

## Research Report

# Methyl-Beta-Cyclodextrin Restores $K_{IR}$ Channel Function in Brain Endothelium of Female Alzheimer's Disease Mice

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### Abstract.

**Background:** As the sixth-leading cause of death in the United States, Alzheimer's disease (AD) entails deteriorating endothelial control of blood flow throughout the brain. In particular, reduced inward-rectifying  $K^+$  ( $K_{IR}$ ) channel function in animal models of aging and AD compromises endothelial function and optimal perfusion of brain parenchyma. Deficient endothelial  $K_{IR}$  channels may result from aberrant interaction with plasma membrane cholesterol as a primary regulator of membrane fluidity and ion channels.

**Objective:** We tested the hypothesis that mild methyl- $\beta$ -cyclodextrin (M $\beta$ CD) treatment to reduce membrane cholesterol may restore endothelial  $K_{IR}$  channel function in brain endothelium of old AD mice.

**Methods:** Membrane potential was continuously measured in isolated endothelial tubes from posterior cerebral arteries of young (1 to 3 months) and old (16 to 19 months) female *3xTg-AD* mice before and after mild treatment with the cholesterol-removing agent M $\beta$ CD (1 mmol/L). Elevated extracellular potassium ( $[K^+]_E$ ; 15 mmol/L) and NS309 (1  $\mu$ mol/L) activated  $K_{IR}$  and  $Ca^{2+}$ -activated  $K^+$  ( $SK_{Ca}/IK_{Ca}$ ) channels respectively before and after M $\beta$ CD treatment.

**Results:**  $SK_{Ca}/IK_{Ca}$  channel function for producing hyperpolarization remained stable regardless of age group and M $\beta$ CD treatment ( $\Delta V_m$ :  $\sim -33$  mV). However, as deficient during AD,  $K_{IR}$  channel function was restored ( $\Delta V_m$ :  $-9 \pm 1$  mV) versus pre-M $\beta$ CD conditions ( $-5 \pm 1$  mV); a progressive effect that reached  $-14 \pm 1$  mV hyperpolarization at 60 min following M $\beta$ CD washout.

**Conclusion:** In female animals, M $\beta$ CD treatment of brain endothelium selectively restores  $K_{IR}$  versus  $SK_{Ca}/IK_{Ca}$  channel function during AD. Thus, the endothelial cholesterol- $K_{IR}$  channel interface is a novel target for ameliorating perfusion of the AD brain.

Keywords: Alzheimer's disease, brain endothelium, cholesterol,  $K^+$  ion channels

## INTRODUCTION

Alzheimer's disease (AD) is the leading type of dementia ( $\sim 6.2$  million people in the United States alone) and continues to rise in parallel with an increasingly aging demographic of individuals of

age 65 years and older [1]. Moreover, females in the general population indicate a higher prevalence, severity, and rate of AD pathogenesis even after correcting for age [2, 3]. Thus, especially in the context of biological sex, the etiology of AD remains obscure with no effective treatment strategies in place accordingly. Emerging evidence suggests maintenance or restoration of cerebrovascular health for optimal brain perfusion as a solution. In particular, how endothelial function may be harnessed to prevent

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dementia per conditions of aging [4, 5] and development of AD pathology [6–8].

In addition to pre-capillary small- and intermediate- $Ca^{2+}$ -activated  $K^+$  ( $SK_{Ca}/IK_{Ca}$ ) channels, endothelial inward-rectifying  $K^+$  ( $K_{IR}$ ) channels are integral to cerebral blood flow regulation [9, 10]. Our group has demonstrated that the hyperpolarizing function of endothelial  $K_{IR}$  channels of posterior cerebral arteries (corresponding to vasodilation and maintenance of cerebral blood flow) is reduced during conditions of aging [5] and AD [7]. Further, another recent study observed lower  $K_{IR2}$  channel current density in brain capillary endothelium in 5xFAD mice (age, 12 to 13 months; severe AD pathology as neuronal loss [11]), with overall consequences of diminished functional hyperemia in response to both whisker stimulation and elevated  $K^+$  [8]. In turn, this study identified a potential therapeutic role for membrane lipid regulation via enhanced phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ) supply to restore deficient cerebrovascular  $K_{IR2}$  channels [8].

As an additional key component of lipid regulation while integral to development of AD pathology [12–14], membrane cholesterol may also inactivate vascular  $K_{IR2}$  channels during conditions of health [15] and hypercholesterolemia [16, 17], with the latter most pronounced for diminished endothelial  $K_{IR2}$  channel function in particular. This regulatory role for cholesterol has not been tested using direct electrophysiological measurements of freshly isolated and intact endothelium in a study model of AD. Thus, we have tested the hypothesis that *mild methyl- $\beta$ -cyclodextrin ( $M\beta CD$ ) treatment to reduce membrane cholesterol may restore  $K_{IR}$  channel function in brain endothelium of old AD mice*. With a central emphasis on females, membrane potential ( $V_m$ ) was measured in posterior cerebral endothelial tubes of young (1 to 3 months) and old (16 to 19 months) *3xTg-AD* mice. The function of  $SK_{Ca}/IK_{Ca}$  and  $K_{IR2}$  channels underlying endothelium-derived hyperpolarization (EDH) [18, 19] were examined before and after mild treatment with the cholesterol-removing agent methyl- $\beta$ -cyclodextrin ( $M\beta CD$ ; 1 mmol/L).  $SK_{Ca}/IK_{Ca}$  channel function was stable throughout conditions regardless of  $M\beta CD$  treatment. Remarkably, reduced  $K_{IR2}$  channel function during advanced AD was restored to pre-AD conditions following presence of  $M\beta CD$ . This selective restoration of  $K_{IR2}$  channel function was likely due to mild membrane cholesterol removal and/or disruption in cerebrovascular endothelium during AD pathology. Altogether, cholesterol- $K_{IR}$  channel interaction plays a key role

in cerebrovascular endothelial  $K_{IR2}$  channel down-regulation during AD and direct modification of membrane cholesterol may ameliorate associated deficits in cerebral perfusion and dementia.

