

Cell Reports

Supplemental Information

**Antagonistic Effects of BACE1  
and APH1B- $\gamma$ -Secretase Control Axonal Guidance  
by Regulating Growth Cone Collapse**

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## Supplemental Information

### Supplemental experimental procedures

**Antibodies and inhibitors:** Antibodies to the following antigens were used: BACE1 (D10E5, Cell Signaling); soluble and full-length CHL1 (AF2147, R&D Systems); Actin (A5441; Sigma); full-length APP, C99 and C83 (B63 antibody (Annaert et al., 2001)); Neuropilin1 (AF566, R&D Systems); PlexinA (MAB5856, R&D Systems); Alexa Fluor 555 Phalloidin (A34055, Invitrogen);  $\beta$ III tubulin (T5076; Sigma);  $A\beta_{x-40}$  (Sandwich ELISA-capturing antibody JRF/cA $\beta$ 40/28, Janssen Pharmaceutica (Beerse, Belgium)); rodent  $A\beta_{1-x}$  (Sandwich ELISA-detection antibody JRF/rA $\beta$ /2, Janssen Pharmaceutica (Beerse, Belgium)); anti-V5 (460705, Invitrogen). Antibody against CHL1CTF $\beta$  was generated and purified by Thermo Fisher Scientific by immunization with a peptide containing the C-terminal DGSFIGAYTGAKKEKGSVE sequence of CHL1. The following inhibitors were purchased:  $\beta$ -secretase inhibitor IV (565788, Calbiochem), metalloprotease inhibitor GM6001 (364206, Calbiochem) and  $\gamma$ -secretase inhibitor IX – DAPT (565770, Calbiochem).

**Plasmids:** Full-length cDNA of mouse CHL1 was purchased from Open Biosystems, amplified and subcloned into a pcDNA3.1/V5-His-TOPO vector (K4800-01, Invitrogen). In the CHL1 double mutant the membrane-proximal RGGKYSV sequence (ERM binding domain) was mutated to AGGKASA and the FIGAY sequence (ankyrin binding domain) was mutated to FIGAA (*Fl-Chl1E+A*) (Schlatter et al., 2008). The specific beta-fragments (products of BACE1 cleavage considering the known BACE1 cleavage site on CHL1) and mutations in CHL1 cDNAs were generated using QuikChange II XL Site-directed Mutagenesis Kit (200522, Stratagene) and the following primers:

*Chl1 C-terminal beta fragment (Chl1CTF $\beta$ ):*

Forward(F): 5'-GATGGATGTAATTGAGACAAGAGGGAGAGA-3'

Reverse(R): 5'-TGCCCCGGAGTGGGAAGGTG-3'

*Chl1 N-terminal beta fragment (Chl1NTF $\beta$ ):*

Forward(F): 5'-CGATGATGGAATTGCCATTATGTGG-3'

Reverse(R): 5'-CGTTATTGAAAAATGCTATCGTTATCACCCC-3'

*Chl1 C-terminal beta fragment mutant for the ERM domain (Chl1CTF $\beta$ E):*

Forward(F): 5'-GTGAAGAGGAACGCAGGTGGAAAGGCTTCAGCAAAAGAAAAGG-3'

Reverse(R): 5'-CCTTTTCTTTTGCTGAAGCCTTCCACCTGCGTTCCTCTTCAC-3'

*Chl1 C-terminal beta fragment mutant for the ankyrin domain (Chl1CTF $\beta$ A):*

Forward(F): 5'-GTTTATTGGCGCAGCCACTGGAGCTAAGGAG-3'

Reverse(R): 5'-CTCCTTAGCTCCAGTGGCTGCGCCAATAAAC-3'

Expression constructs encoding *Chl1D1062H* and pCMV-GFP-*ires*-Cre have been described (Fazzari et al., 2010; Zhou et al., 2012). Expression construct encoding GFP (pCMV-GFP) was purchased from BD Biosciences Clontech (PT3262-5, #6074-1). Expression construct encoding Sema3A-RFP was kindly provided by Dr. Joris De Wit (VIB Center for the Biology of Disease, KU Leuven). The expression of all plasmids was tested by Western blot analysis of the cell lysates (Fl-Chl1, *Chl1D1062H*, Chl1CTF $\beta$ , *Fl-Chl1E+A*, *Chl1CTF $\beta$ E+A*, *Chl1CTF $\beta$ E* and *Chl1CTF $\beta$ A*) and conditioned medium (Chl1NTF $\beta$ ) from COS-1 cells using anti-V5 (460705, Invitrogen) antibody for full-length proteins and C-terminal fragments and anti-CHL1 (R&D) antibody for secreted N-terminal fragment (data not shown). The transfections of the different constructs were performed using TransIT-LT1 reagent (MIR2360, Sopachem) according to the manufacturer's protocol.

