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Bepridil decreases Aβ and calcium levels in the thalamus after middle cerebral artery occlusion in rats

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Abstract

Alzheimer's disease (AD) and cerebral ischaemia share similar features in terms of altered amyloid precursor protein (APP) processing and β-amyloid (Aβ) accumulation. We have previously shown that Aβ and calcium deposition, and β-secretase activity, are robustly increased in the ipsilateral thalamus after transient middle cerebral artery occlusion (MCAO) in rats. Here, we investigated whether the non-selective calcium channel blocker bepridil, which also inhibits β-secretase cleavage of APP, affects thalamic accumulation of Aβ and calcium and in turn influences functional recovery in rats subjected to MCAO. A 27-day bepridil treatment (50 mg/kg, p.o.) initiated 2 days after MCAO significantly decreased the levels of soluble Aβ40, Aβ42 and calcium in the ipsilateral thalamus, as compared with vehicle-treated MCAO rats. Expression of seladin-1/DHCR24 protein, which is a potential protective factor against neuronal damage, was decreased at both mRNA and protein levels in the ipsilateral thalamus of MCAO rats. Conversely, bepridil treatment restored seladin-1/DHCR24 expression in the ipsilateral thalamus. Bepridil treatment did not significantly affect heme oxygenase-1- or NAD(P)H quinone oxidoreductase-1-mediated oxidative stress or inflammatory responses in the ipsilateral thalamus of MCAO rats. Finally, bepridil treatment mitigated MCAO-induced alterations in APP processing in the ipsilateral thalamus and improved contralateral forelimb use in MCAO rats. These findings suggest that bepridil is a plausible therapeutic candidate in AD or stroke owing to its multifunctional role in key cellular events that are relevant for the pathogenesis of these diseases.

Keywords: Alzheimer's disease • amyloid precursor protein • β-amyloid • calcium • β-secretase • transient middle cerebral artery occlusion • sensorimotor function • bepridil • seladin-1/DHCR24

Introduction

Several studies have shown that AD and ischaemic brain injury share similar neuropathological features, including altered APP processing and Aβ accumulation [1–6], and increased neuroinflammation [7]. We have previously shown that APP and Aβ accumulate in the dense plaque-like deposits in the thalamus of rats subjected to transient MCAO [8]. We have also demonstrated that APP processing and the expression of Aβ-degrading enzymes are altered in the ipsilateral thalamus after MCAO. These changes took place after a prolonged time following the ischaemic insult [9]. Furthermore, these alterations in the ipsilateral thalamus coincided with significantly increased β-secretase (BACE) activity, augmented calcium levels and depletion of Golgi-localized γ-ear-containing ARF-binding protein 3 (GGA3), which is a protein responsible for sorting BACE to lysosomal degradation [10]. Based on these findings, it appears that the observed alterations in Aβ accumulation are linked to disrupted calcium homeostasis, which initiates the secondary degeneration known to take place in the thalamus after MCAO [11–13].

As calcium and Aβ are intimately linked in AD pathology [14, 15], multifunctional drugs that modulate both calcium and Aβ dyshomeostasis are potential therapeutic targets. An interesting candidate in this context is bepridil, which is a non-selective calcium channel blocker used to treat angina pectoris [16–19]. Furthermore, bepridil was recently shown to inhibit BACE-mediated

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cleavage of APP both *in vitro* and *in vivo* by increasing the pH in endosomal compartments [20]. Bepridil has also been shown to modulate γ-secretase-mediated cleavage of APP independently of endosomal alkalization [20]. Thus, testing the effects of bepridil in MCAO rats involving both Aβ and calcium dysregulation may provide insights into the underlying pathogenic mechanisms related to the interplay between calcium and Aβ.

Seladin-1/DHCR24 protein encoded by the *DHCR24* gene is a potential protective factor against neuronal damage. This notion stems from the previous studies, which have shown that seladin-1 plays a cytoprotective role in oxidative stress-induced apoptosis by scavenging reactive oxygen species [21]. Moreover, seladin-1 interacts with the p53 tumour suppressor protein [22], which is a redox-sensitive transcription factor involved in the pathogenesis of brain ischaemia and AD. Importantly, seladin-1 expression is down-regulated in large pyramidal neurons in specific regions in AD brain, suggesting that seladin-1 associates with selective neuronal vulnerability [23]. Heme oxygenase-1 (HMOX1) and NAD(P)H quinone oxidoreductase-1 (NQO1) are typical target genes that are up-regulated in response to oxidative and electrophilic stress [24, 25]. Expression of these genes is transcriptionally activated by Nrf2, which is an antioxidant response element-binding transcription factor that translocates into the nucleus upon oxidative and electrophilic stress [26]. Based on their stress-related functions, seladin-1, HMOX1 and NQO1 are feasible markers to be used in the elucidation of neuronal vulnerability and oxidative stress-related responses upon neuronal damage.

In this study, we explored whether daily treatment with bepridil (50 mg/kg/day, p.o.) modulates calcium and Aβ accumulation in the ipsilateral thalamus and whether bepridil affects functional recovery in rats after MCAO. We found that bepridil treatment significantly decreased the calcium levels in the ipsilateral thalamus in MCAO rats, which were in turn correlated with a significant reduction in soluble Aβ42 and Aβ40 levels. Moreover, bepridil treatment appeared to enhance functional recovery in MCAO rats, as indicated by improved forelimb use in the cylinder test. Thus, our results suggest that bepridil is a beneficial multifunctional drug, which may simultaneously modulate pathological accumulation of both calcium and Aβ.

Materials and methods

Animals

Thirty-one male Wistar rats (age 2–3 months, bodyweight 295–344 g) from the Laboratory Animal Centre, Kuopio, were subjected to either MCAO ($n = 23$) or sham operation ($n = 8$). The rats were housed under 12:12 hr light/dark conditions in a temperature-controlled environment (20 ± 1°C). Food and water were available *ad libitum*. All animal procedures were approved by the Animal Ethics Committee (Hämeenlinna, Finland) and conducted in accordance with the guidelines set by the European Community Council Directives 86/609/EEC. All efforts were made to minimize the number of animals used and to ensure their welfare throughout.

Middle cerebral artery occlusion

Focal cerebral ischaemia was induced by the intraluminal filament technique [27]. Anaesthesia was induced in a chamber using 5% halothane in 30% O_2/70% N_2. A surgical depth of anaesthesia was maintained throughout the operation with 0.9–1.3% halothane delivered through a nose mask. Body temperature was monitored and maintained at 37°C using a heating pad connected to a rectal probe (Harvard Homeothermic Blanket Control Unit; Harvard Apparatus, Holliston, MA, USA). The right common carotid artery was exposed through a midline cervical incision under a surgical microscope and gently separated from the nerves. The external carotid artery was closed with a suture and cut with microscissors and electrocoagulated. A heparinized nylon filament (diameter 0.25 mm, rounded tip) was inserted into the stump of the external carotid artery. The filament was advanced 1.8–2.1 cm into the internal carotid artery until resistance was felt. The filament was held in place by tightening the suture around the internal carotid artery and by placing a microvascular clip around the artery. After 120 min. of MCA occlusion, the filament was removed and the external carotid artery was permanently closed by electrocoagulation. The sham-operated rats were treated in a similar manner, except the filament was not placed into the internal carotid artery. Buprenorfin (0.03 mg/kg, s.c.) was used to relieve post-operative pain. In addition, post-operative care of MCAO rats included supplemental 0.9% NaCl (i.p.) and softened food pellets to prevent weight loss.

Study design and drug treatment

Two days after the operation, a modified version of the limb-placing test [28] was used to verify successful MCAO (Fig. 1). Only the rats with a total score less than 10 were included in the study. Based on the limb-placing scores on post-operative day 2, animals were assigned to the groups of MCAO controls (MCAO + vehicle) ($n = 7$), MCAO + bepridil (50 mg/kg p.o., $n = 5$) and SHAM ($n = 6$). From 31 rats, two sham-operated animals died during the operation. Eight rats died within 48 hrs after operation with the main reason being haemorrhage and severe oedema. One MCAO animal died during the bepridil treatment. Two MCAO animals scored over 10 points in the limb-placing test on post-operative day 2 and were excluded from the study. Only the animals that survived through the whole study were included in the analyses of the results.

