism. There was no significant difference in MPP⁺ in the striatum between BMS-470539-treated and vehicle-treated mice 90 minutes after a single 40mg/kg i.p. injection of MPTP-HCl (14.2±2.3 and 17.6±0.6pmol/mg tissue, n=4 and 5, respectively; $p>0.05$).

Discussion

Epidemiological studies have demonstrated a reproducible bidirectional association between melanoma and PD.¹⁻⁴ They also raised the possibility that the *MC1R* gene may mediate the association.^{15,16} Built on the previously characterized skin phenotype and melanoma tendency in redhead *MC1R*^{e/e} mice,^{10,11} the present interdisciplinary study identifies a novel role of *MC1R* in the nigrostriatal dopaminergic system. Our findings demonstrate compromised nigrostriatal dopaminergic system in *MC1R*^{e/e} mice and their greater susceptibility to local and systemic dopaminergic toxins 6-OHDA and MPTP. Furthermore, pharmacological stimulation of MC1R with a selective agonist attenuates MPTP neurotoxicity.

MC1R is expressed in the mouse SN at the protein and mRNA levels. Although juxtaposed to or on the cell surface in some cases, MC1R appears to be located intracellularly, overlapping with cytoplasmic TH fluorescence. Depending on cellular context and cell types, trafficking and localization of GPCRs are tightly regulated and are highly related to their signaling cycle.³⁶ Immunoreactivity of GPCRs detected inside of the cells may reflect not only technical incorporation of fixation and detergent permeabilization but also functional status of the receptors; for example, they are newly synthesized to be transported to the plasma membrane or internalized following agonist stimulation,^{36,37} both often producing a typical punctate staining pattern in the case of MC1R.³⁸ Relative cell surface expression versus intracellular retention has been reported to correlate with function of *MC1R* variants.³⁹ The only neuronal demonstration of MC1R was in cytosome of human periaqueductal gray matter neurons.¹⁴ Intracellular MC1R undergoes oligomerization, which can significantly affect its signaling.⁶ It is unclear whether a band at higher molecular weight in both brain tissues and cells that appears to respond modestly to shRNA represents MC1R oligomers. Further characterization of MC1R subcellular distribution and oligomerization would be helpful in deciphering regulation of its function in the SN dopaminergic neurons.

Loss-of-function mutant *MC1R*^{e/e} mice display loss of nigral dopaminergic neurons, reduced striatal DA content, and impaired locomotor activity under basal conditions. These results reveal a significant role of MC1R signaling in maintaining nigrostriatal dopaminergic neuron survival. The trophic influence of MC1R may be due to its direct actions given its expression in dopaminergic neurons. Dopaminergic neuron-specific disruption of MC1R would differentiate whether local MC1R or rather systemic MC1R is responsible. Although MC1R appears to be expressed in other cells in the SN, numbers of nigral TH-negative neurons in *MC1R*^{e/e} mice remain unchanged. Furthermore, the impaired dopaminergic neuronal integrity is accompanied by increased oxidative damage in the ventral midbrain but not in the striatum. These data suggest that the benefit of MC1R activity may be relatively specific to dopaminergic neurons. However, it is not clear whether MC1R disruption affects only the nigrostriatal system or whether it has broader influence on other dopaminergic cell populations or other central nervous system (CNS) regions. The greater oxidative damage in the midbrain is in agreement with greater oxidative damage in skin in redhead *MC1R*^{e/e} mice,¹¹ supporting a common antioxidant mechanism underlying MC1R-mediated protection in melanocytes and dopaminergic neurons. *MC1R*^{e/e} mice do not develop

dopaminergic deficits until adulthood, indicating age-dependent effects of endogenous MC1R signaling on the nigrostriatal dopaminergic pathway. Aging is a primary risk factor for PD.⁴⁰ Further investigation is needed to elucidate potential interplay between MC1R and aging and age-related factors including oxidative stress. Nevertheless, the demonstrated nigrostriatal dopaminergic deficits in *MC1R*^{e/e} mice suggest possible predisposition of these mice to parkinsonism as a result of MC1R disruption.

