RESEARCH ARTICLE

Brorin is required for neurogenesis, gliogenesis, and commissural axon guidance in the zebrafish forebrain

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Abstract

Bmps regulate numerous neural functions with their regulators. We previously identified Brorin, a neural-specific secreted antagonist of Bmp signaling, in humans, mice, and zebrafish. Mouse Brorin has two cysteine-rich domains containing 10 cysteine residues in its core region, and these are located in similar positions to those in the cysteine-rich domains of Chordin family members, which are secreted Bmp antagonists. Zebrafish Brorin had two cysteine-rich domains with high similarity to those of mouse Brorin. We herein examined zebrafish brorin in order to elucidate its in vivo actions. Zebrafish brorin was predominantly expressed in developing neural tissues. The overexpression of brorin led to the inactivation of Bmp signaling. On the other hand, the knockdown of brorin resulted in the activation of Bmp signaling and brorin morphants exhibited defective development of the ventral domain in the forebrain. Furthermore, the knockdown of brorin inhibited the generation of γ–aminobutyric acid (GABA)ergic interneurons and oligodendrocytes and promoted the generation of astrocytes in the forebrain. In addition, brorin was required for axon guidance in the forebrain. The present results suggest that Brorin is a secreted Bmp antagonist predominantly expressed in developing neural tissues and that it plays multiple roles in the development of the zebrafish forebrain.

Introduction

During embryonic development of the vertebrate brain, the neural plate undergoes regional subdivisions into the forebrain, midbrain, hindbrain, and spinal cord. The forebrain is subdivided into the secondary prosencephalon, consisting of the telencephalon and hypothalamus, and the diencephalon undergoes subdivisions into the thalamus, prethalamus, zona limitans intrathalamic (ZLI), and pretectum [1,2]. The telencephalon is also subdivided into the ventrally positioned subpallial telencephalon and dorsally located pallial telencephalon. Interactions between secreted signaling molecules are crucial for the regionalization and control of cell proliferation and also for the specification of cell fates in the telencephalic and diencephalic...
subdivisions. Bone morphogenetic proteins (Bmps) have numerous roles in neural development and Bmp signaling is involved in growth and patterning in the dorsal telencephalon [3–7]. In addition, the cross-regulation of Bmp, Wnt, and Fgf signaling is required for dorsal telencephalic patterning [5,6]. Bmps are also known to transform the fate of neural precursors from neurogenesis or oligodendroglialogenesis to astrogliogenesis [8–10]. In the ventral region of the forebrain, patterning is coordinated via Hedgehog (Hh) signaling, which is critical for specifying ventral forebrain neurons [11–13].

Bmps are secreted signaling molecules that are members of the TGF-β superfamily [14], and are subjected to regulation by numerous secreted regulators, including Noggin, Follistatin, FSRP, and members of the Chordin family and DAN/Cerberus family [15]. Brorin [also known as von Willebrand factor C domain-containing protein 2 (Vwc2)] was previously identified in mice, zebrafish, and humans [16,17]. Mouse Brorin has two cysteine-rich domains, the cysteines in which are located at similar positions to those in the domains of Chordin family members [16,18]. Brorin has been shown to inhibit the activity of Bmps in vitro [16]. In the mouse, Brorin is predominantly expressed in the neural tissues of embryos and adult animals, and Brorin reportedly promoted neurogenesis, but not astrogliogenesis, in cultured mouse neural precursor cells [16]. However, the role of Brorin in early neural development has not yet been elucidated.

We previously identified zebrafish brorin [17]. In the present study, we investigated zebrafish brorin activity during the embryonic development of the brain. We demonstrated that brorin inhibited Bmp signaling and played a critical role in the development of the ventral domain and specification of γ-aminobutyric acid (GABA)ergic interneurons and oligodendrocyte progenitors in the forebrain. It was also implicated in the suppression of astrocyte generation in the forebrain. Our results indicate that brorin is essential for the appropriate expression of axon guidance molecules and has a role in the formation of forebrain commissural axons.

**Materials and methods**

**Husbandry**

Zebrafish (*Danio rerio*) were maintained, embryos were obtained by natural spawning and cultured, and their developmental stages were evaluated as described previously [19]. These experiments were approved by and conducted according to the guidelines of the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Pharmaceutical Sciences (protocol approval number: 2015–26).

