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BACE1 regulates the proliferation and cellular functions of Schwann cells

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Abstract

BACE1 is an indispensable enzyme for generating β -amyloid peptides, which are excessively accumulated in brains of Alzheimer's patients. However, BACE1 is also required for proper myelination of peripheral nerves, as BACE1-null mice display hypomyelination. In order to determine the precise effects of BACE1 on myelination, here we have uncovered a role of BACE1 in the control of Schwann cell proliferation during development. We demonstrate that BACE1 regulates the cleavage of Jagged-1 and Delta-1, two membrane-bound ligands of Notch. BACE1 deficiency induces elevated Jag-Notch signaling activity, which in turn facilitates proliferation of Schwann cells. This increase in proliferation leads to shortened internodes and decreased Schmidt-Lanterman incisures. Functionally, evoked compound action potentials in BACE1-null nerves were significantly smaller and slower, with a clear decrease in excitability. BACE1-null nerves failed to effectively use lactate as an alternative energy source under conditions of increased physiological activity. Correlatively, BACE1-null mice showed reduced performance on rotarod tests. Collectively, our data suggest that BACE1 deficiency enhances proliferation of Schwann cell due to the elevated Jag1/Delta1-Notch signaling, but fails to myelinate axons efficiently due to impaired the neuregulin1-ErbB signaling, which has been documented.

Keywords

ACE1; Alzheimer's β -secretase; Jagged; Notch; myelination; myelin sheath; node of Ranvier; Schmidt-Lanterman clefts

INTRODUCTION

BACE1, a membrane-bound aspartyl protease, was discovered to be the Alzheimer's β -secretase for cleaving amyloid precursor protein (APP) to generate β -amyloid peptides (A β) (Vassar et al., 1999; Yan et al., 1999; Hussain et al., 1999; Sinha et al., 1999; Lin et al., 2000). BACE1 is highly expressed by neurons and plays important roles in nerve development and function (Vassar et al., 2014). During early murine development, axonal BACE1 is required

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for normal peripheral nerve myelination (Hu et al., 2006; Willem et al., 2006). In BACE1-null mice, myelin sheath thickness is significantly reduced. Peripheral nerve hypomyelination also occurs in BACE1-null zebrafish (van et al., 2013). Genetic deletion of BACE1 also attenuates peripheral nerve remyelination following sciatic nerve crush (Hu et al., 2008).

Hypomyelination in BACE1-null mice is partially attributed to loss of BACE1-mediated cleavage of type III $\beta 1$ neuregulin-1 (Nrg1). Axonally-expressed type III $\beta 1$ Nrg1 controls myelination in the peripheral nervous system (Michailov et al., 2004; Taveggia et al., 2005). Nrg1 has approximately 33 isoforms (Mei and Nave, 2014; Hu et al., 2016) and BACE1 can cleave both types I and III $\beta 1$ Nrg1 (Hu et al., 2008; Fleck et al., 2013; La et al., 2011a). After nerve crush, type I Nrg1 is also required for optimal remyelination of regenerated axons, as mice deficient in axonal Nrg1 are initially hypomyelinated (Stassart et al., 2013; Fricker et al., 2011) but eventually attain levels of remyelination comparable to wild-type (WT) nerves (Fricker et al., 2013). This indicates that axonal Nrg1 is required for initial phases of remyelination. Further studies suggest that type I Nrg1 in Schwann cells, rather than axonal type III Nrg1, contributes to remyelination, as loss of Nrg1 expression in Schwann cells severely impairs remyelination after nerve crush (Stassart et al., 2013). Consistent with this observation, we have recently shown that BACE1 in Schwann cells is required for remyelination, as demonstrated in a nerve transplantation experiment using BACE1-null and WT Schwann cells (Hu et al., 2015). We also showed that nerve injury induces expression of BACE1 and type I Nrg1 in Schwann cells and that BACE1-cleaved Nrg1 mediates remyelination following sciatic nerve crush. Type III Nrg1 is sparsely detected in Schwann cells, indicating that optimal remyelination requires type I Nrg1 in Schwann cells and type III Nrg1 in axons (Stassart et al., 2013).

During remyelination of peripheral nerves in BACE1-null mice, we observed a significant increase in glial fibrillary acidic protein (GFAP) levels in Schwann cells (Hu et al., 2013), which supports the possibility that Schwann cell proliferation is increased in BACE1-null peripheral nerves. Why increased proliferation of Schwann cells leads to hypomyelination, rather than hypermyelination, in BACE1-null mice remains to be determined. Therefore, we investigated Schwann cell maturation, myelin internode ultrastructure, and the molecular mechanism underlying increased proliferation of Schwann cells upon BACE1 ablation. We found that BACE1 deficiency abolished cleavage of Jagged-1/Delta-1 and increased Notch signaling activity, which promotes proliferation of Schwann cells. However, the increased number of Schwann cells failed to enhance myelination due to reduced Nrg1 signaling in BACE1-null axons. BACE1-null mice exhibited a decrease in myelin internodal length and Schmidt–Lanterman incisures. Functional assays revealed significantly impaired peripheral nerve conduction and motor performance on the rotarod test. These studies link alterations in Jag-Notch signaling with the control of Schwann cell proliferation. Collectively, our data show that BACE1 in both axons and Schwann cells is indispensable for normal peripheral nerve function.

MATERIALS AND METHODS

Animals

BACE1-null mice were generated as described in the original publication (Cai et al., 2001) and maintained in a C57BL/6 background. All animal use and procedures were first described and submitted for approval by the Institutional Animal Care and Use Committee at the Lerner Research Institute and in compliance with the guidelines established by the Public Health Service Guide for the Care and Use of Laboratory Animals.

