Bace1 and Neuregulin-1 cooperate to control formation and maintenance of muscle spindles

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The protease β-secretase 1 (Bace1) was identified through its critical role in production of amyloid-β peptides (Aβ), the major component of amyloid plaques in Alzheimer’s disease. Bace1 is considered a promising target for the treatment of this pathology, but processes additional substrates, among them Neuregulin-1 (Nrg1). Our biochemical analysis indicates that Bace1 processes the Ig-containing β1 Nrg1 (IgNrg1β1) isoform. We find that a graded reduction in IgNrg1 signal strength in vivo results in increasingly severe deficits in formation and maturation of muscle spindles, a proprioceptive organ critical for muscle coordination. Further, we show that Bace1 is required for formation and maturation of the muscle spindle. Finally, pharmacological inhibition and conditional mutagenesis in adult animals demonstrate that Bace1 and Nrg1 are essential to sustain muscle spindles and to maintain motor coordination. Our results assign to Bace1 a role in the control of coordinated movement through its regulation of muscle spindle physiology, and implicate IgNrg1-dependent processing as a molecular mechanism.

Introduction

Proteolysis of membrane-tethered molecules is critical for cellular communication. Sheddases, a group of membrane-bound proteases, cleave single-span membrane proteins at the extracellular surface. One of the most studied sheddases, the aspartyl protease Bace1 (β-secretase 1), is an important drug target for Alzheimer’s disease. Bace1 cleaves the amyloid precursor protein (APP) and is responsible for generation of pathogenic Aβ peptides (Luo et al, 2001; Vassar et al, 2009). In addition to APP, around 20 substrates have been identified for Bace1 (Willem et al, 2006; Kandalepas and Vassar, 2012; Kuhn et al, 2012; Zhou et al, 2012). Knowledge of their physiological functions can help to monitor adverse effects caused by Bace1 inhibition in patients.

One Bace1 substrate is Neuregulin-1 (Nrg1), a trophic factor that signals through ErbB tyrosine kinase receptors to regulate nervous system development and regeneration (Falls, 2003; Willem et al, 2006; Birchmeier, 2009; Fricker et al, 2011). Nrg1 is a complex gene encoding more than 15 protein isoforms that are generated by a combination of alternative mRNA splicing and the use of several promoters. All subtypes of Nrg1 proteins present an EGF-like domain required for receptor binding and signalling. Depending on the presence of either an Ig-like or a cysteine-rich domain (CRD) in their amino-terminal (N-terminal) sequences, Nrg1 variants can be classified into IgNrg1 (type I and type II) and CRD-Nrg1 (type III). Few Nrg1 variants are secreted molecules (e.g., glial growth factor, GGF), most being membrane-bound proteins that can be shed by proteases (Falls, 2003). Bace1-dependent shedding of Nrg1 has been implicated in the control of myelination in the peripheral nervous system (Hu et al, 2006; Willem et al, 2006).

Coordinated body movement requires constant input of sensory information to elicit a concerted motor response. Muscle spindles are sensory organs dispersed throughout muscles of vertebrates, which detect muscle stretch, allowing thus the perception of body position ( proprioception) important for coordinated movement (Maier, 1997). The muscle spindle is composed of a bundle of specialized ( intrafusal) muscle fibres, and its formation is induced by contact between TrkC⁺ sensory axons and muscle fibres (Errfors et al, 1994; Farinas et al, 1994; Klein et al, 1994; Tesserarolo et al, 1994; Walro and Kucera, 1999; Chen et al, 2003). Further maturation takes place during late fetal and early postnatal phases when muscle spindles grow and become enclosed by the capsules (Hunt, 1990; Zelena and Soukup, 1993; Maier, 1997). Previous studies showed that the genetic deletion of neuronally produced Nrg1 or its receptor ErbB2 in muscle tissue prevent muscle spindle differentiation (Andrechek et al, 2002; Hippenmeyer et al, 2002; Leu et al, 2003).

Here we show that formation, maturation and maintenance of muscle spindles depend on Bace1. We use mouse mutants to demonstrate that in the absence of Bace1, muscle spindle numbers are reduced and spindle maturation is impaired.

Keywords: Bace1; muscle spindle; Nrg1; proprioception
Moreover, we find that a graded reduction in IgNrg1 signal strength results in increasingly severe deficits in the formation and maturation of muscle spindles. Conversely, we observed supernumerary muscle spindles upon overexpression of a membrane-tethered Nrg1 variant (Ig-containing β1 Nrg1 (IgNrg1(β1))) in sensory neurons, an effect which strictly depended on the presence of Bace1. Strikingly, inhibition of Bace1 activity or ablation of Nrg1 expression in adult mice resulted in a massive reduction of the muscle spindle pool and impaired coordination of movement. Together, these data implicate Bace1-dependent processing of IgNrg1 in ontogenesis and long-term maintenance of muscle spindles.

