Topical application of the pharmacological cold mimetic menthol stimulates brown adipose tissue thermogenesis through a TRPM8, UCP1, and norepinephrine dependent mechanism in mice housed at thermoneutrality

Greg L. McKie | Kyle D. Medak | Hesham Shamshoum | David C. Wright

Abstract

Increasing whole-body energy expenditure via the pharmacological activation of uncoupling protein 1 (UCP1)-dependent brown adipose tissue (BAT) thermogenesis is a promising weight management strategy, yet most therapeutics studied in rodents to date either induce compensatory increases in energy intake, have thermogenic effects that are confounded by sub-thermoneutral housing temperatures or are not well tolerated in humans. Here, we sought to determine whether the non-invasive topical application of the pharmacological cold mimetic and transient receptor potential (TRP) cation channel subfamily M member 8 (TRPM8) agonist L-menthol (MNTH), could be used to stimulate BAT thermogenesis and attenuate weight gain in mice housed at thermoneutrality. Using three different strains of mice and multiple complimentary approaches to quantify thermogenesis in vivo, coupled with ex vivo models to quantify direct thermogenic effects, we were able to convincingly demonstrate the following: (1) acute topical MNTH application induces BAT thermogenesis in a TRPM8- and UCP1-dependent manner; (2) MNTH-induced BAT thermogenesis is sufficient to attenuate weight gain over time without affecting energy intake in lean and obese mice; (3) the ability of topical MNTH application to stimulate BAT thermogenesis is mediated, in part, by a central mechanism involving the release of norepinephrine. These data collectively suggest that topical application of MNTH may be a promising weight management strategy.

KEYWORDS

menthol, thermoneutral, topical, TRPM8, UCP1

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1 | INTRODUCTION

Ambient cold exposure potently activates the sympathetic nervous system (SNS) which in turn drives the β2AdrR- and β3AdrR-dependent activation of non-shivering thermogenesis in humans1–3 and rodent4–6 brown adipose tissue (BAT), respectively. BAT thermogenesis is principally, although not exclusively,7 mediated by uncoupling protein 1 (UCP1)-dependent increases in the dissipation of the mitochondrial proton motive force,8–10 and in pre-clinical rodent models, cold-induced increases in BAT thermogenesis can protect against diet-induced obesity.11–15 These findings have fueled the idea that ambient cold exposure is a promising weight loss treatment for human obesity,16,17 despite evidence that ambient cold exposure has little to no effect on body weight or fat mass in humans.16,18–20

One likely explanation for these discrepant findings between species is that most preclinical studies have been confounded by sub-thermoneutral housing temperatures thereby limiting their translational relevance to human metabolism.21–24 In fact, we have recently demonstrated that when mice are housed at thermoneutrality and their BAT phenotype is humanized,25 a physiologically relevant model of ambient cold exposure causes rapid and persistent hyperphagia that exacerbates, rather than attenuates, diet-induced obesity.26 Similar reports are beginning to emerge27 and thus it should be acknowledged that ambient cold exposure does not protect humanized rodents against diet-induced obesity. As such, the identification of translationally relevant alternative treatment strategies is highly warranted.

Unlike ambient cold exposure, pharmacological sympathomimetics can activate BAT thermogenesis independent of alterations in energy intake.28–32 However, while such thermogenic agents reliably activate BAT in rodents,4,28–30,33–35 there has been limited success to date in humans,2,36–45 likely because these thermogenic agents are specific to the β3AdrR yet human BAT thermogenesis is under β2AdrR control.3 Fortunately, the pharmacological cold mimetic and transient receptor potential (TRP) cation channel subfamily M member 8 (TRPM8) agonist L-menthol (MNTH) retains the thermogenic effects of other sympathomimetics without directly activating any βAdrR36,47 or affecting energy intake31,32 effectively circumventing the aforementioned issues regarding other sympathomimetics.

