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Short Communication

Alzheimer's Disease-Like Impaired Cognition in Endothelial-Specific Megalin-Null Mice

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Abstract. Megalin has been suggested to be involved in Alzheimer's disease (AD), mediating blood-brain barrier (BBB) transport of multiple ligands, including amyloid-β peptide (Aβ), but also neuroprotective factors. Because no transgenic model is currently available to study this concept, we have obtained transgenic mice blocking megalin expression at the BBB. These endothelial megalin deficient (EMD) mice developed increased anxiety behavior and impaired learning ability and recognition memory, similar to symptoms described in AD. Degenerating neurons were also observed in the cerebral cortex of EMD mice. In view of our findings we suggest that, in mice, megalin deficiency at the BBB leads to neurodegeneration.

Keywords: Alzheimer's disease, cognitive impairment, memory, neurodegeneration, transgenic mice

INTRODUCTION

Megalin, also known as low-density lipoprotein receptor-related protein-1 (LRP-2) and glycoprotein 330, is ubiquitously expressed in a variety of tissues and is present in a wide range of different cell types. In the central nervous system, megalin is expressed in brain capillaries and choroid plexus [1], where it is known to function as a blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier clearance transporter for amyloid-β peptide (Aβ) [2, 3].

Megalin is the largest member of the low-density lipoprotein receptor (LDLR) family [4, 5] and recognizes a wide range of structurally and functionally distinct ligands [6]. Among these ligands, vitamin D [7, 8], Sonic hedgehog (Shh) [9–11], lipoproteins (e.g., apolipoprotein E) [12, 13], hormones (e.g., leptin [14] or insulin [15]), and neurothrophic factors [16] (including insulin-like growth factor I) [17], enter from the blood into the brain where they are fundamental for its development, function, and protection against injury.

Together, these observations prompted us to analyze the possible consequence of blocking megalin expression in the endothelium of BBB. For this purpose, we have generated a mutant mouse carrying megalin impairment at the endothelial cells. In the present study, we used the *Cre/loxP* system, whereby

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mice with *loxP*-flanked *megalin* gene were mated with *Tie-Cre* transgenic mice expressing the Cre recombinase exclusively in the endothelial cells. Because megalin is expressed in brain capillaries [1], this model offers the possibility to evaluate the consequence of megalin blockage in the BBB.

METHODS

Transgenic mice

Endothelial megalin deficient (EMD) mice were generated using the Cre-Lox system under the control of the Tie2 promoter [18]. Tie2-Cre transgenic mice were obtained from Howard Hughes Medical Institute, Dallas (kindly provided by Masashi Yanagisawa) and crossed with gp330/megalin-deficient transgenic mice (megalin$^{flox/flox}$) obtained from Max-Delbrück-Centrum, Berlin (kindly provided from Thomas E. Willnow [19]), to generate Tie2-Cre/megalin$^{flox/flox}$, EMD mice. Mice homozygous for the flossed" megalin allele, either with or without the Tie2-Cre transgene (Tie2-Cre^{+}megalin$^{flox/flox}$ or megalin$^{flox/flox}$, respectively) were used. Non-transgenic littermates (i.e., Tie2-Cre^{-} mice) were used as controls (megalin$^{flox/flox}$), and named as wild-type (WT). Tg2576 mice (overexpressing human AβPP695), and AβPP/PS1 mice, a cross between Tg2576 and mutant PS1 (M146L), were used as mouse model of amyloidosis. For experiments, 6 month-old male mice were used from our inbred colony. At the end of experiments, animals were deeply anesthetized and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for immunohistochemical analysis. All animals were handled and cared for according to the Council Directive 2010/63/UE of 22 September 2010.

Culture of brain microvascular endothelial cells (BMECs)

BMECs were isolated and cultured as previously described [20]. Cerebral cortices from 8 week-old EMD mice and from 8 week-old WT mice were used. See Supplementary Material for the detailed protocol.

Behavioral testing

After adaptation to human handling, behavioral tests were conducted over an 11-day period, as previously described [21]. Spontaneous alternation, tested with a T-maze, was the first parameter evaluated, followed on the same day by open-field (days 1–3), the elevated plus maze (days 4 and 5), and object recognition test (days 10 and 11). See Supplementary Material for the detailed protocol.

Immunoassays

Western blotting assays were performed as described previously [22]. Briefly, proteins were isolated from brain tissue or cell cultures by standard methods. Brain tissue was homogenized in Tris-buffered saline (20 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl) containing a mixture of protease inhibitors. Homogenates were centrifuged for 10 min at 10000 rpm at 4°C, and supernatants were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare) and incubated with the specific antibodies. Primary antibodies used were: goat anti-megalin (Santa Cruz Biotechnology) and mouse anti-β-actin (Sigma).

