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A study on the function of Rho guanine nucleotide exchange factors in axon branching of developing cortical neurons

（発達期大脳皮質ニューロンの軸索分岐における Rho グアニンヌクレオチド交換因子の機能に関する研究）

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Abstract

Axon branching is regulated by cytoplasmic signaling molecules via cytoskeletal remodeling. However, what molecular mechanisms are involved in this process remains elusive. It has been shown that active RhoA promotes branch formation of horizontally elongating axons (horizontal axons), which originate from layer 2/3 neurons in the developing mammalian cortex. Based on this finding, I studied the role of RhoA-GEFs, Rho guanine nucleotide exchange factors that activate RhoA, in horizontal axon branching. *In situ* hybridization showed that more than a half RhoA-GEF member was expressed in the developing rat cortex. These RhoA-GEFs were mostly expressed in the macaque cortex as well. An *in vitro* study demonstrated that overexpression of seven RhoA-GEFs promote horizontal axon branching. Moreover, branching patterns were different between overexpressing RhoA-GEFs. In particular, ARHGEF18 markedly increased terminal arbors, whereas ABR increased smaller branches in not only distal but also proximal region of horizontal axons. On the other hand, knockdown of either ARHGEF18 or ABR considerably suppressed axon branching. Taken together, the present study suggests that subsets of RhoA-GEFs differentially promote axon branching of mammalian cortical neurons by activating the downstream Rho small GTPase pathways.

Table of Contents

Abstract.....1

General Introduction.....3

References for General Introduction.....7

Introduction.....13

Materials and Methods.....15

Results.....20

Discussion.....36

References.....41

Tables.....50

Acknowledgement.....53

Publications and conference presentations.....54

General Introduction

A formation of precise neuronal circuits underlies various brain functions, such as cognition, learning, memory, sensory perception and motor behavior. During development, neurons extend an axon along appropriate courses. The axon starts to form terminal branches, once it reaches the target region. The number of synapses increases accompanied by branching, so that the single axon innervates multiple target cells. Thus, axon branching is a crucial step to establish neuronal connectivity in the nervous system.

In general, axon branching is regulated by various extracellular cues, including axon guidance molecules, neurotrophic factors, morphogens, cell adhesion molecules, and extracellular matrix proteins (Gibson and Ma, 2011, Bilimoria and Bonni, 2013). Some of these molecules act as positive cues for axon branching (Cohen-Cory and Fraser, 1995, Lucas and Salinas, 1997, Wang et al., 1999, Alsina et al., 2001, Szebenyi et al., 2001, Krylova et al., 2002, Mann et al., 2002, Ozdinler and Erzurumlu, 2002, Soussi-Yanicostas et al., 2002, Ma and Tessier-Lavigne, 2007, Bodmer et al., 2009, Fukunishi et al., 2011, Spillane et al., 2012, Granseth et al., 2013), while others act as negative regulators (Yamamoto et al., 2000, Yates et al., 2001, Bagri et al., 2003, Liu and Halloran, 2005). For example, Netrin-1, one of axon guidance molecules, promotes axon branching of cortical neurons, whereas another axon guidance molecule, semaphorin 3A, inhibits branching (Dent et al., 2004). Secreted molecules work on relatively distant axons, whereas cell surface molecules work in a cell-cell contact dependent fashion. These molecules are released from the surrounding and target cells in a cell type-specific fashion and/or neuronal activity-dependent manner (Castren et al., 1992, Fukunishi et al., 2011, Schwartz et al., 2011, Hayano et al., 2014). In other words,

various extracellular cues cooperate to control precise axon branching in specific locations.

It is also well known that cytoskeletal remodeling is required when axons form branches (Yu et al., 1994, Gallo and Letourneau, 1998, Dent et al., 1999, Gallo and Letourneau, 1999, Dent and Kalil, 2001, Gallo, 2011, Lewis et al., 2013). An axon initiates branching by forming actin rich protrusion from the axon shaft. Subsequently, microtubules invade these protrusions as branch develops. The remodeling of these cytoskeleton proteins is regulated by Rho family small GTPases (Hall, 1998). Several studies showed that these molecular species regulate axon branching of various neuronal types (Ng et al., 2002, Hall and Lalli, 2010, Spillane and Gallo, 2014). For example, a member of Rho family small GTPases, RhoA, has been reported to promote axon branching of hippocampal and cortical neurons (Ahnert-Hilger et al., 2004, Ohnami et al., 2008). These studies imply the existence of intracellular molecules that mediate convergence of variable extracellular signals onto Rho family small GTPases.

Rho family small GTPases act as molecular switches by cycling between the active GTP-bound state and inactive GDP-bound state (Van Aelst and D'Souza-Schorey, 1997). The activity of Rho family small GTPases is regulated by two families of intracellular molecules, Rho guanine nucleotide exchange factors (RhoGEFs) and Rho GTPase activating proteins (RhoGAPs). RhoGEFs activate Rho family small GTPases by catalyzing the exchange of bound GDP to GTP, whereas RhoGAPs inactivate them by enhancing intrinsic GTPase activity (Jaffe and Hall, 2005). More than 70 RhoGEFs and 80 RhoGAPs have been identified in the human genome, each of which are regulated by various cell surface proteins, such as receptor tyrosine kinase, G-protein coupled receptors, and cell adhesion molecules. Each member of RhoGEFs activates

distinct sets of Rho family small GTPases, while the RhoGAPs are thought to act unselectively on broad range of Rho family small GTPases. Considering the variety of RhoGEFs and their selectivity on the downstream Rho family small GTPases, it is possible that RhoGEFs mediate convergence of various extracellular signals to select Rho family small GTPases to tightly regulate axon branch formation. Although some of them have been implicated to function in dendrite as well as axonal development, little is known about the function in axon branching (Aurandt et al., 2002, Ng and Luo, 2004, Margolis et al., 2010, Oh et al., 2010, Park et al., 2012, Hayashi et al., 2013).