TRPM8 is highly expressed in cutaneous sensory afferent nerves,46,47,51–53 this makes MNTH an ideal candidate for topical application, a non-invasive drug administration route that activates cutaneous sensory afferent neurons,48 has a similar pharmacokinetic profile to oral MNTH administration59 while being less aversive,55 and is associated with greater increases in thermogenesis49,56 and local concentrations of MNTH in subcutaneous adipose tissue.56 Although topical MNTH application has been shown to acutely increase whole-body oxygen consumption and markers of BAT thermogenesis in mice housed at near thermoneutral temperatures,54 it is currently unknown whether topical MNTH treatment activates BAT thermogenesis directly via TRPM8 or indirectly via a TRPM8-dependent increase in SNS activity and if the increase in thermogenesis would be sufficient to attenuate weight gain in mice housed under thermoneutral conditions. Therefore, the aim of this study was to determine if, and how, topical MNTH application stimulates BAT thermogenesis to attenuate weight gain in mice housed at thermoneutrality.

Here, using multiple in vivo and ex vivo approaches in three different strains of mice housed at thermoneutrality, we were able to convincingly demonstrate the following: (1) acute topical MNTH application induces BAT thermogenesis in a TRPM8 and UCP1 dependent manner; (2) MNTH-induced BAT thermogenesis is sufficient to attenuate weight gain over time without affecting energy intake in lean and obese mice; (3) the ability of topical MNTH application to stimulate BAT thermogenesis is mediated, in part, by a central mechanism involving the release of norepinephrine.

2 | MATERIALS AND METHODS

2.1 | Animal husbandry and sacrifice

Male C57BL/6J mice were purchased from the Jackson Laboratory at 8 to 10 weeks of age. UCP1 knockout (KO) mice60 on a C57BL/6J background (JAX stock no. 003124) were purchased from the Jackson Laboratory and breeding pairs of wild-type (WT) and UCP1 KO mice were used to establish a colony at the University of Guelph. TRPM8 KO mice45 on a C57BL/6J background were purchased from the Jackson Laboratory (JAX stock no. 008198) and backcrossed onto a C57BL/6N background for several generations before a colony of WT and TRPM8 KO mice were established at the University of Guelph. TRPM8 KO mice were backcrossed onto a C57BL/6N background given the original mice donated to The Jackson Laboratory Repository were on a C57BL/6N genetic background. Mice were individually housed on a 12:12 h photocycle
at thermoneutrality (29.9 ± 0.8°C; 45% humidity) in shoebox-style cages containing corncob bedding (cat no. 7097; Teklad). UCP1 and TRPM8 mice were born and raised at thermoneutrality and because C57BL/6J mice purchased from JAX were not, they were allowed at least one week to acclimate to thermoneutrality before being used for experiments. All mice had ad libitum access to water and standard rodent chow (cat. no. 7004; Teklad) except under conditions of diet-induced obesity where mice were fed a 45% high-fat diet (Diet Formula: D12451; Research Diets Inc.). All mice were maintained on their respective diets throughout and were 8–12 weeks of age when studied.

Mice were sedated by injection (60 mg/kg; i.p.) of sodium pentobarbital (65.4 mg/ml; Schering Canada Inc.) and sacrificed by exsanguination of the heart. The epididymal and inguinal white adipose tissue depots (eWAT and iWAT, respectively), as well as the interscapular BAT depots, and the liver were excised, snap-frozen in liquid nitrogen, and stored at −80°C unless stated otherwise. Blood was centrifuged at 1000 g for 10 min and the serum was aliquoted and stored at −80°C unless stated otherwise. Blood was centrifuged at 1000 g for 10 min and the serum was aliquoted and stored at −80°C. Mice chronically treated with menthol were sacrificed 48 h following their last treatment to allow for the residual effects of the treatment to wear off. Acutely-treated mice were sacrificed at the indicated time points in the figure captions.

2.2 | Topical menthol application

Weight matched mice were treated with a relative dose (2 g/kg) of MNTH (5% wt/vol; cat no. W266590; Sigma–Aldrich) or its ethanol solvent (CTRL) using a needleless syringe. MNTH or CTRL was selectively applied to the unshaved ventral and dorsal surfaces of restrained mice carefully avoiding orifices, limbs, and the tail. All treatments occurred between 1000 and 1200 h and mice were either treated once for acute terminal experiments or once daily for five consecutive days over two weeks (10 treatments total) for chronic experiments.