Results

**Bace1 mutant mice display coordination defects**
During handling of Bace1<sup>−/−</sup> mutant mice, we noted that their movement was altered. Most notably, they appeared not to be able to hang to an inverted grid, a task requiring motor coordination and/or muscle strength (Coughenour et al., 1977; Landauer et al., 2003). A quantitative assessment demonstrated that wild-type littermates did hang to the grid three times longer than Bace1<sup>−/−</sup> mice; Bace1<sup>+/+</sup> and wild-type animals performed similarly (Figure 1A). We then assessed muscle coordination using gait analysis. The walking pattern was recorded as mice freely walked on a moving belt, and gait analysis monitored movement and position of individual paws using the Treadscan system (Beare et al., 2009). The walking pattern of wild-type mice (Figure 1B and C) shows coordinated alternation of paws, i.e., opposing movements of anterior and posterior limbs on the same side (homolateral coupling value ≈ 0.5; Figure 1D and Supplementary Figure S1A), and opposing movements of left and right limbs of the same axial levels (homologue coupling value ≈ 0.5; Figure 1E and Supplementary Figure S1B and C). Homolateral coupling of Bace1<sup>−/−</sup> mutants was severely affected and deviated considerably from wild-type animals. This reflects a lack of forelimb/hindlimb coordination and resulted in a swaying walking pattern (Figure 1C; quantified in D and Supplementary Figure S1A), and opposing movements of left and right limbs of the same axial levels (homologue coupling value ≈ 0.5; Figure 1E and Supplementary Figure S1B and C). Homolateral coupling of Bace1<sup>−/−</sup> mutants was severely affected and deviated considerably from wild-type animals. This reflects a lack of forelimb/hindlimb coordination and resulted in a swaying walking pattern (Figure 1C; quantified in D and Supplementary Figure S1A). In contrast, homologue coupling was little disturbed at either axial level, indicating correct left/right alternation (Figure 1C; quantified in E and Supplementary Figures S1B and C). Bace1 mutant mice display peripheral hypomyelination but little Schwann cell turnover, similar to Schwann cell-specific coErbB2 (Krox20<sup>cre</sup> ErbB2<sup>flox/flox</sup>) mutant mice (g-ratios P180: control, Bace1<sup>−/−</sup> and coErbB2: 0.68 ± 0.01, 0.75 ± 0.01 and 0.80 ± 0.01, respectively; cf. Garratt et al., 2000; Hu et al., 2006; Willem et al., 2006; Grossmann et al., 2009). In contrast to Bace1 mutants, we did not observe significant changes in motor coordination in coErbB2 mutants (Supplementary Figure S2A–D). Together, our data indicate that motor coordination is disrupted in Bace1<sup>−/−</sup> mutants, and this coordination deficit is not caused by hypomyelination.

**Bace1 mutation affects formation and maintenance of muscle spindles**
Coordination of body movement requires functional proprioception, and muscle spindles are important proprioceptive organs governing the coupling of antagonistic muscles (the spindle structure is shown schematically in Figure 1F). We quantified the amounts of muscle spindles in lower hindlimbs of newborn control and mutant mice at P0, using a combination of morphological criteria (large nuclei, presence of a capsule) and immunohistology with antibodies against Egf3, a muscle spindle-specific transcription factor, collagen IV, a marker for muscle spindle outer capsules, and NF200, a neurofilament isofrom expressed by sensory fibres contacting the spindles (Figure 1G and H; cf. Tourtellotte and Milbrandt, 1998; Tourtellotte et al., 2001; Hippenmeyer et al., 2002). Newborn Bace1<sup>−/−</sup> mice presented a pronounced reduction (45%; P < 0.001) of the number of muscle spindles, and the reduction persisted until adulthood (Figure 1G, Table I). A small but significant reduction (14%, P = 0.01) was observable in heterozygous Bace1<sup>+/−</sup> mutant mice. The overall morphology of persisting muscle spindles was unchanged in Bace1<sup>−/−</sup> mice at P0, but postnatal muscle spindle growth was impaired (Figure 1H, Table II, see also below). We conclude that Bace1 is required for the correct formation and maturation of muscle spindles.

**Bace1 activity is required to sustain muscle spindles and to maintain motor coordination**
We next assessed whether Bace1 activity is needed to sustain mature muscle spindles. Adult (P180) wild type, heterozygous and homozygous Bace1 mutant mice were treated with the pharmacological Bace1 inhibitor Ly2811376 for a period of 29 days (May et al., 2011). Ly2811376 inhibited Bace1 activity effectively in vivo, as assessed by monitoring the Nrg1 processing in the brain (Figure 2A). Ly2811376 treatment led to a regression of adult muscle spindles, notably a loss of 40% of muscle spindles in wild type and heterozygous Bace1 mutant animals, compared to corresponding vehicle-treated groups (Figure 2B; Supplementary Figure S2E). Ly2811376 treatment did not further decrease the muscle spindle pool in homozygous Bace1 mutant mice, demonstrating that this effect was mediated through specific Bace1 inhibition (Figure 2B; Supplementary Figure S2E). Together, these genetic and pharmacological data indicate that Bace1 controls the maintenance of muscle spindles during adulthood, as well as their formation during development.

We next tested whether Bace1 inhibition in the adult affected motor coordination. A quantitative assessment of grip ability demonstrated that animals treated with a Bace1 inhibitor lost their footing 3–4 times faster than animals treated with the vehicle (Figure 2C). Gait analysis also demonstrated that the walking pattern of the mice was aberrant after long-term inhibition with Bace1 inhibitor (Figure 2D–F). Thus, the value of homolateral coupling deviated considerably from the one observed in vehicle-treated animals. This reflects a lack of forelimb/hindlimb coordination and resulted in a swaying walking pattern (Figure 2D; quantified in E and Supplementary Figure S1A). Homologue coupling was little disturbed after Bace1 inhibition (Figure 2D; quantified in F and Supplementary Figure S1B and C). We conclude that long-term treatment of adult mice with Bace1 inhibitor disrupts motor coordination. It is noteworthy that coordination was affected to similar extents in Bace1<sup>−/−</sup> and in Bace1 inhibitor-treated animals, indicating that Bace1 activity is continuously required for motor coordination.
Bace1 processes Nrg1 isoforms

Various Nrg1 isoforms exist (Figure 3A) that can take over distinct functions. Nrg1 isoforms containing an Ig domain (IgNrg1) are produced by proprioceptive neurons and control the induction of the muscle spindle (Hippenmeyer et al, 2002). Bace1 is expressed broadly in sensory neurons.
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Table II Diameter of muscle spindles and intrafusal content at birth and during adulthood

| Spindle diameter (µm) | Control | Bace1−/− | Bace1−/− IgNrg1Δ/Δ | co-IgNrg1 | IgNrg1βSEAP | Bace1−/− IgNrg1βSEAP |
|----------------------|---------|----------|---------------------|----------|-------------|---------------------|
| P0                   | 26.5 ± 0.9 | 23.7 ± 1.4NS | 23.4 ± 0.9NS | 25.9 ± 1.1NS | 26.8 ± 1.5NS | 22.7 ± 0.5NS |
| P30                  | 40.6 ± 0.6 | 32.5 ± 0.8*** | 33.3 ± 0.6*** | 29.4 ± 1.0*** | 42.1 ± 0.6NS | 34.6 ± 1.2** |

| Intrafusal fibres/spindle | Control | Bace1−/− | Bace1−/− IgNrg1Δ/Δ | co-IgNrg1 | IgNrg1βSEAP | Bace1−/− IgNrg1βSEAP |
|---------------------------|---------|----------|---------------------|----------|-------------|---------------------|
| P0                        | 4.0 ± 0.2 | 3.9 ± 0.2NS | 3.1 ± 0.1** | 2.9 ± 0.1*** | 4.3 ± 0.2NS | 4.0 ± 0.1NS |
| P30                       | 3.9 ± 0.1 | 3.9 ± 0.1NS | 3.4 ± 0.1NS | 3.1 ± 0.1** | 4.5 ± 0.2*** | 3.8 ± 0.1NS |