Electrophysiology of nerve conduction

Adult WT and BACE1-null male mice at 1 month of age were euthanized after CO₂ asphyxiation. A lateral incision was made to expose the hind limb muscles. The sciatic nerve was identified, traced along its length, and cut proximally close to the spinal column and distally before the branching of the nerve. The sciatic nerve was gently freed from the surrounding tissue and placed in an interface perfusion chamber (Medical Systems Corp., Greenvale, NY). Nerves were maintained at 37°C and perfused with artificial cerebrospinal fluid (aCSF) that contained (in mM): 124 NaCl, 3.0 KCl, 2.0 CaCl₂, 2.0 MgCl₂, 1.25 NaH₂PO₄, 23 NaHCO₃, and 10 glucose. The aCSF was bubbled with a 5% CO₂-containing gas mixture (95% O₂, 5% CO₂) to maintain pH at 7.45. The nerve was continuously exposed to a moisturized gas mixture containing 95% O₂ and 5% CO₂. Suction electrodes filled with aCSF were attached to the nerve for stimulation and recording of compound action potentials (CAPs) after the nerves were equilibrated for 60 min. CAPs were evoked every 30 s and stimulus strength was adjusted to evoke the maximum CAPs and then increased another 25% to ensure that stimulus strength was always supramaximal (A365R Stimulus Isolator, WPI, Sarasota, FL). All experiments were performed using an Axoclamp 900A amplifier (Molecular Devices Inc, Sunnyvale, CA) interfaced to a personal computer with a Digidata 1400A analog/digital interface and the pCLAMP 10.2 software package (Molecular Devices Inc, Sunnyvale, CA). Signals were amplified 50 times, low-pass filtered at 100 kHz (SR560, Stanford Research Systems, Sunnyvale, CA), and digitized at 50 kHz before being stored for offline analysis. Areas under CAPs were measured to quantify and compare axon function. The CAP area, time to peak (latency), peak amplitude, and peak duration were measured using Clampfit 10.2 software (Molecular Devices Inc, Sunnyvale, CA). CAP areas were normalized to the baseline condition. To study axon function response to a physiological stressor, two sets of high frequency stimulations (100 Hz for 4 min and 100 Hz for 10 min) were applied sequentially after an initial baseline period, with 15 min of recovery in between. After the last high frequency simulation, nerves were allowed to recover for at least 2 h. To determine axon excitability, nerves were stimulated using a range of different stimulus intensities from minimum to maximum with stepwise increments to determine threshold to evoke a CAP and maximal stimulation to generate stimulus intensity curves. All data were normalized to the control values.

Schwann cell primary cultures

Five-day-old pups (P5) were killed by decapitation and cleaned by brief submersion in 70% ethanol. Both sides of sciatic nerves were dissected out and digested with 2 mL of PBS containing 0.125% trypsin and 0.05% collagenase A for 30 min at 37°C. After washing with

DMEM medium containing 10% horse serum and centrifuging at 190g for 5 min, the pallet cells were suspended in Schwann cell basic growth medium (DMEM containing 10% horse serum, 2ng/ml neuregulin1- β 1, 100U/ml penicillin and streptomycin, 2mM L-glutamine, and 0.5 μ M forskolin) and plated on ploy-L-lysine coated 60-mm culture dishes, with medium being changed every three days. Six days after culturing, cells were treated with 4 μ g/ml of anti-mouse Thy-1.2 antibody and 200 μ l/ml rabbit complement serum for 2 h at 37°C to kill fibroblasts and incubated with Schwann cell growth medium (basic growth medium with 20 μ g/ml bovine pituitary and 10 ng/ml FGFb). When reaching ~80% confluence, cells were harvested for western blotting.

Western blotting and antibodies

Proteins were extracted from WT and BACE1-null mice in RIPA buffer [50 mM Tris-HCl at pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1 mM NaF, 1 mM Na₃VO₄ and a protease inhibitor cocktail (Roche)]. Equal amounts of protein (50 μ g) were resolved on a NuPAGE Bis-Tris Gel (Invitrogen, Palo Alto, CA) and transferred onto nitrocellulose membranes (Invitrogen) for western blot analysis. HRP-conjugated secondary antibodies were used and visualized using enhanced chemiluminescence (Thermo Scientific). Jagged1 (1:200), Jagged2 (1:200), and Delta1 (1:200) antibodies were purchased from Santa Cruz (Santa Cruz, CA). Notch1-ICD (1:1,000) was purchased from Cell Signaling (Boston, MA) and β -actin (1:10,000) was purchased from Sigma (St. Louis, MO).

Sciatic nerve teasing and light microscopy

After being perfused with 4% paraformaldehyde/PBS fixing buffer, sciatic nerves were dissected out from both WT and BACE1-null mice at four months old. All nerves were incubated with 1% osmium tetroxide for 2 h at room temperature and then treated with 45%, 66% and 100% glycerin, each for 24 h at 45°C. Each nerve was placed on a glass slide along with a few drops of 100% glycerol and separated from the proximal to the distal using Dumont microforceps (No. 5) under a dissecting microscope, into smaller bundles of axons until individual axons could be separated. Axonal pictures were then taken under a 20 $\times$ light microscope. Using Image J software, the internodal length was measured and the number of Schmidt-Lanterman incisures was calculated per internode.