2.3 | Metabolic phenotyping

Treatment naïve mice were sedated by injection (30 mg/kg; i.p.) of sodium pentobarbital and sacrificed by cardiac exsanguination before 20–30 mg of iWAT, eWAT, BAT, and the liver were harvested, weighed, rinsed, and then incubated in 96-well plates containing 250 µl of M199 (cat no. M7653; Sigma) supplemented with 2% fatty acid-free BSA (cat no. 152401; MP Bio). Plates were then left to equilibrate to 37°C and 5% O2 in a Forma Steri Cycle CO2 incubator (model. 370; Thermo Scientific) for 10–30 min before being treated with 100-µM MNTH, 1-mM MNTH, 10-µM CL 316, 243 (cat no. 17499; Cayman Chemical), 10-µM glucagon (rDNA origin; Eli Lilly), or CTRL. CL 316,243 was used as a positive control for lipolysis in the adipose tissue depots whereas glucagon was used as a positive control for glucose production in the liver. Liver explants were used to directly assess the ability of MNTH to stimulate hepatic glucose production. These MNTH concentrations were chosen based on previous reports demonstrating that 1-µM and 30-µM MNTH is sufficient to induce the expression of UCP1 in differentiated human white adipocytes and primary cultured murine brown
adipocytes, respectively. However, it should also be noted that these MNTH concentrations are 3–4 orders of magnitude greater than what is observed in the serum of mice 60 min following topical application of 10% MNTH. iWAT and eWAT explants were treated for 2 h, whereas BAT and liver explants were only treated for 1 h. At the indicated time points 200 µl of media was collected and put on ice, BAT and liver tissues were rinsed in ice-cold 1x PBS, snap-frozen in liquid nitrogen, and then stored at −80°C. There were 6 biological replicates and at least 2 technical replicates per group per tissue. NEFA, glycerol, and glucose accumulation in the media were normalized to tissue explant mass.

2.6 Adipose tissue organ cultures

Due to the low RNA yields in the iWAT and eWAT explants, the ex vivo experiment was repeated but in iWAT and eWAT adipose tissue organ cultures. Treatment naïve mice were sedated then sacrificed as described above, and the entire iWAT (355 ± 109 mg) and eWAT (426 ± 144 mg) depots were then harvested, weighed, rinsed, and minced in Petri dishes containing 12 ml of M199 supplemented with 2% fatty acid-free BSA, 1% antibiotic/antimycotic (cat no. 30-004-CI; Corning), 100 U/ml insulin (rDNA origin; Eli Lilly), and 2.5 nM dexamethasone (cat. no. D1159; Sigma). Dishes were left to equilibrate to 37°C and 5% O2 for 24 h after which time the media was discarded, the tissue minces were rinsed in ice-cold 1x PBS, and 12 ml of fresh M199 supplemented with 2% fatty acid-free BSA was added to each dish. After equilibrating for 1 h, the dishes were treated with 100 µM MNTH, 1 mM MNTH, 10 µM CL316, 243, or CTRL. After 2 h, 200 µl of media was collected and put on ice, tissue minces were rinsed in ice-cold 1x PBS, snap-frozen in liquid nitrogen, and stored at −80°C. There were 4–5 biological replicates per group per tissue.

2.7 Chemical sympathectomy of brown adipose tissue

BAT sympathectomy was achieved by injection (100 mg/kg; i.p.) of the catecholaminergic neurotoxin 6-hydrobromide (6-OHDA; cat no. 25330; Cayman Chemical) as previously described. For these experiments, one cohort of mice was housed at a sub-thermoneutral room temperature (21°C; 45% humidity) on a 12:12 h photoperiod while the other was housed at thermoneutrality (as described). Forty-eight hours before terminal experiments treatment naïve mice were weight-matched and then injected with vehicle (VEH) or 6-OHDA. After 48 h, the mice housed at room temperature were sacrificed, whereas the mice housed at thermoneutrality were sedated, transferred to metabolic caging, and treated with CTRL or MNTH before undergoing metabolic phenotyping (all as previously described).

2.8 Serum/media biochemistry

Epinephrine and norepinephrine were extracted from serum, acylated, and enzymatically converted before being measured by ELISA (cat. no. KA3768; Abnova) as described elsewhere. Serum insulin (cat no. 10-1247-01; Mercodia) and glucagon (cat. no. 10-1281-01; Mercodia) were also measured by ELISA, whereas NEFA, glycerol, and glucose (cat no. EIAGLUC; ThermoFisher) from serum or media were measured on 96 well plates using a colorimetric assay. All assays were conducted in duplicate and in accordance with the manufacturer’s instructions.