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Expression profiles were assessed over time by RT-PCR using a pair of primers for an 806-bp fragment of brorin (5′-CTCTTGTACACAACTGACG-3′ / 5′-TAGCAGATGGTGCATTGTC-3′) and zebrafish elongation factor 1-α (ef1α) [20].

**Whole mount in situ hybridization**

Whole mount in situ hybridization was performed as described previously using digoxigenin-labeled RNA probes [21]. The brorin probe was synthesized using a plasmid containing full-length cDNA. The following probes were also employed: zebrafish emx1 [22], tbr1 [23], dix2a [24], shh [25], ngn1 [26], ascl1a [27], gad1 [28], plp [29], glula [30], netrin 1a [31], and sema3d [32].
Injection of RNA

The entire coding region of zebrafish brorin cDNA was inserted into a vector (pCS2+) [33]. Using a mMESSAGE mMACHINE kit (Ambion), capped brorin mRNA was synthesized from linearized brorin cDNA derived from pCS2+. mRNA was then diluted with water to 0.4 μg/μl and 1 nl was injected into 2-cell to 4-cell zebrafish embryos.

Injection of morpholino oligonucleotides

After synthesis by Gene-Tools, LLC (Corvallis, OR), morpholino oligonucleotides (MOs) were diluted in Danieau buffer [34]. The two MOs used were splice site-targeted brorin MO1, with a 25-base antisense sequence corresponding to that between intron 1 and exon 2 of the coding region (5’-ATGGAGACACCTAGAAGAACAAACC-3’), and splice site-targeted brorin MO2, with a 25-base antisense sequence corresponding to that between exon 1 and intron 1 of the coding region (5’-CACTTAATGTGCTGCTCATAACCTTA-3’). The control MO sequence was 5’-CCTCTTACCTCAGTTACAAATTATA-3’ [35–37]. Either brorin MO1 (6 ng), brorin MO2 (12 ng), or control MO (12 ng) was injected into 2-cell to 4-cell zebrafish embryos.

In order to investigate the effectiveness of these MOs, RNA was isolated from embryos injected with control MO, brorin MO1, or brorin MO2, and RT-PCR was performed using the above primers.

Immunohistochemistry

Whole mount immunostaining was performed as described previously [20] using rabbit anti-phospho-Smad1/5/8 (Cell Signaling) diluted to 1:100 [38,39] and mouse anti-acetylated tubulin (Sigma) diluted to 1:200 [40]. Alexa Fluor 488 goat anti-rabbit IgG (1:200; Invitrogen) or anti-mouse IgG (1:500; Invitrogen) was employed for the detection of fluorescence.

Results

Characterization of zebrafish brorin

We previously identified the zebrafish brorin gene in a homology-based search of zebrafish nucleotide sequences in GenBank using the amino acid sequence of mouse Brorin [17]. Zebrafish Brorin is presumed to be a secreted protein composed of 309 amino acids with a putative 22-amino acid signaling sequence at its amino-terminus (Fig 1A). In its core region, it has two cysteine-rich domains with high similarity to those of mouse Brorin (Fig 1A). While the 127-amino acid sequence of the amino-terminal region shares less similarity with that of mouse Brorin, the other regions of zebrafish Brorin and mouse Brorin are highly similar (~82% identity) (Fig 1A) [16].

The coding region of zebrafish brorin is divided into 2 introns. The coding region of mouse Brorin is also divided into 2 introns, with similar positions to those of zebrafish brorin (Fig 1A) [16]. Zebrafish brorin is closely linked to the ikzf1 and fingl1 genes on chromosome 13, while mouse Brorin is closely linked to the Ikcj1 and Fingl1 genes at A2 on chromosome 2 (Fig 1B). These results also indicate that zebrafish brorin is a zebrafish ortholog of mouse Brorin.

Pattern of brorin expression in the brain

The expression of brorin in the brains of zebrafish embryos has already been reported at 36 hours post fertilization (hpf) [17]; however, its expression has not yet been examined at different stages of embryonic development. The time course of brorin expression during embryonic development was initially examined using RT-PCR. A RT-PCR analysis was performed using samples ranging between 3hpf and 3dpf. A low level of brorin expression was initially detected.
at 18 hpf, after which its expression gradually increased and was detected until at least 72 hpf (Fig 2A).