Redhead *MC1R*^{e/e} mice display higher susceptibility to 6-OHDA and MPTP, two established neuronal toxins that are commonly used to model dopaminergic neurodegeneration of human PD.²² Their exacerbated dopaminergic deficits manifest at anatomical, neurochemical, and functional levels after intrastriatal infusion of 6-OHDA or systemic administration of MPTP. The greater susceptibility is likely related to preexisting dopaminergic neuron dysfunction and oxidative damage. In addition, MC1R has been reported to play a central role in inflammatory responses.^{23,34} Oxidative stress and inflammatory responses are known to be involved in 6-OHDA and MPTP toxicity as well as in the pathophysiology of human PD.^{23,34} Dopaminergic neurodegeneration in the context of *MC1R* disruption may also directly or indirectly involve pigmentation pathway itself. A recent study intriguingly reported increased SN echogenicity in fair skin human subjects.⁴¹ Further investigations are essential to answer important questions regarding downstream intracellular pathways and mediators.

The enhanced vulnerability of redhead *MC1R* mutant mice in models of PD is consistent in parallel with previously reported lower threshold for melanoma induction in these mice,¹¹ supporting protective effects of MC1R in both melanocytes and dopaminergic neurons against tumorigenesis and neurodegeneration, respectively. Together with the epidemiological associations of redhead *MC1R* loss-of-function variants and high risk of melanoma,^{7,8} and possibly high risk of PD,^{15–21} the predisposition of *MC1R* redhead mutation to melanoma and PD in animal models supports a potential role of MC1R as the biological basis of the melanoma and PD link. Future studies should focus on epidemiological, biological, and clinical interactions between *MC1R* and PD genetic factors,² particularly α -synuclein,⁴² and leucine-rich repeat kinase 2 (*LRRK2*).⁴³ α -Synuclein has been implicated in melanoma pathophysiology,^{44,45} and *LRRK2* is the most commonly mutated among PD-associated genes in melanomas.⁴⁶

Identifying the role of endogenous MC1R in adult dopaminergic system maintenance and defense presents a unique opportunity to explore the potential of MC1R as a novel therapeutic target for PD. BMS-470539, a selective MC1R agonist²³ with modest blood–brain barrier penetrance, is found to protect dopaminergic neurons against MPTP-induced cell loss in the SN and DA depletion in the striatum. The neuroprotective action of pharmacological MC1R activation provides further evidence that compromised dopaminergic neuronal integrity in redhead *MC1R* mutant mice and their exacerbated dopaminergic neurotoxicity in models of PD are likely to be MC1R specific. Studies are underway to investigate specificity and efficacy of MC1R neuroprotection by complementary genetic *MC1R* overexpression. MC1R agonists are currently undergoing clinical testing for depigmentation disorders and an acute phototoxicity related condition.

^{47,48} Whether systemic MC1R activation is required for its CNS effects, more CNS-penetrant versions may be needed to target neuronal MC1R for the treatment of PD.

Our findings that genetic inactivation of MC1R signaling impairs nigrostriatal dopaminergic neuron survival and that pharmacological activation of MC1R is neuroprotective uncover a protective role of MC1R in the nigrostriatal dopaminergic system. They provide evidence for targeting MC1R as a novel therapeutic strategy for PD. Together with epidemiological associations between MC1R, melanoma,^{7,8} and PD,^{15–21} and the known roles of MC1R in melanocytes and melanoma pathogenesis,^{1,5,60,11} our data support a shared mechanistic link between melanoma and PD.