2.9 Brown adipose tissue preparation

Weighed BAT depots were homogenized in a cell lysis buffer solution containing 1-mM EDTA (cat no. FNN0011; Invitrogen), phenylmethylsulfonyl fluoride (cat no. P7626; Sigma), and protease inhibitor cocktail (cat no. P8340; Sigma). BAT homogenate was centrifuged at 1500 g for 10 min, aliquoted, and then epinephrine and norepinephrine concentrations were measured with an ELISA (as described above) and expressed relative to tissue mass.

2.10 Quantitative reverse transcription PCR

RNA was extracted using 1-ml TRIZole and Bio Basic EZ-10 Spin Column Total RNA Miniprep Super Kits (cat no. BS784; Bio Basic). Complementary DNA was synthesized from total RNA using Superscript II (cat no. 4368814; Invitrogen), phenylmethylsulfonyl fluoride (cat no. P7626; Sigma), and protease inhibitor cocktail (cat no. P8340; Sigma). BAT homogenate was centrifuged at 1500 g for 10 min, aliquoted, and then epinephrine and norepinephrine concentrations were measured with an ELISA (as described above) and expressed relative to tissue mass.

2.11 Immunoblotting

BAT was homogenized, protein content was determined, and samples were prepared as previously described.
Equal amounts of protein were resolved using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique, wet transferred onto nitrocellulose membranes, blocked, and incubated in primary and secondary antibodies as previously described. Signals were detected using enhanced chemiluminescence on a FluorChem FC2 machine and protein content (density) was quantified with Ponceau S staining used as a loading control. Phosphorylated PKA substrates (cat. no. 9624) and horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074) were purchased from Cell Signaling Technology.

### 2.12 Statistical analyses

Data were screened for outliers using the extreme studentized deviate method with subsequent analyses being conducted in GraphPad Prism (version 9.1.2; GraphPad Software LLC). To compare the mean difference between two unrelated groups an unpaired, two-tailed, t-test was used. To compare the mean difference between three or more unrelated groups an ordinary one-way ANOVA was used. Comparisons between groups differing among two or more factors were made using a two-way ANOVA. Post hoc, the Holm-Sidak test was used to correct for multiple comparisons. Simple linear regression was used to model the relationship between variables where indicated. The p-value was set at .05 a priori and all data are presented as the mean ± SD.

### 3 RESULTS

#### 3.1 Topical menthol application increases brown adipose tissue thermogenesis and attenuates weight gain

Conflicting reports regarding the expression of TRPM8 in peripheral tissues have contributed to the lack of understanding in terms of whether MNTH acts directly or not to increase thermogenesis. To address this issue and confirm the utility of our route of administration, we first sought to determine where and to what extent TRPM8 was expressed in mice housed at thermoneutrality, using mice exposed to a chronic sub-thermoneutral cold stress as a reference. Interestingly, TRPM8 expression was similar between housing temperatures in tissues examined with the exception of iWAT (Figure 1A). Given there were no differences in the cutaneous expression of TRPM8 between mice housed at sub-thermoneutral and thermoneutral housing temperatures (Figure 1A), and the fact that humans express TRPM8 in cutaneous sensory afferent nerves, we reasoned that topical MNTH application could represent a translationally relevant therapeutic approach for weight management if it proved efficacious at inducing thermogenesis in mice at thermoneutrality.

Next, we administered CTRL or MNTH to treatment naïve mice singly housed at thermoneutrality and tracked changes in their core body temperature over 60 min to assess the acute effects of topical MNTH application on energy homeostasis. The change in core body temperature over time was significantly greater in the mice treated with MNTH relative to CTRL (Figure 1B), and using infrared thermographic imaging, we demonstrated that the MNTH-induced increase in core body temperature was paralleled by increases in BAT activity (Figure 1C,D). Weight-matched mice that underwent metabolic phenotyping (Figure 1E) clearly demonstrated that MNTH increased whole-body oxygen consumption (Figure 1F) and energy expenditure (Figure 1G) relative to CTRL-treated mice, suggesting that the topical application of MNTH is sufficient to acutely increase BAT thermogenesis. Interestingly, all of this occurred in the absence of any observable increase in the phosphorylation of PKA substrates in BAT 60 min following topical MNTH application (Figure 1A–C).