The spatiotemporal pattern of brorin expression in the embryonic zebrafish brain was subsequently investigated by whole mount in situ hybridization. A low level of brorin expression was initially observed in the diencephalon primordium at 16 hpf (Fig 2B). Its expression was also detected in the diencephalon primordium at 18 hpf (Fig 2C). At 24 hpf, brorin expression was detected in the telencephalon and prethalamic/alar hypothalamic region, (Fig 2D), as well as in several patches of cells in the hindbrain and spinal cord (Fig 2F and 2G). A low level of brorin expression was also noted in the posterior part of the midbrain, olfactory placode, and pituitary gland at 24 hpf (Fig 2D and 2E). At 36 hpf, brorin expression was detected in the ventral telencephalon, prethalamic/alar hypothalamic region, olfactory placode, hindbrain, and spinal cord (Fig 2H and 2I and data not shown). A low level of brorin expression was also found in the posterior tubercular and pituitary gland at 36 hpf (Fig 2H). This expression of brorin at 36 hpf was consistent with our previous findings [17]. Its expression in the forebrain, hindbrain, and spinal cord persisted until at least 48 hpf (Fig 2I and 2J and data not shown). Although the strong expression of brorin was still noted in the brain, greatly diminished in the olfactory placode (Fig 2K).
Fig 2. Pattern of brorin expression in zebrafish embryos. (A) Amplification of brorin by RT-PCR at the indicated stages (the lower panel shows ef1α as a control). (B-K) Expression of brorin in zebrafish embryos at
Inhibition of brorin functions in zebrafish embryos

In order to assess the role of brorin during the development of zebrafish, knockdown experiments were performed with MOs. Two different splice site-targeted MOs (MO1 and MO2) for brorin were injected into 2-cell embryos to examine whether splicing of the brorin mRNA precursor was efficiently blocked (Fig 3A). Amplified cDNA from brorin MO1-injected embryos was shorter than wild-type cDNA and underwent abnormal splicing to yield a truncated translation product (Fig 3A–3C). The expression of mature brorin mRNA was markedly decreased in embryos injected with brorin MO2 (Fig 3B). New bands at higher or lower molecular weights, which are indicative of cryptic splicing products or exon skipping, were not detected, suggesting the degradation of incorrectly spliced transcripts by nonsense-mediated decay. These results indicate that the two non-overlapping MOs both effectively blocked the maturation of brorin mRNA.

Embryos injected with control MO developed normally, whereas brorin morphants were morphologically defective in the formation of the boundary between the telencephalon and diencephalon, and tectal ventricle at 24 hpf (MO1, n = 175/185 and MO2, n = 29/29) (Fig 3D–3F). Furthermore, we investigated whether brorin RNA rescues the phenotype of brorin MO-injected embryos. We found that the co-injection of brorin RNA with brorin MO1 prevented the development of brain defects caused by brorin MO1 (n = 11/12) (Fig 3G). Thus, these results suggest that brorin is required for normal brain development.

Effects of brorin on Bmp signaling

Since mouse Brorin has been shown to antagonize Bmp signaling in vitro [16], we investigated the effects of brorin knockdown on the Bmp signaling pathway in order to elucidate the mechanisms underlying the phenotypes of brorin morphants. The binding of Bmps to their receptors induces the phosphorylation of Smad proteins, after which phosphorylated Smad (pSmad) is translocated into the nucleus to regulate the transcription of various target genes [41]. Therefore, we examined the phosphorylation of Smad proteins using an antibody that recognizes phosphorylated Smads 1, 5, and 8 in embryos injected with brorin MO at 24 hpf. At 24 hpf, pSmad was detected in dorsal cells in the brains of wild-type embryos (Fig 4A). In brorin morphants, pSmad was increased in the dorsal region of the brain (MO1, n = 11/11 and MO2, n = 18/18) (Fig 4B, and data not shown). This result indicates that the inhibition of brorin leads to the activation of Bmp signaling.