Bepridil (Bepridil hydrochloride; Sigma-Aldrich, Saint Louis, MO, USA) was dissolved first in ethanol (final volume 5%) and then in a mixture of polyethylene glycol (Macrogol 400, Fargon, 50%) and 0.9% NaCl (45%). Bepridil was administered at a dose of 50 mg/kg (15 ml/kg, p.o.) once a day for 27 days as indicated previously [20]. The drug treatment was started on post-operative day 2 to avoid interference with the maturation of acute ischaemic damage.

Behavioural assessments

The behavioural tests selected for this study are sensitive to detect treatment effects and are not affected by repeated testing. In MCAO rats, a spontaneous recovery of function is typically observed in the limb-placing test, whereas the sensorimotor impairment is more permanent when the beam-walking test and cylinder test are applied. All behavioural tests and analyses were carried out in a blinded manner.

A modified version of the limb-placing test was used to assess hindlimb and forelimb responses to tactile and proprioceptive stimulation [28, 29]. The rats were habituated for handling, and tested before ischaemia induction on post-operative days 2, 4, 7, 10, 14, 21 and 28 (Fig. 1). The test consisted of seven limb-placing tasks. Tactile stimulation was elicited by contacting the tested limb with the table surface, and proprioceptive stimulation was explored by pulling down the tested limb (i.e. limb position in space). The following scores were used to detect impairment of the forelimb and hindlimb function: 2 points, the rat performed normally; 1 point, the rat performed with a delay or more than 2 sec. and/or incompletely; 0 point, the rat did not perform normally. The maximum score was 14. Both sides of the body were tested.

The cylinder test was used to assess the imbalance between the impaired and non-impaired forelimb use [30]. For the test, the rat was placed in a transparent cylinder (Ø 20 cm) and videotaped during the light hours of the light/dark cycle. A mirror was placed at 45° angle beneath the cylinder so that behaviour could be filmed from below the cylinder. Exploratory activity for 1–3 min. was analysed by using a video recorder with slow motion capabilities. The number of contacts by both forelimbs and by either the impaired or unimpaired forelimb were counted. The cylinder test was carried out 1 day before the MCAO operation and on post-operative days 14 and 28. The cylinder score for the impaired forelimb was calculated as: [(contralateral contacts + ½ × bilateral contacts)/total contacts] × 100%.

The sensorimotor functions of the hindlimbs were tested using a tapered/ledged beam [31]. Before ischaemia induction, the rats were pre-trained for 3 days to traverse the beam. The beam-walking device consists of a tapered beam with underhanging ledges on each side to permit foot faults without falling. The end of the beam was connected to a black box (20.5 × 25 × 25 cm) with a platform at the starting point. A bright light was placed above the start point to motivate the rats to traverse the beam. Each rat's performance was videotaped and analysed later by calculating the slip ratio for the impaired (contralateral to lesion) forelimb and hindlimb. More slips indicate a greater degree of impairment. Steps onto the ledge were scored as a full slip. A half slip was scored if the limb touched the side of the beam. The beam-walking test was carried out 1 day before the MCAO operation and on post-operative days 14 and 28. The slip ratio was calculated as: [(full slips + ½ × half slips)/total steps] × 100%. The mean of three trials was used for statistical analyses.

Thalamic tissue samples

Tissue samples from the ipsilateral and contralateral thalamus of MCAO- or sham-operated rats were weighed and mechanically homogenized in 400 μl of Dulbecco's phosphate buffered saline (DPBS; Lonza, Basel, Switzerland) in an ice bath. Approximately 10-fold excess of PBS/mg tissue was used. The tissue homogenates were subdivided into three fractions as follows. Ten per cent of a homogenate was mixed with 500 μl of TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) for total RNA extraction; 45% was suspended in DPBS containing EDTA-free protease and phosphatase inhibitors (1:100; Thermo Scientific, Rockford, IL, USA) (total protein I) and 45% suspended in DPBS without inhibitors (total protein II). All aliquots were stored at -70°C for further analyses. Total protein I fractions were subjected to Western blotting and Aβ analyses. Total protein II fractions were used for calcium measurement and α-, β- and γ-secretase enzyme activity assays. The data in the ipsilateral thalamus were normalized to the contralateral thalamus (=100%) to minimize individual variation between rats.

RNA extraction and qPCR analysis

Total RNA was extracted from homogenized thalamic tissues using the TRIzol® reagent according to the manufacturer's instructions. Equal quantities of total RNA samples were subjected to cDNA synthesis using Superscript III reverse transcriptase (Invitrogen). Subsequently, SYBR Green Master PCR Mix (Applied Biosystems, Foster City, CA, USA) and target-specific PCR primers for seladin-1/DHCR24 (5′-CAAGCCGTGGTTCTTTA AGC-3′ and 5′-CATCCAGCCAAAGAGGTAGC-3′), TNF-α (5′-CGAGTGACAA GCCTGTAGCC-3′ and 5′-GTGGGTGAGGAGCACGTAGT-3′), L-type calcium channel LTCC (5′-TTCGATGTGAAGGCACTGAG-3′ and 5′-TATGCCCTC CTGGTTGTAGC-3′), HMOX1 (5′-GAAGAAGATTGCGCAGAAGG-3′ and 5′-TGTGTTCCTCTGTCAGCAGTG-3′), NQO1 (5′-GCCCGGATATTGTAGCTGAA -3′ and 5′-GTGGTGATGGAAAGCAAGGT-3′) and GAPDH (5′-CTTCTGAG TGGCAGTGATGG-3′and 5′-ACATCAAATGGGGTGATGCT-3′) were used for the amplification of cDNA samples by real-time quantitative PCR (7500 Fast Real Time PCR System; Applied Biosystems). PCR primers were designed to amplify a region extending across at least two different exons. A standard curve method was used to obtain seladin-1, TNF-α, LTCC, HMOX1, NQO1 and GAPDH levels. The mRNA levels of each gene were normalized to those of GAPDH from the same samples.

Western blotting

Total proteins were extracted using TPER protein extraction buffer (Pierce/Thermo Scientific, Rockford, IL, USA) containing protease and phosphatase inhibitors (Pierce) from an aliquot of total protein I fraction. After protein quantification using BCA protein assay (Pierce), the proteins (30–50 μg/lane) were separated on 4–12% Bis-Tris-polyacrylamide gel electrophoresis (PAGE; Invitrogen) and blotted onto Immun-Blot polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). Primary antibodies against APP C-terminus (A8717; Sigma-Aldrich), GGA3 (BD Biosciences, Dako, Glostrup, Denmark), glial fibrillary acidic protein (GFAP; Dako), seladin-1/DHCR24 (C59D8, Cell Signaling, Danvers, MA, USA), insulin-degrading enzyme (IDE, ab32216; Abcam, Cambridge, MA, USA), neprilysin (NEP, AF1126; R&D Systems, Minneapolis, MN, USA), low-density lipoprotein receptor-related protein (LRP) (ab92544; Abcam, Cambridge, MA, USA) and GAPDH (ab8245; Abcam) were used for immunoblotting. After incubation with appropriate secondary horseradish peroxidase-conjugated antibodies (GE Healthcare), the membranes were subjected to a chemiluminescent substrate (ECL™ Advance Western Blotting Detection Kit; Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA), and protein bands were detected with an ImageQuant RT ECL Imager (GE Healthcare). Western blot images were quantified using Quantity One software (Bio-Rad). SH-SY5Y human neuroblastoma cells overexpressing an APP751 isoform were used as a control for size comparison of the bands [32].