Morphological analyses

For confocal microscopy experiments, the indicated genotypes of mice were perfused with 4% paraformaldehyde. The middle part of sciatic nerves (6 mm length) was dissected out from both side nerves. The nerve segments were cut on a cryostat (Microm GmbH, Walldorf, Germany). Serial 14 μ m longitudinal or transversal sections were selected at five-section intervals for immunofluorescent staining with specific primary antibodies. After washing with PBS three times, sections were incubated with the secondary antibody goat anti-mouse or rabbit IgG (1:400) conjugated with Alexa fluor 488 or 568 (Molecular Probes). All nerve images were captured by a Leica SP5 confocal microscope and cells were counted using Image J software. EGR2 (1:500), Sox2 (1:500), and NCAM (1:500) antibodies were purchased from Millipore (Billerica, MA). Sox10 (1:500) and BrdU (1:200) antibodies were purchased from Abcam (Cambridge, MA).

For three-dimensional electron microscopy (3D-EM), animals were first subjected to transcardial perfusion with 4% paraformaldehyde and 2.5% glutaraldehyde for fixation. Sciatic nerves were then surgically removed and immersed in fixative solution overnight at 4°C and then processed for EM examination.

Statistical Analyses

Statistical analyses were performed using Microsoft Excel software (Microsoft Corp) or Graphpad Prism 4.0 (GraphPad Software, Inc). All data were analyzed for statistical significance using an *F*-test for equal variance followed by a two-tailed Student's *t*-test or verified by an ANOVA test. Significant *P* values are denoted by the use of asterisks in the text and figures (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). All data values are expressed as mean ± s.e.m.

RESULTS

BACE1 deficiency increases the number of Schwann cells

To determine whether BACE1 deficiency affects proliferation of Schwann cells, we examined sciatic nerves from BACE1-null and WT mice at 1 month of age and labeled Schwann cell nuclei with an antibody specific to EGR2 (also known as Krox-20), which is a master regulatory gene for myelination in Schwann cells (Topilko et al., 1994). When cross-sections of nerves were examined, a greater number of EGR2⁺ cells was found in one-month-old BACE1-null nerves compared to their WT littermates (Figure 1A), as the average number of EGR2⁺-cells was 127.50±3.78 in BACE1-null nerves per examined section *vs.* 101.33±4.98 in WT controls. We further examined EGR2⁺ cells using fixed longitudinal sections and found that the number of EGR2⁺ cells in BACE1-null nerves was increased compared to controls (Figure 1B). The average number of EGR2⁺ cells remained increased in BACE1-null nerves at 3 months of age (Figure 1C, 133.63±5.20 in BACE1-null sections *vs.* 109.63±2.69 in WT sections; *n*=7 sections/animal, 3 animals in each group, *P*<0.01, Student's *t*-test). Although WT nerves are thicker than BACE1-null nerves, the average number of myelinating Schwann cells, in which EGR2 is mainly expressed (Jessen and Mirsky, 2002), was greater in BACE1-null nerves than in WT controls in both age groups (Figure 1D).

Furthermore, we stained nerve sections from the same age groups with an antibody to Sox10, which is a critical regulator of Schwann cell myelination (Britsch et al., 2001; Kuhlbrodt et al., 1998; Lopez-Anido et al., 2015). We found that the number of Sox10-labeled Schwann cells was greater in one-month-old BACE1-null nerves (113.47±4.56 per cross-section) compared to WT control nerves (95.65±3.87; Figure 1E; *n*=7 sections, 3 animals in each group, *P*<0.01, Student's *t*-test). In three-month-old nerves, the number of Sox10-labeled Schwann cells was also greater in BACE1-null (122.08±4.93 per cross-section) compared to WT (103.26±3.79). Overall, the average number of Sox10-labeled Schwann cells was significantly greater in BACE1-null nerves compared to WT controls (Figure 1F, *n*=7 sections, 3 animals in each group, *P*<0.01, Student's *t*-test). Collectively, these results indicate that BACE1 deficiency increases the number of myelinating Schwann cells through adulthood.

We also asked whether BACE1 deficiency affects the maturation of Schwann cells. To address this question, we stained fixed sciatic nerves from postnatal day 8 (P8) mice with antibodies specific to Sox2, which is a marker for undifferentiated stem cells and is expressed by Schwann cell precursors and immature Schwann cells (Le et al., 2005). P8 BACE1-null nerves had a greater number of Sox2-positive Schwann cells, as viewed in both transverse (Figure 2A) and longitudinal sections (Figure 2B), compared to WT nerves. At one month of age, the overall number of Sox2-positive Schwann cells was reduced in sciatic nerves, but BACE1-null nerves still had a higher population of Schwann cells (Figure 2C–D; 31.47 ± 2.62 per WT vs. 43.53 ± 2.97 per BACE1-null cross-section; $n=7$ sections, 3 animals in each group, $P<0.01$, Student's *t*-test) compared to WT nerves. Increased Sox2-positive Schwann cells in BACE1-null nerves will presumably contribute to the increase in myelinating Schwann cells, which are differentiated from immature Schwann cells (Jessen and Mirsky, 2005).