Equatorial diameter and number of intrafusal fibres per muscle spindle was determined in 3–6 animals per genotype and per age and is expressed as mean ± s.e.m. Asterisks indicate significance of the differences observed when mutants were compared to control littermates.

(Willem et al., 2006). In situ hybridization combined with immunohistochemistry showed that Bace1 and IgNrg1 are co-expressed in NF200+ large diameter sensory neurons at birth; thus proprioceptive neurons co-express Bace1 and IgNrg1 (Figure 3B). Quantification demonstrated that the vast majority of sensory neurons as well as IgNrg1+ sensory neurons in dorsal root ganglia (DRG) co-expressed Bace1 (93.7 ± 1.2% and 99.8 ± 0.2%, respectively). Various IgNrg1 isoforms exist, called z1/2 and β1–4 that differ in the EGF-like domains and in sequences carboxy-terminal (C-terminal) thereof (Supplementary Figure S3A; Falls, 2003). We analysed their expression in DRG at P0 using semi-quantitative PCR (qPCR). This showed that among the IgNrg1 isoforms, β variants and particularly, the β1 isoform are expressed at highest levels (Figure 3C).

IgNrg1β1 isoforms contain a predicted Bace1 cleavage site (Figure 3D and Hu et al, 2008). However, IgNrg1 isoforms are produced by few sensory neurons, whereas CRD-Nrg1 is expressed broadly in sensory neurons (Figure 3B, cf. Meyer et al, 1997; Hippenmeyer et al, 2002). This hampers a direct biochemical analysis of IgNrg1 processing in sensory ganglia in vivo. We therefore analysed processing of HA-tagged β1 isoforms in cultured HEK293 cells, and tested whether the predicted Bace1 cleavage sites present in β1 variants of Ig- or CRD-containing Nrg1 are recognized. Full-length and processed C-terminal fragment of Nrg1β1 proteins containing CRD or Ig domains were observed in the absence of transfected Bace1 cDNA, using an antibody against the C-terminus of Nrg1 (Figure 3E). In the presence of Bace1, the full-length protein almost disappeared, whereas the processed C-terminal fragment accumulated (Figure 3E). Thus, Bace1 cleaves the Nrg1β1 sequence, regardless of whether it is present in a CRD or Ig isoform.

IgNrg1β2 and IgNrg1β3 represent a membrane-tethered and a secreted isoform, respectively, and are produced at lower levels than IgNrg1β1 in DRG (Figure 3A and C). We compared processing of the two membrane-tethered isoforms IgNrg1β1 and IgNrg1β2 in transfected HEK293 cells and analysed the release of their HA-tagged extracellular fragment, which contains the receptor-binding EGF domain (Figure 3F). IgNrg1β1 was constitutively processed, but co-transfection of Bace1 resulted in a further increase in the amount of the C-terminal and HA-tagged N-terminal fragments, as well as a decrease of full-length protein, indicating increased processing. In contrast, IgNrg1β2 was neither processed constitutively, nor in a Bace1-dependent manner (Figure 3F). The release of the N-terminal fragments was quantified using Nrg1 constructs carrying alkaline phosphatase in their extracellular domain (Figure 3G; secreted alkaline phosphatase or SEAP, cf. Willem et al, 2006). Co-transfection of IgNrg1βSEAP and Bace1 increased the amount of released alkaline phosphatase six-fold, which was abolished by the Bace1 inhibitor C3 but not by the metalloproteinase inhibitor GM6001 (Figure 3G; Willem et al, 2006; Freese et al, 2009). In contrast, released alkaline phosphatase from the corresponding IgNrg1β2SEAP was low and little affected by Bace1 activity. We conclude that the major Ig variant expressed in sensory neurons, Nrg1β1, is a substrate of Bace1.

We next tested whether endogenous levels of Bace1 can process IgNrg1β1. Hippocampal neurons were transfected with an IgNrg1β1 expression vector (Figure 3H and I). The majority of cellular IgNrg1β1 was processed, and only little full-length protein was observable. In the presence of the Bace1 inhibitor C3, IgNrg1β1 cleavage was reduced, resulting in accumulation of Nrg1 FL and slightly reduced levels of the 65-kDa C-terminal fragment (Figure 3H). HA-tagged Nrg1β1 in the supernatant was detected using HA-specific antibodies, which revealed mildly reduced quantities of the N-terminal fragment in case of Bace1 and metalloproteinase inhibition (Figure 3I). The combination of Bace1 and metalloproteinase inhibitors acted synergistically. In addition, the N-terminal fragment was immunoprecipitated using HA-specific antibodies and detected on western blot using HA and 4F10 antibodies. We took advantage of the 4F10 antibody, which recognizes a Nrg1β1-specific epitope only when exposed by Bace1 cleavage (Fleck et al, 2013). This revealed that the neuronal production of the Nrg1β1 4F10-specific epitope was impaired upon inhibition of Bace1, but not of metalloproteinases (Figure 3I). These data indicate that endogenous amounts of Bace1 suffice to cleave IgNrg1β1 in primary hippocampal neurons, and support the notion that in such neurons IgNrg1β1 is cleaved by Bace1 and metalloproteinases.