Aβ and soluble APP measurements

Soluble or insoluble Aβ x-40 and Aβ x-42 (Aβ40 and Aβ42) levels were measured from the total protein I fraction. Protein aliquots were first ultracentrifuged (100,000 ×*g*, 50.4 Ti rotor; Beckman Coulter, Palo Alto, CA, USA) for 2 hrs at 4°C, and the supernatant (=soluble fraction) was collected. Subsequently, the remaining pellet was resuspended in guanidine buffer (5 M guanidine-HCl / 50 mM Tris-HCl, pH 8.0), incubated for 2 hrs at room temperature on a shaker and diluted 1:50 in BSAT-DPBS

(5% BSA/0.03% Tween-20 in DPBS, pH 9.0) containing protease and phosphatase inhibitors. Finally, the suspension was centrifuged for 20 min. at 15700 $\times g$ and the supernatant (=insoluble fraction) was collected for Aβ measurements. Both insoluble and soluble Aβ40 and Aβ42 levels were determined using a monoclonal and HRP-conjugated antibody-based Human/Rat β Amyloid 40 (294-62501) and Human/Rat β Amyloid 42 (High-Sensitive; 290-62601) ELISA Kit (Wako, Osaka, Japan). After 30-min. incubation at room temperature, the reaction was terminated and the absorbance was measured at 450 nm with an ELISA microplate reader (Wallac/Perkin Elmer, Waltham, MA, USA). Aβ concentrations were normalized to tissue weights in each sample. Soluble sAPPα and sAPP total (=sAPPα + sAPPβ) levels were detected from the same protein fraction as soluble Aβ using Western blot analysis with 6E10 (Signet Laboratories, Dedham, MA, USA) and 22C11 (Mab348; Millipore, Darmstadt, Germany) antibodies, respectively. Figure S1A illustrates the epitope sites for antibodies 6E10 and 22C11 used for the detection of sAPPα and total sAPP (sAPPtot), respectively.

Calcium measurements

Tissue homogenate samples (20 μl) were digested (CEM MDS-2000 microwave digestor) in 100 μl suprapur nitric acid (Merck, Whitehouse Station, NJ, USA). After digestion, 100 μl 2% Lanthanum solution (Riedel de Haen, Honeywell Riedel-deHaën, Hanover, Germany) was added and the samples were diluted to a volume of 1 ml with Milli-Q water. Calcium measurements were carried out on a ZEEnit 700 atomic absorption spectrometer (Analytik Jena AG, Jena, Germany) with a calcium hollow cathode lamp at wavelength 422.7 nm using an air-acetylene flame and SFS6 injection module. Calcium concentrations were normalized to tissue weights in each sample.

α-, β- and γ-secretase assays

α- and β-Secretase Activity Kits (FP001 and FP002; R&D Systems) were used to measure α- and β-secretase activities from the thalamic tissue homogenates (total protein II fraction) according to the manufacturer's instructions. Briefly, equal amounts of membrane protein fractions were incubated at 37°C for 2 hrs with the secretase-specific substrate peptides conjugated to fluorescent reporter molecules EDANS and DABCYL. Subsequently, the emitted light (510 nm) was detected on a fluorescence microplate reader (Wallac) after EDANS excitation at 355 nm. γ-Secretase activity was measured from the thalamic tissue homogenates as previously described [33]. In brief, 60 μg of solubilized membrane protein preparation was incubated at 37°C overnight in 150 μl of assay buffer containing 50 mM Tris-HCl, pH 6.8, 2 mM EDTA, 0.7% CHAPSO (w/v) and 8 μM fluorogenic γ-secretase substrate (NMA-GGVVIATVK(DNP)- D R D R D R-NH 2, cat nr. 565764; Calbiochem, Darmstadt, Germany). At the same time, γ-secretase inhibitor L685,458 was used to validate the specificity of the γ-secretase activity assay. After incubation, reactions were centrifuged at 15,700 × g for 10 min. and transferred to a 96-well plate. Fluorescence was measured using a plate reader (Fluorstar Galaxy; MTX Lab Systems, Inc., Vienna, VA, USA) with an excitation wavelength of 355 nm and an emission wavelength of 440 nm. The background fluorescence from substrate samples was subtracted in the final analysis.

Statistical analyses

Statistical analyses were performed using the SPSS program (version 14.0, SPSS; IBM, Armonk, NY, USA). Non-parametric Mann–Whitney *U*-tests (equal variances not assumed) and one-way ANOVA with *post hoc* tests (LSD) were used for statistical analyses of biochemical data. First, results obtained from ipsilateral and contralateral sides were compared within the individual treatment groups. Subsequently, ipsilateral or contralateral sides were compared between treatment groups (MCAO, MCAO + bepridil and sham-operated rats). Differences in the limb-placing scores between experimental groups were analysed using Mann–Whitney *U*-tests. Beam-walking and cylinder data for the overall group effect were analysed using analysis of variance for repeated measures (MANOVA). Comparisons between groups were then made using an independent samples *t*-test. Correlations were determined using Pearson's correlation coefficient. Values are indicated as mean ± standard deviation (S.D.). The level of statistical significance was set to $P < 0.05$.

Results

Bepridil treatment decreases Aβ42 and Aβ40 levels in the ipsilateral thalamus of MCAO rats

Our previous studies have shown that focal ischaemia in rats robustly enhances APP processing and the accumulation of Aβ peptide in the thalamus after 30 days of MCAO [8, 9, 34]. Here, we wanted to explore whether bepridil, which was recently shown to inhibit β-secretase cleavage and to modulate γ-secretase cleavage of APP [20], affects Aβ levels in the thalamus after MCAO and subsequent functional recovery of rats. To address these questions, we treated MCAO rats with bepridil (50 mg/kg, p.o.) or vehicle once a day for 27 days starting after post-operative day 2 (Fig. 1). During this period, vehicle- and bepridil-treated MCAO rats as well as sham-operated rats underwent behavioural testing before being killed (Fig. 1).

According to our biochemical analyses, soluble Aβ40 and Aβ42 levels were significantly increased in the ipsilateral thalamus of vehicle-treated MCAO rats, whereas bepridil treatment prevented this increase in both soluble Aβ40 and Aβ42 levels (Fig. 2A and B). After normalization to the contralateral side (=100%), which was carried out to minimize individual variation between rats, soluble Aβ40 and Aβ42 levels in the ipsilateral thalamus of bepridil-treated MCAO rats were decreased by 46% and 57% when compared with vehicle-treated MCAO rats respectively. Also, a similar decrease in guanidine-soluble (insoluble) Aβ42 levels was observed after bepridil treatment in the ipsilateral thalamus of MCAO rats (Fig. 2C). Bepridil treatment did not significantly affect soluble Aβ40 and Aβ42 or insoluble Aβ42 levels in the contralateral thalamus when compared with vehicle-treated or sham-operated rats (Fig. 2). These data suggest that bepridil treatment decreases soluble Aβ42 and Aβ40 levels in the ipsilateral thalamus in MCAO rats.

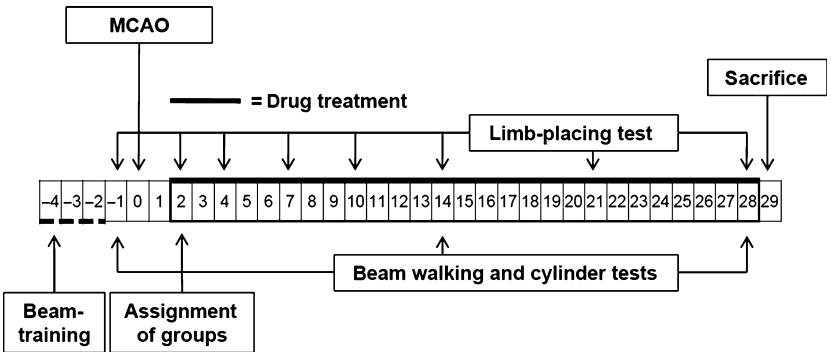

Fig. 1 Study design of bepridil treatment in MCAO rats. Rats were habituated to handling and behavioural testing before baseline assessment. Two days after the MCAO, rats were assigned to behaviourally equal treatment groups based on the limb-placing test. Rats were treated daily for 27 days with vehicle or bepridil (50 mg/kg/day, p.o.) starting at post-operative day 2. During this period, sensorimotor impairment was assessed using the limb-placing test, the ledged/tapered beam-walking test and the cylinder test. Rats were killed for biochemical analyses at post-operative day 29.