## METHODS

### *Animal care and use*

All animal care use and experimental protocols for this study were approved by the Institutional Animal Care and Use Committee of Loma Linda University and performed in accord with the National Research Council's "Guide for the Care and Use of Laboratory Animals" (8th Edition, 2011). Experiments were performed using young Pre-AD (1–3 months; females, 31 animals) and old AD (16–19 months; females, 39 animals) *3xTg-AD* mice [20] generated from homozygous breeding pairs obtained from the Jackson Laboratories [Wilmington, MA, USA; (B6;129-Tg (APP<sup>Swe</sup>,tauP301L) 1Lfa Psen1<sup>tm1Mpm</sup>/Mmjax); Mutant Mouse Resource and Research Center (MMRRC) stock #034830]. All animals were housed on a 12:12 h light-dark cycle at 22–24°C with fresh water and food available *ad libitum*. For individual experiments, animals were selected randomly with respect to group and biological sex.

### *Solutions and reagents*

Preparation and composition of all solutions have been previously described for measurements of  $V_m$  in intact endothelium freshly isolated from mouse posterior cerebral arteries [5, 7, 21, 22]. Briefly, physiological salt solution (PSS; pH 7.4) was prepared for continuous superfusion of cerebral endothelial tubes [(in mmol/L): 140 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 10 Glucose]. For dissection of brain and arteries, PSS lacking CaCl<sub>2</sub> contained 0.1% bovine serum albumin. For isolation of intact endothelium, an enzyme cocktail [0.31 mg/mL papain (P4762, Sigma), 0.5 mg/mL dithioerythritol (D8255, Sigma), 0.75 mg/mL collagenase (C8051, Sigma), and 0.13 mg/mL elastase (E7885, Sigma)] was prepared in dissociation PSS containing reduced  $Ca^{2+}$  (0.1 mmol/L CaCl<sub>2</sub>) and 0.1% bovine serum albumin. The 15 mmol/L KCl solution was prepared with an equimolar decrease of NaCl accordingly in order to maintain osmolarity. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA)

or ThermoFisher Scientific (Pittsburgh, PA, USA) unless otherwise indicated.

#### *Dissection of cerebral arteries and isolation of endothelial tubes*

Sequential dissection of mouse brain for preparation of posterior cerebral arteries and isolation of arterial endothelium have been previously described and illustrated in our previous studies [5, 7, 21, 22]. Briefly, following isoflurane inhalation for anesthesia and decapitation, the brain was acutely removed and placed in chilled (4°C) dissection PSS. Posterior cerebral arteries were carefully dissected from the brain and cut into segments. Arterial segments were partially digested in dissociation PSS containing the enzyme cocktail at 34°C for 10 to 12 min. Following digestion, arterial segments were triturated using borosilicate glass capillary tubes [1B100-4, World Precision Instruments (WPI), Sarasota, FL, USA] with an internal tip diameter of 80–120  $\mu\text{m}$  to remove adventitia, smooth muscle cells, and internal elastic lamina to isolate intact endothelial tubes. Endothelial tubes were continuously superfused with PSS at 37°C.

#### *Recording of intracellular $V_m$*

Recording of  $V_m$  in mouse cerebral endothelium has been previously described and illustrated [5, 7, 21, 22]. Briefly,  $V_m$  of isolated endothelial tubes was recorded with Axoclamp electrometers (2B and/or 900A; Molecular Devices, Sunnyvale, CA) using microelectrodes pulled (P-97, Sutter Instruments) from glass capillary tubes (GC100F-10, Warner Instruments, Holliston, MA, USA). Under continuous superfusion with PSS, an endothelial cell was penetrated with a microelectrode backfilled with 2 mol/L KCl (tip resistance:  $\sim 150\text{ M}\Omega$ ) while viewing the endothelial tube at  $400\times$  magnification. Amplifier outputs were connected to a data acquisition system (Digidata 1550A; Molecular Devices), whereby all data were acquired at a 10 Hz frequency on a Hewlett-Packard personal computer using Axoscope software (Molecular Devices). Each experiment included a continuous intracellular  $V_m$  recording for  $\sim 2\text{ h}$ .

#### *Cholesterol assay of cerebral blood vessels*

Cholesterol content was determined in total blood vessel content in the brain using Cholesterol Quantification Assay Kit (Sigma) in accord with

manufacturer's instructions. Brain microvessel fragments were isolated from the mouse brains using a previously illustrated protocol [23]. Cholesterol content ( $\mu\text{mol/L}$ ) was normalized to total phospholipid ( $\mu\text{mol/L}$ ) determined from the same blood vessel isolates using Phospholipid Assay Kit (Sigma) according to manufacturer's instructions.