BACE1 deficiency promotes proliferation of Schwann cells

To determine whether the increased number of Schwann cells in BACE1-null nerves is due to increased Schwann cell proliferation, we performed BrdU pulse labeling experiments using P8 mice. Twenty-four hours after intraperitoneal injection of BrdU, we examined BrdU-positive nuclei in fixed sciatic nerve sections. We detected more BrdU-positive nuclei in BACE1-null nerves compared to WT littermate controls (Figure 3A–B; 30.14 ± 2.36 BrdU⁺-cells per cross-section in WT vs. 51.60 ± 2.54 in BACE1-null sciatic nerves, $n=7$ sections, 3 animals in each group, $P<0.01$, Student's *t*-test), indicating more dividing cells in BACE1-null nerves. Co-staining of sections with an antibody to Sox2 confirmed that the majority of BrdU⁺-cells were attributable to Schwann cells (Figure 3C–D; 18.27 ± 1.78 double positive-cells in WT vs. 25.49 ± 2.05 in BACE1-null; $n=7$ sections, 3 animals in each group, $P<0.01$, Student's *t*-test). Hence, increased dividing cells were attributed to proliferation of Schwann cells.

BACE1 deficiency shortens internodes and paranodes

We next investigated whether increased myelinating Schwann cells would alter internodal length and/or paranodal structures in BACE1-null sciatic nerves. Fixed sciatic nerve sections were labeled with an antibody specific to caspr, which is an axonal membrane protein normally localized in paranodal axolemma (Dupree et al., 1999). The number of paranodes was dramatically increased in BACE1-null sciatic nerves compared to WT controls (Figure 4A: 120.6 ± 21.9 per $100,000 \mu\text{m}^2$ in WT vs. 286.9 ± 46.0 in BACE1-null sciatic nerves, $n=5$ animals, $P<0.01$, Student's *t*-test), suggesting a shortened internodal length in BACE1-null mice. When internodal length was examined in teased sciatic nerve fibers (Figure 4B), the internodes of BACE1-null sciatic nerves were visibly shorter compared to WT controls. Further quantification revealed that the internodal length of WT sciatic nerves was $618.0 \pm 23.0 \mu\text{m}$, while that of BACE1-null sciatic nerves was $461.0 \pm 20.0 \mu\text{m}$ on average ($P<0.01$, $n=40$).

Morphologically, nodes of Ranvier are arranged as short gaps between two adjacent myelinated segments or internodes (Girault and Peles, 2002; Poliak and Peles, 2003). The above caspr staining of nodes indicated that paranodal staining was reduced in intensity and

asymmetrically distributed across BACE-null nodes compared to WT nodes (red circles, Figure 4A). To further examine the nodal ultrastructure, we examined nodes using serial blockface scanning electron microscopy (SEM), which provides serial-sectioned images of entire paranodal/nodal regions with nanometer resolution. Surprisingly, there appeared to be no major differences in paranodal ultrastructure between WT and BACE1-null axons (data not shown). Myelin lamellae terminate as cytoplasmic loops on the paranodal axolemma (see example in Figure 4C). Although outpocketing and crenellation of juxtaparanodal compact myelin were seen in BACE-null axons, major disruptions, such as paranodal loops displaced from the axolemma or terminating internodally, tomacular overgrowth, or myelin breakdown, were not detected. Despite no obvious ultrastructural differences, measurements of paranodal distance (first to last paranodal loop) revealed shorter paranodal length in large sizes of BACE-null axons (diameter $>2\text{ }\mu\text{m}$) compared to controls, but not obvious in smaller axons (Figure 4D). Reduced caspr staining at BACE-null nodes is likely to reflect fewer paranodal loops due to fewer compact myelin lamellae. Despite no overt change in nodal ultrastructure, we cannot eliminate the possibility that the molecular architecture of paranodal loops is disrupted without major changes in axoglial organization.

In Figure 4B, we also noted less Schmidt–Lanterman incisures within an internode in BACE1-null sciatic nerves (Figure 4B; 20.9 ± 1.2 in WT vs. 13.4 ± 0.7 in BACE1-null nerves; $P<0.01$, $n=40$). Reduced numbers of Schmidt–Lanterman incisures were also evident when fixed BACE1-null sciatic nerves were stained with an antibody specific to N-CAM, a neuronal surface adhesion molecule (Figure 4E). Collectively, BACE1 deficiency results in shortened internodal lengths, decreased Schmidt–Lanterman incisures, and altered nodes of Ranvier in large axons.

BACE1 deficiency impairs nerve conduction

To determine the impact of altered Schwann cell proliferation on axonal excitability and conduction properties, we measured compound action potentials (CAPs) evoked in isolated sciatic nerves from BACE1-null and WT mice (Figure 5A). Sciatic nerves were placed in between two suction electrodes delivering stimulation to the proximal side and recording evoked responses from the distal side. This recording configuration allows stimulation of all axons in the sciatic nerve while recording a CAP elicited from all axons, thereby providing information on the number, speed, and spatiotemporal summation of axonal function in a nerve bundle. Recordings from sciatic nerves obtained from WT mice showed rapidly rising CAPs with a single sharp peak, while those evoked from BACE1-null nerves showed slowly rising CAPs with lower amplitude and a blunt peak. Quantification showed increased time to CAP peak (latency) (Figure 5A, measurement depicted in magenta) by more than 3-fold in BACE1-null nerves compared to WT controls (Figure 5B, 1.0 ± 0.3 in 4 WT vs. 3.4 ± 0.3 in 6 BACE1-null sciatic nerves, $P<0.001$, two-tailed Student's t-test). CAP peak amplitude (Figure 5A, depicted in green) in BACE1-null nerves was also decreased by more than 50% (Figure 5C, 1.0 ± 0.2 in 4 WT vs. 0.4 ± 0.1 in 6 BACE1-null sciatic nerves, $P<0.05$, two-tailed Student's t-test). As a result of a slower spatiotemporal summation of axon function, CAP duration (Figure 5A, depicted in blue), was increased by 2.5 fold in BACE1-null nerves (Figure 5D, 1.0 ± 0.1 in 4 WT vs. 2.5 ± 0.1 in 6 BACE1-null sciatic nerves, $P<0.001$, two-tailed Student's t-test). Due to the dramatic increase in CAP duration, which

compensated for the reduction in CAP peak amplitude, CAP area measurements (which take peak amplitude and duration into account) were similar between the two groups (Figure 5E, 1.0 ± 0.26 in 4 WT vs. 0.74 ± 0.08 in 6 BACE1-null sciatic nerves, $P=0.33$, two-tailed Student's t-test).