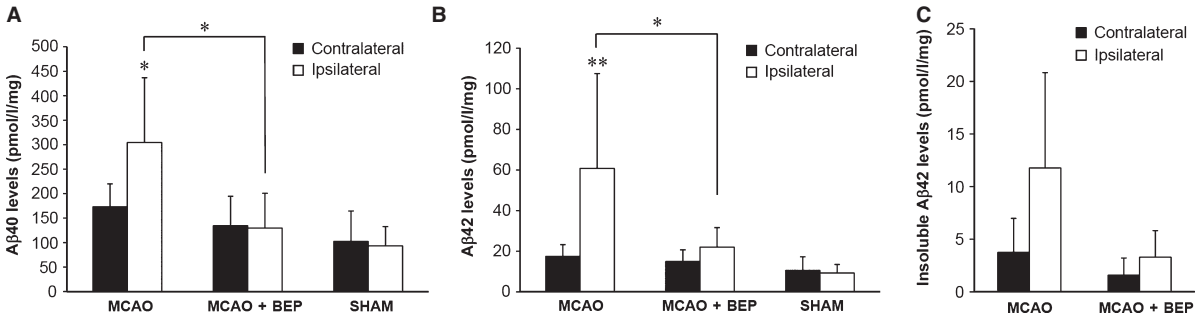

Fig. 2 Bepridil treatment decreases soluble Aβ40 and Aβ42, and insoluble Aβ42 levels in the ipsilateral thalamus of MCAO rats. Twenty-nine days after MCAO, soluble Aβ40 (**A**) and Aβ42 (**B**) levels were measured from the ipsilateral and contralateral thalamus of MCAO rats and statistical comparison was performed between ipsilateral and contralateral sides. Soluble Aβ40 and Aβ42 levels were significantly decreased in the ipsilateral thalamus after the treatment of MCAO rats with bepridil (MCAO+BEP) as compared to the ipsilateral thalamus of vehicle-treated (MCAO) rats. SHAM represents sham-operated rats. (**C**) The levels of insoluble Aβ42 were decreased in the ipsilateral thalamus in MCAO+BEP rats as compared with the ipsilateral thalamus of vehicle-treated (MCAO) rats. Data are shown as mean ± S.D. $*P < 0.05$, $**P < 0.01$, $n = 4–7$.

Bepridil treatment decreases calcium levels in the ipsilateral thalamus of MCAO rats

The enhanced accumulation of Aβ was previously observed to coincide with increased calcium levels in the ipsilateral thalamus in MCAO rats [9, 34]. Thus, we next assessed whether bepridil, which is also a non-selective calcium channel blocker, affects calcium levels in MCAO rats. As previously stated [9], we observed a robust increase in the calcium levels in the ipsilateral thalamus when compared with the contralateral side in MCAO rats (Fig. 3A). Importantly, calcium levels in the ipsilateral thalamus were significantly decreased by 71% in MCAO rats treated with bepridil as compared to vehicle-treated MCAO rats. When compared with the sham-operated rats, however, calcium levels in the ipsilateral thalamus of bepridil-treated MCAO rats were still significantly higher, indicating that bepridil treatment did not fully normalize calcium levels. Conversely, calcium levels were similar in the contralateral thalami among vehicle-treated, bepridil-treated and sham-operated rats. Correlation analyses revealed a positive correlation between the levels of Aβ42 and calcium ($r = 0.85$, $P < 0.01$) (Fig. 3B), and between Aβ40 and calcium ($r = 0.74$, $P < 0.01$; data not shown) in the ipsilateral thalamus. Collectively, these data suggest that bepridil treatment reduces calcium levels in the thalamus of MCAO rats, which coincides with decreased levels of thalamic Aβ.

Bepridil treatment moderately affects APP processing in the ipsilateral thalamus in MCAO rats

Next, we wanted to explore whether the bepridil-related changes are linked to altered expression and/or processing of APP in the ipsilateral thalamus. Consistent with previous findings [9], we observed a significant increase (~1.4-fold) in APP C-terminal fragment (CTF) levels in the ipsilateral thalamus when compared with contralateral thalamus

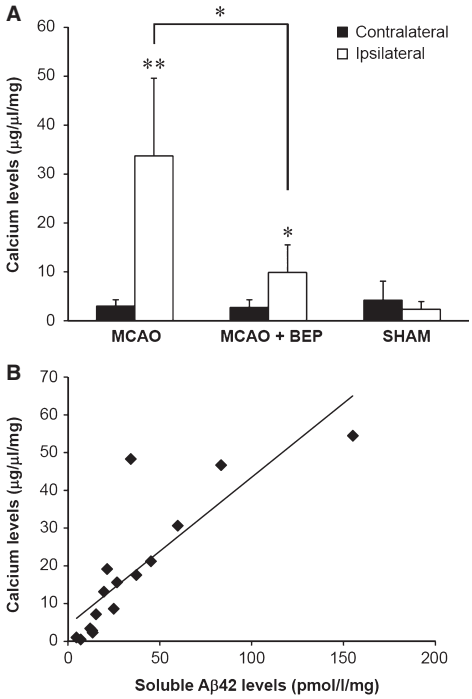

Fig. 3 Bepridil treatment decreases calcium levels in the ipsilateral thalamus in MCAO rats. (**A**) Calcium levels in the ipsilateral thalamus were significantly decreased after treating MCAO rats with bepridil (MCAO +BEP) for 27 days as compared with the vehicle-treated MCAO rats (MCAO). Statistical comparison was also performed between ipsilateral and contralateral sides. No difference in calcium levels between the ipsilateral and contralateral thalamus was detected in sham-operated rats (SHAM). Data are shown as mean ± S.D.. $*P < 0.05$, $**P < 0.01$, $n = 5–7$. (**B**) Correlation analysis between calcium levels and soluble Aβ42 levels in both the ipsilateral and contralateral thalamus. There was a significant correlation between increased calcium and increased soluble Aβ42 levels in the ipsilateral thalamus at 29 days after MCAO ($r = 0.85$, $P < 0.01$).

after MCAO (Fig. 4A). Furthermore, total APP (APPtot = APP695 immature + APP695 mature) levels were significantly decreased in the ipsilateral thalamus. In the bepridil-treated MCAO rats, however, we did not observe significant APP-related alterations in the ipsilateral thalamus (Fig. 4A). As increased β-secretase (BACE) activity is associated with the depletion of the BACE trafficking protein, GGA3, in the ipsilateral thalamus of MCAO rats [9], we assessed whether bepridil treatment affected the full-length protein levels of GGA3 (Fig. 4A). GAPDH-normalized GGA3 levels were decreased by an average of 50% in the ipsilateral thalamus in both vehicle- and bepridil-treated MCAO rats. Similar to a previous report [10], the reduction in full-length GGA3 protein levels coincided with an increase in a GGA3-specific cleavage product of ~50 kDa in both vehicle- and bepridil-treated samples (data not shown). This indicates that full-length GGA3 was proteolytically cleaved as also shown earlier [9, 10]. Consistent with the decreased full-length GGA3 levels, we also observed an increase in BACE activity (~1.8-fold) in the ipsilateral thalamus in both vehicle- and bepridil-treated MCAO rats (Fig. 4B). These changes were not observed in the ipsilateral thalamus of sham-operated rats (Fig. 4A and B). α- and γ-Secretase activities were not significantly altered in the ipsilateral thalamus in either vehicle- or bepridil-treated MCAO rats (Fig. 4B).

To further assess possible APP-related alterations in the bepridil-treated MCAO rats, the levels of soluble APP (sAPP) were measured (Fig. 4C). GAPDH-normalized total sAPP (sAPPtot) levels were significantly increased particularly in the ipsilateral thalamus in vehicle-treated MCAO rats (~2.3 fold) but also in the bepridil-treated MCAO rats (~1.8 fold). Moreover, sAPPtot levels showed a positive correlation with BACE activity in the ipsilateral thalamus ($r = 0.55$, $P < 0.05$; data not shown). As a cell culture medium sample from SH-SY5Y cells overexpressing the APP751 isoform (SH-SY5Y-APP751) was used as a control in Western blots, we were able to explore the isoform expression profiles of sAPPtot in the thalamic samples (Fig. 4C). In addition to the expected total sAPP695 (sAPP695tot), we observed in the ipsilateral thalamus of vehicle- and bepridil-treated MCAO rats the appearance of an APP-specific band, which was similar in size to the total sAPP751 (sAPP751tot) in the SH-SY5Y-APP751 culture medium sample. This is consistent with our previous findings with the APP-Kunitz protease inhibitor domain-specific antibody that revealed an up-regulation of APP751 isoform-specific expression in the ipsilateral thalamus 30 days after MCAO [9]. Detection of sAPPα from rat thalamus using the 6E10 antibody revealed only a moderate staining in Western blot gels. In contrast, the human-derived sAPP751α was abundantly detected in the cell culture medium sample from SH-SY5Y-APP751 cells (Fig. 4C). This result is consistent with the data in mouse brain, which showed unsubstantial sAPPα staining with 6E10 antibody (Figure S1), indicating that 6E10 antibody does not efficiently detect rodent-specific sAPPα. Taken together, these results suggest that the treatment of MCAO rats with bepridil affects APP processing by moderately altering APP CTF and total APP levels in the ipsilateral thalamus.