IgNrg1β1 isoforms are required for motor coordination
To directly define the function of IgNrg1 isoforms in motor coordination, we generated a new mutant allele in which one of the exons encoding Ig sequences is ‘floxed’ or constitutively deleted (IgNrg1βbox and IgNrg1β; Supplementary Figure S3B). IgNrg1βbox or re-combined IgNrg1βbox mutations specifically interfere with the production of IgNrg1, but not CRD-Nrg1 transcripts (see Materials and methods for more details). We then generated Wnt1Cre/+IgNrg1βbox/A mice; Wnt1Cre is expressed in neural crest cells that give rise to sensory neurons (Danielian et al, 1998; Le Douarin and Kalcheim, 1999). Semi-quantitative RT-PCR indicated that expression of IgNrg1 and particularly IgNrg1β1...
expression of CRD-Nrg1 transcripts was unaltered in DRG of co-IgNrg1 mice (Figure 4A).

Conditional IgNrg1 mutants displayed pronounced curled tails and uncoordinated tail movements, which we attributed to an almost complete depletion of the spindle pool in tail muscles (Supplementary Figure S4B). Besides, co-IgNrg1 mice lost their grip five times faster than their control littermates in the inverted grid test (Figure 4B). Moreover, gait analysis showed that co-IgNrg1 mutants, like the Bace1 mouse described above, suffered from pronounced deficits in forelimb/hindlimb coordination (Figure 4C and D). In addition, a pronounced deficit in left/right coordination was apparent in hindlimbs, resulting in frequent hopping movement (Figure 4E, summarized in Figure 7I). We conclude that, similar to Bace1 mutants, coordinated movement is severely disrupted in co-IgNrg1 mice.

We next analysed muscle spindles in IgNrg1 mutants (Figure 5A; Supplementary Figure S5A and Supplementary Table II). We observed a significant (30%, \(P = 0.002\)) reduction and a massive loss of spindles (84%, \(P<0.001\)) in heterozygous IgNrg1 \(+/-\) and co-IgNrg1 newborn mice, respectively. No obvious alteration was apparent in the morphology of Golgi tendon organs and Pacinian corpuscles in co-IgNrg1 mutants, nor did we detect changes in myelination of peripheral nerves (Supplementary Figure S5B and C). Strikingly, only in the presence of Bace1 supernumerary neurotrophin 3 (NT3) in the supernumerary muscle spindles. (Figure 5B–D), which is possibly caused by expression of 

Maturation of muscle spindles depends on Bace1 and Nrg1

Muscle spindles are induced before birth and mature during postnatal life, when they can be detected by a combination of morphological criteria and immunohistology (Maier, 1997;...
Figure 3 Nrg1β is a substrate of Bace1. (A) Structure of Nrg1 isoforms containing cysteine-rich domain (CRD) or Iq-like (Ig) domains. Full-length (Nrg1 FL), N-terminal (Nrg1 NtF) and C-terminal (Nrg1 CtF) fragments analysed in (E, F, H, I) are indicated. Green arrows show the Bace1 cleavage site present in β1 isotypes, yellow star the position of the HA-tag used for biochemical analysis; C-terminal transmembrane domain TM.

(B) In situ hybridization (Bace1, IgNrg1) combined with immunohistology (neurofilament 200; NF200) demonstrates co-expression of Bace1 and IgNrg1 in sensory neurons. Left: Bace1 is broadly expressed in sensory neurons. Right: IgNrg1 and Bace1 (insert) are co-expressed in NF200⁺ sensory neurons. (C) qPCR of DRG mRNA encoding CRD-Nrg1 and α/β IgNrg1 isoforms (P0). (D) Sequence alignment of α/β Nrg1 isoforms; predicted Bace1 cleavage sites are indicated. Asterisk indicates stop codon in β3. (E, F) Western blot analysis of Bace1-dependent processing of (E) Ig- and CRD-Nrg1β1, (F) IgNrg1β1 and IgNrg1β2 in HEK293 cells. Antibodies are indicated (αCNX: anti-calnexin). Full-length precursors (Nrg1 FL; IgNrg1: 110 kD, CRD-Nrg1: 130 kD) and processed C-terminal fragment (Nrg1 CtF: 65 kD) are detected in RIPA lysates using an antibody against the Nrg1 C-terminus; HA-tagged N-terminal fragments (Nrg1 NtF) are detected in the supernatant using anti-HA. Calnexin serves as loading control. NT, non-transfected. (G) Quantification of Bace1-dependent shedding of IgNrg1β1SEAP and IgNrg1β2SEAP. The N-terminal sequence of IgNrg1SEAP variants contains alkaline phosphatase whose enzymatic activity is detected in supernatants in the absence/presence of Bace1 cDNA, C3 or GM6001 inhibitors. (H) Western blot analysis of IgNrg1β1 cleavage in primary neurons. Detected are full-length and C-terminal IgNrg1β1 in RIPA lysates using an antibody recognizing the Nrg1 C-terminus. (I) The N-terminal fragment of IgNrg1β1 was directly detected in supernatant by western blotting using anti-HA (upper panels). Alternately, supernatant was immunoprecipitated using anti-HA (middle and lower panels), and Nrg1 NtF fragments were detected using anti-HA and 4F10 antibodies; the latter identifies a Bace1-specific Nrg1 cleavage product. Asterisks indicate cross-reactive proteins. Scale bar, 50 μm (B).
spindles characterized by a small diameter (<30 μm; Figure 6B) and few intrafusal fibres (≤3 fibres/spindle; Figure 6D). Conversely, neuronal overexpression of IgNrg1β resulted in higher proportions of muscle spindles with unusually large diameter (>50 μm; Figure 6C) and high intrafusal fibre numbers (>4 fibres/spindle; Figure 6E). The outer capsule morphology and the innervation of remaining muscle spindles appeared unchanged in all genotypes (Figure 6A). Thus, Bace1 as well as IgNrg1 govern the maturation of muscle spindles.