Consistent with the altered axonal conduction properties, deletion of BACE1 caused a drastic decrease in axon excitability, as demonstrated by a prominent rightward shift in stimulus intensity plots (Figure 5F). The threshold to evoke a CAP increased from 50 μ A to 100 μ A in BACE1-null mice (Figure 5F, arrows). This shift suggests a direct role for BACE1 and resultant hypomyelination in regulating axon excitability and conduction.

BACE1 deficiency impairs rotarod performance and metabolic responses

Considering the altered nerve structure and myelination as well as the alterations in nerve conduction, we asked whether BACE1-null mice would behave differently on the rotarod performance test, which evaluates balance, endurance, and motor coordination. BACE1-null mice showed significantly impaired behaviors on the rotarod test, as the latency of falling from the rotarod was significantly reduced in two age groups of BACE1-null mice (Figure 6A: 134.2 ± 13.2 s in 3-month-old WT vs. 103.5 ± 10.1 s in BACE1-null mice; 164.2 ± 14.1 s in 10-month-old WT vs. 125.6 ± 12.7 s in BACE1-null mice, $n=20$ mice in each group, $P<0.01$). Impaired rotarod performance in BACE1-null mice is consistent with the corresponding impairment in nerve conduction properties.

On the other hand, the impaired performance of BACE1-null mice on the rotarod test might also be attributable to altered energy utilization, especially during high physical activity (Tekkok et al., 2005; Baltan, 2015; Brown et al., 2003a). High frequency stimulation (HFS) is a physiological challenge that can assess the ability of peripheral nerves to switch from glucose to lactate as an energy source (Evans et al., 2013a). Exposing sciatic nerves obtained from WT nerves to HFS (100 Hz) for 4 min (short) or 10 min (long) showed that CAPs instantly recovered to baseline levels after 4 min HFS and recovered to $90 \pm 1\%$ after 10 min. In contrast, HFS stimulation for 4 min drastically suppressed CAPs in BACE1-null mice down to 17% of control levels, which recovered to about 30%. A 10 min HFS stimulation reduced CAPs to just 12% of baseline levels (Figure 6B, 0.99 ± 0.01 in 3 WT vs. 0.12 ± 0.11 in 6 BACE1-null nerves, $p=0.0008$, two-tailed Student's t-test). Axons alternate between glucose and lactate as an energy source, but prefer lactate during intense activity or when glucose levels are low (Brown et al., 2003; Tekkok et al., 2005; Baltan, 2015). Irreversible depression in axon function following increased activity suggests that; 1) BACE1 deletion may impair glycogen storage in Schwann cells so that lactate cannot be produced, 2) glycogen is present and converted to lactate but cannot be delivered to axons due to a reduction and/or depletion of MCT1 (Morrison et al., 2015), and 3) lactate is produced and delivered properly but axonal mitochondria cannot use lactate due to dysfunction. BACE1 seems to play an important role in axonal metabolic responses during a physiological challenge such as HFS; therefore, the above mechanisms are currently under further investigation.

BACE1 deficiency alters Jag-Notch signaling function

To understand the molecular mechanism underlying increased Schwann cell proliferation, we examined changes in relevant proteins. Sciatic nerves in 1-month-old mice were used for western blot analysis. We found that BACE1-null samples showed significantly elevated levels of full length Jag1 and Delta1, but not full length Jag2 (Figure 7A). This is consistent with previous studies demonstrating that Jag1 is more effectively cleaved by BACE1 than Jag2 (Hu et al., 2013; He et al., 2014). Jag1 and Delta1 are membrane-anchored ligands of Notch molecules and their binding to Notch induces activation of Notch by inducing release of Notch Intracellular Domain (NICD). Levels of NICD were significantly increased in BACE1-null nerves compared to WT controls (Figure 7A, 7C). In addition, levels of Jag1, Delta1, and NICD were significantly increased in Schwann cells isolated from P5 BACE1-null mice (Figure 7B). Further quantification confirmed a significant increase in NICD levels in BACE1-null Schwann cells (Figure 7D), suggesting that reduced processing of these Notch ligands by BACE1 increases Notch signaling activity on the cell surface. This is mechanistically important, as our data are in line with a prior report that enhanced Notch activity promotes Schwann cell proliferation (Woodhoo et al., 2009).