In order to establish whether Brorin inhibits the Bmp signaling pathway in vivo, we performed gain-of-function experiments. In early zebrafish embryos, Bmps are expressed in the ventral margin of the blastula and a ventral-to-dorsal gradient of Bmp activity is essential for patterning of the dorsoventral axis. The non-axial region of the tail is lost in the zebrafish mutants bmp2b/swirl, bmp7/snailhouse, and smad5/somitabun [42–45]. Similar phenotypes have been observed after the misexpression of a Bmp inhibitor such as noggin1 [46]. Accordingly, the inhibition of Bmp signaling prevents tail development. At 24 hpf, embryos injected with brorin RNA exhibited morphological abnormalities in the brain and defects in the tail (n = 39/43) (Fig 5A–5D). In order to investigate the effects of brorin overexpression on the Bmp signaling pathway, we examined the phosphorylation of Smad1/5/8 in embryos injected

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Fig 3. Inhibition of brorin function in zebrafish embryos. (A) The coding region of brorin is divided into two introns, with open boxes and black lines indicating exons and introns, respectively. MO indicates the target position of brorin MO. (B) brorin cDNA was amplified from the cDNA of wild-type embryos or brorin MO-injected embryos by RT-PCR using the P1 and P2 primers, the positions of which are indicated by arrows (A). (C) The nucleotide sequences of brorin cDNAs were elucidated. Numbers show the nucleotide sequence of the coding region and amino acid sequence, and arrowheads indicate splice sites between exons one and two. (D-G) Lateral views of control MO-injected (D), brorin MO1-injected (E), brorin MO2-injected (F), and
brorin RNA at 8 and 24 hpf. Consistent with the above results, pSmad 1/5/8 staining was not detected in the ventrolateral domain of embryos injected with brorin RNA at 8 hpf \((n = 22/25)\) (Fig 5E and 5F). At 24 hpf, pSmad was detected in the dorsal cells of the forebrain and eye in wild-type embryos, but not in embryos injected with brorin RNA \((n = 11/11)\) (Fig 5G and 5H). Furthermore, a decrease in pSmad in the ventral part of the somite was observed in brorin RNA-injected embryos that exhibited a mild defect in the tail, whereas high levels of pSmad were detected in wild-type embryos \((n = 19/22)\) (Fig 5I and 5J). These results indicate that the overexpression of brorin leads to the inactivation of Bmp signaling.

Dorsalization is also caused by the inhibition of the Wnt signaling pathway and embryos lacking wnt8 display a similar phenotype to that caused by the inhibition of the Bmp signaling pathway [46]. Therefore, in order to investigate the effects of brorin knockdown on the Wnt signaling pathway, we examined the expression of axin2, which is a direct target gene of the canonical Wnt signaling pathway, in brorin morphants at 24 hpf. In brorin morphants, the expression of axin2 was not increased in the brain \((\text{MO1}, n = 13/13)\) (Fig 4C and 4D). These results indicate that Brorin inhibits Bmp signaling, but not canonical Wnt signaling.

### Effects of brorin knockdown on patterning in the forebrain

The pattern of brorin expression in the embryonic zebrafish brain and phenotypic changes in brorin MO-injected embryos suggest that brorin is involved in the formation of the forebrain. Bmp signaling participates in forebrain patterning [3–7]. Therefore, in order to investigate the
Fig 5. pSmad distribution in brorin RNA-injected embryos. (A-D) Lateral views of wild-type (A, C) and brorin RNA-injected (B, D) embryos at 24 hpf. (E, F) Pattern of pSmad expression in wild-type (E) and brorin
involvement of *brorin* in forebrain regionalization, we examined the expression of telencephalon marker genes in *brorin* morphants. In wild-type embryos, the expression of *emx1* and *tbr1* (pallial telencephalon marker genes) was not detected in the subpallial region of the telencephalon at 24 hpf. In *brorin* morphants, the ectopic expression of *emx1* and *tbr1* was detected in the subpallial domain of the telencephalon (MO1, *n* = 22/22 and *n* = 14/14, respectively) (Fig 6A–6D). In contrast, the expression of *dlx2a*, which is normally detected in the ventral telencephalon, was reduced in *brorin* morphants at 24 hpf (MO1, *n* = 22/23 and MO2, *n* = 9/12) (Fig 6E and 6F and data not shown). Furthermore, a reduction in *dlx2a* expression was observed in the ventral telencephalon of *brorin* morphants at 18 hpf (MO1, *n* = 15/15) (Fig 6G and 6H). These results indicate that *brorin* is required for the development of the subpallial telencephalon. Furthermore, we investigated whether the knockdown of *brorin* had an effect on diencephalic specification at 24 hpf. The expression of *dlx2a* is normally detected in the prethalamus, but was weakly expressed in *brorin* morphants (MO1, *n* = 22/23 and MO2, *n* = 9/12) (Fig 6E and 6F and data not shown). A reduction in *dlx2a* expression in the prethalamus was also observed in *brorin* morphants at 18 hpf (MO1, *n* = 15/15) (Fig 6G and 6H). However, the expression of *shh* in the floor plate and hypothalamus was unaffected in *brorin* morphants (MO1, *n* = 19/19) (Fig 6I and 6J). These results indicate that *brorin* is required for the complete initiation of *dlx2a* expression in the forebrain.