**BACE1 inhibitor *in vivo* treatments:** Wild-type 5-days old C57Bl/6 mice were subcutaneously dosed with compound J (CpdJ; (Esterhazy et al., 2011)) at 30 mpk or vehicle (20% Captisol) as

control. After 12 h, this treatment was repeated once to prolong the drug effects *in vivo*. Mice were sacrificed 24 h after the first treatment and the brains were snap frozen in liquid nitrogen and stored at -80 °C for further analysis.

**Brain homogenates:** To prepare brain homogenates, each hemisphere from P7 mice was homogenized in ice-cold TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) using a glass-Teflon homogenizer by 12 strokes at 750 rpm. Homogenates were centrifuged at 14000g for 15 min to separate the supernatants (TBS fraction) and the cell pellets. The obtained cell pellets were lysed in DIP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) on ice for 20 min and cleared by centrifugation at 14000g for 15 min. The DIP fractions were analyzed by western blot to detect the full-length or C-terminal fragments of the proteins of interest (Fl-CHL1, Fl-APP and APP-CTFs). Additionally, the DIP fractions were subjected to immunoprecipitation followed by western blot using CHL1CTF $\beta$  antibody (Thermo Fisher Scientific) to detect full-length and specially the carboxyl-terminal fragments of CHL1.

**Preparation of primary neuronal cultures:** Thalamic (ventral basal area), hippocampi or brains were dissected, trypsinized and dissociated by gentle trituration. Cells were re-suspended in MEM (31095-029; Invitrogen) containing 10% horse serum (26050088; Invitrogen), penicillin-streptomycin and 0.6% glucose, and plated into 6-well plates pre-coated with 0.5 mg/ml poly-L-lysine (P2636-1g; Sigma). 2 h after plating, the culture medium was replaced by Neurobasal medium (21103-049, Invitrogen) with B27 supplement (17504-044, Invitrogen). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. For western blotting, thalamic or mixed brain neuronal cultures were prepared from E14 mouse embryos as described (Leyva-Diaz et al., 2014; Wright et al., 2007; Zhou et al., 2012). After 48 h in culture, the cells were treated overnight with 1  $\mu$ M  $\beta$ -secretase inhibitor IV (CIV; 565788, Calbiochem), 50  $\mu$ M

metalloprotease inhibitor (GM6001; 364206, Calbiochem) or 10  $\mu$ M  $\gamma$ -secretase inhibitor (DAPT; 565770, Calbiochem). Next day the cells were treated with lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA and 1% Triton X-100) on ice for 20 min and cleared by centrifugation at 14000g for 15 min. Buffers were supplemented with complete protease (11836145001, Roche) and phosphatase inhibitor cocktails (4906837001, Roche).

**Thalamic explants in collagen:** The ventral area of thalami from E14 mouse embryos from WT, *Bace1*<sup>-/-</sup> and *Chl1*<sup>-/-</sup> mice were dissected into small pieces and placed into droplets of 2 mg/ml collagen (354236, Corning) next to Sema3A-secreting COS-1 aggregates and cultured in Neurobasal medium (21103-049, Invitrogen) with B27 supplement (17504-044, Invitrogen). The explants were maintained at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. WT thalamic explants were also co-cultured with non-transfected COS-1 aggregates as negative control. Some WT thalamic co-cultures were treated with 1  $\mu$ M  $\beta$ -secretase inhibitor IV (CIV). In order to prepare Sema3A-secreting aggregates few droplets of dissociated COS-1 cells previously transfected with Sema3A-RFP were cultured overnight at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (10270-106, Invitrogen). Sema3A-RFP transfections were performed using TransIT-LT1 reagent (MIR2360, Sopachem) according to the manufacturer's protocol. After 48-72 h the co-cultures were fixed with 4% paraformaldehyde in 1X phosphatase-buffered saline, immunostained with anti-tubulin  $\beta$ III antibody (T5076; Sigma) and observed under a Nikon A1R Eclipse Ti confocal microscope. The level of Sema3A-induced repulsion is represented by a proximal/distal (P/D) ratio, which compares axonal growth on proximal and distal side of the thalamic explant towards the COS-1 aggregate. Explants were divided into four sectors (Figure 1A1) and the guidance effect was quantified by the fluorescence intensity

(measured with Image J software) of the quadrants that are proximal and distal to the COS-1 cell aggregate.