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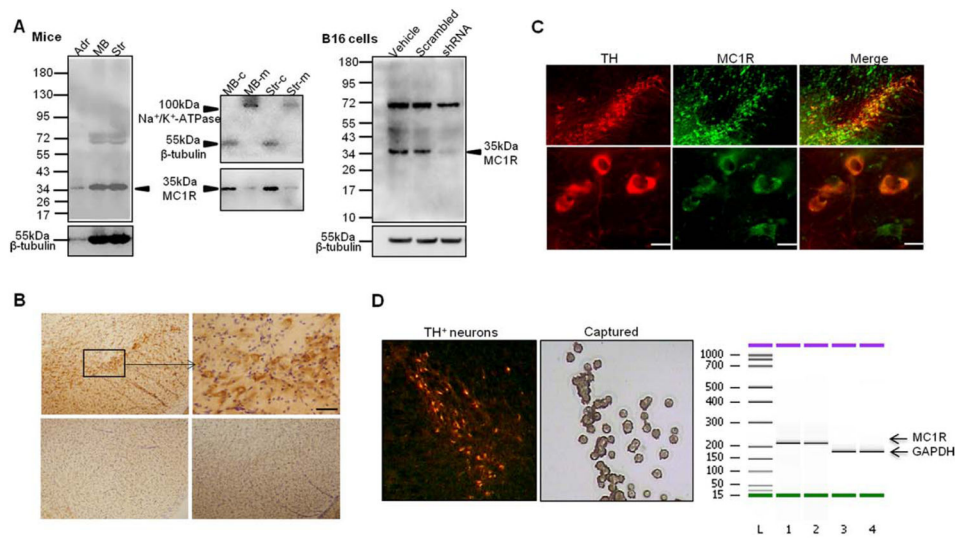
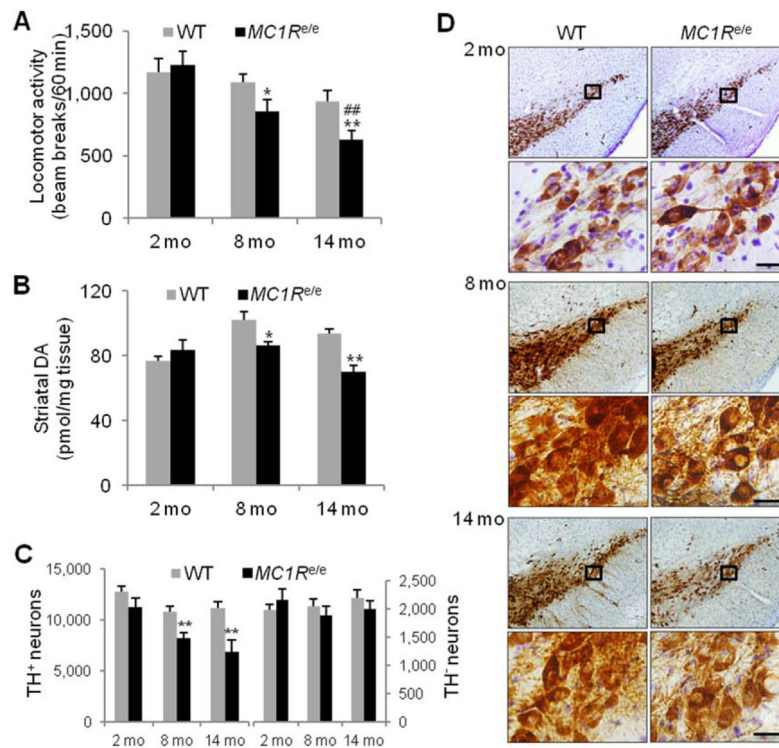
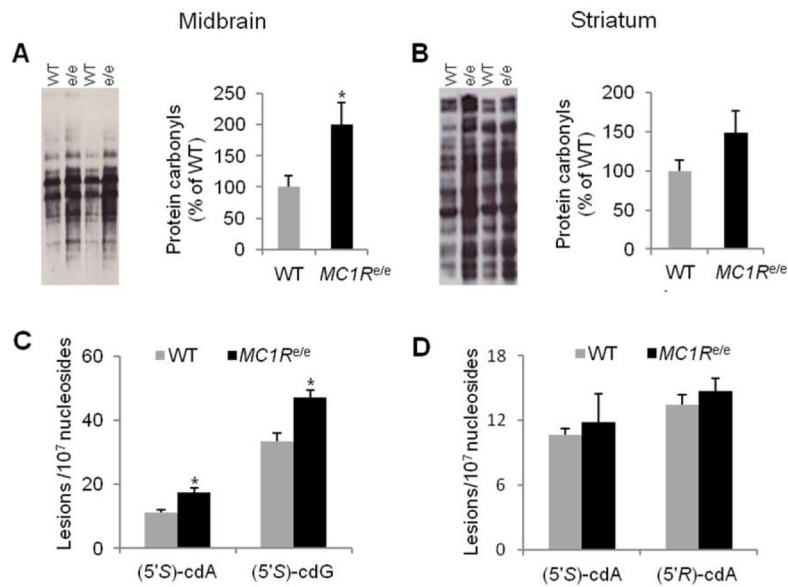


FIGURE 1.