#### *Cholesterol imaging of endothelial cell tubes*

Endothelial cell tubes were secured on a glass coverslip with glass micropipettes using two micro-manipulators and superfused with PSS while gradually raising the bath temperature to 37°C. Endothelial cell membrane cholesterol was detected by incubating cells with Filipin-III (50  $\mu\text{g/ml}$ , Sigma) at 37°C for  $\sim 1.5\text{ h}$  under light-protected conditions. The cells were then washed with fresh PSS and images were acquired using a fluorescent microscope (Eclipse Ti-S; Nikon), a 60X objective [Plan Apo  $\lambda$  (numerical aperture: 0.95); Nikon], a solid-state light source containing a violet channel (MIRA Light Engine; Lumencor, Inc., Beaverton, OR, USA), a 16-megapixel monochrome camera (DS-Qi2, Nikon), and Nikon imaging software (NIS Elements-F 4.60.00). For unpaired image acquisitions prior to staining with Filipin, endothelial cell tubes were treated with methyl- $\beta$ -cyclodextrin (M $\beta$ CD) for 20 min followed by 1 h washout period at 37°C. For ideal visibility, all images illustrating Filipin staining were pseudo-colored green.

#### *Pharmacology*

An effective half-maximal concentration of NS309 (EC<sub>50</sub>,  $\sim 1\text{ }\mu\text{mol/L}$ ; 3895, Tocris, Bristol, England) was used to directly evaluate function of SK<sub>Ca</sub>/IK<sub>Ca</sub> channels to produce hyperpolarization [5, 22]. As consistent with our previous studies [22], DMSO solvent ( $\leq 0.1\%$ ) for working stocks of NS309 was found to have no effect on endothelial  $V_m$  on its own for  $\leq 5\text{ min}$  applications used for respective drug treatments in cerebrovascular endothelial tubes. Elevated level of extracellular KCl ( $[\text{K}^+]_o$ ; 15 mmol/L) was used to stimulate endothelial  $K_{IR2}$  channels for hyperpolarization [22, 24]. M $\beta$ CD (1  $\mu\text{mol/L}$ ; 332615, Sigma) was prepared in PSS to reduce cholesterol in the endothelial cell membrane [25, 26]. Respective  $\text{K}^+$  channel activating agents were applied before and after washout of a 20 min application of M $\beta$ CD treatment. Resting  $V_m$  was typically allowed  $\geq 2\text{ min}$  to stabilize before application of

a pharmacological agent, whereby each application was allowed sufficient time ( $\geq 3$  min) to record peak  $V_m$  responses. In between individual applications, the endothelial tube was washed with PSS to baseline conditions.

#### Data and statistical analysis

For quantification of cholesterol levels normalized to total phospholipid content,  $n = 1$  entails one experiment using a total blood vessel sample pooled from three mouse brains of each group (pre-AD versus AD). For  $V_m$  recordings, data analyses included resting  $V_m$  (mV) and change in  $V_m$  ( $\Delta V_m$ ) = peak response  $V_m$  – preceding baseline  $V_m$ . Summary data represent values averaged over 10 s during stable recordings. Filipin-III image acquisitions and  $V_m$  recordings represent the number ( $n$ ) of independent experiments, each representing one endothelial tube from one mouse.

For simultaneous comparisons among both age groups  $\pm$  M $\beta$ CD treatment, statistical analysis included a two-way Analysis of Variance (ANOVA) with Tukey's *post hoc* correction for multiple comparisons. One-way ANOVA with Tukey's *post hoc* examined statistical significance for respective AD groups for pre-M $\beta$ CD conditions versus post-M $\beta$ CD conditions at both 30 min and 60 min time points. The two-tailed unpaired *t*-test was used for select comparisons between two groups as pre-AD versus AD data sets (e.g., cholesterol measurements, pre- or post-M $\beta$ CD treatment). Two-tailed paired student's *t*-tests were used to analyze select comparisons for pre-M $\beta$ CD versus post-M $\beta$ CD within the Pre-AD or AD group. All graph preparation and statistical analyses were performed using GraphPad Prism (La Jolla, CA, USA). Differences between groups were accepted as statistically significant with  $p < 0.05$ . All summary data are presented as the mean  $\pm$  SEM. Note that the absence of statistically significant differences does not necessarily rule out physiological or pathological importance of respective study group comparisons that indicate  $p > 0.05$ .