**Live-cell imaging of thalamic neurons:** For live-cell imaging WT thalamic neurons were plated into 96-well plates (655180, Greiner bio-one) pre-coated with 0.5 mg/ml poly-L-lysine. After 48 h in culture, the neurite growth was recorded using a InCell Analyzer 2000 (GE Healthcare). The cells were treated with 5 nM Sema3A-Fc and images were acquired every 2 min for 30 min. The cells were then washed three times with warm Neurobasal medium and recovery was recorded every 2 min for 30 min in Neurobasal medium supplemented with B27 supplement and either treated with  $\gamma$ -secretase inhibitor (DAPT) or vehicle (DMSO). Per well ten fields of view were recorded. To correct for drift during the time-lapse due to the washing the sequences were registered. To facilitate the registration and consequent analysis the images were processed to enhance the edges. Neurite length was measured (Fiji/ImageJ software) at 0 min, 30 min after Sema3A treatment and at 30 min after the washing step and treatment with vehicle or DAPT and was then normalized to the 0 min neurite length for each cell (% of control). In total ~ 40 cells per well were analyzed in three independent treatments.

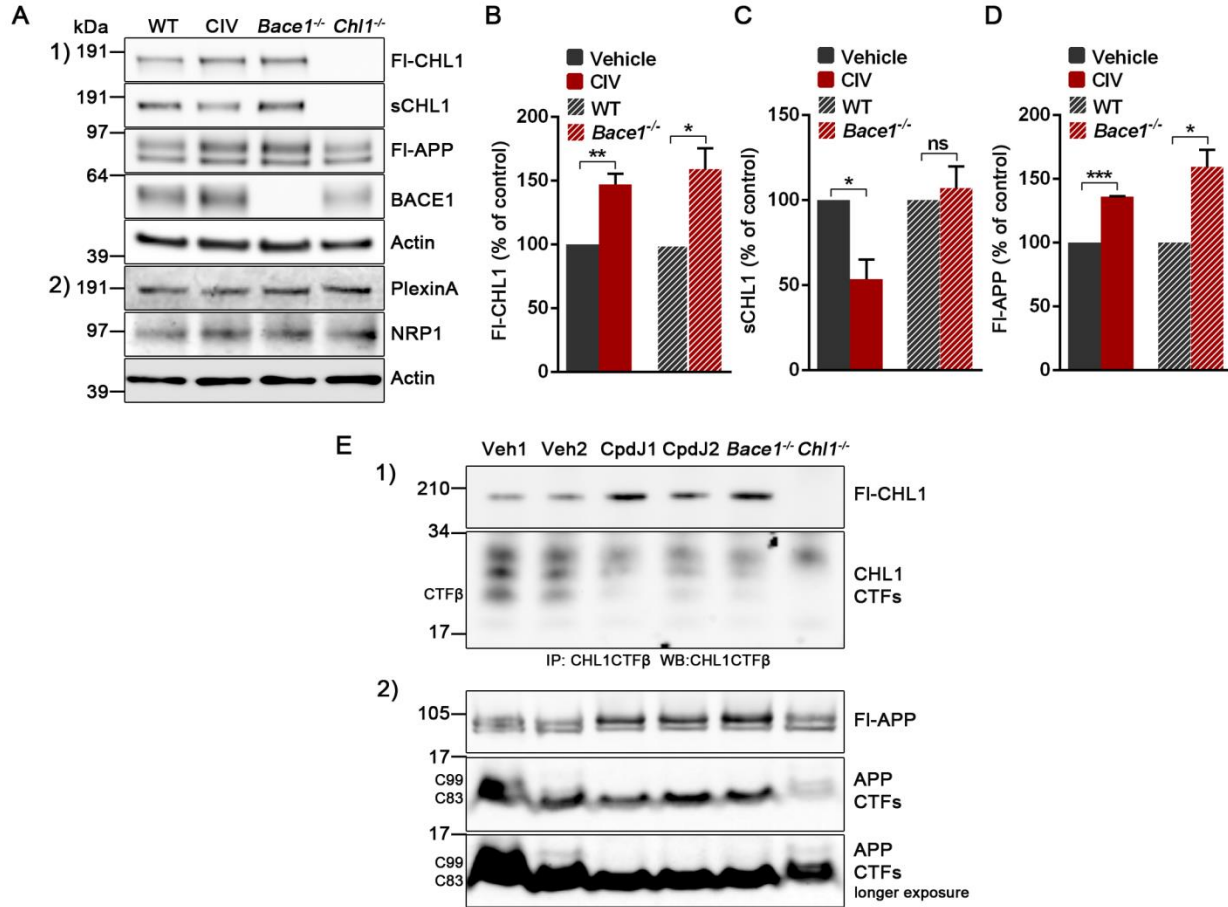
**Quantification of soluble A $\beta$  peptides using sandwich ELISA:** After 48 h in culture, cells were treated overnight with 0.003, 0.01, 0.03, 0.1, 0.3, 1 or 3  $\mu$ M  $\beta$ -secretase inhibitor IV (CIV). The conditioned media were collected and analyzed by sandwich ELISA for soluble amyloid-beta peptide 1-40 (A $\beta$ <sub>1-40</sub>). NUNC Ninety-six-well plates (life technologies) were coated with 1.5 mg/ml A $\beta$ <sub>x-40</sub> capture antibody (JRF/cA $\beta$ 40/28) dissolved in coating buffer (10 mM Tris-HCl, 10 mM NaCl, 10 mM NaN<sub>3</sub>, pH 8.5). After overnight incubation at 4 °C, the plates were rinsed with 0.05% Tween 20 in 1X Phosphate Buffered Saline and blocked with casein buffer (1% Casein in 1X Phosphate Buffered Saline, pH 7.4) for 4 h at room temperature. Synthetic mouse A $\beta$ <sub>1-40</sub> peptide was diluted in casein buffer and used as a standard. Standard and samples were