**Maintenance of muscle spindles requires continuous Nrg1 signalling**

Bace1 inhibition in the adult results in motor coordination deficits and in a depletion of the muscle spindle pool (Figure 2B–F). We next tested whether Nrg1 is also continuously needed for motor coordination and muscle spindle maintenance. We used for this a previously established colony of cre-ER<sup>Tm</sup>+/<sup>+IgNrg1<sup>b</sup>+/<sup>-</sup> mice (Fricker et al, 2013) hereafter called coTxNrg1 mice. In such animals, a ubiquitously expressed tamoxifen-inducible cre induces recombination, and recombination deletes sequences encoding the EGF domain present in all Nrg1 isoforms. The mutation was introduced at P70, after somatic growth ended, and eliminated expression of Nrg1 transcripts in DRG of coTxNrg1 animals at P100 (see Figure 7A for an outline of the experiment and Figure 7B for quantification of Nrg1 transcripts). coTxNrg1 mice displayed profoundly impaired grip and did hang 3–4 times less long in the inverted grid test than control littermates. Motor coordination was strongly impaired, as assessed using a beam-walking test (Figure 7C and D; Supplementary Figure S7). We also analysed the muscle spindles in coTxNrg1 mutants: coTxNrg1 mice possessed less than half as many muscle spindles as control littermates (Figure 7E and F), and remaining spindles were shortened (Figure 7G; spindle length: 1805 ±106 and 1593 ±56 μm in control and coTxNrg1 mice, respectively; P = 0.048). In addition, the morphology of residual muscle spindles was profoundly altered and displayed thinner and discontinuous collagen IV staining (Figure 7H), which are signs of outer capsule degeneration (cf. Maier, 1997; Elsohemy et al, 2009). Further, intrafusal calbindin staining was weak in control mice of this age, and no longer observable by immunohistology in coTxNrg1 animals (Figure 7H). NF200<sup>+</sup> terminals were however still present, indicating that sensory innervation was preserved in remaining spindles (arrowheads in Figure 7H). The analysis of inducible mutant mice thus demonstrated that continuous Nrg1 signalling is essential to sustain muscle spindles and maintain motor coordination in the adult.

**Discussion**

Bace1 is well known for its role in Alzheimer’s disease, and is required for APP cleavage and production of β peptides (Luo et al, 2001). In this study, we show that Bace1 controls muscle spindle ontogenesis and maintenance in the adult. We combined complex genetic analyses, biochemical studies and pharmacological interference to provide evidence that these functions depend on cleavage of membrane-tethered IgNrg1 by Bace1. Our findings identify Bace1 as an important molecule in proprioception.
Bace1 and Nrg1 control ontogenesis and maintenance of muscle spindles

Our genetic study provides direct evidence that an Ig-containing isoform of Nrg1 supplies the muscle spindle-inducing activity. This is in accordance with the previous work that demonstrated a role of Nrg1 but not of CRD-containing isoforms in spindle induction (Hippenmeyer et al., 2002).

IgNrg1\(^{b1}\) is the dominantly expressed Ig-containing isoform of Nrg1 in DRG and thus expected to participate in muscle spindle induction. Our analysis of mice overexpressing membrane-tethered IgNrg1\(^{b1}\) in neurons indicates that this isoform induces supernumerary muscle spindles, but this depends on the presence of Bace1.

A direct contact exists between proprioceptive axons and muscle during muscle spindle differentiation (Ernfors et al., 1994; Farinas et al., 1994; Klein et al., 1994; Tessarollo et al., 1994; Walro and Kucera, 1999; Chen et al., 2003). Membrane of sensory axons and muscle fibres are thus in close apposition. Nevertheless, our data show that membrane-tethered IgNrg1\(^{b1}\) isoforms are not able to elicit a response without being shed. Further, we find that Nrg1 ablation or Bace1 inhibition in the adult result in a regression of the muscle spindle pool, indicating that Bace1-dependent Nrg1 activity is required to sustain muscle spindles even in the presence of an intact nerve. Previous studies had demonstrated that a continuous contact between the intrafusal fibres and sensory axons is a prerequisite for the maintenance of muscle spindles in adults. For instance, adult nerve transection in rats results in acute morphological changes followed by a massive loss of muscle spindles (Copray et al., 1999; Elsohemy et al., 2009). However, the molecular identity of the signal(s) provided by sensory axons had not been defined, and our data indicate that Nrg1 provides a key signal for spindle maintenance.

Functions of neuronally produced Nrg1 isoforms during development and adulthood

We show here that IgNrg1\(^{b1}\) requires Bace1 for its activity as spindle-promoting factor. IgNrg1\(^{b1\ E15}\) mice ectopically express this particular isoform and display supernumerary muscle spindles, a phenotype that is completely reversed when Bace1 is ablated. This is accompanied by increased survival of proprioceptive neurons in IgNrg1\(^{b1\ E15}\); it is interesting to note that supernumerary muscle spindles in IgNrg1\(^{b1\ E15}\) mice express NT3, a known rate-limiting factor for survival of proprioceptive neurons (Ernfors et al., 1994; Klein et al., 1994; Tessarollo et al., 1994). The expression of NT3 in such supernumerary muscle spindles might account...
for the enhanced survival of proprioceptive neurons during the selection phase. Several findings indicate that not all Nrg1 activity present at sensory–muscular contact sites is provided by IgNrg1ββ and depends on Bace1 for signalling. Previous data had indicated that the induction of muscle spindles strictly depends on neuronal Nrg1, i.e., no spindles form in the absence of Nrg1 (Hippemeyer et al., 2002), but we observed remaining muscle spindles in Bace1 mutants, although their number and size were reduced. Thus, Bace1-independent Nrg1 isoforms appear to participate in muscle spindle induction, for instance the secreted IgNrg1ββ (GGF) isoform that is also produced at low levels in sensory neurons. Alternately, metalloproteinases that are known to process Nrg1 might contribute to IgNrg1 shedding in vivo (Montero et al., 2000; Shirakabe et al., 2001; Horiuchi et al., 2005; La Marca et al., 2011; Luo et al., 2011). Sensory neurons provide the IgNrg1 signal for spindle induction. Because of their limited availability, the relative overabundance of CRD-Nrg1, and the lack of IgNrg1-specific antibodies, we were unable to directly assess IgNrg1 processing in sensory neurons. However, our experiments indicate that the endogenous Bace1 and metalloproteinases cooperatively cleave IgNrg1ββ in hippocampal neurons, similar to the shedding of CRD-Nrg1 described recently (Fleck et al., 2013). Further work is required, for instance by the use of mouse genetics, to assess the contribution of metalloproteinases to IgNrg1ββ cleavage in sensory neurons.