## RESULTS

The goal of this study was to ascertain the mechanism of how cerebrovascular endothelial  $K_{IR}2$  channel function is reduced during AD pathology as observed in our recent study [7]. In accord with our current hypothesis, we examined the potential impact of membrane cholesterol on endothelial  $K^+$  channel

function for hyperpolarization of  $V_m$  as components of EDH during development of AD pathology. As the most impacted biological sex in the clinical AD population, we chose to center the current studies on use of females [2, 3]. It should be noted that female *3xTg-AD* mice also live consistently to  $\geq 16$  months in age [27, 28]. Relatively high concentrations of M $\beta$ CD (5 mmol/L, 60 min) enhance endothelial  $K_{IR}2$  channel currents by  $>3$ -fold during conditions of hypercholesterolemia [16]. Thus, we applied a relatively mild M $\beta$ CD treatment (1 mmol/L, 20 min; [25]) in an attempt to restore deficient  $K_{IR}2$  channel-dependent hyperpolarization in cerebral endothelium during AD to pre-AD conditions if possible. Continuous  $V_m$  recordings were applied in endothelial tubes (width:  $\sim 90$   $\mu$ m, length:  $\geq 300$   $\mu$ m) freshly isolated from *3xTg-AD* mouse posterior cerebral arteries for  $\sim 2$  hr [7]. Changes in  $V_m$  were recorded in response to NS309 (SK $Ca$ /IK $Ca$  channel opener) and 15 mmol/L KCl ( $K_{IR}2$  channel activator) before and after treatment of endothelial cells with M $\beta$ CD followed by washout for up to 60 min. With application of this procedure, a significant shift in  $V_m$  was not evident during the presence and washout of M $\beta$ CD alone ( $\Delta V_m$ :  $-1 \pm 1$  mV). Here, we demonstrate selective restoration of  $K_{IR}2$  channel function in freshly isolated and intact cerebrovascular endothelium of old female AD animals via targeting membrane cholesterol.

#### Total cholesterol content in cerebral blood vessels during conditions of pre-AD and AD

Cholesterol dyshomeostasis is known to underlie conditions of atherosclerosis per aging [29] while the presence of the apolipoprotein E (*APOE*)  $\epsilon 4$  allele in particular is a major risk factor for development of AD [14, 30] and associated cerebrovascular pathology [31]. Thus, we first sought to determine cholesterol levels in isolated cerebral vessels of *3xTg-AD* animals during pre-AD and AD conditions. Although the mean value for the total cholesterol:phospholipid ratio is  $\sim 40\%$  higher for AD ( $0.345 \pm 0.120$ ) versus Pre-AD ( $0.242 \pm 0.068$ ), this difference is not statistically significant ( $p = 0.48$ ; unpaired *t*-test) (Fig. 1;  $n = 5$  independent experiments for each group, 3 animals pooled per experiment; 15 animals total per group) and should be interpreted with caution. Thus, these data suggest that cerebral blood vessels may or may not be enriched with excessive levels of cholesterol among individual animals during conditions of AD.

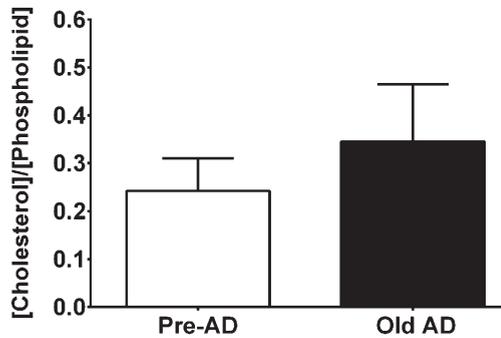


Fig. 1. Cerebrovascular cholesterol content among pre-AD and AD groups. Data represent molar ratio of cholesterol content per total phospholipid content for respective groups.  $n$  = number of experiment for each group (Pre-AD: 5 & Old AD: 5), whereby each group consisted of pooled purified cerebral blood vessels isolated from three female mice.

#### Membrane cholesterol in endothelial cell tubes during conditions of pre-AD and AD

Presence of cholesterol inclusion bodies or “crystals” appear in endothelial cells during conditions of disease such as atherogenesis [26] and can be removed using M $\beta$ CD [32]. With use of Filipin to stain membrane cholesterol [26, 32], we observed fluorescent crystals in endothelial tubes during AD pathology (Fig. 2F;  $15.3 \pm 0.7$  crystals/mm<sup>2</sup>) that were significantly reduced in number (Fig. 2H;  $3.6 \pm 0.9$  crystals/mm<sup>2</sup>) following mild M $\beta$ CD treatment (1  $\mu$ mol/L; 20 min incubation, 1 h washout) or in young, pre-AD animals regardless of M $\beta$ CD treatment (Fig. 2B, D;  $0.2 \pm 0.1$  crystals/mm<sup>2</sup>). These data demonstrate that membrane cholesterol crystals are a feature of cerebrovascular endothelium during AD pathology which may impact function of  $K^+$  channels crucial to endothelial function underlying perfusion of the brain.

#### Effect of M $\beta$ CD on cerebrovascular endothelial $SK_{Ca}/IK_{Ca}$ channel function

Endothelial  $SK_{Ca}/IK_{Ca}$  channels are integral to a major blood flow regulation pathway as EDH in cerebral arteries [22, 33] and arterioles [34]. We have previously found that  $SK_{Ca}/IK_{Ca}$  channel function is preserved in females while modestly enhanced ( $\sim 20\%$ ) in males during AD pathology [7]. Accordingly, we found that peak hyperpolarization to NS309 (1  $\mu$ mol/L) as  $SK_{Ca}/IK_{Ca}$  channel activation was not altered by advanced AD pathology or M $\beta$ CD treatment (1  $\mu$ mol/L) (Fig. 3;

$p > 0.05$ ; ANOVA or  $t$ -tests).  $SK_{Ca}/IK_{Ca}$  channel-mediated hyperpolarization was as follows across female groups: Pre-AD (Pre-M $\beta$ CD,  $34.2 \pm 1.2$  mV & Post-M $\beta$ CD,  $33.0 \pm 0.7$  mV;  $n = 11$ ) and Old AD (Pre-M $\beta$ CD,  $33.1 \pm 1.6$  mV & Post-M $\beta$ CD,  $31.1 \pm 1.0$  mV;  $n = 12$ ) (Fig. 3B). Altogether, these data demonstrate that cerebrovascular endothelial  $SK_{Ca}/IK_{Ca}$  channel stability during AD pathology is not altered by M $\beta$ CD treatment.