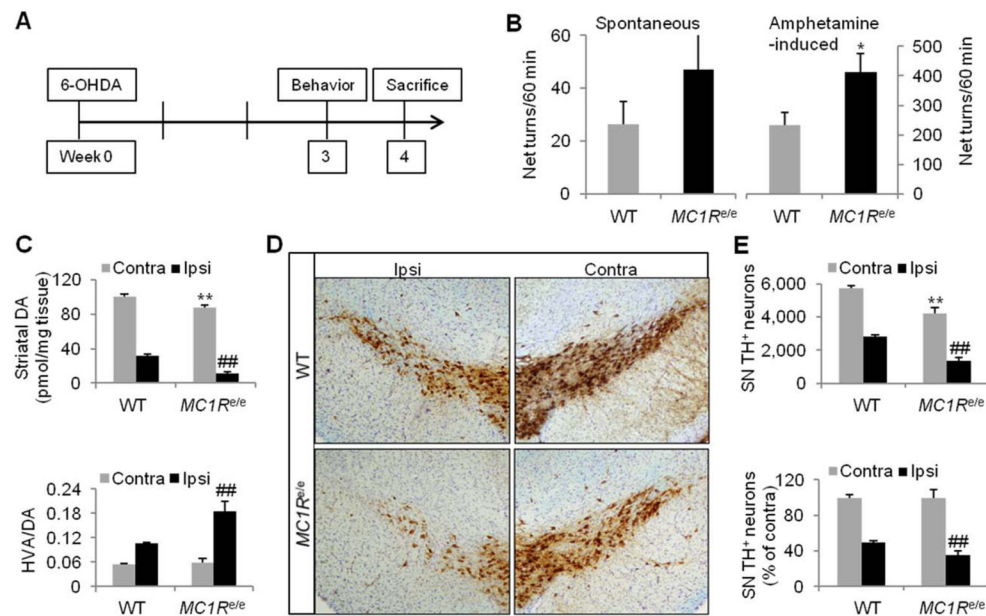
Expression of MC1R in the substantia nigra (SN) in adult naive C57BL/6J mouse brain. (A) Western blot using anti-MC1R antibody with total tissue lysate (left panel), membrane (m) or cytosolic fraction (c) (middle panel) isolated from ventral midbrain (MB) and the striatum (Str), or B16 mouse melanoma cells treated with shRNA targeting mouse MC1R or scrambled construct (right panel). Adrenal (Adr), where MC1R is known to be expressed, serves as a control.¹² β -Tubulin and Na⁺/K⁺-adenosine triphosphatase (ATPase) are loading controls for total and cytosolic proteins, and membrane proteins, respectively. (B) Immunostaining of MC1R and Nissl counterstaining in a ventral midbrain coronal section (top panel). Cells in the box in the SN pars compacta are shown at higher magnification on the right. Scale bar=25 μ m. Bottom panels show ventral midbrain coronal sections incubated with blocking peptide (left) or no primary antibody (right). (C) Fluorescence double labeling for tyrosine hydroxylase (TH; red) and MC1R (green) in the SN. Scale bars=10 μ m. (D) Laser capture microdissection of TH-positive neurons from the SN and reverse transcriptase polymerase chain reaction analysis of MC1R mRNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as internal control.