For immunohistochemistry assays, fixed brains were cut on a vibratome (Leica Microsystems) at 50 μm, and tissue sections were incubated overnight at 4°C with goat anti-megalin antibody (Santa Cruz Biotechnology) in 0.1 M phosphate buffer containing 0.5% bovine serum albumin and 0.5% Triton X-100. Rodhamine-labeled tomato lectin (Vector Laboratories) was used as endothelial cell marker. Images were captured using a Zeiss LSM 510 Meta scanning laser confocal microscope (Carl Zeiss Microimaging, GmbH). The specificity of the staining was tested by omission of primary antibodies.

Fluoro-Jade B labeling has been shown to stain degenerated, but not healthy, neurons [23], even in chronic neurodegenerative processes [24]. Fluoro-Jade B (Histochem, Jefferson, AR) staining was carried out as described previously [25]. Morphometrical analysis, using ImageJ software (NIH Image), was done, and results expressed as number of Fluoro-Jade B-positive cells.

Data analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using a two-way ANOVA followed by Tukey's post hoc test for multiple comparisons. All calculations were made using SPSS v15.0 software. Statistical significance was set at $p < 0.05$.

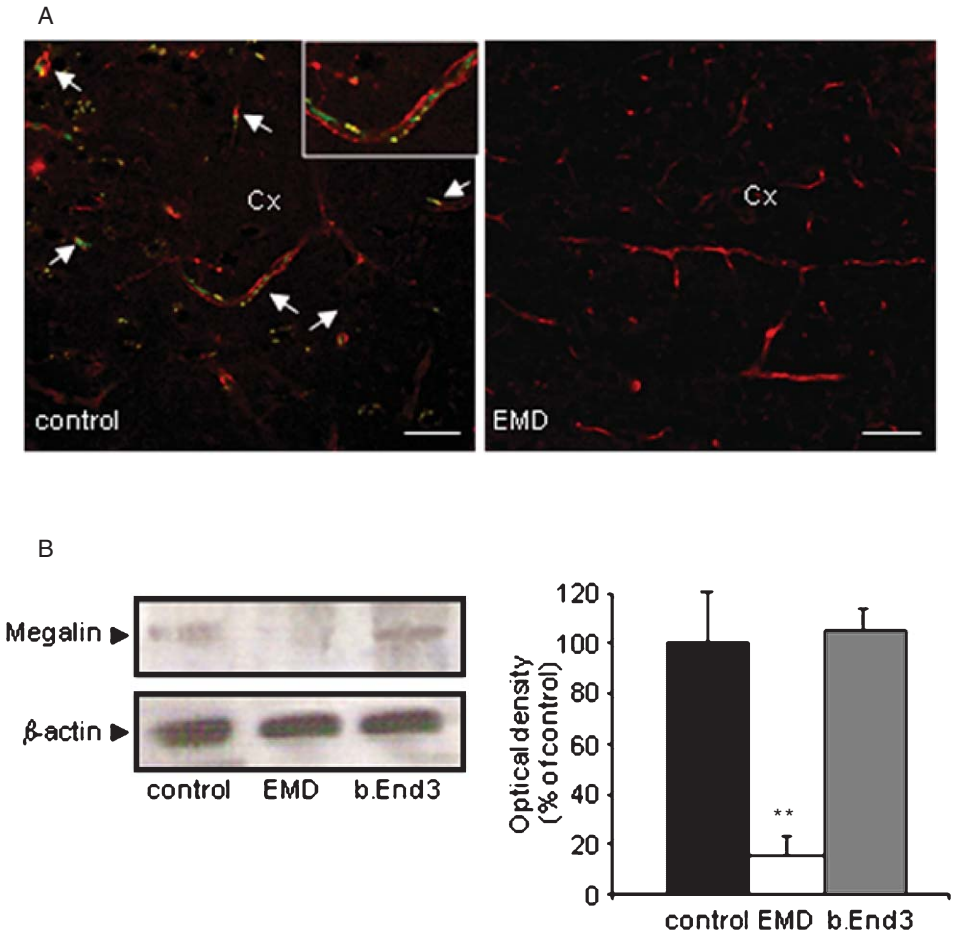

Fig. 1. Megalin expression in brain microvessels in EMD and wild-type mice. A) Double staining for megalin (green) and lecting (red) in brain tissue sections in wild-type and EMD mice. Scale bar, 20 μm. B) Megalin brain capillary expression in control group and EMD mice by western blot analysis. Scanning densitometry of megalin band related to β-actin in control and EMD mice. Data are expressed as mean ± SEM. $^{**}p < 0.01$ versus control group, $n = 3$ independent experiments. EMD, endothelial megalin deficient; Cx, cerebral cortex; b.End3, brain endothelial cell line.

RESULTS

Generation of EMD mice

Immunohistochemistry was used to verify that our experimental system blocked megalin protein expression in microvasculature. Confocal microscopical analysis indicated that megalin in brain capillaries of EMD mice was lacking compared to WT mice (Fig. 1A).