We observed subtle differences in maturation of muscle spindles in Bace1 and IgNrg1 mutant mice; for instance, IgNrg1 but not Bace1 mutants frequently display spindles with unusually few intrafusal fibres. Thus, Bace1-dependent and -independent IgNrg1 isoforms might assume slightly different functions during muscle spindle maturation. Alternatively, these differences in muscle spindle morphology might represent a response to graded differences in Nrg1 signals. In particular, co-IgNrg1 animals display a very severe reduction in spindle numbers (84% reduction), significantly smaller spindle diameter and fewer intrafusal fibres in remaining spindles; compound Bace1−/−IgNrg1+/− mutants display reduced in numbers (71% reduction) and display milder changes in spindle size and intrafusal fibre numbers; finally, in Bace1 mutant and inhibitor-treated mice, spindles are reduced in numbers (~50%), but numbers of intrafusal fibres are comparable to those in wild-type mice. Thus, a graded lowering of Nrg1 signals in this series of mutants appears to be translated into more and more pronounced reduction in numbers of muscle spindles and into increasingly pronounced severities of morphological deficits in remaining spindles.

Nrg1 generated by sensory neurons exerts two unrelated functions, muscle spindle induction and myelination, and both are disrupted in Bace1 mutant mice. This argues strongly for a Bace1-dependent shedding of Nrg1 in the control of these events. In particular, our biochemical data show that Bace1 processes CRD-Nrg1ββ sequences in cultured HEK293 cells. Unprocessed CRD-Nrg1 has an apparent MW of 130–140 kD when expressed in cultured cells, and a Nrg1 isoform of 130 kD accumulates in the brain of Bace1−/− or Ly281376-treated mice (Willem et al., 2006 and this study). Furthermore, hypomyelination is observed in Bace1−/− and CRD-Nrg1+/− mice (Michailov et al., 2004; Willem et al., 2006). Together, these experiments indicated that CRD-Nrg1 requires Bace1-dependent processing to control myelination. A recent report demonstrated that hypermyelination associated with overexpression of CRD-Nrg1 is not completely blocked by a Bace1 mutation, although a trend that did not reach a statistical significance was observed (Velanac et al., 2012). Thus, other substrates but CRD-Nrg1 might cause or participate in the impaired myelination observed in Bace1 mutants. Alternately, the transgenic overexpression of CRD-Nrg1 might bypass the processing by Bace1. In this context, it is noteworthy that Bace1-dependent processing only occurs during transit of substrates through endosomes, whereas
Figure 7 Continuous Nrg1 signalling is required to sustain muscle spindles in the adult. (A) Schematic outline of Nrg1 ablation and spindle analysis in adult coTxNrg1 mice (Tx, tamoxifen). (B) qPCR analysis of Nrg1 transcripts in DRG of control and coTxNrg1 mice at P100. (C, D) Performance of coTxNrg1 mice (P120) on the beam walk test. coTxNrg1 mice display frequent missteps and distinctive hopping movements; the latter is also a feature of co-IgNrg1 mice. (E) 3D reconstruction of muscle spindles (white lines) in the tibialis anterior muscle of control and coTxNrg1 mice (P180). (F) Quantification of numbers of muscle spindles in tibialis anterior muscle of control (black bars) and coTxNrg1 (red bars) animals. (G) Distribution of muscle spindle length in coTxNrg1 and control mice. (H) Immunohistological analysis of muscle spindles from control and coTxNrg1 animals. The presence of NF200+ endings indicates that sensory projections contacted spindles in both genotypes (arrowheads), but calbindin was not detectable in intrafusal fibres of coTxNrg1 mice. In addition, collagen IV staining in outer capsules was weak and interrupted, indicating that capsules degenerated in remaining spindles of coTxNrg1 mice. (I) Schematic summary of gait impairment in Ly2811376-treated, Bace1−/−, co-IgNrg1, and coTxNrg1 animals. Each panel displays the directions of paw movements (arrows) during a series of two steps. Both Bace1−/− mice and Bace1-inhibited mice display defective anterior/posterior coordination resulting in a swaying walk. In addition, co-IgNrg1 and coTxNrg1 mice show more pronounced deficits of posterior homologue coupling resulting in frequent hopping. Scale bars, 1.5 mm (E); 15 μm (H).
other proteases like those of the metallocarboxypeptidase family are active at the plasma membrane (Edwards et al., 2008; Willem et al., 2009; Weber and Safig, 2012). In such case, metallocarboxypeptidases could contribute to enhanced and Bace1-independent Nrg1 signalling in mice overexpressing CRD-Nrg1. Alternatively, transgenic CRD-Nrg1 overexpression might saturate ErbB signalling; hence Schwann cells would no longer be affected by a reduction of bioactive Nrg1 caused by Bace1 mutation.

Two isoforms, Ig- and CRD-Nrg1, are expressed in sensory neurons (Meyer et al., 1997). We show here that IgNrg1 induces formation of muscle spindles, but is dispensable for myelination. In contrast, CRD-Nrg1 regulates the development of Schwann cells and myelination, but is not required for muscle spindle formation (Meyer et al., 1997; Wolpowitz et al., 2000; Hippenmeyer et al., 2002). The question arises of how Ig- and CRD-Nrg1 isoforms produced in the same sensory neurons exert their distinct roles. Both Nrg1 isoforms are cooperatively cleaved by Bace1 and metallocarboxypeptidases (this work, Willem et al., 2006; Fleck et al., 2013). Further, similar downstream signalling molecules such as ErbB2 and CREB participate in myelination and in muscle spindle induction (Garratt et al., 2000; Andrechek et al., 2002; Leu et al., 2003; Arthur-Farraj et al., 2011; Herndon et al., 2013). Divergent intra- or intercellular trafficking mechanisms might exist that result in separate subcellular localization and function of Ig- and CRD-Nrg1 isoforms.