To address this issue, I focused on horizontal connections in the mammalian neocortex. Cortical neurons in the upper layers extend a primary axon which travel perpendicularly to the laminar structure towards the white matter, and axon collaterals (horizontal axons) which emit from the primary axon in layers 2/3 and 5 and extend horizontally to cortical layers. These horizontal axons form terminal branches to make synaptic connections with distant cortical neurons, contributing to horizontal spreading of the sensory information (Callaway and Katz, 1990; Ruthazer and Stryker, 1996; Shouval et al., 2000). Horizontal axon branching is found in the mammalian cortex from the rodents to primates, and has a typical feature in branch development, including neuronal activity dependence, and dynamic refinement of necessary branches after formation of exuberant arbors. These aspects suggest that a common mechanism works for establishing horizontal connections.

A previous study has shown that active RhoA promotes horizontal axon branching (Ohnami et al., 2008). I hypothesized that RhoA-activating RhoGEFs (RhoA-GEFs) promotes horizontal axon branching via activation of RhoA. Among the known RhoGEFs, twenty-eight members are reported to function on RhoA, as well as

other Rho family small GTPases (Rossman et al., 2005, Schiller, 2006, Garcia-Mata and Burridge, 2007, Loirand et al., 2008). In this study, I investigated molecular mechanisms of axon branching with focusing on RhoA-GEFs.

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Introduction

Axon branching is an essential process to form synaptic connections with multiple target cells. This process is known to be regulated by various target-derived factors such as secreted molecules, extracellular matrix and cell surface molecules (Bilimoria and Bonni, 2013; Gibson and Ma, 2011). These molecules are released from the surrounding and target cells, and bind to their specific receptors on growing axons to regulate branch formation. Such ligand-receptor interactions cause cytoskeletal changes via cytoplasmic signaling pathways (Gallo, 2011; Hall and Lalli, 2010; Kalil and Dent, 2014; Ng et al., 2002; Spillane and Gallo, 2014). However, the molecular mechanism that underlies the cytoplasmic signaling is almost unknown.

Horizontal connections in the mammalian cortex are one of the suitable systems in which to investigate the molecular mechanisms in CNS neurons. Horizontally elongating axons (horizontal axons) originate from layer 2/3 neurons and form terminal branches to innervate distant layer 2/3 cells, which contributes to horizontal spreading of the sensory information (Callaway and Katz, 1990; Ruthazer and Stryker, 1996; Shouval et al., 2000). Horizontal axon branching is well conserved in mammalian species, suggesting that a common mechanism works for establishing horizontal connections. A previous study has shown that horizontal axon branching is established via a remodeling process with branch addition and elimination. Evidence has further demonstrated that RhoA, a member of small GTPase, positively regulate horizontal axon branching (Ohnami et al., 2008). A plausible mechanism is that each signal of target-derived and intrinsic factors may regulate RhoA activation via the corresponding signaling pathways. However it is unanswered what upstream molecules activate RhoA to promote horizontal axon branching.

In the present study, I focused on Dbl family Rho guanine nucleotide exchange factors (RhoGEFs) as the upstream molecules, which activate Rho GTPases by catalyzing exchange of GDP to GTP (Jaffe and Hall, 2005). Twenty-eight members have been identified to activate RhoA (hereafter referred as RhoA-GEFs), as well as other Rho GTPases (Rossman et al., 2005). I hypothesized that RhoA-GEFs are involved in horizontal axon branching. First, RhoA-GEF expression in the developing cortex was investigated by *in situ* hybridization. Next, the function of RhoA-GEFs on horizontal axon branching was investigated through in gain-of-function and loss-of-function studies *in vitro*. The results demonstrate that some RhoA-GEFs are involved in horizontal axon branching in a distinct fashion and that these RhoA-GEFs are expressed in the mammalian cortex beyond species, suggesting that RhoA-GEFs are evolutionally conserved and contribute to cortical axon branching during development.

Materials and Methods

Animals

Sprague–Dawley (SD) rats were purchased from Japan Lab Animals and used for *in situ* hybridization and cortical slice culture experiments. Four macaque monkeys (*Macaca mulatta*) were used for *in situ* hybridization. All experiments were performed according to the guidelines established by the animal welfare committees of Osaka University, the National Institute for Basic Biology, Japan, and the Japan Neuroscience Society.

Preparation of Plasmid constructs

Full-length ORF clones of human *RhoA-GEFs* were purchased from Promega, and were subcloned into pCAG-HA to obtain hemagglutinin (HA) -tagged RhoA-GEF expression vectors. A list of ORF clones are given in Table 1. The shRNAs for rat *Arhgef18* and *Abr* were designed and cloned into piGENE mU6 vector. The target sequence for *Arhgef18* is 5'-GCAGCAGAGCAAGAAGTTTCA-3' and that for *Abr* is 5'-GGAGAAGTTCAAAGTCTGG-3'. All plasmids were isolated (HiPure Plasmid Maxiprep Kit, Invitrogen) and suspended in Hanks' solution.

Probe preparation

The cDNA fragments of rat and macaque *RhoA-GEFs* were amplified by RT-PCR from total RNA which was isolated from P8 rat cortex and P0 macaque cortex, respectively. The primers used for PCR are listed in table 2 and 3. The cDNA fragment was then cloned into pGEM-T vector. To produce linearized template for the synthesis of RNA probes, the inserts were PCR-amplified with primers containing T7 and SP6

promoter sequences (T7 sequence, TTGTAAAACGACGGCCAGTG; SP6 sequence, TGACCATGATTACGCCAAGC), and the PCR products were purified (QIAquick PCR Purification Kit, Qiagen). DIG-labeled RNA probe was synthesized (DIG RNA Labeling Mix, Roche) following the manufacture's instruction.