Furthermore, BMEC extracts were subjected to immunoblotting analysis. Whereas megalin was readily detectable in BMEC cultures from WT mice (the control group), it was lacking in BMEC cultures from EMD mice (Fig. 1B). For a positive control, we have used a brain endothelial cell line (b.End3) which expressed megalin [21]. The western blot analysis indicated that megalin in brain capillaries of EMD mice was decreased by >80% compared to WT mice (Fig. 1B).

Phenotypic characterization of EMD mice

We then tested mice for impaired behavior. In the open-field, 6 month-old EMD mice spent less time ($p = 0.007$) with fewer entries ($p = 0.001$) in the central region than WT mice on day 1 and 2 (Fig. 2A). This exploratory tendency in EMD mice was comparable with that carried out in Tg2576 and AβPP/PS1 mice (Fig. 2A), indicating stronger anxiety behavior [26]. Non-spatial visual recognition memory was studied using the object recognition test. Impaired retention

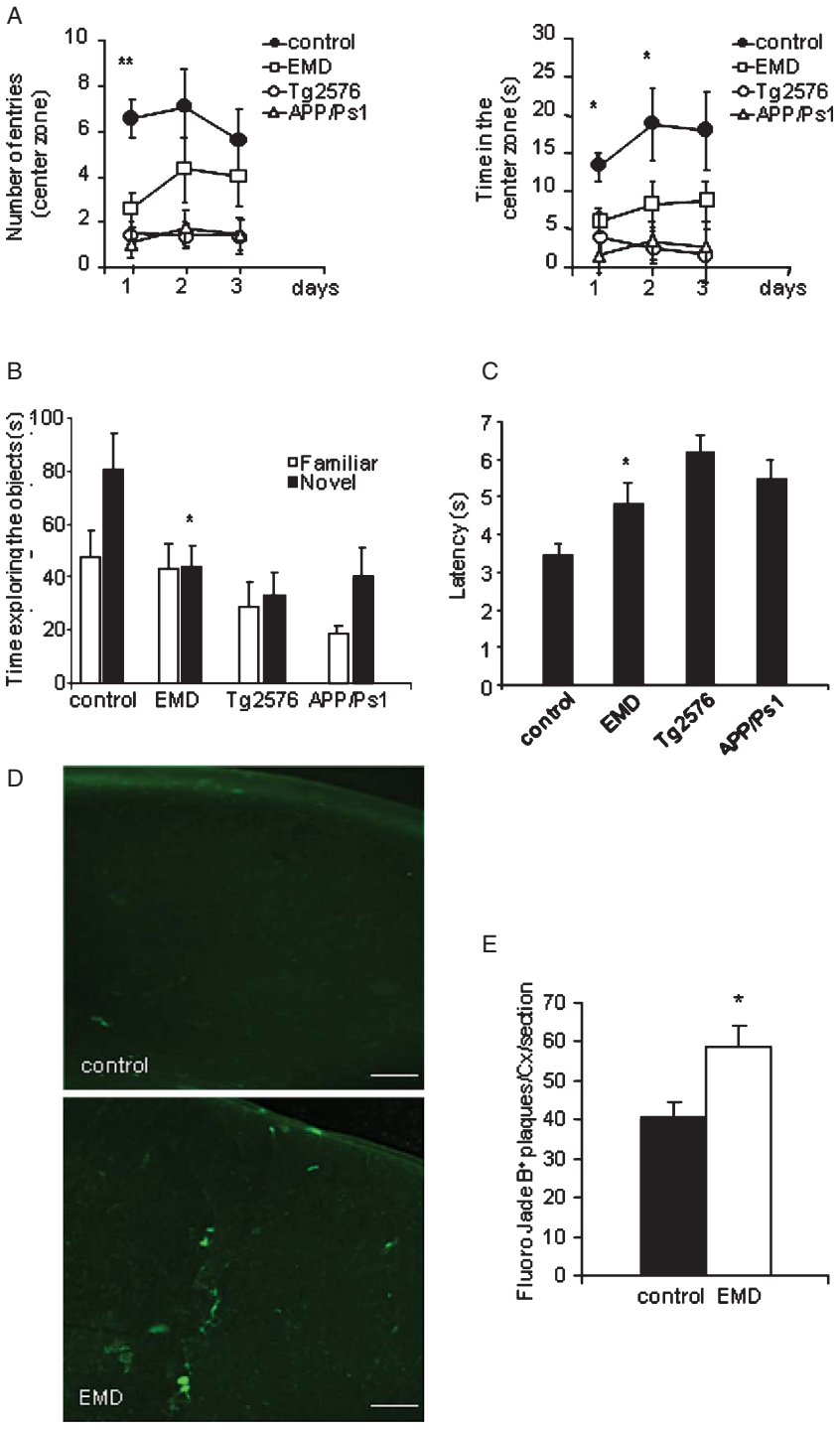

Fig. 2. Phenotypic characterization of EMD and wild-type mice. A) Behavior in the open field was similarly impaired in EMD mice as that in Tg2576 and AβPP/PS1 mice compared to WT mice, as determined by the number of entries and the time in center area. B) In the novel-object recognition test, EMD mice showed a significant decline in performance, as determined by the time exploring the novel object. C) An increase in the latency in the T-maze was observed in EMD mice versus wild-type animals. D) Photomicrographs show fluorescent Fluoro-Jade B staining, mainly in the cerebral cortex of EMD mice. Scale bar, 20 μm. E) The histogram shows the number of Fluoro-Jade B-positive cells in EMD mice compared with WT mice. Stereological analysis revealed a significant increasing in the number of neurodegenerative cells in the cerebral cortex of EMD mice. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control group. WT, wild type; EMD, endothelial megalin deficient.

in both animal models of amyloidosis, Tg2576 and AβPP/PS1 mice, was replicated in 6 month-old EMD mice, displaying less time exploring the novel object compared with WT mice ($p = 0.024$; Fig. 2B). In the T-maze test, time to make a decision was increased in EMD mice compared to WT mice ($p = 0.046$; Fig. 2C). EMD mice showed choice trial latency similar to that observed inTg2576 and AβPP/PS1 mice (Fig. 2C).

Neuronal degeneration was visualized using Fluoro-Jade B staining. Widespread Fluoro-Jade B-positive neurons were detected in the cerebral cortex of 6 month-old EMD mice (Fig. 2D). Stereological analysis of multiple stained section revealed that the number of Fluoro-Jade B-positive neurodegenerative neurons was significantly increased in EMD mice compared with WT mice ($p < 0.05$) (Fig. 2E).

DISCUSSION