**FIGURE 2.**

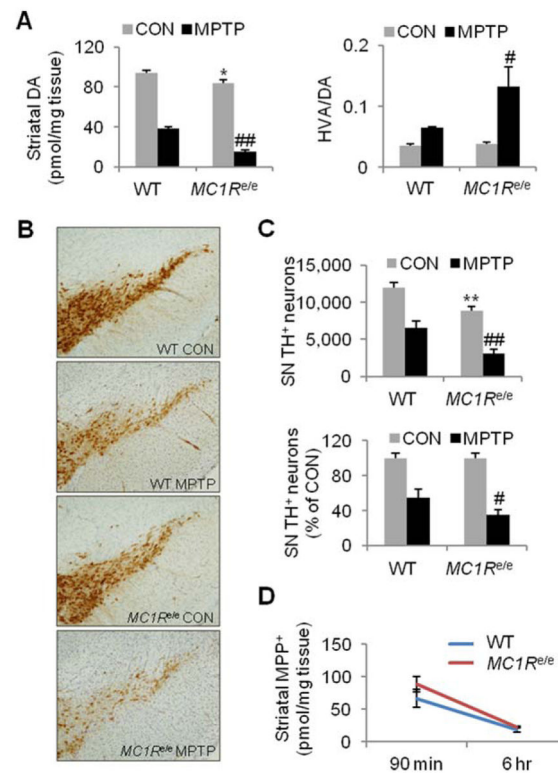
Compromised nigrostriatal dopaminergic integrity in *MC1R^{e/e}* mice. (A) Locomotor activity in *MC1R^{e/e}* mice and littermate wild-type (WT) controls assessed by adjacent beam breaks during the first hour of the dark cycle in an open field chamber (n=15, WT and *MC1R^{e/e}* 2- and 8-month-old mice; n=14 and 13, WT and *MC1R^{e/e}* 14-month-old mice). * $p=0.05$, ** $p=0.01$ versus WT littermates; ### $p=0.01$ versus 2-month-old self-control *MC1R^{e/e}* mice by 2-way analysis of variance (ANOVA) followed by Tukey post hoc test. $p=0.038$ for genotype effect; $p=0.0002$ for time effect; $p=0.11$ for interaction. (B) Striatal dopamine (DA) content in *MC1R^{e/e}* mice and WT littermates determined by high-performance liquid chromatography coupled with electrochemical detection (n=5, WT and *MC1R^{e/e}* at all 3 age points). * $p < 0.05$, ** $p < 0.01$ versus WT littermates, 1-way ANOVA followed by Tukey post hoc test. (C) Stereological quantification of tyrosine hydroxylase (TH)-positive and TH-negative (Nissl-positive) neurons in substantia nigra pars compacta in *MC1R^{e/e}* mice and littermate WT controls (n=5, WT and *MC1R^{e/e}* 2- and 14-month-old mice; n=6, WT and *MC1R^{e/e}* 8-month-old mice). ** $p=0.01$ versus WT littermates, 1-way ANOVA followed by Tukey post hoc test. (D) Representative sets of nigral sections from *MC1R^{e/e}* mice and littermate WT controls stained for TH. Cells in the black boxes at indicated age points are shown at higher magnification in the lower panels. Scale bars=20 μm .