Mapping the cleavage site of Delta1 by BACE1

We have previously confirmed cleavage of Jag1 by BACE1 (He et al., 2014). To confirm the cleavage of Delta1 by BACE1, we synthesized one long peptide with 40 amino acids (GYVCECARGYGGPNCQFLPELPPGPAVVDL-TEKLEGQGG), which spans the immediate ectodomain region, for mapping the cleavage site as discussed previously (Hu et al., 2008; Luo et al., 2011). This peptide potentially contains the putative cleavage sites based on our initial sequence analysis. Cleavage of this peptide, which showed high purity and had the expected molecular weight (Figure 8A), by recombinant BACE1 in our enzymatic assay showed that BACE1 cleaved Delta1 mainly between DL-TE (Figure 8B). This cleavage site is 9 amino acids upstream of the Delta1 transmembrane domain and is comparable to that in Jag1 and Nrg1 (Hu et al., 2008; He et al., 2014). We also observed another cleavage product between VC-EC at the N-terminal side of this peptide, as the cleaved product matched molecular mass (Figure 8B). In the case of APP and Jag1, two BACE1 cleavage sites were also present (Liu et al., 2002; Qahwash et al., 2004; He et al., 2014). While future mutagenesis will be performed to explore which site is predominant, this mapping study shows that Delta1 has BACE1 cleavage sites similar to Jag1.

DISCUSSION

BACE1 is a membrane-anchored aspartyl protease; its physiological substrates are membrane-bound proteins (Hemming et al., 2009; Yan et al., 2001; Kuhn et al., 2012; Dislich et al., 2015). While BACE1 inhibition will benefit Alzheimer's patients by reducing excessive accumulation of A β peptides, BACE1 inhibition also likely affects cellular processes associated with inhibited cleavages of other BACE1 substrates. Previous studies have focused on the role of BACE1-cleaved Nrg1 in axons (Hu et al., 2016; Fleck et al., 2012). We show here that BACE1 regulates peripheral nerve myelination by directly altering the size and functions of myelinating Schwann cells. This study provides the first evidence

that BACE1 also controls myelination by directly regulating Jag1/Delta1-Notch activity in Schwann cells.

In developing peripheral nerves, immature Schwann cells are known to differentiate into mature myelinating Schwann cells and non-myelinating Schwann cells (Jessen and Mirsky, 2005; Monk et al., 2015). Non-myelinating Schwann cells usually bundle small axons (<1 mm diameter) into Remak bundles, while myelinating Schwann cells associate with and ensheath larger axons in a 1:1 ratio (Roberts and Lloyd, 2012). Although each Schwann cell ensheathes only one axon, one axon is longitudinally and continuously wrapped by multiple Schwann cells, with gaps formed by nodes of Ranvier. Proper myelination of axons is essential for saltatory conduction of neuronal action potentials. The controlling signals for myelination are derived from both axons and Schwann cells. Neurotrophic factors such as type III $\beta 1$ Nrg1 in axons provide instructive signals to Schwann cells to regulate the myelination process. Specifically, the level of axonal type III $\beta 1$ Nrg-1 isoform controls myelin sheath thickness by binding of the neuronal Nrg-1 to ErbB receptors on Schwann cells, which induces expression of myelin proteins and ensheathment of axons (Taveggia et al., 2005; Michailov et al., 2004). BACE1 in axons cleaves this Nrg1 isoform and this cleavage is indispensable, as BACE1-null nerves display hypomyelination (Luo et al., 2011; Willem et al., 2006; Hu et al., 2006; Fleck et al., 2013; La et al., 2011b). Neuronal overexpression of an equivalent form of BACE1-cleaved Nrg1 type III variant induces hypermyelination *in vivo* and restores myelination of NRG1 type III-deficient neurons (Velanac et al., 2012).

On the other hand, intrinsic signals from Schwann cells are equally important for proper myelination and we have provided experimental evidence of this in this study. First, we showed that BACE1 is expressed by cultured primary Schwann cells (Figure 7B). A previous study suggested that BACE1 was not readily detected in Schwann cells by *in situ* hybridization (Willem et al., 2006), and this is related to high expression of BACE1 in neurons. By using a sensitive western blot assay, we detected BACE1 in cultured primary Schwann cells in the absence of axons. We previously also showed that injury induces expression of BACE1 in Schwann cells, likely due to inflammatory responses (Hu et al., 2015). Second, we observed increased Schwann cell proliferation in BACE1-null nerves during development (Figures 1–2) and after injury (data not shown). During normal nerve development, the number of Schwann cells is often matched to the number and length of axons (Jessen and Mirsky, 1998). The increased number of Schwann cells is unlikely due to reduced signaling from axonal Nrg1, as Nrg1 promotes the maturation and survival of Schwann cells (Zorick and Lemke, 1996; Nave and Salzer, 2006; Garratt et al., 2000). Instead, it is likely due to autocrine signaling from the enhanced Jag1/Delta1-Notch pathway, as all of these molecules are intrinsically expressed by cultured Schwann cells (Figure 7). This speculation is supported by a prior publication showing that Notch signals the generation of Schwann cells from Schwann cell precursors and regulates the size of the Schwann cell pool by controlling proliferation (Woodhoo et al., 2009). We showed that full length Jag1 and Delta1 are elevated in BACE1-null nerves and Schwann cells. Our previous mapping study confirmed that Jag1 is a natural substrate of BACE1 (He et al., 2014) and that abolished cleavage of Jag1 in BACE1-null Schwann cells increases the level of full length Jag1. This study provides further evidence that Delta1 is also an effective substrate of