These results indicate that megalin blockade in brain endothelial cells triggered brain disturbances in rodents that are reminiscent of those found in AD. In this report we present behavioral characterization of EMD mice to 6 months of age. We used cre/loxP-mediated conditional megalin-deficient mice, and with this method we could block megalin expression specifically in the brain capillary endothelial cells that are forming BBB, and originate many of the changes seen in AD brains, including behavioral impairments and neurodegeneration. Behavioral impairment was found in the hippocampal-dependent T-maze and in the hippocampal-dependent novel object recognition task. Our data agree with a study showing that selective decreased expression of LRP-1, another member of LDLR family, in the BBB reduced learning ability and memory [26].

Curiously, neuropathological analysis of brains from EMD mice does not reveal any AD-related pathology. The lack of pathological hallmarks, including amyloid deposits, in the present model may question a significant pathogenic role of BBB megalin dysfunction in AD. It seems likely that additional factors are required to develop these pathogenic alterations. This is not surprising since under normal conditions rodents do not develop plaques [27]. In agreement with the present findings, cognitive impairment may develop with brain amyloidosis without plaques [28], and amyloid plaques are not always associated with cognitive deterioration [29].

The cognitive deficits were accompanied by increased diffuse neurodegeneration in the cerebral cortex of EMD mice. It has been suggested that neurodegeneration in the cortex and hippocampus may be one mechanism underlying the post-traumatic behavioral deficits [30, 31]. Diffuse neurodegeneration in the retrosplenial cortex may explain the deficits in working memory. Animals with lesions of the cerebral cortex make more errors in the eight-arm radial arm maze task of working memory compared to controls [32, 33]. It must be noted that neither overt neuronal loss nor caspase activation (data not shown) was observed, despite the presence of increased Fluoro-Jade-B staining, suggesting that neuronal dysfunction, rather than neuronal loss, may underlie the behavioral deficits seen.

Alternatively, altered cognition after megalin deletion may be related to an as yet uncharacterized role of neurotrophic growth factors in maintaining cognitive status independent of amyloidosis. In this regard, it has been shown that insulin-like growth factor I, insulin, and leptin play an important role in maintaining normal cognitive performance in rodents [34, 35] and possibly in humans [36, 37]. The signaling pathways downstream to neurotrophic and insulin signaling are defective in brain aging and AD, and these responses are likely to contribute to defects in synaptic plasticity, learning, and memory [38]. On the other hand, and based on megalin-dependent trafficking of Shh [9–11], and its role on neuronal injury [39], we cannot exclude a deficit on Shh presence and subsequent behavior response. We propose that in EMD mice these signaling pathways may be altered resulting in the behavioral impairment. However, this hypothesis requires further study. In conclusion, in this study, we demonstrated that EMD mice display phenotypic changes consistent with selective behavioral impairments, closely resembling those found in AD.

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SUPPLEMENTARY MATERIAL

Supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-131604.

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