Given the acute effects of MNTH on BAT thermogenesis, we next sought to determine whether chronic application of MNTH would affect body mass. To test this, we treated mice with CTRL or MNTH 5 days/week for 2 consecutive weeks tracking changes in body mass and food intake throughout. As expected, MNTH treatment significantly attenuated increases in body mass over time (Figure 1H) and total body mass gain (Figure 1I) with no effects on energy intake (Figure 1J). Chronic MNTH treatment induced the expression of UCP1, but not PGC-1a or TRPM8, in BAT (Figure 1K). This is consistent with the previous literature.
demonstrating that the genetic deletion of PGC-1a in BAT does not affect UCP1 expression. There were no effects of chronic MNTH treatment on any transcript in either iWAT (Figure 1L) or eWAT (Figure 1M), and the ability of MNTH to induce UCP1 expression in BAT but not iWAT or eWAT is consistent with a growing body of literature contending the physiological relevance of the browning of white adipose tissue. Importantly, the ability of MNTH to attenuate weight gain without affecting energy intake was preserved in obese mice that had been fed a high-fat diet for 6 weeks (Figure S2A–C). Collectively, our data demonstrate that topical MNTH application increases energy expenditure and is an efficacious approach to limit weight gain in lean and obese mice housed at thermal neutrality.

### 3.2 The effects of topical menthol application are TRPM8 and UCP1 dependent

Despite expectedly low levels of TRPM8 expression in peripheral tissues at thermoneutrality (Figure 1A), we were able to demonstrate that the acute thermogenic effects of topical MNTH application are indeed TRPM8 dependent, as TRPM8 KO mice failed to exhibit the MNTH-induced increase in whole-body oxygen consumption and energy expenditure present in WT mice (Figure 2A–D). Importantly, this confirms the specificity of MNTH for TRPM8 and that topical MNTH application does not elicit thermogenesis via TRPM8-independent effects.

Next, we administered CTRL or MNTH to treatment naïve UCP1 WT and KO mice, tracked changes in
their core body temperature over 60 min, and coupled this with infrared thermographic imaging to determine whether the acute effects of topical MNTH application on BAT thermogenesis were also UCP1 dependent. As a positive control, some mice received an injection (1 mg/kg; i.p.) of CL 316,243. MNTH-treated UCP1 WT mice exhibited significantly greater increases in core body temperature than did CTRL and even CL-treated mice, with this effect being expectedly absent in the UCP1 KO mice (Figure 2D). Infrared thermographic imaging confirmed our prior findings that the source of the increase in core body temperature following MNTH treatment is BAT, as MNTH increased BAT surface temperature in WT but not KO-treated mice (Figure 2E–H), clearly demonstrating that the effects of MNTH on BAT thermogenesis are UCP1 dependent. Metabolic phenotyping further confirmed this as weight-matched mice treated with MNTH increased whole-body oxygen consumption and energy expenditure in a UCP1-dependent manner (Figure 2J–L). As a positive control for the application of MNTH on UCP1 KO mice undergoing metabolic phenotyping we measured blood glucose in a separate cohort of UCP1 KO mice 60 min post-MNTH application and observed an effect of MNTH to increase blood glucose (CTRL: 6.5 ± 0.6 mmol/L, MNTH: 7.6 ± 1.0 mmol/L; p = .016), clearly indicating that UCP1 KO mice are still responsive to MNTH despite an inability to increase energy expenditure. Taken together, these data clearly indicate that acute topical MNTH application activates BAT thermogenesis in a TRPM8 and UCP1-dependent manner.

Given the dependence on UCP1 for increases in energy expenditure with acute MNTH treatment, we next sought to confirm whether the attenuation of body weight gain previously observed with short-term MNTH treatment was also UCP1 dependent. To test this, we treated weight-matched UCP1 WT and KO mice with CTRL or MNTH, and in line with our prior data, UCPI WT but not KO mice treated with MNTH exhibited an attenuated increase in body mass and total body mass gain over the 2 weeks (Figure 2L,M), independent of significant alterations in food intake (Figure 2N).