**Effects of *brorin* knockdown on development of GABAergic neurons, oligodendrocytes, and astroglia**

GABAergic interneurons and oligodendrocytes originate from the subpallial telencephalon and ventral thalamus of the forebrain, and Dlx2 participates in the specification of GABAergic interneurons and oligodendrocytes [47–51]. The reduced expression of *dlx2a* in *brorin* morphants suggests an effect on the specification of GABAergic interneurons and oligodendrocytes in the ventral forebrain. In the forebrain, achaete-scute complex (*ascl*) 1a is expressed by GABAergic interneurons and their precursors [52]. *gad1*, which encodes glutamic acid decarboxylase, is also specifically expressed by GABAergic interneurons [28]. In order to examine whether the knockdown of *brorin* affects the differentiation of forebrain GABAergic interneurons, the expression of *ascl1a* and *gad1* was analyzed in *brorin* morphants at 24 hpf and 28 hpf, respectively. While *ascl1a* expression was normally detected in the ventral telencephalon and diencephalon, it was severely reduced in these regions in *brorin* morphants at 24 hpf (MO1, *n* = 20/20) (Fig 7A and 7B). In addition, *gad1* expression was normal in the subpallial telencephalon and nucleus of the tract of the postoptic commissure (POC), but was markedly reduced in *brorin* morphants at 28 hpf (MO1, *n* = 17/17 and MO2, *n* = 7/7) (Figs 7C and 7D and S1A Fig). The reduction in *gad1* expression in *brorin* morphants was prevented by the co-injection of *brorin* RNA with *brorin* MO1 (*n* = 15/15) (S1B Fig). These results demonstrate that the specification of forebrain GABAergic interneurons is suppressed in *brorin* morphants. We then investigated whether the knockdown of *brorin* affected neuronal differentiation in the pallial telencephalon at 24 hpf. The expression of *ngn1*, which is a basic helix-loop helix (bHLH) proneural gene, was analyzed in *brorin* morphants. In wild-type embryos, a narrow region that did not express *ngn1* was observed in the pallial telencephalon, while the expression of *ngn1* was up-regulated and the region that did not express *ngn1* was undetectable in the pallial telencephalon of *brorin* morphants (MO1, *n* = 16/16) (Fig 7E and 7F). However, the...
Fig 6. Telencephalic and diencephalic gene expression in *brorin* morphants. (A-D) The expression of *emx1* (A, B) and *tbr1* (C, D) in wild-type embryos (A, C) and *brorin* morphants (B, D) at 24 hpf. Arrows in
expression of ngn1 was not detected in the subpallial telencephalon of brorin morphants, in contrast to other pallial telencephalon markers (MO1, n = 16/16) (Fig 7E and 7F). These results suggest that neuronal differentiation in the pallial telencephalon and subpallial telencephalon is affected in brorin morphants. In addition to the pallial telencephalon, ngn1 is normally expressed in the ventral diencephalon; however, its expression in the ventral diencephalon was reduced in brorin morphants (MO1, n = 15/16) (Fig 7E and 7F). This result indicates that neuronal differentiation in the ventral diencephalon is affected in brorin morphants and is consistent with the above results.

We also examined the involvement of brorin in the development of oligodendrocytes. The expression of PLP (proteolipid protein)/DM20, a marker of oligodendrocyte differentiation,
was analyzed in brorin morphants. plp expression was strongly reduced in the brains of brorin morphants at 4.5 dpf (MO1, n = 16/16 and MO2, n = 7/7) (Fig 7G and 7H and S1C Fig). On the other hand, the co-injection of brorin RNA with brorin MO1 prevented the reduction in plp expression caused by brorin MO1 (n = 8/11) (S1D Fig). This result indicates that the development of oligodendrocytes in the brain is suppressed by the knockdown of brorin. We also examined whether the knockdown of brorin affected the development of astroglia. The expression of glula (glutamine synthetase) was analyzed in brorin morphants because Glul (Glns) is predominantly expressed in astrocyte precursors and astrocytes [30]. glula expression was markedly up-regulated in the brains of brorin morphants at 3 dpf (MO1, n = 9/10 and MO2, n = 9/11) (Figs 7I and 7J and S1E Fig). The co-injection of brorin RNA with brorin MO1 suppressed the increased expression of glula caused by brorin MO1 (n = 11/12) (S1F Fig). These results demonstrate that astroglial development is facilitated by the knockdown of brorin.