mixed with the detection antibody (JRF/rA $\beta$ /2) and added to the ELISA plate. After overnight incubation at 4 °C, the plate was washed and developed with 0.2 mg/ml of 3,5,3',5'-tetramethylbenzidine (TMB, Sigma) dissolved in 100 mM sodium acetate (NaAc, pH 4.9) supplemented with 0.03% H<sub>2</sub>O<sub>2</sub>. The reactions were allowed to proceed for maximum 15 min at room temperature. The reactions were stopped by adding 2 N H<sub>2</sub>SO<sub>4</sub> and the plate was read on a Perkin Elmer Envision 2103 multilabel reader at 450 nm.

### **Accession numbers section**

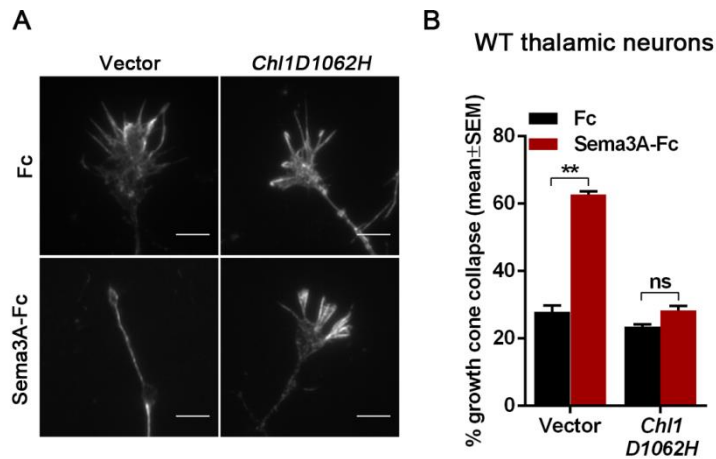
DNA sequence of mouse CHL1: GenBank:BC131670.1 (cDNA clone MGC:150204)