BACE1 and two potential cleavage sites were identified (Figure 8). It appears that Jag2 is mostly expressed by axons, as its expression in Schwann cells was extremely low. The elevation of both Jag1 and Delta1 would induce sequential proteolytic cleavages of Notch for enhanced release of NICD (Kopan and Ilagan, 2009), and enhanced NICD promotes Schwann cell proliferation (Woodhoo et al., 2009). Third, the size of Schwann cells appeared to be smaller in BACE1-null nerves. During development, Schwann cell size is determined by the regulation of cell growth, and cell division proliferation is inversely correlated (Roberts and Lloyd, 2012) such that enhanced cell proliferation will restrict cell size due to contact inhibition. Consistent with this notion, we observed a gross reduction in internodal length in BACE1-null nerves, either in developmental stages or in remyelinating stages (Figure 4B–D) in addition to the hypomyelination in BACE1-null nerves. Consistently, we observed a reduced number of Schmidt–Lanterman incisures, which are correlated to the volume of Schwann cell cytoplasm. As similarly observed (Willem et al., 2006), small axons in BACE1-null Remak bundles are usually poorly separated by Schwann cell cytoplasm, and this may also be related to smaller cytoplasm of BACE1-null Schwann cells. Although the molecular mechanism for such changes in Schwann cell size is not fully understood, the possible contribution of LDL receptor related protein-1 (LRP1) may be tested in the future. It has been shown with *in vitro* assays that BACE1 cleaves LRP1 (von et al., 2010; von Arnim et al., 2005), and deletion of LRP1 in Schwann cells shows similar defects in Remak bundles (Orita et al., 2013). On the other hand, the possible contribution of BACE1-cleaved Nrg1 in the control of this process cannot be excluded, as Nrg1 hemi-mice also showed defects in Remak bundles (Taveggia et al., 2005; Fricker et al., 2009). Hence, BACE1 is required as a check point protein for regulating Schwann cell proliferation.

BACE1 deficiency appears to disrupt normal functions of Schwann cells as demonstrated in our electrophysiological recordings and behavioral testing. The ability of peripheral nerves to switch from glucose to lactate as an energy source is a physiological response to increased stress, which relies on the Schwann cell-axon lactate shuttle or normal mitochondrial functions in Schwann cells (Evans et al 2013), and this ability is defective in BACE1-null nerves (Figure 6B). Glucose and lactate are equally efficient in supporting peripheral and central nerve function (Brown et al., 2012; Brown et al., 2004; Evans et al., 2013b; Tekkok et al., 2005; Baltan, 2015). Excess glucose is stored as glycogen in Schwann cell cytoplasm (Brown et al., 2012), and glycogen is broken down into lactate to support axon function when ambient glucose levels are low/depleted or when there is an increase in neuronal/axonal activity (Evans et al., 2013a; Brown et al., 2003b; Brown et al., 2005). We directly tested the ability of axons to use lactate as an energy substrate by exposing the sciatic nerves to high-frequency stimulation (HFS), a physiological stimulus, to assess Schwann cell functions. In control nerves, axon function returns to normal levels following HFS, as axon metabolism can effectively switch from glucose to lactate, but axon function failed to recover to baseline in BACE1-null nerves. Furthermore, BACE1-null mice show weak performance on the rotarod test, which requires endurance. The major irreversible depression in BACE1-null nerve function suggests several possibilities: 1) deletion of BACE1 may impair glycogen storage in Schwann cells so that lactate cannot be efficiently produced, 2) glycogen is present and converted to lactate but cannot be delivered to axons due to a reduction and/or depletion of monocarboxylate transporter 1 (Morrison et al., 2015),

and 3) lactate is produced and delivered properly, but axonal mitochondria cannot use lactate due to dysfunction. Further studies are required to delineate how BACE1 deficiency disrupts axon function, and whether it is directly due to hypomyelination or occurs indirectly by affecting Schwann cell function.

Hypomyelination found in BACE1-null mice was initially attributed to the reduced cleavage of type III Nrg1 (Hu et al., 2006; Willem et al., 2006), and growing evidence supports the requirement of BACE1-dependent Nrg1 signaling functions in the control of myelin sheath thickness [see reviews by (Fleck et al., 2012; Hu et al., 2016; Mei and Nave, 2014; Birchmeier and Nave, 2008)]. Results from this study further suggest BACE1 as a check point protein by regulating Schwann cell myelination and cellular functions through the Nrg1-Erb2/3 and Jag1/Delta1-Notch pathways via either paracrine or autocrine fashions. BACE1 deficiency enhances proliferation of Schwann cell due to the elevated Jag1/Delta1 levels, but fails to myelinate axons efficiently due to impaired the paracrine Nrg1 signaling. Axon-Schwann cell metabolic coupling is also disrupted, although the detailed molecular mechanisms remain to be established.

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Abbreviation

BACE1	β -site APP cleaving enzyme-1
Nrg1	neuregulin-1
CNS	central nervous system
PNS	peripheral nervous system
Jag1	Jagged-1
NICD	Notch intracellular domain

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Main point

This study demonstrates BACE1 in the controls Schwann cells proliferation, via activating the Jag1/Delta1-Notch signaling. This enhanced proliferation of Schwann cells correlates with altered intermodal length, nodal structure and nerve function.

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Figure 1. BACE1 deficiency enhances developmental Schwann cell proliferation

Cross (A) or longitudinal (B) sections of one-month-old WT and BACE1-null mouse sciatic nerves were fixed and stained with an antibody specific to EGR2 to label Schwann cells. (C) Similar staining experiments with EGR2 antibody were conducted using three-month-old mice. (D) The increases in EGR2-positive Schwann cells in BACE1-null sciatic nerves at both ages were significant as quantified by average numbers from 7 sections in each animal ($n=3$ animals, $** P<0.01$, Student's t -test). (E) The same groups of nerve samples were also examined with an antibody to Sox10 to label mature Schwann cells. (F) Increases in Sox10-positive Schwann cells in BACE1-null sciatic nerves at both ages were evident as shown in average numbers from 7 sections in each animal ($n=3$ animals, $** P<0.01$, Student's t -test). Scale bar is 30 μ m.