In situ *hybridization*

In situ hybridization was performed on developing rat and macaque brains, as previously described (Fengyi et al., 2000; Hayano et al., 2014; Komatsu et al., 2005; Zhong et al., 2004). Briefly, rat brains were harvested and fixed in PFA (4% PFA in PBS) at 4 ^{o}C overnight. After cryopreservation with 30% sucrose in PBS, the brains were sectioned into 20-μm-thick coronal sections using cryostat (Leica CM1850). The sections were subjected to re-fixation, acetylation, and pre-hybridization followed by hybridization with the DIG-labeled probe (4 μg/mL) at 60 ^{o}C. After extensive washing, the sections were incubated with alkaline phosphatase (AP)-conjugated anti-DIG antibody (1:2000, Roche) at 4 ^{o}C overnight. Finally, the hybridized probes were visualized with AP substrate (BM Purple, Roche) at RT.

Macaque brains were sectioned into 40-μm-thick coronal sections using sliding microtome (ROM-380, YAMATO, Japan). Free-floating sections were subjected to re-fixation, proteinase K (0.5/mL) treatment, acetylation, pre-hybridization, followed by incubation in hybridization buffer containing 1 μg/mL DIG-labeled probes at 60 ^{o}C. The sections were washed and then treated with RNase buffer (10 mM Tris-HCl, pH8.0, 1 mM EDTA, 500 mM NaCl) containing 20 μg/mL RNase A. After washing, the hybridized probe was detected with AP-conjugated anti-DIG antibody (1:1000, Roche) and NBT/BCIP AP substrate (Roche).

Organotypic slice culture of cortex

Organotypic slice culture of cortex was performed as described previously (Yamamoto et al., 1989; Yamamoto et al., 1992). In brief, about 300-μm-thick coronal slices were dissected from sensory cortices of P1 rat and were placed on collagen-coated culture membrane (Millicell-CMPICMORG50, Millipore). The cortical explants were cultured in the medium containing DMEM/F12, 10% modified N2 supplement, and 5% FBS for the first 3 days. A half of the medium was changed to DMEM/F12 with 10% modified N2 supplement at 3 days *in vitro* (DIV) and every other day from 7 DIV to 14 DIV. At 14 DIV, the cultures were fixed, immunostained and imaged for the analysis of horizontal axon morphology.

Pharmacological treatment of cultured cortical slices

To investigate the involvement of RhoA signaling in RhoA-GEF function, the Rock inhibitor, Y-27632 was added at a concentration of 50 μM to the culture medium during the second week in culture. Y-27632-containing medium was exchanged every other day.

In vitro *electroporation*

In order to transfect EGFP expression vector, overexpression and knock-down constructs, the cortical slice cultures were subjected to *in vitro* electroporation at 4-5 DIV as described previously (Matsumoto et al., 2015; Uesaka et al., 2008). Briefly, a small amount of plasmid solution containing pTα1-EGFP (2 μg/μL), mixture of pTα1-EGFP plus pCAG-HA-RhoGEF (4 μg/μL), or mixture of pTα1-EGFP plus shRNA expression plasmid (1 μg/μL) was pressure ejected using fine glass capillary

onto the upper part of the cultured slice. Immediately, five trains of 200 square pulses (1 msec duration at 200 Hz, 250 μA) were delivered with another glass micropipette (inner diameter of 150 μm) to the site.

Immunohistochemistry

The procedure of the immunostaining for the slice culture experiment was described previously (Yamada et al., 2010). In brief, Cultured slices were fixed with PFA at 14 DIV, washed, and incubated with rat anti-GFP (1:1000, Nacalai, GF090R) and mouse anti-HA (1:200, Cell signaling, 6E2) in blocking solution (5% normal goat serum, 0.3% Triton X-100 in PBS) at 4 ^{o}C overnight. After extensive washing, the slices were incubated with appropriate secondary antibodies for 2 hr at RT. After washing, the slices were mounted in the medium containing 2.3% DABCO, 1μg/mL DAPI and 50% glycerol.

Image acquisition and analysis

Fluorescently labeled neurons were imaged by confocal microscopy (Nikon C1). For cortical slice cultures, tiled z-stack images were acquired with 10x objective (NA = 0.45, pinhole size 30 μm) at 5-μm steps. The images were stitched using Stitching plugin on Fiji image processing software.

Fluorescently labeled horizontal axons were traced in 2D-projected images using Simple Neurite Tracer plugin of Fiji image processing software. Labeled neurons that met following criteria were chosen for the analysis. First, their soma locates within 100-400 μm from the pial surface. Second, they have prominent apical dendrite and spine-like protrusions. Third, they have single primary axons that extended vertically

from the soma to the white matter.

Data were collected from more than three independent experiments. The “total length” was measured as the length of an entire horizontal axon process. The “distance from origin to tip” was defined as a linear distance between the origin of horizontal axon and the farthest tip. The “tip length” was obtained by measuring the length from a tip to the first branch point (terminal segment). Quantification was achieved by using L-measure software (Scorcioni et al., 2008). Statistical analysis was performed on *R* software. Sample numbers are presented as the number of horizontal axons for quantification of “the number of branch points”, “total length”, and “distance from origin to tip”. The sample number for tip length analysis was presented as the number of terminal segments. Tukey Honest Significant Difference or Steel-Dwass tests were used for multiple comparison statistical tests. All statistical values are presented as mean ± SEM. An appropriate statistical test was selected for each analysis.

Results

Gene expression of RhoA-GEFs in the developing mammalian cortex

It is known that there are twenty-eight RhoA-GEFs out of approximately seventy RhoGEF members (Garcia-Mata and Burridge, 2007; Loirand et al., 2008; Rossman et al., 2005; Schiller, 2006). First, I examined which RhoA-GEFs are expressed in the developing sensory cortex, including visual and somatosensory area. For this, gene expression of all the twenty-eight RhoA-GEFs was investigated by *in situ* hybridization at postnatal day (P) 14, when horizontal axon branches are formed (Uesaka et al., 2005).