Functions of Bace1 in the mature nervous system
We demonstrate here that continuous Bace1 activity is required in the adult to maintain muscle spindles and therefore maintain accurate proprioception and coordinated movement. Bace1 was assigned various developmental functions, for instance in myelination and vascularization of the eye (Hu et al., 2006; Willem et al., 2006; Cai et al., 2012). Further, behavioural deficits (prepulse inhibition, novelty-induced hyperactivity, social recognition) were observed, which might originate from disrupted synapse formation and maturation (Stefansson et al., 2002; Laird et al., 2005; Savonenko et al., 2008; Wang et al., 2008). Bace1 inhibition is considered a promising route for the treatment of Alzheimer’s disease, and is expected to interfere with Aβ generation and the formation of pathogenic Aβ aggregates (Haass, 2004; Luo et al., 2011). To this date, several Bace1 inhibitors are tested in clinical phase II and planned for phase III. Adverse effects due to the inhibition of physiological functions of the protease must be considered. The data presented here indicate that one unwanted side effect of a long-term inhibition of Bace1 in adults might be disrupted muscle spindle functions, which are expected to impair coordinated movement.

Materials and methods

Animal strains and generation of IgNrg1£lox and IgNrg1b mice
Bace1b/− and IgNrg1bOv (Nrg1 type I β1 overexpression under the control of the Thy 1.2 promoter) strains have been described (Michailov et al., 2004; Dominguez et al., 2005). Exons 3 and 4 of Nrg1 encode the like-domain (IgNrg1b). IgNrg1b£lox mutants contain LoxP sites 5′ and 3′ of exon 4, which were inserted by homologous recombination into embryonic stem (ES) cells. Targeted ES cells were used to generate a mutant strain using standard techniques. The IgNrg1b£ strain was generated by crossing IgNrg1b£ox with Deleter™ animals (Schwenk et al., 1995), in which cre recombinase is expressed in all tissues including germ cells, thus inducing complete recombination. We obtained IgNrg1b£ mice upon backcrossing Deleter™IgNrg1b£ox animals with wild-type animals. The Wnt1-Cre strain expresses cre in neural crest cells, causing recombination in the progenitors that give rise to sensory neurons (Danielian et al., 1998; Le Douarin and Kalcheim, 1999). Wnt1£IgNrg1b£ox/A (co-IgNrg1b) mice were used for further analysis. Knockout IgNrg1b£ox/A (here also called IgNrg1b£) animals (here also called IgNrg1b£) present severe hypomyelination similar to Bace1−/− mice, and were previously described (Garratt et al., 2000). Phenotypes were analysed on a mixed C57Bl/6×129Ola background and mutants always compared with littermates.

Bace1 inhibition in adult mice
In vivo pharmacology studies were conducted with wild type, heterozygous and homozygous Bace1 mutant mice (EPO Berlin-Buch GmbH). Briefly, the Bace1 inhibitor Ly281376 (Eli Lilly) was prepared as a 160 μg/μl stock solution in pharmasolve (ISP technologies Inc), and aliquots were diluted 1/16 in corn oil (Ly281376 concentration: 10 μg/μl). The animals were treated daily with Ly281376 at a dose of 100 μg/g of body weight/day for 29 consecutive days, or a corresponding volume of vehicle solution, a treatment that did not change body weight. Animals were sacrificed within 4 h after the last treatment. Brain tissue was snap frozen and kept at −80°C until further processing.

Ablation of Nrg1 expression during adulthood
The cre-ER™ and IgNrg1b£ox/£ox strains have been described (Yang et al., 2001; Hayashi and McMahon, 2002). Briefly, in cre-ER™ mice the cre-ER™ fusion protein is ubiquitously expressed under the control of CAG promoter (CMV enhancer/chicken beta-actin promoter, cf. Hayashi and McMahon, 2002). We used a previously established colony of cre-ER™IgNrg1b£ox/£ox mice, in which cre-mediated recombination of Nrg1b£ alleles only occurs after tamoxifen binds to the oestrogen receptor domain (ER©/©) of cre-ER™ (Fricker et al., 2013). Recombined IgNrg1b£ alleles generate truncated Nrg1 mRNA, and the resulting truncated EGF domain (EGF©/©) is unprocessed. Ten-week-old cre-ER™IgNrg1b£ox/£ox animals were treated with tamoxifen (Fricker et al., 2013). Animals were kept for further 16 weeks before dissection. Cre-ER™IgNrg1b£ox/£ox animals treated with tamoxifen are referred as coTxIgNrg1b, and were compared with three control groups (cre-ER™IgNrg1b£ox/£ox vehicle treated; cre-ER™IgNrg1b©/© tamoxifen treated; Nrg1b£ox/£ox tamoxifen treated). All control groups displayed similar characteristics and were therefore grouped for the spindle length analysis.

Animal handling and assessment of motor coordination
The motor coordination of 5–7 months’ old Ly281376-treated, Bace1 and co-IgNrg1b mutant mice were assessed using gait analysis (Treadscan, Clever Sys Inc. Beare et al., 2009) and inverted grid tests (Coughenour et al., 1977; Landauer et al., 2000), to assess the motor coordination of the cohort of 4-month-old coTxIgNrg1b using beam walking (as described in Carter et al., 2001; Baskin et al., 2003; Taylor et al., 2005). All experiments were conducted in accordance with institutional German regulations and home office guidelines in UK, respectively.

Double in situ hybridization and immunohistochemistry
Fluorescent double in situ hybridization and immunohistochemical analyses were performed as described (Wende et al., 2012) and acquired using Zeiss LSM700 confocal microscope. The sequences of IgNrg1 and Bace1 ISH probes correspond to the 5′UTR and type I-specific coding sequence of Nrg1, or the coding sequence and 3′ UTR of Bace1, respectively. Antibodies used were Egr3 (rabbit, 1:500, Santa Cruz), Neuronfilament 200 (chicken, 1:20,000, Millipore), calbindin (rabbit, 1:500, Swant), calretinin (rabbit, 1:500, Millipore), S100 (rabbit, 1:1,000, Dakocytomation), TrkC (rat, 1:500, R&D Systems), Islet1/2 (mouse, 1:200, DSHB). DAPI (250 nM, Invitrogen) was used as nuclear counter stain. Co localization of Bace1 and IgNrg1 transcripts in DRG neurons was performed on eight DRG sections from four animals. TrkC± and Islet1± sensory neurons were quantified from serial sections (12 μm) of L5 DRG, in the five centromost sections of 3–6 animals/genotype/age. Only TrkC± cells displaying nuclei in the focal plane were counted.