#### Impact of M $\beta$ CD treatment on cerebrovascular endothelial $K_{IR2}$ channel function

$K_{IR2}$  channel function is crucial for cerebral vasodilation and blood flow [10, 15] and is impacted by conditions of hypercholesterolemia [16, 17]. Also, we have recently found reduced  $K_{IR2}$  channel function with advancing age [5] while exacerbated by conditions of AD [7] in both biological sexes. Thus, we tested whether treating with a relatively low dose of M $\beta$ CD, an agent that lowers membrane cholesterol [25, 26], could restore  $K_{IR2}$  channel function in endothelial tubes from AD mice. Relative to  $SK_{Ca}/IK_{Ca}$  channel activation, there is a modest  $V_m$  hyperpolarization response to activation of cerebrovascular endothelial  $K_{IR2}$  channels with 15 mmol/L KCl ( $\Delta V_m \sim -5$  to  $-10$  mV; [5, 22]) (Fig. 4A). In female animals, we have found that hyperpolarization to 15 mmol/L KCl decreased by  $\sim 40\%$  ( $p < 0.05$ , unpaired  $t$ -test) from young pre-AD ( $7.3 \pm 0.6$  mV) to old AD ( $4.5 \pm 0.8$  mV) (Fig. 4B). As an approximate wild-type reference to the AD animals, age-matched (16–19 months) C57BL/6 females respond to 15 mmol/L KCl with  $7.0 \pm 1.1$  mV of hyperpolarization ( $n = 5$ ) [5]; similar to the young Pre-AD group. Following M $\beta$ CD treatment (30 min washout), both pre-AD and AD hyperpolarization responses were similar as  $\sim 9 \pm 1$  mV (pre-AD,  $9.3 \pm 1.2$  mV & AD,  $8.5 \pm 0.8$  mV). In such manner, a statistically significant increase by  $\sim 2$ -fold ( $p < 0.05$ ; two-way ANOVA & paired  $t$ -test) was only observed in AD animals versus paired pre-M $\beta$ CD measurements (Fig. 4A, B). Furthermore, we found that hyperpolarization responses were additionally enhanced following a 60-min washout time period after M $\beta$ CD treatment, whereby hyperpolarization responses for both pre-AD ( $13.3 \pm 1.9$  mV) and AD ( $13.6 \pm 1.2$  mV) females were statistically significant versus respective pre-M $\beta$ CD conditions ( $p < 0.05$ ; One-way ANOVA). As a reference, an approximate time period of incubation with PSS (washout solution and vehicle for M $\beta$ CD) did not significantly