The result showed that *ABR* and *NGEF* were most strongly expressed, while eleven members including *ARHGEF2*, *ARHGEF11*, *ARHGEF12, MCF2L, ARHGEF18,* and *BCR* were moderately expressed (Figure 1). Most of them were broadly expressed in all cortical layers irrespective of expression levels, although a few showed lamina-specific expression patterns (e.g. *ARHGEF2* and *NGEF*). The expression levels of the other members such as *ARHGEF28, ARHGEF40,* and *VAV2* were very low, almost the same as those obtained with sense probes. Thus, thirteen RhoA-GEF members were certainly expressed in the developing rat cerebral cortex.

RhoA-GEF expression was also investigated in the developing macaque cortex, because horizontal axon connections are evolutionarily conserved across the mammalian species (Callaway, 1998; Callaway and Katz, 1990; Larsen and Callaway, 2006; Lohmann and Rörig, 1994). *In situ* hybridization was performed on the occipital cortex of P0 macaque monkey (Figure 2A), based on the previous observations that horizontal axons in macaque brain are still immature at the late embryonic stages (Callaway, 1998; Callaway and Wiser, 1996) and that the synaptic density increases

even at the postnatal stages (Bourgeois and Rakic, 1993).

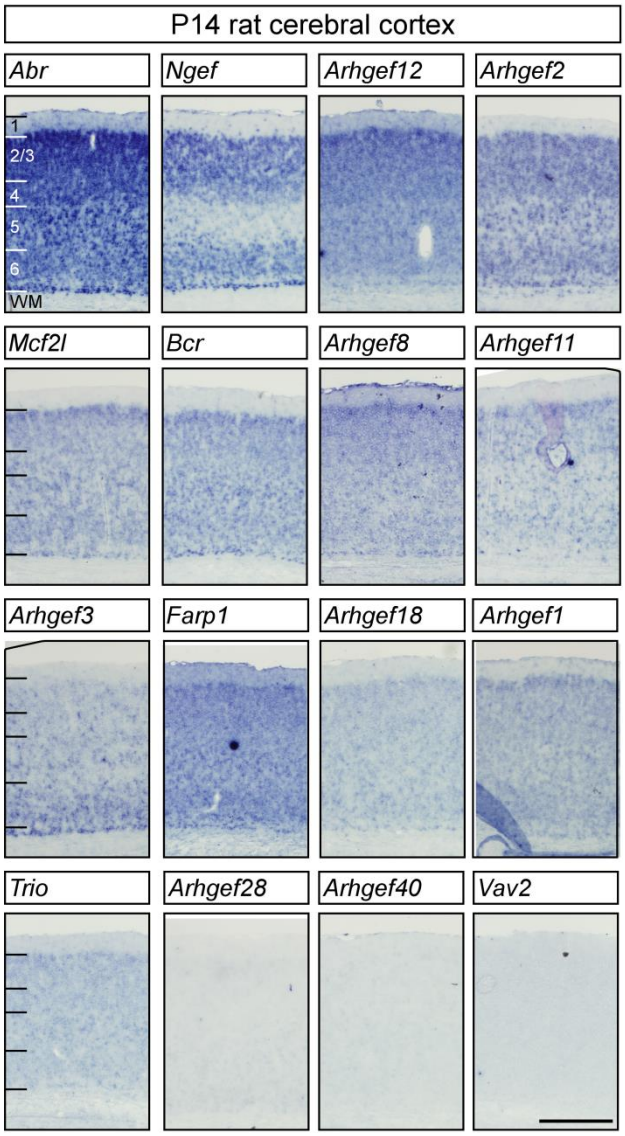

Figure 1. Thirteen RhoA-GEFs are expressed in the developing rat cerebral cortex when horizontal axons form branches. *In situ* hybridization was performed on P14 rat cerebral cortex. Sixteen representative RhoA-GEF members out of 28 were shown. Horizontal lines on the left indicate the boundary of each cortical layers as depicted in top left. Scale bar = 500 μm.