Figure 2. BACE1 deficiency increases immature Schwann cells

(A–B) Postnatal day 8 (P8) and (C) P30 mice were fixed and stained with antibodies to Sox 2. Both cross (A, C) and longitudinal (B) sections were examined by Sox2 staining. (D) Significant increases in precursor and immature Schwann cells in BACE1-null nerves compared to WT nerves were revealed by quantification. Scale bar is 30 μ m.

Figure 3. BACE1 deficiency increases dividing Schwann cells

(A–B) P8 mice received two injections of BrdU and sciatic nerves were dissected from the treated mice 24 hrs post-injection. BrdU-positive nuclei were detected by an antibody specific to BrdU and quantified based on average numbers from 7 sections in each animal (n=3 animals, ** $P<0.01$, Student's t -test). (C) BrdU-positive dividing Schwann cells were analyzed by double-labeling fixed sections with Sox2 and BrdU antibodies. The double-labeled cells were quantified and are shown in D (n=3 animals, ** $P<0.01$, Student's t -test). Scale bars are 30 μ m.

Figure 4. Shortened internodal and paranodal lengths in BACE1-null nerves

(A) Fixed sciatic nerves from one-month-old mice were labeled with an antibody to Caspr, which is localized in the paranodal section and is circled in red. BACE1-null nerves showed increased numbers of nodes and reduced overall paranodal lengths. Scale bar is 40 μm . (B) Teased nerves were prepared from four-month-old mice to compare internodal length flanked by two nodes (green arrow) as well as Schmidt–Lanterman incisures (red arrow). As shown, internodal lengths were shortened, while Schmidt–Lanterman incisures within an internode were decreased in BACE1-null nerves compared to control nerves. The average internodal length was $618.0 \pm 23.0 \mu\text{m}$ in WT axons compared to $461.0 \pm 20.0 \mu\text{m}$ in BACE1-null axons, while the average number of Schmidt–Lanterman incisures within an internode was 20.9 ± 1.2 in WT axons compared to 13.4 ± 0.7 in BACE1-null axons (** $P < 0.01$, $n = 40$). Scale bar is 50 μm . (C) Axons of BACE-null sciatic nerves were examined using 3D EM (a–b, composites from multiple slices; c, single slice) and showed normal nodal ultrastructure, including regularly spaced paranodal loops (*) that closely apposed the axolemma (arrowheads). Some fibers (a) had comparatively short paranodal regions (small arrows) compared with most others (b). Abbreviations: m, compact myelin; c endoneurial collagen fibrils. Scale bars are 1 μm in a and b, 0.25 μm in c. (D) The paranodal length was compared by utilizing the captured 3D–EM images and plotting correlations with axonal width. Larger axons tended to have shorter paranodal length in BACE1-null nerves compared to those in smaller axons. (E) Sciatic nerves from three-month-old mice were fixed and labeled with antibody to NCAM, which also marks Schmidt–Lanterman incisures. There were less

Schmidt–Lanterman incisures in all examined BACE1-null sciatic nerves compared to WT controls.

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Figure 5. BACE1 deficiency impairs axon excitability and conduction

(A) Representative traces of compound action potentials (CAPs) from WT (left) and BACE1-null (right) demonstrate rapidly rising CAPs with sharp peaks in control nerves versus slowly rising, dispersed CAPs with a blunt peak in sciatic nerves from BACE1-null mice. CAPs evoked in sciatic nerves from BACE1-null mice display (B) a prolonged latency (time to peak), (C) a lower amplitude, (D) a longer duration, (E) an unchanged CAP area, and (F) a rightward shift in axonal excitability. Linear regression lines fitted to the slopes (not shown) demonstrated a sharp relationship in WT mice (Slope = 1.3 ± 0.16) compared to BACE1-null mice (slope = 0.17 ± 0.00 , $P < 0.001$, Student's unpaired t -test). The increased threshold to evoke a response in BACE1-null mice (arrows) is noted (* $P < 0.05$ and *** $P < 0.001$, Student's unpaired t -test).

Figure 6. BACE1 deficiency impairs rotarod test performance and axonal metabolic responses
(A) Two groups of mice (3 and 10 months old) were tested on a rotarod apparatus. BACE1-null mice in both age groups showed significantly weaker performance, as they took less time to fall off the rotarod (** $P < 0.01$, $n = 20$ pairs of mice, Student's t -test). **(B)** Time course plots of CAP area (normalized to baseline) from BACE1-null and WT sciatic nerves show that a short (4 min) or long (10 min) HFS (100 Hz stimulation) irreversibly suppresses axon function in sciatic nerves from BACE1-null mice due to impaired ability to use lactate as a fuel to maintain axon function.

Figure 8. Mapping the cleavage site of Delta1 by BACE1

(A) A synthetic peptide (calculated molecular weight of 4135.6 g/mol) corresponding to the sequence upstream of the transmembrane domain was purified and confirmed by mass spectrometry. This peptide was incubated with recombinant BACE1 for enzymatic assays. (B-C) Mapping analysis of BACE1-cleaved peptides in the enzymatic reaction by MOLDI-TOF mass spectrometry revealed one major C-terminal peak with a molecular weight of 918.5 and corresponding to the peptide **TEKLEGQGG** (B). In the N-terminal side, BACE1 can also cleave between the VC-EC site and produce a smaller product with a molecular weight of 2852.3, as well as the longer one of 3232.4 as illustrated next to the peak (C).