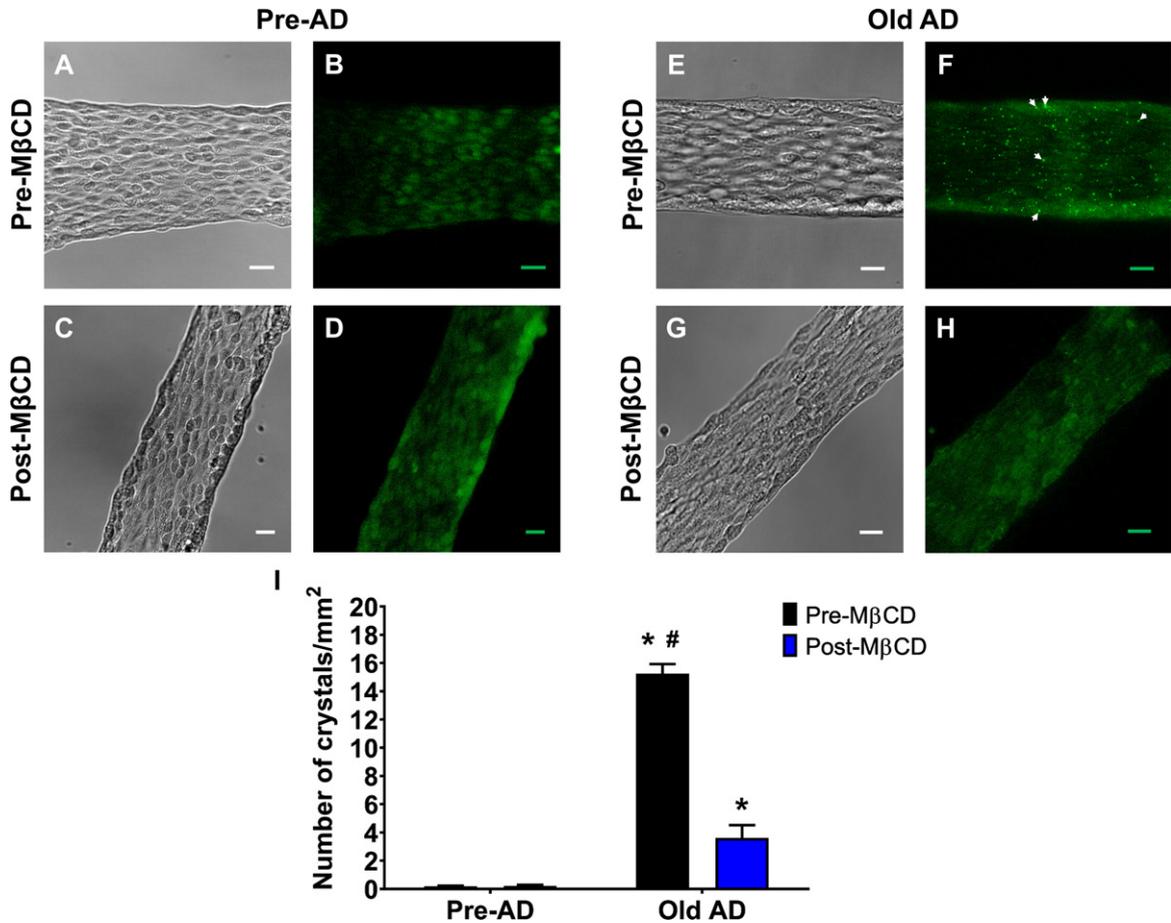


Fig. 2. Presence of cholesterol crystals in AD animals and reduction following M $\beta$ CD treatment. Representative (A, C, E, G) brightfield and (B, D, F, H) Filipin-III images (green pseudo-color) of cerebrovascular endothelial cell tubes isolated from female animals. A, B) Pre-AD images without M $\beta$ CD treatment. C, D) As shown in (A) & (B) respectively after M $\beta$ CD treatment (1 mmol/L, 20 min incubation, & 1 h wash). E-H) As shown in (A), (B), (C), and (D) respectively for AD animals. White arrows indicate cholesterol “crystals” in endothelial tubes during AD conditions in the absence of M $\beta$ CD treatment. The scale bar throughout panels represents 20  $\mu$ m. I) Summary data for number of crystals per unit area ( $\text{mm}^2$ ).  $n$  = number of animals and respective cerebral endothelial tubes (Pre-AD & Old AD: 4; Pre-AD & Old AD after M $\beta$ CD: 3). \* $p$  < 0.05 (two-way ANOVA & paired  $t$ -test), Pre-AD or Old AD female Post-M $\beta$ CD versus respective Pre-M $\beta$ CD group; # $p$  < 0.05 (two-way ANOVA & paired  $t$ -test), Old AD female Pre-M $\beta$ CD versus Old AD female Post-M $\beta$ CD.

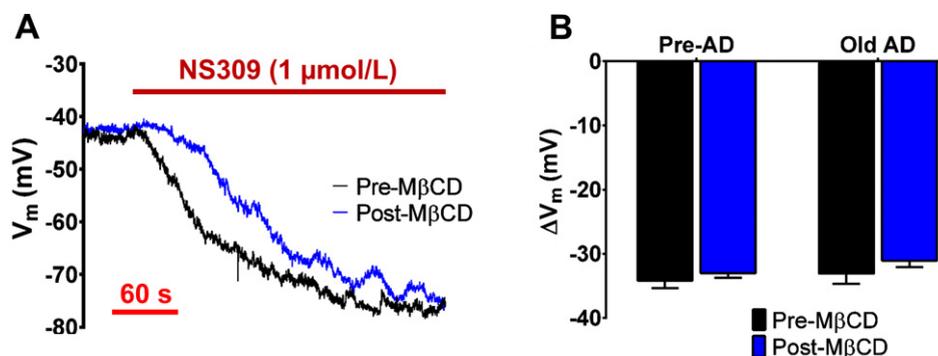


Fig. 3.  $SK_{Ca}/IK_{Ca}$  channel function is not impacted by conditions of AD or membrane cholesterol regulation. A) Representative recordings of  $SK_{Ca}/IK_{Ca}$  channel hyperpolarization in Old AD females in response to NS309 (1  $\mu$ mol/L) before and after 30 min washout of M $\beta$ CD (1 mmol/L, 20 min). B) Summary data for peak  $\Delta V_m$  in response to  $SK_{Ca}/IK_{Ca}$  activation in Pre-AD versus Old AD females before and after M $\beta$ CD treatment (30 min washout).  $n$  = number of animals and respective cerebral endothelial tubes (Pre-AD: 11; Old AD: 12).