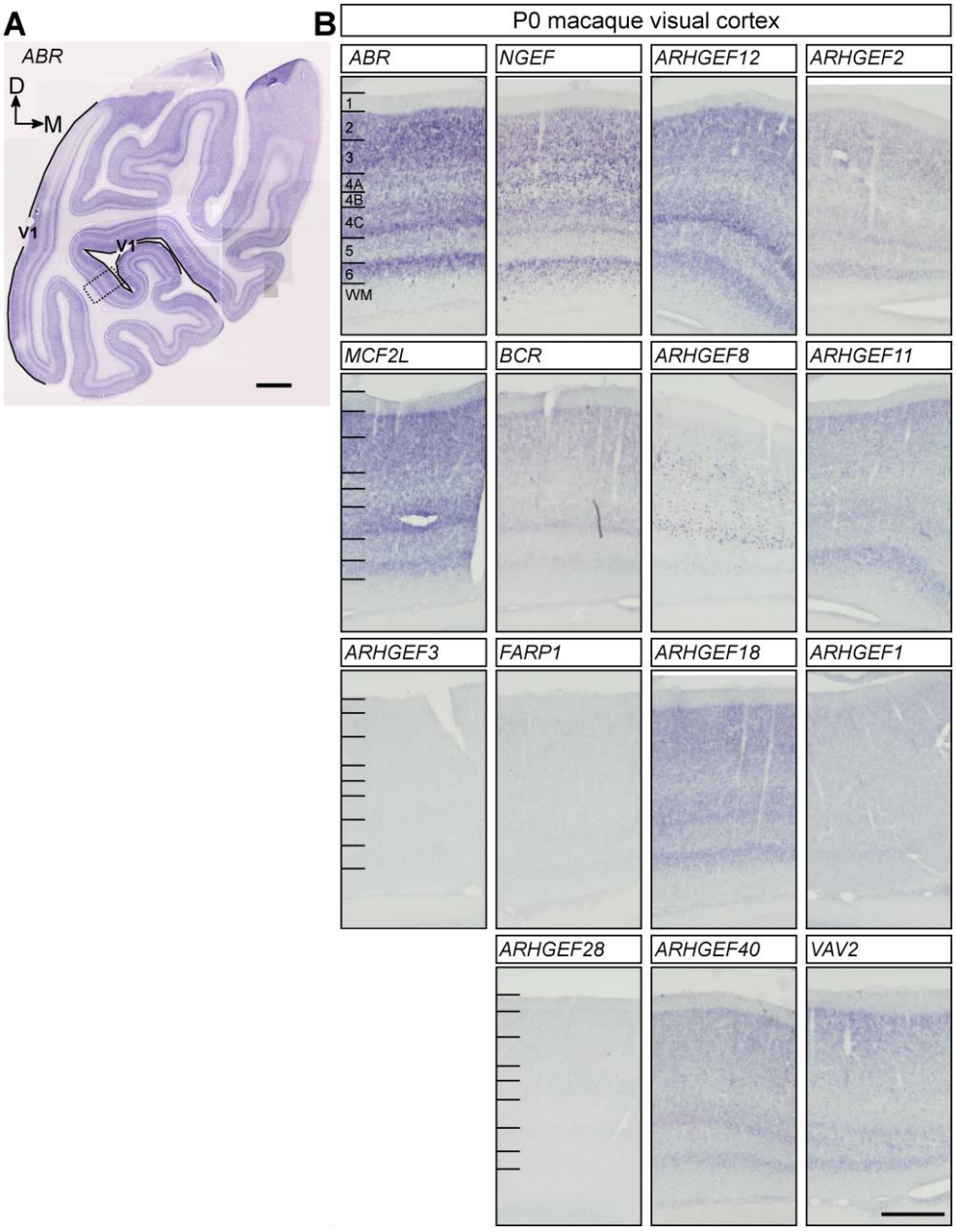

Figure 2. Twelve RhoA-GEFs are expressed in developing macaque neocortex. *In situ* hybridization was performed on P0 macaque cortex. (A) Overall view of the cortical section of P0 macaque neocortex. V1, primary visual cortex, Scale bar = 2 mm. (B) The same set of representative RhoA-GEFs is shown in the same order as in the Figure 1. Horizontal lines on the left indicate the boundary of each cortical layers as depicted in top left. Scale bar = 500 μm.

The result showed that twelve RhoA-GEF members were clearly expressed in the macaque primary visual cortex (Figure 2B). As is the case with expression in rat cortex, *ABR* and *NGEF* were strongly expressed in macaque cortex, while *ARHGEF2, ARHGEF11, ARHGEF12, MCF2L*, *ARHGEF18,* and *BCR* were expressed moderately. Thus, overall tendency in the gene expression of RhoA-GEFs were similar between the two species, although some members showed different gene expression in terms of expression levels (*ARHGEF3*, *ARHGEF40*, and *VAV2*) and expression pattern (*ARHGEF8*). Thus, it is likely that the majority of RhoA-GEFs are expressed in the both mammalian the species.

Overexpression of seven RhoA-GEFs increases horizontal axon branches

To investigate the possible role of RhoA-GEFs in horizontal axon branching, an overexpression study was performed for 17 RhoA-GEF members (Table 1), which were expressed in either of the two species, by using organotypic cortical slice cultures. This culture technique is useful to assess the molecular function in the cellular environment which is close to that *in vivo* (Yamamoto et al., 1992). As illustrated in Figure 3A, coronal slices were dissected from rat sensory cortices at P1 and cultured on the membrane filter. A few days before the onset of the horizontal axon branching, at 4-5 days *in vitro* (DIV), a plasmid encoding enhanced green fluorescent protein (EGFP) under a neuron-specific promoter (pTα1-EGFP) was transfected into a small number of upper layer neurons together with or without each RhoA-GEF expression plasmid. The morphology of individual horizontal axons was analyzed at 14 DIV .

In the control, where only EGFP was transfected, labeled upper layer neurons showed typical pyramidal morphology with apical and basal dendrites, and primary