Bepridil treatment does not affect IDE, NEP, or LRP expression, or the inflammatory response in the ipsilateral thalamus in MCAO rats

Apart from altered APP processing and Aβ production, it is also possible that alterations in Aβ degradation and/or clearance account for the observed decrease in Aβ levels in bepridil-treated MCAO rats. To address this possibility, we investigated the protein levels of IDE, NEP and LRP, which are involved in the degradation and clearance of Aβ (Fig. 5A). Consistent with our previous observations [9], IDE levels were significantly increased in the ipsilateral thalamus of vehicle-treated MCAO rats. Bepridil treatment did not further affect this increase. NEP and LRP levels were unchanged in the ipsilateral thalamus in vehicle- and bepridil-treated MCAO rats as well as in sham-operated rats (Fig. 5A).

As MCAO promotes astrogliosis in the rat thalamus [8, 9], we next assessed the effects of bepridil treatment on the inflammatory response by determining the levels of GFAP and TNF-α expression in

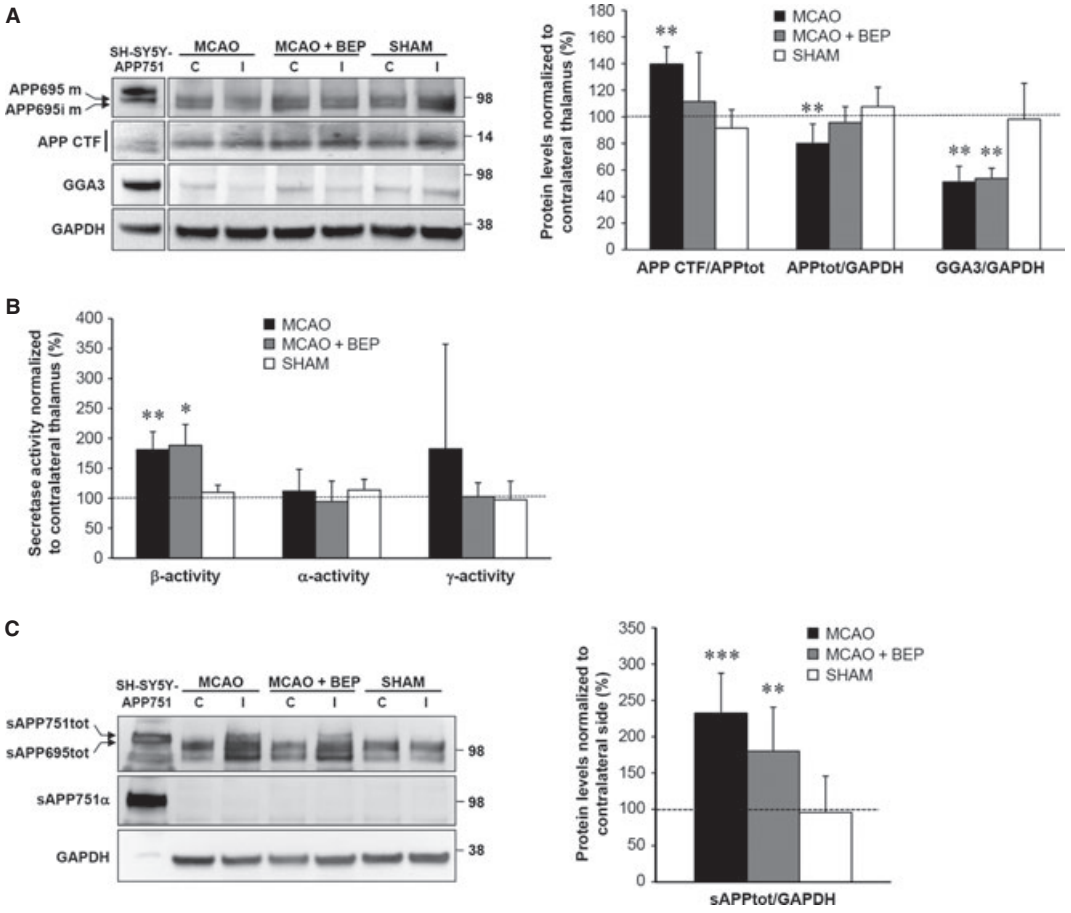

Fig. 4 Bepridil treatment alters APP processing in the ipsilateral thalamus of MCAO rats. (**A**) Western blot showing the levels of the APP695 isoform (m, mature; im, immature) and APP C-terminal fragments (CTF) as well as GGA3 in the contralateral (C) and ipsilateral (I) thalamus of the vehicle-treated (MCAO) rats, bepridil-treated (MCAO+BEP) MCAO rats and sham-operated (SHAM) rats. Protein lysate from human SH-SY5Y cells stably overexpressing the APP751 isoform (SH-SY5Y-APP751) was used as a control. Total APP (APPtot = APP695m + APP695im) and GGA3 protein levels were normalized to GAPDH levels. APP CTF levels were normalized to APPtot. Levels of each protein in the ipsilateral side were normalized to the levels in the contralateral thalamus (=100%). Statistical comparison was performed between ipsilateral and contralateral sides as well as between different treatment groups. (**B**) α-, β- and γ-secretase activity measurements from the thalamus. β-secretase activity was significantly increased in the ipsilateral thalamus of both vehicle- and bepridil-treated MCAO rats, but not in the sham-operated rats, when normalized to the contralateral thalamus (=100%). α- and γ-secretase activities did not show statistically significant changes when normalized to the contralateral thalamus. (**C**) Western blot analysis of total soluble APP (sAPPtot) and soluble APPα (sAPPα) levels in the contralateral (C) and ipsilateral (I) thalamus in the vehicle- and bepridil-treated as well as sham-operated MCAO rats. GAPDH-normalized sAPPtot levels in the ipsilateral thalamus were compared relative to the contralateral thalamus (=100%). Cell culture medium from human SH-SY5Y cells overexpressing APP751 (SH-SY5Y-APP751) was used as a control. Data are shown as mean ± S.D. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $n = 5–7$.

the ipsilateral thalamus (Fig. 5B). Western blot analysis revealed a ~10-fold increase in GFAP protein expression in the ipsilateral thalamus of vehicle-treated MCAO rats. (Fig. 5B). Similarly, we also observed a ~3.5-fold increase in the GAPDH-normalized TNF-α mRNA levels in the ipsilateral thalamus in vehicle-treated MCAO rats. Bepridil did not reverse the robust up-regulation of these markers of astrogliosis and inflammation after MCAO. Taken together, these results suggest that bepridil treatment does not affect the expression of well-known regulators of Aβ degradation or clearance, nor does it alleviate astrogliosis or TNF-α activation in the ipsilateral thalamus in MCAO rats.