3.3 Menthol does not directly affect indices of adipose tissue thermogenesis or hepatic glucose production

Based on our data indicating that TRPM8 expression is low in peripheral tissues of mice housed at thermoneutrality, and previously published observations by others indicating that topical MNTH application drives TRPM8-dependent c-Fos gene expression in the central nervous system, we hypothesized that topical MNTH application activates BAT thermogenesis indirectly by increasing SNS activity. As such, we conducted a series of ex vivo tissue explant and organ culture experiments to rule out any direct effects of MNTH on adipose tissue metabolism, using a physiological and pharmacological dose of MNTH. Since lipolysis and FFA re-esterification increase during states of adrenergic stress, and because the thermogenic function in brown adipocytes is fueled by intracellular lipolysis and the uptake of circulating lipids, we first treated BAT, iWAT, and eWAT explants with CTRL, MNTH, or CL 316,243 and assayed the media to measure the accumulation of NEFA and glycerol. CL 316,243 served as a positive control. There were no direct effects of MNTH treatment on the accumulation of NEFA or glycerol within the media of BAT (Figure 3A), iWAT (Figure 3B), or eWAT (Figure 3C); however, CL 316,243 did expectedly increase both markers of lipolysis in all three tissues. Similarly, there were significant effects of CL 316,243 treatment, but not MNTH, on the induction of UCP1 gene expression in BAT explants (Figure 3D), and iWAT (Figure 3E) and eWAT (Figure 3F) organ cultures. This is in contrast to reports that MNTH, at similar concentrations in vitro, elicits increased UCP1 gene expression in differentiated human white adipocytes and primary-cultured murine brown adipocytes. There were no effects of MNTH or CL 316,243 on PGC-1a expression in BAT, iWAT, or eWAT (Figure 3D–F). The ability of CL 316,243 to increase UCP1 gene expression in BAT independent of alterations in PGC-1a expression aligns with our earlier observations in vivo (Figure 1K) and existing literature. Based on the concentrations used here and the time points at which media and tissues were collected, the inability of MNTH to directly stimulate adipose tissue lipolysis and the expression of genes encoding proteins involved in thermogenesis ex vivo strongly suggests that in vivo topical MNTH application stimulates BAT thermogenesis indirectly.

In an additional ex vivo experiment, we tested the ability of MNTH to directly stimulate glucose production in liver explants, as we observed that topical MNTH application acutely elevated blood glucose concentrations in vivo (Figure S3A) independent of alterations in serum insulin or glucagon (Figure S3B,C). In ex vivo liver explants, MNTH failed to stimulate glucose production directly as the accumulation of glucose within the media only increased following glucagon treatment (Figure S3D). These data further demonstrate that MNTH does not elicit direct effects, at least based on the concentrations used and the time points measured, again suggesting that topical MNTH acts indirectly, in this case, to increase blood glucose, via a mechanism that likely involves the SNS.
3.4 Chemical ablation of catecholaminergic neurons in brown adipose tissue diminishes the thermogenic effects of topical menthol application

To determine whether topical MNTH application elicits BAT thermogenesis via the SNS we used 6-OHDA, a well-characterized model of chemical sympathectomy, to chemically ablate catecholaminergic neurons within BAT and to reduce circulating norepinephrine concentrations. To confirm the efficacy of this approach we first housed mice at a sub-thermoneutral temperature to elicit chronic adrenergic stress and then harvested serum and BAT 48 h after weight-matched mice were treated with VEH or 6-OHDA (Figure 4A). Relative to VEH-treated mice, 6-OHDA expectedly lowered serum (Figure 4B) and BAT (Figure 4C) norepinephrine concentrations with no effect on serum epinephrine (Figure 4D), clearly highlighting the efficacy of using 6-OHDA to chemically ablate catecholaminergic neurons within BAT and reduce circulating norepinephrine concentrations.