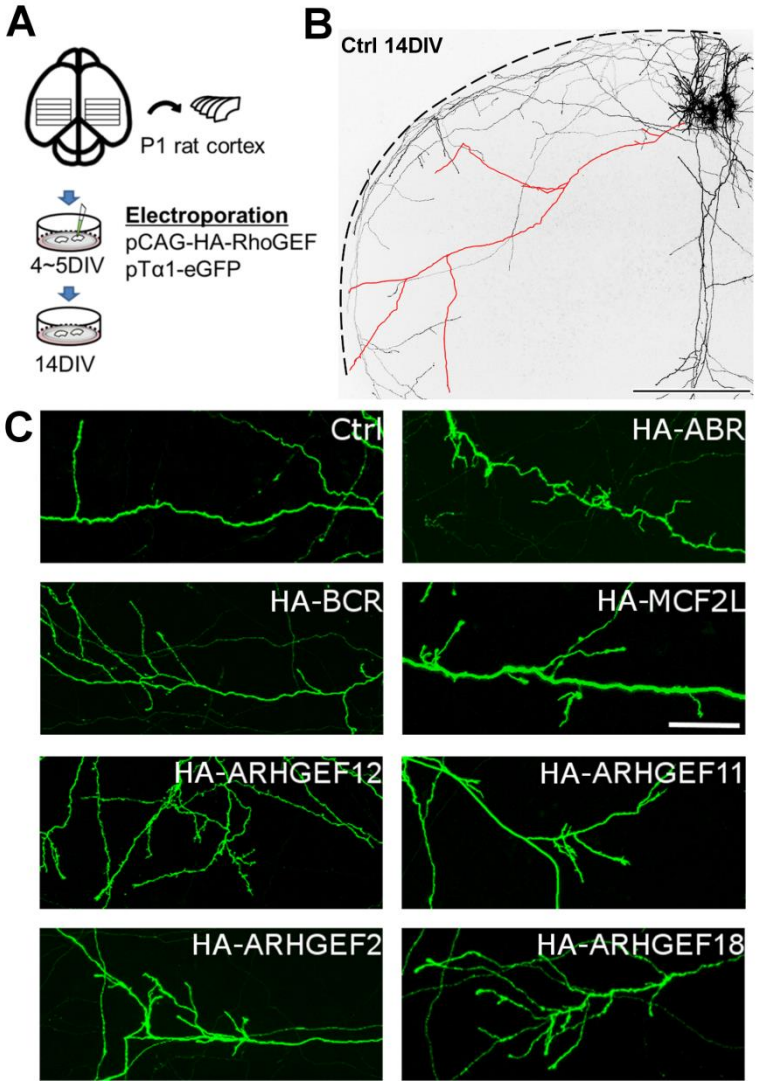

Figure 3. Overexpression of 7 RhoA-GEFs markedly increased horizontal axon branches. (A) A schematic illustrating experimental design of slice culture experiments. (B) Example photo micrograph showing the cultured cortical slice in control experiment at 14 days in vitro (DIV). Single horizontal axon was colored in red for visibility. Broken line indicates the pial surface. Scale bar = 500 μm. (C) Example photo micrographs showing a part of horizontal axon at 14 DIV. The control (Ctrl) and 7 RhoA-GEF members that increased branches markedly were shown. Scale bar = 100 μm.

descending axons which ran perpendicularly to the pial surface (Figure. 3B). Horizontal axons were found to extend from the proximal part of the primary descending axons, and form several branches (Figure 3B), as reported previously (Ohnami et al., 2008; Uesaka et al., 2005). Horizontal axon branches were found to increase dramatically when several RhoA-GEFs were overexpressed with EGFP (Figure 3C). The overexpression was confirmed by immunohistochemical staining for HA tag (Figure 4). To quantify axon branches, individually distinguishable horizontal axons were traced from the origin to axonal tips (Figure 5A, see also a red axon in Figure 3B). Quantitative analysis showed that the branch number was significantly increased when either ABR, BCR, MCF2L, ARHGEF2, ARHGEF11, ARHGEF12, or ARHGEF18 was overexpressed (Figure 5B, Ctrl, 6.4±0.9, n = 31; HA-ABR, 35.6±19.0*, n = 7; HA-BCR, 51.7±12.1***, n = 6; HA-MCF2L, 15.8±5.2*, n = 6; HA-ARHGEF2 17.2 ±5.0*, n = 6; 21.0±5.1**, HA-ARHGEF11, n = 6; HA-ARHGEF12, 27.3±10.1***, n = 7; HA-ARHGEF18, 18.7±3.4***, n = 12, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ against Ctrl, Pairwise Wilcoxon rank-sum test with *p*-value adjusted by Holm's method). Thus, horizontal axon branching was promoted by overexpression of these seven RhoA-GEFs.

ARHGEF18 and ABR overexpression induces different branching patterns

As shown in Figure 5A, it is likely that axonal branching patterns are somewhat different between overexpressing RhoA-GEFs. The horizontal axons transfected with ARHGEF12 or ARHGEF18 appeared to form many branches in the distal part of horizontal axons, whereas overexpression of ABR or BCR increased short branches in an axon shaft as well. Morphological aspects of horizontal axons were

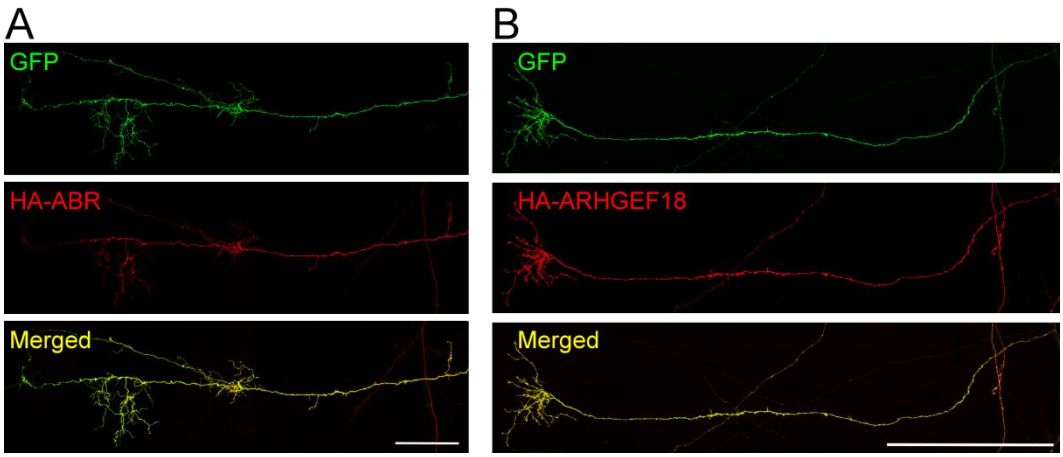

Figure 4. The expression of transfected gene was confirmed by immunohistochemistry using antibody against tagged hemagglutinin. The expression of the 7 RhoA-GEFs that increased branching was found entirely throughout horizontal axon process as exampled by ABR (A) and ARHGEF18 (B). Scale bars = 200 μm

further investigated, focusing on ABR and ARHGEF18, whose overexpression caused marked effects. In spite of the increase in the number of branch points, total length was not obviously increased both in ABR- and ARHGEF18-overexpressing axons (Figure 6A, Ctrl, 2429±256 μm, n = 31; HA-ABR, 2656±809 μm, n = 7; HA-ARHGEF18, 3017±550 μm, n = 12, $p > 0.05$, Tukey test). In contrast, tip lengths became significantly shorter in overexpressing neurons (Figure 6B, Ctrl, 127.8±11.7 μm, tip number = 210; HA-ABR, 22.8±1.7 μm***, tip number = 280; HA-ARHGEF18, 51.7 ±4.2 μm***, tip number =263, *** $p < 0.001$ against control, Tukey test). Noticeably, tip length was much shorter in ABR overexpressing (Tukey test, $p < 0.01$) than ARHGEF18 overexpressing axons.