Bepridil restores seladin-1 mRNA and protein levels but does not affect HMOX1- or NQO1-mediated oxidative stress response in the ipsilateral thalamus in MCAO rats

Previous studies have demonstrated that seladin-1 expression is linked to neurodegeneration in AD. More specifically, seladin-1 mRNA levels are selectively down-regulated in the brain regions affected in AD [23, 35]. Conversely, overexpression of seladin-1 protects neuro-

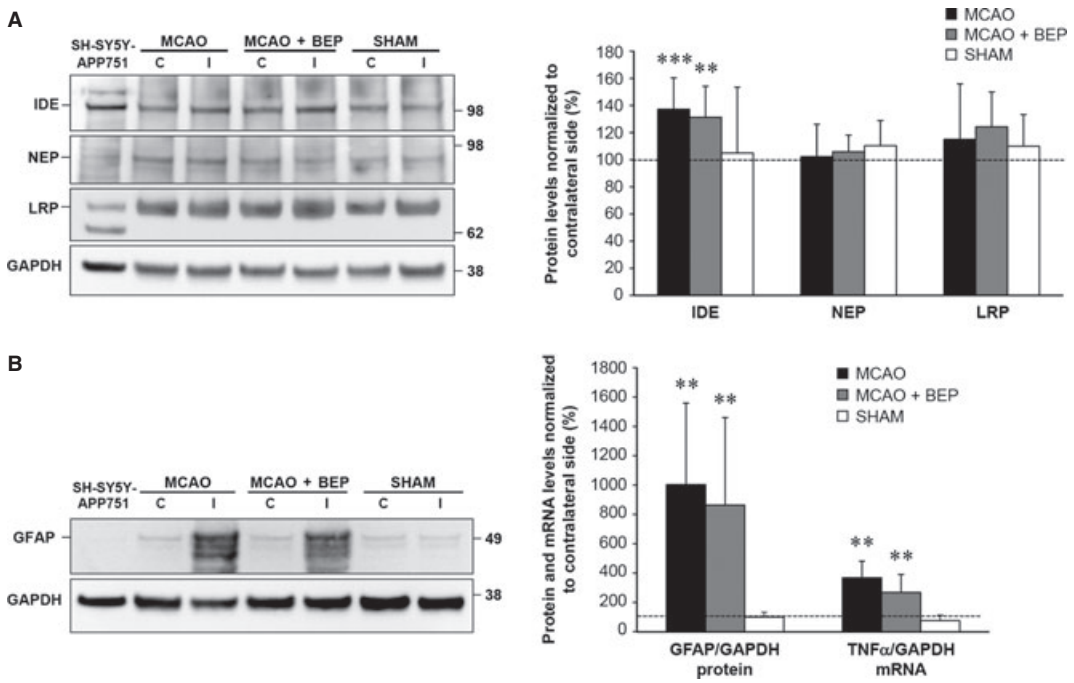

Fig. 5 Bepridil treatment does not affect IDE, NEP or LRP expression, or the inflammatory response in the ipsilateral thalamus in MCAO rats. (**A**) Western blot showing the protein levels of IDE, NEP and LRP in the contralateral (C) and ipsilateral (I) thalamus of the vehicle- (MCAO) and bepridil-treated (MCAO+BEP) MCAO rats as well as sham-operated (SHAM) rats. Total protein lysate from human SH-SY5Y cells stably overexpressing the APP751 isoform (SH-SY5Y-APP751) was used as a control. GAPDH-normalized protein levels in the ipsilateral thalamus were compared relative to the contralateral thalamus (=100%). Statistical comparison was performed between ipsilateral and contralateral sides as well as between different treatment groups. (**B**) Western blot analysis of GFAP levels in the contralateral (C) and ipsilateral (I) thalamus of the vehicle- (MCAO) and bepridil-treated (MCAO+BEP) MCAO rats as well as sham-operated (SHAM) rats. TNF-α and GAPDH mRNA levels were quantified using qPCR. GAPDH-normalized GFAP and TNF-α levels in the ipsilateral thalamus were compared relative to the contralateral thalamus (=100%). Data are shown as mean ± S.D. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $n = 5–7$.

nal cells from Aβ-induced toxicity *in vitro*, indicating that seladin-1 encompasses neuroprotective functions in cellular stress [23, 36]. Given the established association of seladin-1 down-regulation with neuronal degeneration, we set out to assess whether seladin-1 mRNA levels are altered in the ipsilateral thalamus in MCAO rats. Quantitative PCR analysis revealed a significant 30–40% decrease in GAPDH-normalized seladin-1 mRNA and protein levels in the ipsilateral thalamus after MCAO in rats treated with vehicle (Fig. 6A). Interestingly, a similar decrease in seladin-1 mRNA and protein levels was not observed in the ipsilateral thalamus of bepridil-treated MCAO rats. Furthermore, a trend towards a statistically significant difference ($P = 0.057$) in seladin-1 mRNA levels between vehicle- and bepridil-treated MCAO rats was observed. To further explore the functional role of seladin-1 down-regulation in thalamic pathology, we performed correlation analysis between seladin-1 and calcium as well as seladin-1 and Aβ levels (Fig. 6B). There was a significant inverse correlation between GAPDH-normalized seladin-1 mRNA and calcium ($r = -0.70$, $P < 0.01$) as well as insoluble Aβ42 levels ($r = -0.64$, $P < 0.05$). Expression of HMOX1 and NQO1 is up-regulated as a consequence of oxidative or electrophilic stress through Nrf2-Maf-mediated transcriptional activation [24, 25]. Elucidation of GAPDH-normalized HMOX1 and NQO1 mRNA levels revealed that these stress-related target genes were strongly up-regulated in the ipsilateral thalamus of both vehicle- and bepridil-treated MCAO mice (Fig. 6C). Collectively, these data suggest that bepridil treatment restores seladin-1 levels, but does not significantly affect HMOX1- or NQO1-mediated oxidative stress response in the ipsilateral thalamus of MCAO rats.

Bepridil does not affect voltage-dependent L-type calcium channel subunit α-1C (LTCC) mRNA levels in the ipsilateral thalamus in MCAO rats

To elucidate the underlying molecular mechanisms of reduced calcium levels in bepridil-treated MCAO rats, we determined the mRNA levels of the voltage-dependent L-type calcium channel subunit α-1C (LTCC) in the thalamus. The rationale for this approach originates from a previous study, which showed that bepridil treatment affected LTCC expression in canine atria [37]. We observed a significant decrease in LTCC mRNA levels in the ipsilateral thalamus in rats with MCAO. There was no difference between vehicle- and bepridil-treated