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**Muscle spindle identification and quantification**

The number of muscle spindles per section were counted in lower hindlimbs or the tibialis anterior of 4–6 animals per genotype/treatment. Wild-type littermates were used as controls. The lower hindlimb (P0/P30) or the tibialis anterior (P30/P180) were sectioned transversally (60 μm), every fourth section was stained, and five (P0 animals) or 10 (adult animals) sections of the center of the muscle were evaluated per animal. Muscle spindles were defined as encapsulated structures displaying specific morphological hallmarks (nuclear clustering, periaxial space of Sherrington) and containing at least two intrafusal fibres expressing Eg3 (P0) or calbindin (adult). Morphological criteria and immunohistological signals were used to prevent an underestimation of muscle spindle numbers caused by loss of innervation or reduction of Eg3/ calbindin expression in mutant strains. Expression of calbindin in intrafusal fibres was particularly weak in P180 control mice, and absent in age-matched coTxNrg1 mutants. We therefore used morphological criteria (nuclear aggregation, space of Sherrington) to identify muscle spindles in P180 animals.

Muscle spindle diameter and intrafusal fibre counts were obtained from immunohistological sections and were determined at the level of the annulospiral endings (equatorial plane), identified by NF200 staining; 25 muscle spindles/animal were analysed. The area enclosed by the outer capsule of the spindle was measured using ImageJ software 1.46j. Intrafusal fibres were identified using Eg3/ calbindin staining and cell morphology. Each point represents mean ± s.e.m. of data obtained from 5–6 animals per genotype.

For 3D reconstructions of the tibialis anterior muscle, pictures of sections were assembled as a stack. On individual pictures, the surface of the muscle was outlined and the position of muscle spindles was indicated. Muscle spindles were then aligned for reconstructions using ImageJ 1.46j. Muscle spindle length was estimated on reconstructed muscle, data points represent mean ± s.e.m. of 3–6 animals per condition.

**Cell culture and biochemical analyses**

The Bace1 plasmid was described in Westmeyer et al. (2004). For HEK293 cell culture experiments, IgNrg1β/2 (type I isoforms) and CRD-Nrg1β cDNAs were inserted into pSecTag and pcDNA3.1 expression plasmids, respectively. IgNrg1B and β2 carry an HA tag close to the N-terminus, while CRD-Nrg1β bears its HA tag between EGF and transmembrane domains (Fleck et al., 2013). In constructs used for SEAP assay, the alkaline phosphatase sequences substituted sequences of the type I-specific and Ig domains of Nrg1 (Willen et al., 2006). The sequences of all constructs were verified by sequencing.

HEK293 cells were cultured in DMEM + 10% FCS + penicillin/streptomycin (Gibco-BRL Invitrogen) and transfected (5 μg DNA) using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. The hippocampal neurons were isolated from embryo using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. The hippocampal neurons were isolated from embryo-

Bacteroides distasonis were plated at 17 700 cells per cm² onto 6 cm dishes coated with poly-L-lysine (1 mg/ml; Sigma) and cultured in Neurobasal medium supplemented with 2% B27 and 0.5 mM l-glutamine (all from Invitrogen). We transfected primary neurons after 4 days of culture with pSecTag-HA-IgNrg1β plasmid (5 μg DNA) using Lipofectamine 2000. After 9 days in culture, cells were incubated for 24 h in the presence/absence of Bace1 (C3; 0.2 μM) or metalloproteasine (GM6001; 20 μM) inhibitors (EMD Biosciences, Merck). Supernatants and cells were independently collected either 24 h post-transfection (HEK293) or following the inhibitor treatment (primary neurons) for western blot analysis of Nrg1 cleavage. Supernatants were cleared by centrifugation and cells were lysed in RIPA buffer containing protease inhibitor mix (Sigma P8340). Immunoprecipitation from neuronal supernatants was performed with anti-HA Agarose (Sigma A2095; 25 mg/ml) and washed according to standard protocols (Fleck et al., 2013). Western blots were performed as described (Fleck et al., 2013) using the 4F10 antibody, which recognizes an epitope that is exclusively presented by Bace1-cleaved N-terminal Nrg1 fragments (1:40) and commercial antibodies directed against C-terminal Nrg1 (sc348, Santa Cruz, 1:2000), Bace1 (D105E, Cell signalling, 1:1000), calnexin (AF18, Enzo, 1:5000) and HA (3F10-HRP coupled, Roche, 1:10 000). Secondary anti-IgG-HRP antibodies were from Santa Cruz (1:2000) or Promega (1:10 000).

For SEAP analysis, the medium was changed 24 h after transfection and cells were incubated for 16 h in the presence/absence of 5 μM C3 and 20 μM GM6001 (EMD Biosciences, Merck). The SEAP assay was performed as described (Willem et al., 2006).

**Semi-quantitative PCR**

qPCR was essentially performed as described (Wende et al., 2012). The primers used are shown in Supplementary Table I. In Nrg1 isoform analyses, the amount of CRD-Nrg1 transcripts in control DRG was set to 100%, and amounts of other isoforms in control and mutant DRG were displayed as proportion of this value.

**Statistics**

Data are presented as mean ± s.e.m. The differences observed were assessed by ANOVA followed by 2-tailed unpaired Student’s modified t-test. Significance is displayed as follows: *P* < 0.05; **P** < 0.01; ***P** < 0.001. Supplementary Table II details the statistical analysis for the quantification of muscle spindles in newborn Bace1 and IgNrg1 mutants presented in Figure 5A.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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**Author contributions**

CC performed histological and behavioural analyses, MW, ST and CH performed and analysed biochemical experiments; FRF and DLB provided tissue from and performed behavioural testing of coTxNrg1 mice; HW generated the IgNrg1 strain and performed ISH experiments. AW-G supervised Ly2811376 treatment; KAN and PS provided IgNrg1 Bace1 controls spindle development and maintenance

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).
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