**FIGURE 3.**

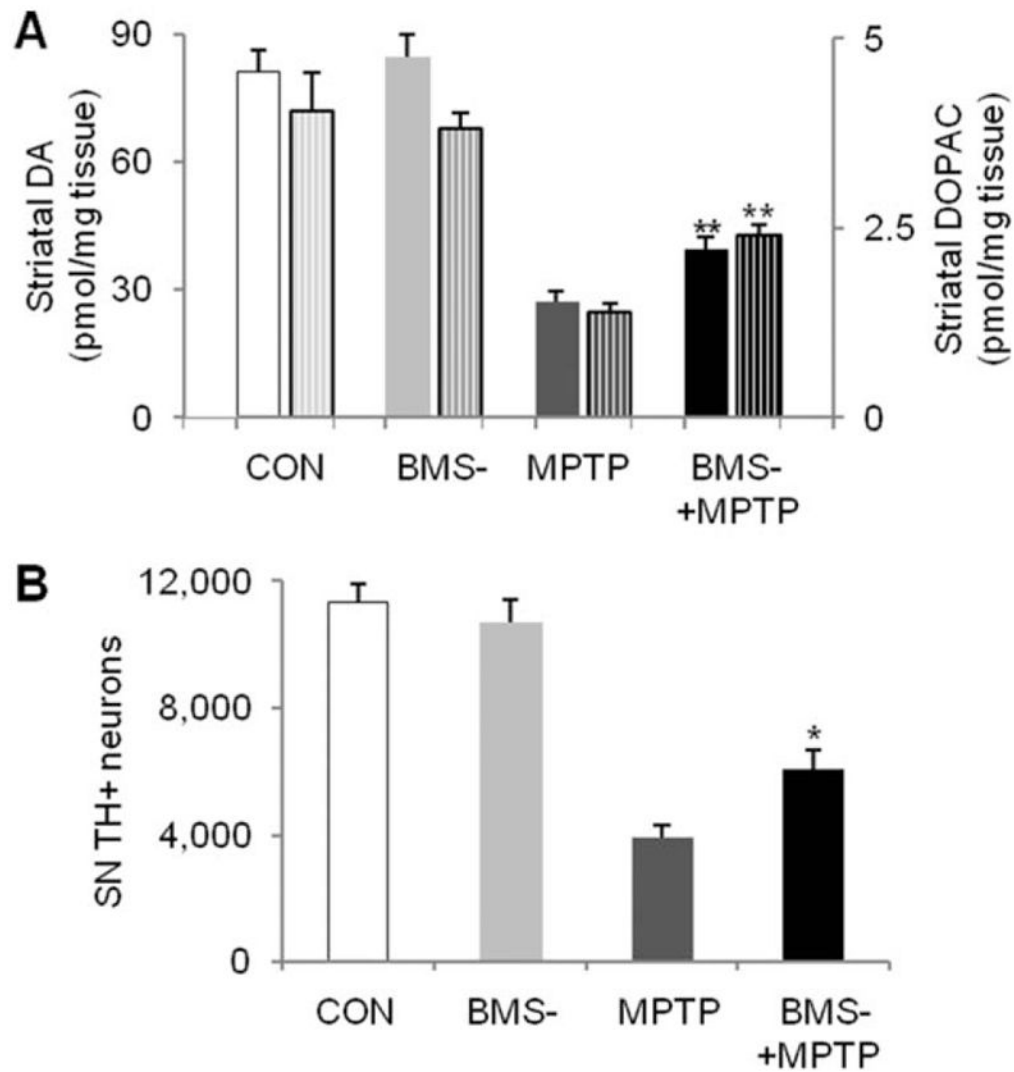
MC1R^{e/e} mice have greater oxidative damage in the ventral midbrain. (A, B) Protein carbonyls assessed by Oxyblot in (A) the ventral midbrain and (B) the striatum in adult (3–6-month-old) *MC1R^{e/e}* mice and littermate wild-type (WT) controls (n=4, WT and *MC1R^{e/e}* ventral midbrain; n=5, WT and *MC1R^{e/e}* striatum). * $p < 0.05$ versus WT by Student *t* test. (C, D) DNA damage markers 8,5'-cyclo-2'-deoxyadenosine (cdA) and 8,5'-cyclo-2'-deoxyguanosine (cdG) assessed by liquid chromatography–tandem mass spectrometry in (C) the ventral midbrain and (D) the striatum in adult *MC1R^{e/e}* mice (5–8 months old) and littermate WT controls (n=4, WT and *MC1R^{e/e}* mice, pooled from 12 animals of each genotype). * $p < 0.05$ versus WT by Student *t* test. [Color figure can be viewed at wileyonlinelibrary.com]