Effects of brorin knockdown on axon guidance

We investigated the involvement of brorin in the formation of commissures because its expression was confined to the region adjacent to the anterior commissure (AC) and tract of the POC in the forebrain by 36 hpf. We used an antibody against acetylated α-tubulin to examine the formation of forebrain commissures in brorin morphants. In wild-type embryos, neurons of the dorsorostral cluster in the telencephalon projected contralaterally to form the AC at 28 hpf [53,54]. Furthermore, they extended axons ventrally towards the ventrorostral cluster forming the supraoptic tract (SOT) [53,54]. Neurons of the ventrorostral cluster in the diencephalon projected contralaterally to form the POC [53,54]. Although axons from the nucleus of the tract of the AC in the telencephalon and axons from the nucleus

Fig 8. Defects in axon guidance in brorin morphants. Fluorescent immunolabeling of axons (αAT) in wild-type embryos (A, C) and brorin morphants (B, D) at 28 hpf. The arrow and arrowhead in panel A indicate the AC and POC, respectively. The arrow in panel C indicates the SOT. A and B are frontal views; C and D are lateral views, with the anterior to the left.

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of the tract of the POC in the diencephalon were present in *brorin* morphants, these axons did not extend across the midline and forebrain commissures were not formed (MO1, *n* = 23/23) (Fig 8B). In addition, the SOT was not detected in *brorin* morphants (MO1, *n* = 23/23) (Fig 8D). These results indicate that the knockdown of *brorin* affects axon guidance in the forebrain.

In order to clarify whether *brorin* is involved in establishing the commissural axon growth substrate, we analyzed the expression of axon guidance molecules (*netrin1a* and *sema3d*) in *brorin* morphants. In zebrafish, *netrin1a* is normally absent from the diencephalon, in which the POC forms, whereas *netrin1a* expression was up-regulated in the telencephalon and expanded across the optic recess into the ventral thalamus in *brorin* morphants (MO1, *n* = 16/17 and MO2, *n* = 8/9) (Figs 9A and 9B and S1G Fig). The co-injection of *brorin* RNA with *brorin* MO1 suppressed the increased expression of *netrin1a* caused by *brorin* MO1 (*n* = 10/13) (S1H Fig). In contrast, *sema3d* expression was normally detected at the midline of the diencephalon immediately ventral to the POC, but was reduced in the diencephalon in *brorin* morphants (MO1, *n* = 15/15) (Fig 9C and 9D).

**Discussion**

**brorin** inhibits Bmp signaling

Bmps play a crucial role in the diverse processes of morphogenesis and development [15, 56], and are subjected to negative or positive regulation by various secreted regulators, including Chordin family members [15]. The Chordin family of proteins possesses three to eighteen cysteine-rich domains, each consisting of 10 cysteine residues [18, 57, 58]. Mouse Brorin is a neural-specific secreted antagonist of Bmp signaling with two cysteine-rich domains in its core region, and the cysteine residues in these domains are located at similar positions to those in other Chordin family members [16]. Among the Chordin family members, these cysteine-rich
domains are the most similar to those of Crossveinless-2, which functions as a Bmp antagonist and pro-Bmp factor [16,58]. However, the amino acid sequence of mouse Brorin does not share structural similarities with other members of the Chordin family, and Brorin is a unique member of this family. The core region of zebrafish Brorin also has two cysteine-rich domains. The amino acid sequence of the 127-amino acid amino-terminal region of zebrafish Brorin was less similar to that of mouse Brorin, although the other regions of zebrafish Brorin (182 amino acids) were highly similar to mouse Brorin. We concluded that Brorin is a zebrafish ortholog of mouse Brorin based on the conservation of the intron-exon organization and syntenic relationship. Our results suggested that the functional region of Brorin was located in the core region containing the two cysteine-rich domains.