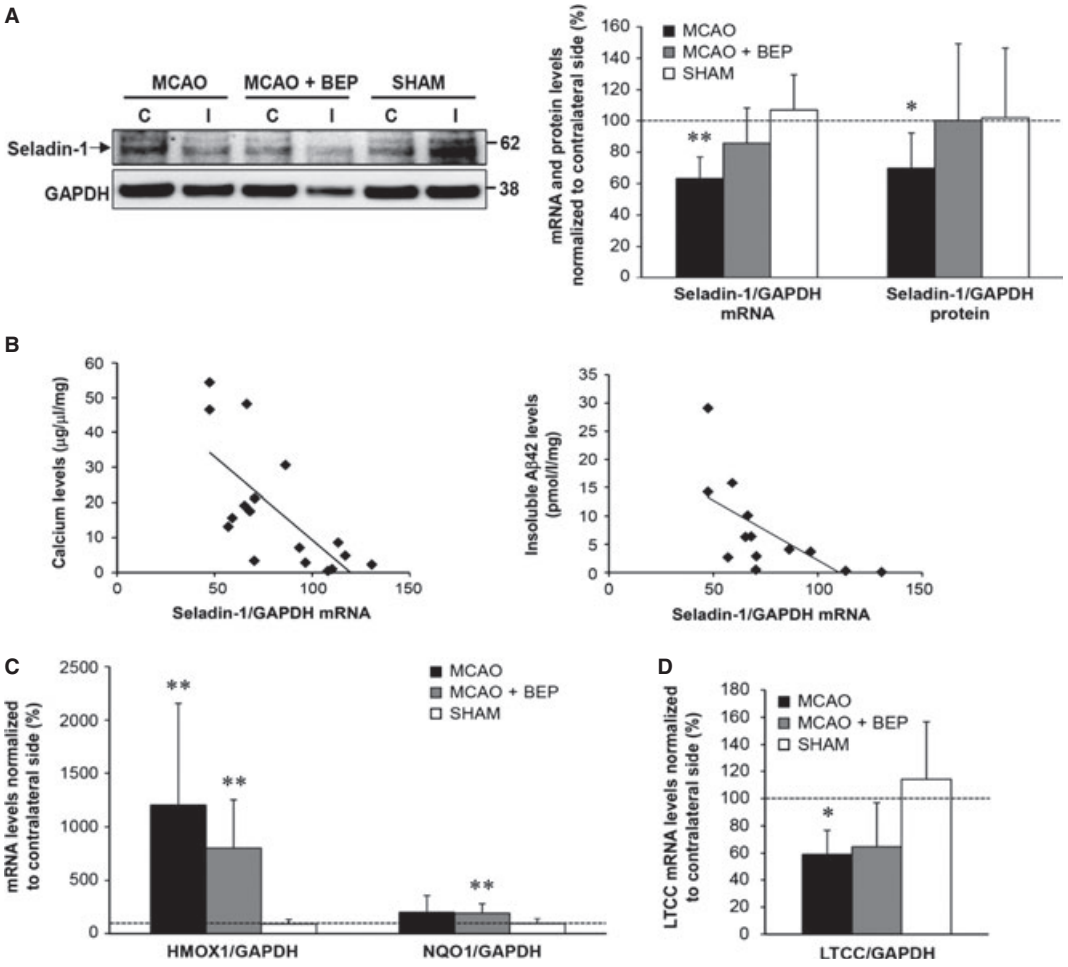

Fig. 6 Bepridil increases seladin-1 mRNA expression but does not affect HMOX1- or NQO1-mediated oxidative stress response in the ipsilateral thalamus in MCAO rats. (**A**) qPCR and Western blot analysis of seladin-1 mRNA and protein levels in the vehicle- (MCAO) and bepridil-treated (MCAO +BEP) MCAO rats as well as sham-operated (SHAM) rats. GAPDH-normalized mRNA and protein levels of seladin-1 in the ipsilateral thalamus were compared to the contralateral thalamus (=100%). Statistical comparison was performed between ipsilateral and contralateral sides as well as between different treatment groups. $^{**}P < 0.01$, $^{*}P < 0.05$ (ipsilateral *versus* contralateral side). (**B**) Correlation analysis of seladin-1 mRNA levels with calcium ($r = -0.70$, $P < 0.01$; left) and insoluble Aβ42 ($r = -0.64$, $P < 0.05$; right) levels in the ipsilateral thalamus. qPCR analysis of GAPDH-normalized HMOX1 and NQO1 (**C**) as well as LTCC (**D**) mRNA levels in the vehicle- (MCAO) and bepridil-treated (MCAO+BEP) MCAO rats as well as sham-operated (SHAM) rats. Contralateral thalamus was set to 100%. $^{**}P < 0.01$, $^{*}P < 0.05$ (ipsilateral *versus* contralateral), $n = 4$–7, data are shown as mean ± S.D.

MCAO rats (Fig. 6D), indicating that bepridil treatment does not affect the mRNA levels of LTCC in the ipsilateral thalamus.

Bepridil treatment improves the behavioural recovery of MCAO rats in the cylinder test

The sensorimotor impairment and recovery of function after MCAO was assessed by three behavioural tests. Spontaneous forelimb use was measured by the cylinder test at baseline (1 day before the operation) and at 14 and 28 days after MCAO (Fig. 7). All the animals used both forelimbs for postural support prior to surgery and MCAO rats showed decreased use of their impaired forelimb (i.e. contralateral to lesion) at 14 and 28 days after the operation. There was no significant group effect in forelimb use. However, ANOVA for repeated measures showed a significant group × time interaction ($P < 0.05$), indicating that sensorimotor recovery was different between the groups. A more detailed analysis revealed that bepridil-treated MCAO rats significantly increased the use of the impaired forelimb on post-operative day 28 as compared with vehicle-treated MCAO rats. The limb-placing test was used to assess forelimb and hindlimb responses to tactile and proprioceptive stimulation. The

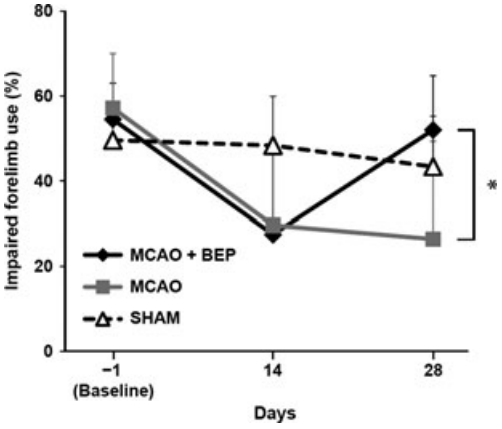

Fig. 7 Bepridil treatment improves sensorimotor recovery in rats following MCAO. The cylinder test was used to assess spontaneous forelimb use during exploration. Fourteen days after MCAO, all rats showed decreased use of the forelimb contralateral to their lesion. At the end of the follow up, bepridil-treated MCAO rats (MCAO+BEP) showed recovery in the contralateral forelimb use as compared with vehicle-treated MCAO rats (MCAO). The imbalance in forelimb use was calculated as follows: [(contralateral contacts + ½ × bilateral contacts)/total contacts] × 100%. Data are shown as mean ± S.D. $*P < 0.05$ (compared to vehicle group), $n = 5–7$.

test showed that MCAO rats were initially severely impaired but then made a partial recovery. A significant overall group effect was explained by the difference between sham-operated and MCAO rats throughout the follow up ($P < 0.01$). MCAO groups with or without bepridil treatment did not differ from each other (data not shown). The tapered/ledged beam-walking test was used as a measure of hindlimb function. The special feature of this test is that the ledge allows the rat to display a deficit normally hidden by compensatory adjustment. All rats showed an increase in slips (foot faults) with no apparent recovery after MCAO. A significant overall group effect resulted from the fact that MCAO rats made significantly more slips compared with sham-operated rats ($P < 0.05$). There was no difference between vehicle- and bepridil-treated MCAO rats during the follow up (data not shown).

Discussion

We have previously shown that APP and Aβ, or their fragments, aggregate in the dense plaque-like deposits in the thalamus of rats subjected to transient MCAO [8]. Subsequently, we demonstrated that APP processing and the expression of Aβ-degrading enzymes is altered following MCAO in the ipsilateral thalamus, long after the initial ischaemic insult [9]. These alterations coincided with significantly augmented calcium levels, depletion of the BACE trafficking protein GGA3 and increased BACE activity in the ipsilateral thalamus. Based on these findings, we suggest that the observed alterations resulting in Aβ accumulation are linked to disrupted calcium homeostasis, which initiates the secondary degeneration taking place in the thalamus after MCAO [11–13]. Owing to the intimate link between calcium and Aβ in AD pathology [14, 15], drugs modulating both calcium and Aβ dyshomeostasis are therapeutically attractive. In this context, bepridil is a promising candidate as it is a non-selective calcium channel blocker [16, 18, 19], which also inhibits β-secretase-mediated APP cleavage both *in vitro* and *in vivo* by increasing the endosomal pH [20].

Here, we show that chronic treatment of MCAO rats with bepridil significantly decreased both soluble Aβ40 and Aβ42 levels as well as insoluble Aβ42 level in the ipsilateral thalamus. Furthermore, bepridil treatment significantly decreased the calcium levels and this decrease strongly correlated with the decrease in Aβ42 and Aβ40 levels in the ipsilateral thalamus. We also observed a significant increase in APP CTF production in vehicle-treated MCAO rats in the ipsilateral thalamus but not in bepridil-treated MCAO rats. Consistent with the increased APP CTF production, sAPPtot levels measured from the thalamic soluble protein fraction were also significantly increased in the vehicle-treated MCAO rats. A similar, although ~30% less extensive, increase was observed in the ipsilateral thalamus in bepridil-treated rats. The *in vitro* β-secretase activity assay in thalamic tissue homogenates indicated that BACE activity was significantly increased in the ipsilateral thalamus after MCAO, similar to our previous findings [9]. Bepridil treatment did not affect this increase. A recent report showed that BACE activity was not blocked by bepridil in an *in vitro* activity assay in HEK293 AP-APP cell lysates [20]. Thus, it is improbable that bepridil-mediated changes in BACE activity would be detected in tissue homogenates using a similar *in vitro* approach. There were no statistically significant changes in α- or γ-secretase activities either in vehicle- or bepridil-treated MCAO rats. Taken together, these data suggest that bepridil treatment affected APP processing in the ipsilateral thalamus of MCAO rats and these changes may be linked to the reduced Aβ levels. However, as calcium levels were simultaneously decreased, it is difficult to determine whether the changes in calcium homeostasis contributed to the production of Aβ or whether the BACE cleavage of APP was directly inhibited by bepridil. Alternatively, it is possible that modulation of the Aβ levels occurred concurrently *via* these two independent mechanisms.