Spatial distributions of horizontal axon branches were further analyzed quantitatively. The distance between the origin and the tip of horizontal axons was divided by 6 equally spaced concentric circles, and the number of branch points in each

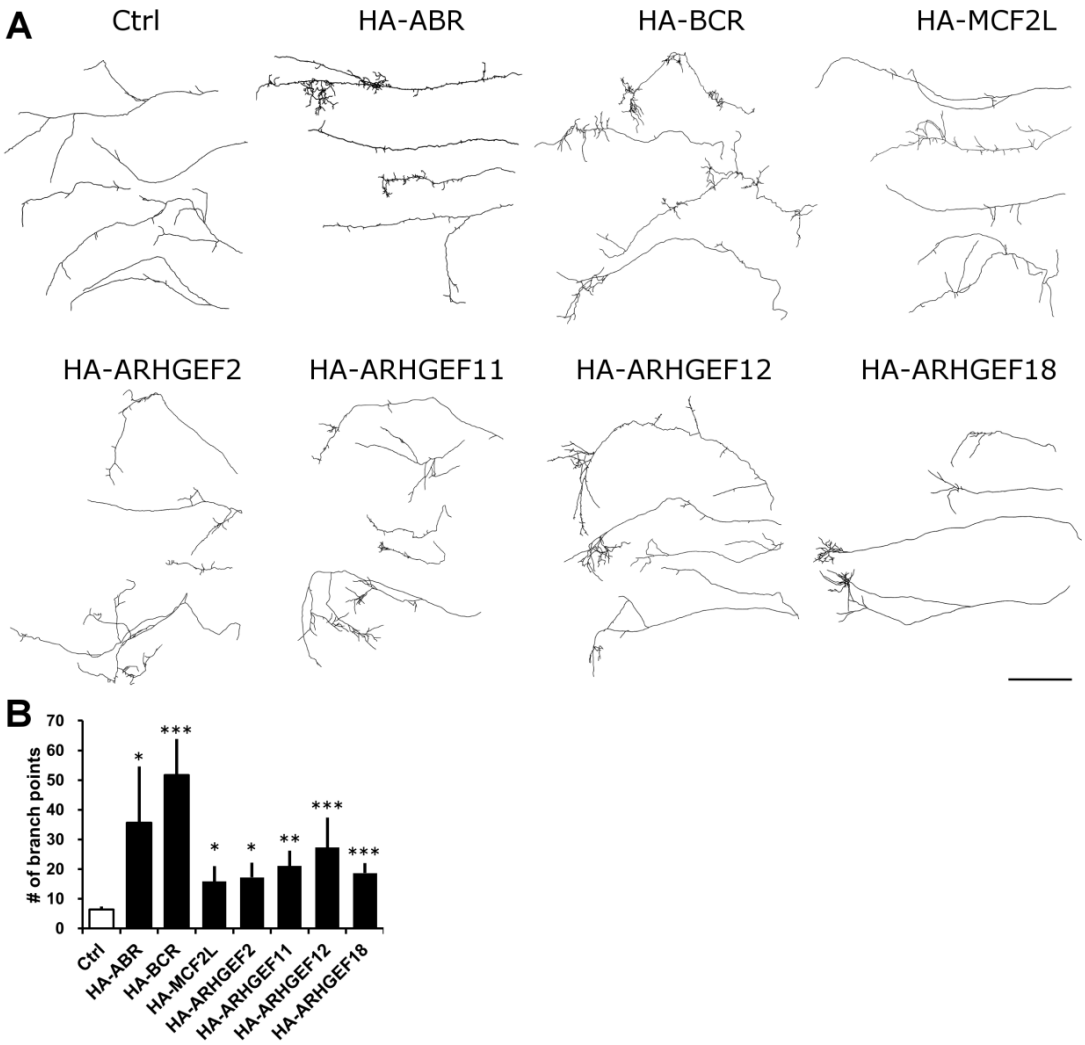

Figure 5. The overall morphology was analyzed by tracing individural horizontal axons entirely. (A) Representative drawing images of horizontal axons at 14DIV. Individual horizontal axons are aligned so that they elongate from right to left direction. Scale bar = 500μm. (B) Quantitative analysis of the number of branch points per horizontal axon. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Wilcoxon rank sum test with p-values adjusted by Holm's method for multiple comparison.