We then repeated this experiment with mice that had been housed at thermoneutrality and pretreated with VEH or 6-OHDA 48 h prior to being transferred to metabolic cages within an environmental enclosure. After acutely acclimating to the metabolic cages, weight-matched mice (Figure 4E) were treated topically with CTRL or MNTH, and respiratory gases were continuously recorded for 60 min, at which time tissues were harvested. At the whole-body level, the ability of topical MNTH application to increase oxygen consumption (Figure 4F) and energy expenditure (Figure 4G) occurred independent of 6-OHDA pre-treatment, although 6-OHDA did appreciably attenuate the effects of MNTH on both parameters. However, at the tissue level, the effects of topical MNTH application on serum and BAT norepinephrine were highly dependent on whether mice were pretreated with VEH or 6-OHDA, as 6-OHDA pre-treatment significantly attenuated the MNTH-induced increase in both serum (Figure 4H) and BAT (Figure 4I) norepinephrine concentrations. 6-OHDA pre-treatment did not affect serum epinephrine concentrations (Figure 4I), but it did blunt MNTH-induced increases in lipolysis (Figure S4A,B). These data affirm that topical MNTH application elicits BAT thermogenesis, at least in part, via a SNS-dependent mechanism involving the release of norepinephrine, and this is reflected in the strong positive association between whole-body energy expenditure and BAT norepinephrine levels (Figure 4K) that appears to be driven by the topical application of MNTH and intact BAT SNS innervation.

4 DISCUSSION

This study attempted to determine if, and how, topical MNTH application stimulates thermogenesis in physiologically humanized BAT of mice housed at thermoneutrality. Using multiple complimentary approaches to quantify thermogenesis in vivo, multiple models to quantify direct effects of MNTH ex vivo, and three different strains of mice, we were able to convincingly demonstrate the following: (1) acute topical MNTH application induces BAT thermogenesis in a TRPM8- and UCP1-dependent manner; (2) MNTH-induced BAT thermogenesis is sufficient to attenuate weight gain over time without affecting energy intake in both chow and high-fat diet-fed mice; (3) the ability of topical MNTH application to stimulate BAT thermogenesis is mediated, in part, by a central mechanism involving the release of norepinephrine. These observations collectively support the generation of a working model whereby the topical application of MNTH to the skin activates TRPM8 to stimulate the afferent transmission of this signal to the SNS, the release of norepinephrine from catecholaminergic neurons innervating BAT, and the downstream activation of UCP1-dependent thermogenesis (Figure 5).

Accumulating evidence supports a role for pharmacologically targeting TRPM8 with MNTH to mimic the thermogenic effects of ambient cold exposure, and
our data clearly expand on this literature by establishing the mechanism through which topical MNTH application works in a translationally relevant preclinical model of human metabolism. To date, only one other study has reported topically treating mice housed at a near thermoneutral temperature with MNTH, but unlike our current work at thermoneutrality, this study only made acute thermogenic measures under chow-fed conditions and
Interestingly, they anesthetized mice with isoflurane, a known inhibitor of BAT thermogenesis,\(^74,75\) prior to each topical MNTH application.\(^54\) More recently, two studies have demonstrated that a high-fat diet supplemented with MNTH (0.5%–1%) reduced body weight gain over 12–28 weeks in male mice,\(^31,50\) and while this dietary MNTH-induced attenuation of weight gain was TRPM8-dependent\(^31\) and associated with increases in UCP1-dependent thermogenesis,\(^31,50\) both studies housed mice at sub-thermoneutral temperatures confounding measures of thermogenesis.\(^21,26\) Moreover, food intake was either not reported\(^50\) or was only measured during the first and last week of the 28-week dietary intervention\(^31\) excluding the possibility that MNTH, which causes a dose-dependent taste aversion over 50 µg/ml in mice,\(^55\) could have attenuated weight gain via subtle effects on energy intake over time. In line with our route of administration, Vizin and colleagues topically treated chow-fed male rats with 5% MNTH once daily for 9 consecutive days and found that MNTH acutely increased whole-body oxygen consumption while attenuating weight gain over time without affecting energy intake.\(^32\) While this latter study was conducted at sub-thermoneutral housing temperatures, Tajino and colleagues previously reported that the acute topical application of 1–10% MNTH to 8-week-old male C57BL/6 mice housed at a near thermoneutral temperature-induced autonomic heat gain responses including dose-dependent increases in core temperature and whole-body oxygen consumption.\(^54\) Importantly, this latter study confirmed that the thermogenic effects induced by the topical application of MNTH were associated with increases in the activation of sensory neurons in dorsal root ganglia,\(^34\) demonstrating that topical MNTH application is sufficient to activate the SNS. Given this, and our data demonstrating that the central release of norepinephrine plays an important part in mediating the thermogenic effects of MNTH, it would seem as though topical MNTH application attenuates weight gain over time via a coordinated mechanism involving a skin-brain-BAT signaling axis. However, future mechanistic studies directly measuring the central effects of MNTH are needed to confirm this.