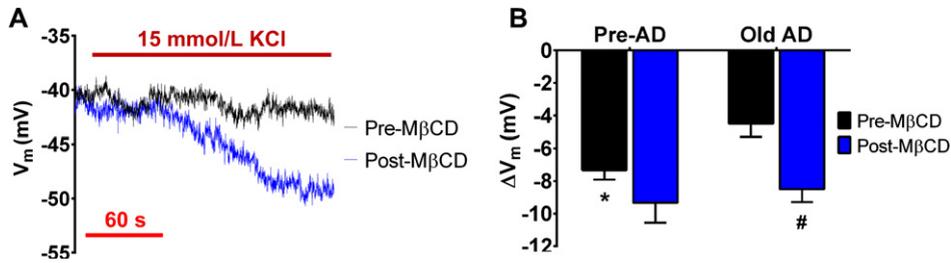


Fig. 4. Reduction of membrane cholesterol restores endothelial  $K_{IR2}$  channel function during AD pathology. A) Representative recordings of  $K_{IR2}$  channel-mediated hyperpolarization in Old AD females in response to 15 mM KCl before and after 30 min washout of M $\beta$ CD (1 mmol/L, 20 min). B) Summary data for peak  $\Delta V_m$  in response to  $K_{IR2}$  channel activation in Pre-AD versus Old AD females before and after M $\beta$ CD treatment (30 min washout). Note that both Pre-AD and Old AD females show similar hyperpolarization to 15 mM KCl as  $\sim 9$  mV following M $\beta$ CD treatment.  $n$  = number of animals and respective cerebral endothelial tubes (Pre-AD: 9; Old AD: 12). \* $p$  < 0.05 (unpaired  $t$ -test) for Old AD Female Pre-M $\beta$ CD versus Pre-AD Female Pre-M $\beta$ CD; # $p$  < 0.05 (two-way ANOVA & paired  $t$ -test), Old AD female Post-M $\beta$ CD versus Old AD female Pre-M $\beta$ CD.

alter hyperpolarization to 15 mM KCl ( $n=4$  AD animals; 0 h,  $4.3 \pm 0.6$  mV & 1.5 h,  $5.3 \pm 0.9$  mV). Altogether, these data suggest that a relatively low concentration of M $\beta$ CD, an agent known to reduce membrane cholesterol, can restore (with potential to further enhance) cerebrovascular endothelial  $K_{IR2}$  channel function during conditions of advanced AD in females.

## DISCUSSION

Understanding blood flow regulation throughout the cerebrovascular network is crucial because chronic, inadequate delivery of oxygen and nutrients throughout the brain contributes to neurodegenerative pathology such as AD [6, 35]. Further, with recognition of the endothelium as an organ that “feeds” all other organs throughout the body [18], naturally, endothelial function has become pivotal for fundamental studies of brain health [9, 36, 37], the aging brain [4, 5, 38], and select conditions of AD [7, 8]. In particular for AD, there are remaining knowledge gaps for potentially altered functions of cerebrovascular endothelial ion channels per blood flow regulation, primarily as the transient receptor potential [39–41] and  $K^+$  channels (e.g.,  $K_{Ca}$  and  $K_{IR}$  subtypes; [7, 8, 39, 42]). In a recent study [7], we have demonstrated that endothelial  $K_{IR}$  channel function of posterior cerebral arteries is reduced during AD by  $\sim 50\%$ , whereas  $SK_{Ca}/IK_{Ca}$  channels are relatively stable. This general finding has also been reinforced for  $K_{IR}$  channel function in capillary endothelium during AD [8], altogether establishing a premise for mechanistic investigation and corresponding therapeutic strategies involving cerebrovascular  $K^+$  channels. With a basis of  $K_{IR}$

channels modulated by membrane cholesterol [17, 43] and cholesterol dysregulation playing a key role in AD etiology [14, 31], the goal of this study was to investigate whether mild M $\beta$ CD treatment to reduce membrane cholesterol would significantly improve cerebrovascular  $K_{IR}$  channel function in old  $3xTg-AD$  animals. Whereas  $SK_{Ca}/IK_{Ca}$  channel function was not altered as otherwise stable from pre-AD to AD, treatment with M $\beta$ CD effectively restored  $K_{IR}$  channel function to the level of pre-AD, supporting cholesterol- $K_{IR}$  channel interaction as a key therapeutic target during neurodegenerative disease.

### *Endothelial membrane cholesterol and use of cyclodextrin for treatment of AD*

Cholesterol is essentially present in the plasma membrane of all mammalian cells as one of the major lipid components for cellular growth and function, whereby its excess beyond physiological levels is generally associated with the development of disease including AD [14, 44–46]. Although not statistically significant, we have found that the mean for cerebrovascular cholesterol (normalized to total phospholipid content) was  $\sim 40\%$  higher in AD versus pre-AD animals (Fig. 1). Thus, our data for cellular cholesterol content suggest that cerebral blood vessels may or may not be enriched with excessive levels of cholesterol among individual animals during conditions of AD. However, with use of fluorescent Filipin staining, we observed apparent cholesterol crystals in endothelial membranes of AD animals that were substantially reduced in number by  $\sim 75\%$  following M $\beta$ CD treatment (Fig. 2). In females, elevated cholesterol levels in human frontal cortex [47] and rodent endothelial plasma membranes [48] have

been shown during conditions of aging as a key risk factor for cognitive decline towards AD [1].

Cyclodextrins such as M $\beta$ CD are cyclic oligosaccharide compounds and have been widely used to bind to cholesterol in a concentration-, time-, temperature-, and cell type-dependent manner [25]. With a reference point of 5 mmol/L M $\beta$ CD (30 to 60 min incubation) for patch-clamp measurements of rodent vascular cells (brain [15], mesentery [16]), we determined that 1 mmol/L M $\beta$ CD (20 min) was sufficient for restoration of reduced  $K_{IR}$  channel-dependent hyperpolarization for AD animals following 30 min of M $\beta$ CD washout. In addition to the precision in time for various drug-response measurements, it should be noted that a major strength of our electrophysiological abilities encompasses a scale of hours (versus seconds or minutes) [21] and thus, we encompassed paired examinations of Pre-M $\beta$ CD and Post-M $\beta$ CD conditions throughout individual endothelium preparations.