## Supplemental Data

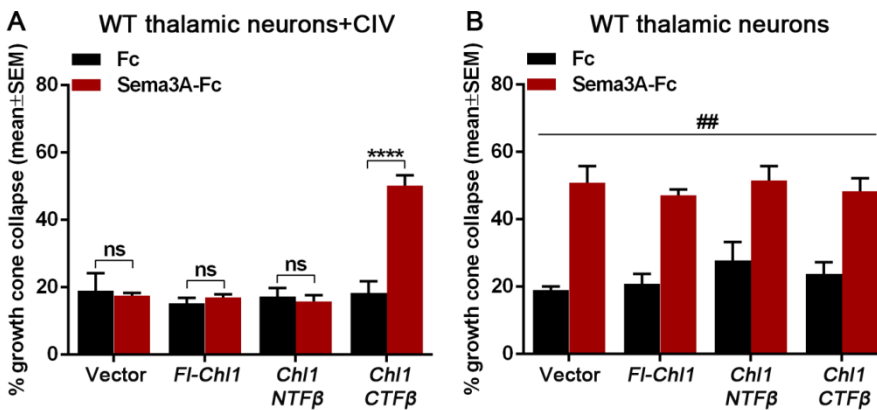


**Figure S1 CHL1 processing by BACE1 - related to Figure 1** (A) Western blot analysis of the conditioned medium and cell lysates from WT, BACE1 inhibitor-treated (1  $\mu$ M CIV), *Bace1*<sup>-/-</sup> and *Chl1*<sup>-/-</sup> thalamic neurons (B-D) Quantification of the western blot analysis for full-length CHL1 (FI-CHL1), soluble CHL1 (sCHL1) and full-length APP (FI-APP). Results are presented as mean  $\pm$  standard error of the mean (SEM). (E) Although the levels of endogenous CHL1CTF $\beta$  are below detection limits in young WT thalamic neurons, we were able to detect these fragments and their significant reduction after BACE1 inhibition in brain homogenates of 7-days old mice. The weak remaining band in the *Bace1*<sup>-/-</sup> is likely reflecting residual cleavage by a metalloprotease known to cleave CHL1 (see results section).

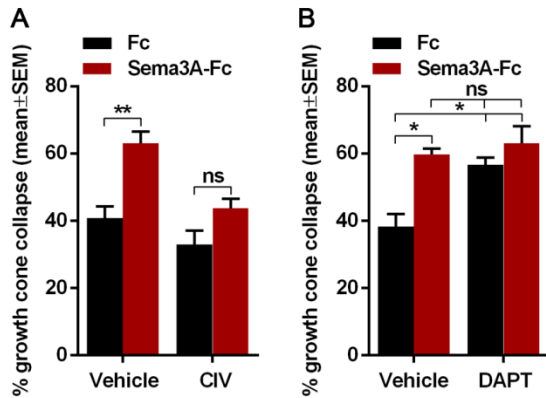




**Figure S2** CHL1 processing by BACE1 is required for Sema3A-induced growth cone collapse in thalamic neurons - related to Figure 2 (A, B) Analysis of Sema3A-induced growth cone collapse in WT thalamic neurons transfected with empty vector or *Chl1D1062H*. Results are presented as mean±standard error of the mean (SEM). Scale bars 5  $\mu$ m.



**Figure S3** CHL1CTF $\beta$  fragment is required for Sema3A-induced growth cone collapse in thalamic neurons - related to Figure 3 (A) Quantification of Sema3A-induced growth cone collapse in BACE1 inhibitor-treated (1  $\mu$ M CIV) and (B) WT thalamic neurons transfected with empty vector, *Fl-Chl1*, *Chl1NTF $\beta$*  or *Chl1CTF $\beta$* . Results are presented as mean±standard error of the mean (SEM).



**Figure S4 CHL1 processing by BACE1 and  $\gamma$ -secretase also regulates Sema3A-induced growth cone collapse in hippocampal neurons - related to Figure 1 and Figure 4 (A)** Quantification of Sema3A-induced growth cone collapse in WT and BACE1 inhibitor-treated (1  $\mu$ M CIV) hippocampal neurons **(B)** Quantification of Sema3A-induced growth cone collapse in WT and  $\gamma$ -secretase inhibitor-treated (10  $\mu$ M DAPT) hippocampal neurons. Results are presented as mean $\pm$ standard error of the mean (SEM).

**Movie S1 Growth cone collapse and recovery when thalamic neurons are treated with vehicle – DMSO - related to Figure 4** Sema3A was added to cultured WT thalamic neurons for 30 min to induce growth cone collapse and neurite retraction and was then removed. Within 30 min of Sema3A washout WT thalamic neurite growth began to recover.

**Movie S2 Growth cone collapse and absence of recovery when thalamic neurons are treated with  $\gamma$ -secretase inhibitor – DAPT - related to Figure 4** Sema3A was added to cultured WT thalamic neurons for 30 min to induce growth cone collapse and neurite retraction and was then removed. Within 30 min of Sema3A washout the WT thalamic neurons treated with the  $\gamma$ -secretase inhibitor (DAPT) continued to retract their neuritis.

### **Supplemental references**

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