While our data is the first short-term study in mice housed at thermoneutrality to demonstrate the efficacy of topical MNTH application for weight management, humans express TRPM8 not only in peripheral sensory nerves,\(^51–53\) but also in the liver, colon, and prostate.\(^76\) Furthermore, agonizing TRPM8 within these tissues produces desirable physiological outcomes in multiple models of disease, including the dose-dependent reduction of cellular viability in human malignant melanoma cells,\(^77\) improved hepatic steatosis scores in models of high-fat diet-induced obesity,\(^30,73\) reduced inflammation in multiple models of
ulcerative colitis, and suppressed cellular migration in human prostate cancer cells. Perhaps most importantly, topical application of MNTH to humans is associated with vasodilation and increased vascular conductance, which is in contrast to other sympathomimetics such as the FDA-approved drug mirabegron that induces BAT thermogenesis but also produces unwanted cardiovascular effects. This suggests that TRPM8 plays a role in multiple physiological processes beyond the detection of ambient cold and highlights the potential utility of targeting this receptor to produce favorable clinical outcomes. Despite this, and evidence that single nucleotide polymorphisms in the TRPM8 gene are associated with alterations in cholesterol and waist circumference, the ability of MNTH to improve metabolic outcomes in humans remains unknown as MNTH has only been studied in human clinical trials to date for indications associated with pain (see clinical trial identifiers within). Taken together, it seems warranted that future studies examine the clinical efficacy of MNTH for metabolic dysfunction and weight management.

FIGURE 4 Chemical ablation of catecholaminergic neurons in BAT diminishes the thermogenic effects of topical MNTH application in male C57BL/6J mice. (A) Body mass at room temperature housed mice prior to treatment with VEH or 6-OHDA. (B and C) Norepinephrine concentrations in the serum and BAT of mice housed at room temperature 48 h following treatment with VEH or 6-OHDA. (D) Epinephrine concentrations in the serum of mice housed at room temperature 48 h following treatment with VEH or 6-OHDA. (E) Body mass of VEH or 6-OHDA-treated mice immediately prior to metabolic phenotyping and treatment with CTRL or MNTH. (F and G) Whole-body oxygen consumption and energy expenditure averaged over the 60 min post-treatment in mice that had been pre-treated with VEH or 6-OHDA 48 h prior. (H and I) Norepinephrine concentrations in the serum and BAT of CTRL or MNTH-treated mice that had been pre-treated with VEH or 6-OHDA 48 h prior. (J) Epinephrine concentrations in the serum of CTRL or MNTH-treated mice that had been pre-treated with VEH or 6-OHDA 48 h prior. (K) Simple linear regression showing a strong positive significant relationship between energy expenditure and BAT norepinephrine concentrations. All data are presented as mean ± SD. Experiments in panels E–K were conducted in at least two independent cohorts of mice.
5 | CONCLUSION

In conclusion, topical application of the pharmacological cold mimetic MNTH attenuates weight gain in mice with physiologically humanized BAT, independent of alterations in energy intake or nutritional status, by stimulating BAT thermogenesis through a mechanism that is dependent upon TRPM8, UCP1, and the central release of norepinephrine.

DISCLOSURES

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Greg L. McKie and David C. Wright designed all experiments. Greg L. McKie, Kyle D. Medak, and Hesham Shamshoum performed experiments. Greg L. McKie conducted analyses, generated manuscript figures, and drafted the manuscript. Greg L. McKie, Kyle D. Medak, Hesham Shamshoum, and David C. Wright edited the manuscript and approved the final version submitted for publication.

ETHICS APPROVAL

The Animal Care Committee at the University of Guelph approved all procedures used in this study which followed the Canadian Council on Animal Care (CCAC) guidelines.

DATA AVAILABILITY STATEMENT

Data are available upon request from the corresponding author.

ORCID

Greg L. McKie https://orcid.org/0000-0002-6469-0747
Hesham Shamshoum https://orcid.org/0000-0002-6518-3800

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SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

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