**FIGURE 4.**

MC1R^{e/e} mice are more susceptible to dopaminergic toxin 6-hydroxydopamine (6-OHDA). (A) Adult (6–8-month-old) *MC1R^{e/e}* and littermate wild-type (WT) mice were infused with 10 μ g 6-OHDA into the left striatum. Rotational behavior was assessed at 3 weeks, and animals were sacrificed at 4 weeks after 6-OHDA lesion. (B) Spontaneous and 5mg/kg amphetamine-induced net ipsilateral rotations in *MC1R^{e/e}* mice and littermate WT controls (n=12, WT and *MC1R^{e/e}*). **p* < 0.05 versus WT by Student *t* test. (C) Striatal dopamine (DA) measured by high-performance liquid chromatography (HPLC) and DA turnover rate (homovanillic acid [HVA]/DA ratio, HVA determined by HPLC) on contralateral (Contra) unlesioned side and ipsilateral (Ipsi) lesioned side in *MC1R^{e/e}* mice and WT littermates (n=12, WT and *MC1R^{e/e}*). ***p* < 0.01 versus WT Contra side; ##*p* < 0.01 versus WT Ipsi side, 1-way analysis of variance (ANOVA) followed by Tukey post hoc test. (D) Disruption of substantia nigra (SN) dopaminergic neurons (stained positive for tyrosine hydroxylase [TH]) on the Ipsi lesioned side of an *MC1R^{e/e}* mouse after 6-OHDA lesion. (E) Stereological quantification of nigral TH-positive neurons expressed as absolute counts and percentage of Contra side in *MC1R^{e/e}* mice and WT littermates (n=12, WT and *MC1R^{e/e}*). ***p* < 0.01 versus WT Contra side; ##*p* < 0.01 versus WT Ipsi side, 1-way ANOVA followed by Tukey post hoc test for absolute counts and Student *t* test for normalized counts. [Color figure can be viewed at wileyonlinelibrary.com]

**FIGURE 5.**

MC1R^{e/e} mice are more susceptible to dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Adult (6–8-month-old) *MC1R^{e/e}* and littermate wildtype (WT) mice were injected intraperitoneally (i.p.) with 20mg/kg MPTP-HCl or control vehicle (saline) once daily for 4 days and sacrificed 7 days after the last MPTP administration. (A) Dopamine (DA) in the striatum measured by high-performance liquid chromatography (HPLC) and DA turnover rate (homovanillic acid [HVA]/DA ratio, HVA determined by HPLC) in MPTP or vehicle control (CON) *MC1R^{e/e}* mice and WT littermates (n=6, WT and *MC1R^{e/e}* CON; n=8 and 7, WT and *MC1R^{e/e}* MPTP). * $p < 0.05$ versus WT CON; # $p < 0.05$, ## $p < 0.01$ versus WT MPTP, 1-way analysis of variance (ANOVA) followed by Tukey post hoc test. (B) Substantial loss of TH-positive neurons in the substantia nigra (SN) of an *MC1R^{e/e}* mouse after MPTP subacute treatment. (C) Stereological quantification of nigral tyrosine hydroxylase (TH)- positive neurons expressed as absolute counts and percentage of CON group in *MC1R^{e/e}* mice and WT littermates (n=6, WT and *MC1R^{e/e}* CON; n=8 and 7, WT and *MC1R^{e/e}* MPTP). ** $p < 0.01$ versus WT CON; # $p < 0.05$, ## $p < 0.01$ versus WT MPTP, 1-way ANOVA followed by Tukey post hoc test for absolute counts and Student *t* test for normalized counts. (D) Striatal 1 methyl-4-phenylpyridinium (MPP⁺) detected by HPLC in *MC1R^{e/e}* and littermate WT mice following 20mg/kg MPTP-HCl intraperitoneal administration (n=6 and 8, WT and *MC1R^{e/e}*, both 90 minutes and 6 hours). [Color figure can be viewed at wileyonlinelibrary.com]

**FIGURE 6.**

MC1R agonist protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity. Adult (3-month-old) C57Bl/6 mice were administered with a single dose of MPTP-HCl at 40mg/kg or vehicle (saline) intraperitoneally, and BMS-470539 (BMS-) 100mg/kg or vehicle (water) was injected subcutaneously 10 minutes before and 60 minutes after MPTP. Mice were sacrificed 7 days later. (A) Striatal dopamine (DA) and metabolite 3,4-dihydroxyphenylacetic acid (DOPAC; patterned columns, the right y-axis) were analyzed by high-performance liquid chromatography. (B) Numbers of nigral tyrosine hydroxylase (TH)-positive neurons were counted by stereological method. * $p < 0.05$, ** $p < 0.01$ versus MPTP group, 1-way analysis of variance followed by Tukey post hoc test ($n=5, 4, 6, 7$ for control [CON], BMS-, MPTP, and BMS-+MPTP groups, respectively). SN=substantia nigra.