*SK<sub>Ca</sub>/IK<sub>Ca</sub> channel function is unaffected, whereas K<sub>IR</sub> channel function is restored by mild M $\beta$ CD treatment of cerebrovascular endothelial membrane cholesterol during AD*

Endothelial SK<sub>Ca</sub>/IK<sub>Ca</sub> channels are vital for cerebral blood flow regulation [33, 34], whereby their function is not impaired by conditions of AD alone [7]. Accordingly, the current study demonstrates that M $\beta$ CD has no significant impact on SK<sub>Ca</sub>/IK<sub>Ca</sub> channel activity to produce membrane hyperpolarization (Fig. 3), suggesting that membrane cholesterol does not play a major role for regulation of cerebrovascular SK<sub>Ca</sub>/IK<sub>Ca</sub> during health and disease. Overall, this lack of effect of M $\beta$ CD treatment on SK<sub>Ca</sub>/IK<sub>Ca</sub> channel function is consistent with previous studies (reviewed in [49]). As highly expressed in smooth muscle cells [50], large conductance K<sub>Ca</sub> (BK<sub>Ca</sub>) channels are suppressed [51] or activated in response to membrane-enriched cholesterol depending on expression of *KCNMB1* and trafficking of BK<sub>Ca</sub>  $\beta_1$  subunits [52].

$K_{IR}$  channels are integral to neurovascular coupling and the overall coordination of cerebral precapillary vasodilation and blood flow [10, 53] with a unique capacity for also boosting SK<sub>Ca</sub>/IK<sub>Ca</sub> channel hyperpolarization [54, 55]. Elevated membrane cholesterol suppresses endothelial  $K_{IR}2$  channel currents [16, 17, 45] by interacting with a CD loop region of the C-terminus as a cytosolic component of the channel [56]. Remarkably, we found that  $K_{IR}2$

channel-dependent hyperpolarization increased progressively with time of washout following M $\beta$ CD treatment to  $\sim 9$  mV and  $\sim 14$  mV for both female Pre-AD and AD animals at 30 min and 60 min respectively (Fig. 4 and Results text). The enhancement of hyperpolarization responses in pre-AD animals was unexpected but previous evidence suggests that, even in healthy humans, normal cholesterol levels can suppress endothelial function [57]. Also, we should note that mechanisms of local control of  $K_{IR}$  channels can occur via glycocalyx regulation as well [43]. Relative contributions of membrane cholesterol-dependent versus -independent modulation of endothelial ion channels remain to be resolved for conditions of AD.

*Experimental considerations and potential treatment strategies*

Although  $K_{IR}2$  channels are also present on cerebrovascular smooth muscle cells [10, 15], we studied the key component of coordinated electrical signaling of cerebral blood flow control as the endothelium [9, 22]. In addition to retention of *in vivo* properties as  $V_m$  regulation and intercellular electrical coupling [22], the mechanical stability of endothelial cells (versus smooth cells) for continuous, long-term electrophysiological recordings is an experimental advantage as well. It should be noted that another recent study found that cholesterol primarily regulates cerebral arterial smooth muscle versus endothelial  $K_{IR}2$  channels in young (10 to 12 weeks) female rats [15]. Thus, with knowing that cholesterol can also impact smooth muscle  $K_{IR}2$  channels in intact cerebral arteries [15], we believe that the current study demonstrates the general concept that mild treatment with M $\beta$ CD can alleviate suppressed  $K_{IR}2$  channel function during conditions of AD [7, 8]. Therapy via PIP<sub>2</sub> restoration may also restore capillary endothelial  $K_{IR}2$  channel function for improved neurovascular coupling and cerebral blood flow during AD [8]. However, PIP<sub>2</sub> is also a key dynamic component of constitutive, moment-to-moment physiology (e.g., intracellular Ca<sup>2+</sup> signaling), whereby its hydrolysis following activation of G<sub>q</sub>-protein coupled receptors [58], can notably facilitate TRPV4 activation while suppressing  $K_{IR}2$  channels [59]. Although such investigations of PIP<sub>2</sub> are mechanistically informative, we suggest that a mild reduction of membrane cholesterol may serve as a more effective strategy for improving endothelial  $K_{IR}2$  channel function with significantly

less impact on constitutive events governing basal cerebral blood flow.

## SUMMARY AND CONCLUSIONS

Impairment of cerebral blood flow during AD is due, in large part, to endothelial dysfunction in the brain. Therefore, it is crucial to locate mechanisms and therapies that center on improvement of cerebral perfusion. With following up from our recent studies demonstrating a functional decrease of endothelial  $K_{IR2}$  channels, the current study was strategically undertaken to restore  $K_{IR2}$  channel-dependent hyperpolarization in response to elevated extracellular  $K^+$ . We found that mild application of a cholesterol removal agent as M $\beta$ CD progressively restored and even enhanced  $K_{IR2}$  channel-dependent hyperpolarization while  $SK_{Ca}/IK_{Ca}$  channels were unaffected. Further, this finding was observed in females as the representative biological sex of the clinical AD population [2, 3]. Altogether, these findings suggest that the endothelial cholesterol- $K_{IR}$  channel interface may be a novel therapeutic target for ameliorating cerebrovascular function to optimize perfusion of the AD brain.

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## CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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