Exogenous Brorin has been shown to inhibit the phosphorylation of Smad by Bmp2 and Bmp6 as well as osteoblastic differentiation in vitro [16]. In zebrafish, Bmps are essential for ventralization of the embryo and the inhibition of Bmp signaling results in embryos that are devoid of the non-axial region of the tail [42–46]. We found that the overexpression of brorin inhibited the phosphorylation of Smad at the gastrulation stage and led to defects in the tail in zebrafish embryos. Furthermore, we observed the loss of pSmad in the forebrain of brorin RNA-injected embryos and an increase in pSmad in the brains of brorin morphants at 24 hpf. Embryos injected with brorin RNA and brorin morphants both exhibited morphological abnormalities in the brain. On the other hand, the Wnt signaling pathway was not promoted by the knockdown of brorin, although the inhibition of Wnt signaling leads to the dorsalization of zebrafish embryos and results in a similar phenotype to that caused by the inhibition of Bmp signaling. These results indicate that Brorin inhibits Bmp signaling, but not Wnt signaling, suggesting that it acts as a Bmp antagonist in vivo.

**brorin is involved in forebrain development**

In the telencephalon, *dlx2a* expression was decreased in the ventral region at 24 hpf by the knockdown of brorin. In addition, brorin morphants exhibited the ectopic expression of markers of the pallial telencephalon in the ventral telencephalon along with the reduced expression of a marker of the subpallial telencephalon. These results indicate that brorin is required for the development of the subpallial telencephalon. Furthermore, the activation of Bmp signaling was observed in the dorsal region of the brain in brorin morphants at 24 hpf. Bmp activity from the roof plate was previously shown to be involved in patterning of the dorsal telencephalon [4–7]. These findings suggest that alterations in gene expression in the subpallial telencephalon of brorin morphants are due to increases in Bmp activity in the dorsal region of the brain.

Wnt and Fgf are also involved in forebrain patterning and previous studies reported that the cross-regulation of Bmp, Wnt, and Fgf signaling in the early telencephalon is required to pattern the cerebral cortex [4–6]. Excess Fgf8 has no effects on *Bmp4* expression in the cortical hem, whereas an increase in Bmp activity suppresses *Fgf8* expression in the anterior telencephalon and anterior neural ridge [6]. On the other hand, the inhibition of Bmp signaling results in the loss of expression of *Wnt* genes in the cortical hem and excess Fgf8 also suppresses the expression of *Wnt* genes [6,7]. However, *axin2* expression was not increased in the forebrain of brorin morphants. Since Bmp antagonists other than brorin are also expressed in the forebrain, we speculate that the activation of Bmp signaling caused by the inhibition of brorin alone may be insufficient to activate Wnt signaling. Furthermore, the inhibition of both *fgf3* and *fgf8* has been shown to suppress *tbr1* expression [59]. However, the expansion of *tbr1* expression was detected in the telencephalon of brorin morphants. Therefore, patterning of the telencephalon by brorin may not be mediated through the Fgf signaling pathway. A direct role
for Shh in patterning of the dorsal telencephalon has also been reported [60]. However, the phenotype of brorin RNA-injected embryos was not similar to that of shh RNA-injected embryos, because the overexpression of shh results in abnormalities in the forebrain and eyes, but not in the tail [61, 62]. Thus, brorin has been suggested to play a role in patterning of the telencephalon by inhibiting Bmp signaling and the inactivation of Bmp signaling by brorin is not mediated through Shh.

In the diencephalon, dlx2a expression was also decreased in the prethalamus at 18 and 24 hpf by the knockdown of brorin, whereas shh expression was unaffected in the hypothalamus. These results demonstrate that brorin is required for the complete initiation of dlx2a expression in the prethalamus. Thus, we expect brorin to be involved in patterning of the forebrain through its inhibition of Bmp signaling.

**brorin is required for the specification of oligodendrocyte progenitors and GABAergic interneurons and inhibits astrogliogenesis in the forebrain**

Previous studies reported that Ngn1 confers neuronal identity on uncommitted precursors and is essential for neurogenesis [63–65]. Brorin was shown to be involved in neuronal differentiation in vitro [16]. In brorin morphants, the expression of ngn1 was reduced in the ventral diencephalon, but was up-regulated in the pallial telencephalon. These results suggest that brorin modulates neuronal differentiation in the telencephalon and diencephalon. The ectopic expression of Dlx2 in cortical explants results in the induction of the GABAergic marker GAD1, and Ascl1 is also required for proper GABAergic specification [66, 67]. The present study showed that the expression of dlx2a and ascl1a was reduced in the forebrains of brorin morphants. Furthermore, the knockdown of brorin resulted in a marked reduction in the expression of gad1 in the ventral telencephalon and diencephalon. Accordingly, brorin appears to play a crucial role in the differentiation of GABAergic interneurons.