Apart from decreased Aβ generation, it is possible that changes in Aβ degradation or clearance also contributed to the present data. IDE and NEP are the enzymes that are intimately involved in Aβ degradation in the brain [38]. We found that IDE protein levels were increased in the ipsilateral thalamus of both vehicle- and bepridil-treated MCAO rats while no alterations were observed in the levels of NEP. Also, there were no significant changes in vehicle- or bepridil-treated MCAO rats in the expression of LRP, which is a key component in the receptor-mediated clearance of Aβ [39]. Together, these results suggest that bepridil treatment does not affect the expression of well-known players involved in the degradation and clearance of Aβ in the ipsilateral thalamus of MCAO rats. However, we cannot rule out the possibility that some of the other enzymes or proteases within the brain involved in degrading or clearing Aβ [38] may have been affected by bepridil after MCAO. Also, it is still possible that bepridil treatment may modulate the activity of IDE and NEP as well as the function of

LRP through mechanism that does not involve expressional alteration.

It is well established that astrogliosis and pro-inflammatory cytokine expression are induced in cerebral ischaemia [8, 40, 41]. In accordance with those data, we detected an increase in GFAP and TNF-α expression in the ipsilateral thalamus after MCAO. However, bepridil did not mitigate astrogliosis or the inflammatory response after MCAO. Previous reports show that reduced seladin-1 mRNA levels are linked to neurodegeneration in AD [23, 35] and that seladin-1 overexpression protects neuronal cells from Aβ-induced toxicity *in vitro* [23, 36]. In this study, we found that seladin-1 mRNA and protein levels were significantly reduced in the ipsilateral thalamus in vehicle-treated MCAO rats but not in the bepridil-treated MCAO rats. Correlation analysis revealed that there was a significant inverse correlation between seladin-1 mRNA and calcium as well as insoluble Aβ42 levels. This suggests that seladin-1 expression is down-regulated in response to calcium and Aβ dysregulation in the ipsilateral thalamus in MCAO rats, in a similar manner to the affected brain regions in AD [23, 35]. Alternatively, it is possible that reduced seladin-1 mRNA and protein levels may simply reflect neuronal loss in the thalamus of MCAO rats. *In situ* hybridization analysis of human cortical brain sections has previously demonstrated a neuron-specific expression pattern for seladin-1 [23]. Although bepridil treatment restored seladin-1 levels, the elevated expression of oxidative stress-induced genes HMOX1 and NQO1 did not indicate significant alleviation in the stress response after bepridil treatment. Nevertheless, it should be emphasized that HMOX1 mRNA levels were reduced on average 30% in the ipsilateral thalamus of bepridil-treated MCAO rats as compared with vehicle-treated MCAO rats, implying that bepridil may have some oxidative stress-relieving effects. Consequently, this may have affected, e.g. the APP processing phenotype observed in the ipsilateral thalamus of bepridil-treated rats. Taken together, our results suggest that restored seladin-1 mRNA and protein levels in the ipsilateral thalamus of bepridil-treated MCAO rats could associate with the improved neuronal survival. Nevertheless, inflammatory- or oxidative stress-related responses were not significantly mitigated in the ipsilateral thalamus of bepridil-treated MCAO rats.

Depending on the prevailing model system, Aβ plaques have been shown to impair calcium homeostasis in the mouse models of AD [42], whereas sustained increase in cytosolic calcium levels induced by KCl-mediated depolarization in neuronal cultures may trigger intraneuronal Aβ1-42 production and neuronal death [43]. Furthermore, as soluble Aβ oligomers interact with certain lipids and receptors at the plasma membrane, it has been suggested that the increased calcium influx could result from disrupted membrane lipid integrity, Aβ-mediated pore formation or modulation of ion channels [14]. Also, APP regulates intracellular trafficking of voltage-gated L-type calcium channel ($Ca_v1.2$) by retrieving $Ca_v1.2$ to the intracellular compartments (away from the plasma membrane) [44]. This means that alterations in APP expression or its processing may affect the levels of $Ca_v1.2$ at the plasma membrane and thus modulate calcium influx. Previously, bepridil treatment was shown to up-regulate mRNA expression of the α-1C subunit of $Ca_v1.2$ in a canine model of atrial tachycardia [37]. Although the mRNA levels of the α-1C subunit were reduced in the ipsilateral thalamus in vehicle-treated MCAO rats in this study,

Table 1 Summary of the effects of bepridil treatment on factors relevant for AD pathogenesis in the thalamus in MCAO rats.

	Ipsilateral *versus* contralateral thalamus, 30 days after MCAO	
	Vehicle	**Bepridil (50 mg/kg, p.o.)**
Calcium-related changes		
Calcium	↑↑	↑
LTCC mRNA	↓↓↓	(↓)
Aβ pathology		
Soluble Aβ40	↑	+\–
Soluble Aβ42	↑↑	+\–
Insoluble Aβ42	(↑)	+\–
APP processing		
APPtot	↓↓	+\–
APP CTFs	↑↑	+\–
sAPPtot	↑↑↑	↑↑
GGA3	↓↓	↓↓
β-secretase	↑↑	↑
α-secretase	+\–	+\–
γ-secretase	(↑)	+\–
Aβ degradation		
IDE	↑↑	↑↑
NEP	+\–	+\–
LRP	+\–	+\–
Inflammation		
Astrocytes (GFAP)	↑↑	↑↑
TNF-α mRNA	↑↑	↑↑
Survival		
Seladin-1 mRNA	↓↓↓	(↓)
Oxidative stress		
HMOX1	↑↑↑	(↑↑)
NQO1	↑	↑

The arrows refer to *P* values and change between the ipsilateral and contralateral thalamus. Arrows in parentheses indicate the minor changes with no statistical significance.

bepridil treatment did not affect the expression of this subunit. This indicates that there may be a feedback mechanism that down-regulates $Ca_v1.2$ expression when calcium homeostasis is impaired in the thalamus. It is also possible that bepridil does not exert its effects through the inhibition of voltage-gated calcium channels, but alternatively *via* inhibition of sodium-calcium exchangers that also allow calcium influx [45]. This idea is supported by findings from anoxic myelinated CNS axons, in which the influx of calcium was blocked by bepridil but not by specific inhibitors of the L-type calcium channels. These data suggest that calcium entry during CNS anoxia takes place primarily through the reverse operation of sodium–calcium exchangers [45]. The effects of bepridil treatment on different factors relevant for AD pathogenesis are summarized in Table 1.

The reduction in Aβ and calcium levels by bepridil treatment also improved behavioural recovery in MCAO rats. Improved limb-placing scores and, particularly, improved sensorimotor performance in the cylinder test in bepridil-treated MCAO rats were observed on post-operative day 28, and this was possibly related to the delayed nature of pathology in the thalamus [46]. Although the present data do not prove causal relationships, they are consistent with the organization and integration of sensory, motor and cognitive pathways within the thalamus, which are impaired by Aβ and calcium aggregation [47]. Increasing pharmacological evidence show that drugs that prevent or lessen secondary pathology in the thalamus also improve behavioural performance [48]. For example, the recent work by [49] shows association with improved sensorimotor outcome and reduced Aβ, neuronal loss and inflammatory response in the ipsilateral thalamus in MCAO rats treated by γ-secretase inhibitor. Less is known about functional significance of calcium aggregation in the thalamus. Thalamic calcification may initially have a protective role by removing excessive intracellular and/or extracellular Ca^{2+} [50], but eventually this may lead to widespread functional impairment. Thus, prevention of long-term calcification by drugs, which chelate calcium phosphate may alleviate neurological dysfunction as shown by Loeb *et al.* [51]. In conclusion, further assessments related to bepridil, such as dose-dependency studies are still needed to comprehensively illuminate the link between the mitigation of Aβ and calcium pathology as well as the functional recovery in MCAO rats.

In conclusion, our data indicate a close interplay between calcium and Aβ pathology. This emphasizes the importance of targeting therapeutic interventions to the prevention of calcium dyshomeostasis in diseases, such as AD. In this context, bepridil is a potential candidate because of its multifunctional ability to modulate both calcium homeostasis and BACE1 function.

Acknowledgements

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Assessment of 6E10 and 22C11 antibodies used to detect soluble APP (sAPP) species (sAPPtot and sAPPα).

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