PLP is expressed in oligodendrocyte progenitor cells [68–70]. The knockdown of brorin resulted in a marked reduction in plp expression, in addition to the decreased expression of dlx2a, in the brain. However, the expression of glula, a marker of the astroglial lineage, was increased in the forebrain of brorin morphants. These results demonstrated that the knockdown of brorin suppresses oligodendroglial specification and promotes astrogliogenesis in the forebrain. Thus, brorin is required for the specification of oligodendrocyte progenitors and is involved in suppressing the development of astroglia in the forebrain. The knockdown of brorin led to the activation of Bmp signaling. The repression of the Bmp pathway is known to be required for oligodendroglial specification during development of the vertebrate brain [10]. Bmp signaling inhibits the specification of oligodendrocytes from neural progenitor cells and promotes the generation of astrocytes [8–10]. Consequently, Bmps promote the differentiation of glial progenitors toward the astroglial lineage. Accordingly, brorin may regulate glial cell differentiation toward an oligodendroglial fate by repressing the Bmp pathway.

**brorin is required for the appropriate expression of axon guidance molecules and axon guidance**

The AC, POC, and SOT were absent in brorin morphants, demonstrating that the loss of brorin influences commissure formation. In vertebrates, commissural axon crossing is regulated by a combination of attractive and repulsive cues [71–74]. Netrins attract commissural axon growth cones toward the midline of the central nervous system (CNS), whereas Semaphorins typically repel growth cones [75–77]. In brorin morphants, the expression of sema3d was lost in the diencephalon, whereas it was unaffected in the midbrain. Furthermore, the expression of netrin1a was increased in the telencephalon and expanded across the commissure region in
the diencephalon, indicating that brorin is required for the proper expression of netrin1a and sema3d in the forebrain. Accordingly, brorin may regulate commissure formation by modulating the expression of axon guidance molecules. However, Brorin itself may be an axon guidance molecule because Bmps have been shown to influence axon guidance in the CNS by acting directly on axons [78].

Conclusions

brorin inhibits Bmp signaling in the zebrafish and is involved in the development of the forebrain, including the specification of GABAergic interneurons and oligodendrocytes as well as the inhibition of astrocyte generation. Furthermore, brorin is required for the appropriate expression of axon guidance molecules and commissure formation in the forebrain. These results implicate brorin in regionalization, cell-type specification, and axon guidance through the repression of Bmp signaling during zebrafish forebrain development. The amino acid sequence of Brorin is similar to that of Brorin-like/vwc2l, and we previously reported the expression of brorin-like and phenotype of brorin-like knockdown [17]. In the forebrain, the expression pattern of brorin is similar to, but distinct from that of brorin-like. At 36 hpf, brorin expression was detected in the ventral telencephalon, while brorin-like was not expressed in the telencephalon [17]. On the other hand, brorin and brorin-like were both expressed in the prethalamic/alar hypothalamic region [17]. In addition, the phenotype of brorin knockdown is similar to, but distinct from that of brorin-like. Therefore, we conclude that brorin has unique roles in the development of the forebrain. However, brorin and brorin-like may in part function redundantly during forebrain development. This will be addressed in a future study.

Supporting information

S1 Fig. Effects of brorin knockdown on the specification of GABAergic interneurons, oligodendrocytes, and astrocytes, and axon guidance. The expression of gad1 (A, B), plp (C, D), glula (E, F), and netrin1a (G, H) in brorin MO2-injected (A, C, E, G) and brorin MO1- and brorin RNA-injected (B, D, F, H) embryos is displayed at the indicated stages. (TIF)

Author Contributions

Conceptualization: AM NI.
Data curation: AM.
Formal analysis: AM YM.
Funding acquisition: AM.
Investigation: AM YM HF KN.
Methodology: AM.
Project administration: AM.
Resources: AM YM HF KN MK.
Supervision: AM.
Validation: AM MK NI.
Visualization: AM YM.
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