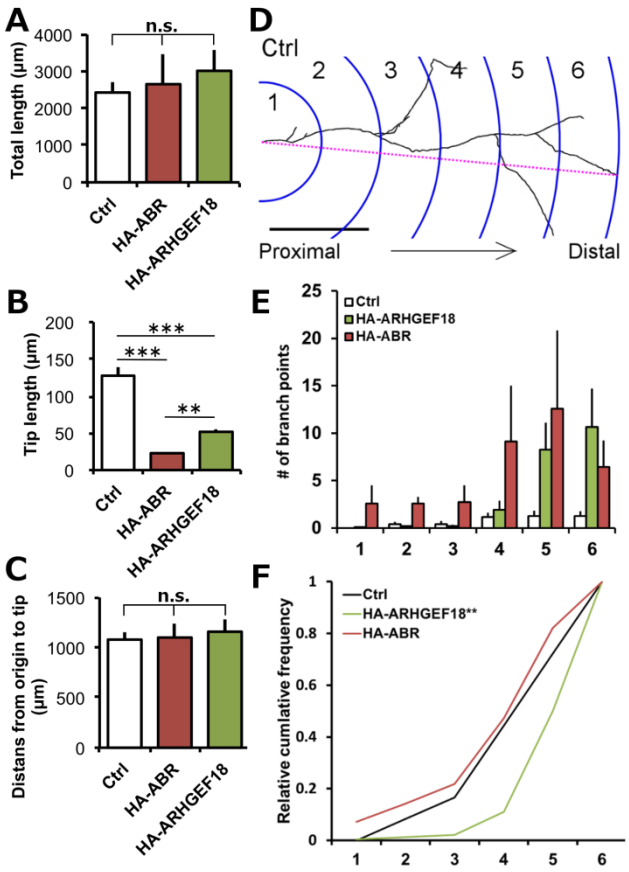

Figure 6. ABR and ARHGEF18 induced distinct aspects of horizontal axon morphology. (A-C) Quantitative analysis of the distance from the origin and the tip, total length, and tip length, respectively. ** $p < 0.01$, *** $p < 0.001$, n.s. not significant, Tukey test. (D) The distribution of branch points along horizontal axon shaft was analyzed quantitatively. The distance from the origin to the tip of the horizontal axon was divided by equally spaced 6 concentric circles, and then the number of branch points in each annulus was quantified. (E) The number of branch points in each annulus. (F) Relative cumulative frequency plots of branch points over each annulus. ** $p < 0.01$, Kolmogorov-Smirnov test.

annulus was quantified (Figure 6D). As shown in Figure 6E, control horizontal axons formed more branches in the distal part compared to the proximal part. Overexpression of ABR increased branches both in the proximal and distal parts. In contrast, branches in the distal but not in the proximal part were dramatically increased when ARHGEF18 was overexpressed. The difference was analyzed by plotting relative cumulative frequency of branch points (Figure 6F). The result clearly showed that the branch distribution of ARHGEF18 overexpressing horizontal axons was shifted towards the terminal domain of horizontal axons (** $p < 0.01$, Kolmogorov-Smirnov test), whereas that of ABR overexpressing axons did not change significantly. This was not due to the changes in horizontal axon extension because the distance between the origin and the tip was not different significantly between control, ABR overexpressing, and ARHGEF18 overexpressing horizontal axons (Figure 6C, Ctrl, 1080±73 μm, n=31; HA-ABR, 1100 ±137 μm, n= 7; HA-ARHGEF18, 1158±130 μm, n=12, $p>0.05$, Tukey test). Thus, these results indicate that ARHGEF18 and ABR induce distinct aspects of horizontal axon branching.

Downstream effector of ARHGEF18 and ABR for axon branching

As it is known that ARHGEF18 and ABR act not only on RhoA but also on other Rho family small GTPases (Blomquist et al., 2000; Heisterkamp et al., 1993; Kaartinen et al., 2001; Nagata and Inagaki, 2005; Niu et al., 2003; Tan et al., 1993), I sought to investigate if RhoA acts as the downstream molecule for branch promoting activity of ARHGEF18 and ABR. For this purpose, cultured cortical slices were treated with the Rock inhibitor, Y-27632, during the second week in culture after overexpression of ARHGEF18 or ABR.

As shown in Figure 7A, Y-27632-treated ARHGEF18-overexpressing axons formed much fewer branches, compared to non-treated axons (see also HA-ARHGEF18 in Figure 5A). Quantitatively, the number of branch points was dramatically decreased in Y-27632-treated axons (Figure 7B, Ctrl, 6.4±0.9, n = 31; HA-ARHGEF18, 18.7± 3.4, n = 12; HA-ARHGEF18 + Y-27632, 3.8±0.9, n = 12, $p < 0.001$, Steel-Dwass test). In agreement with the previous observation (Ohnami et al., 2008), the value was even smaller than control, although the difference was not statistically significant ($p = 0.25$ against control). In addition, the tip length of Y-27632 treated horizontal axons increased compared to control and non-treated axons (Figure 7C, Ctrl, 127.8±11.7 μm, tip number = 210; HA-ARHGEF18, 51.7±4.2 μm, tip number =263; HA-ARHGEF18+Y-27632, 384.1±55.3 μm, tip number = 55, $p < 0.001$ against Ctrl and HA-ARHGEF18, Tukey test). The total length was not affected significantly (Figure 7D, Ctrl, 2429±256 μm, n = 31; HA-ARHGEF18, 3017±550 μm, n = 12; HA-ARHGEF18+Y-27632, 3372±577 77, n =12). These results suggest that ARHGEF18 requires RhoA-Rock signaling for horizontal axon branching.

In contrast to ARHGEF18, Y-27632 treatment of ABR-overexpressing neurons did not completely suppress branch promoting activity of ABR (Figure 7E). Quantitative analysis showed that Y-27632-treated horizontal axons formed branches, which is not different significantly compared to untreated ABR-overexpressing axons (Figure 7F, HA-ABR, 35.6±19.0, n = 7; HA-ABR+Y-27632, 26.8±9.8, n = 8, $p = 0.97$, Steel-Dwass test). In addition, the value was significantly higher compared to control ($p = 0.016$). In contrast, the tip length became significantly longer in Y-27632 treated axons than untreated axons, but was significantly shorter than control (Figure 7G, HA-ABR, 22.8±1.7 μm, tip number = 280; HA-ABR+Y-27632, 61.6±4.9 μm, tip

number = 222, $p < 0.001$, Tukey test). The total length of HA-ABR+Y-27632 increased slightly compared to HA-ABR (Figure 7H, HA-ABR, 22.8±1.7 μm, tip number = 280; HAABR+Y-27632, 3829±1217 μm, n = 8), although the difference was not statistically significant ($p = 0.5$ against HA-ABR, Tukey test). Thus, it is unlikely that RhoA signaling is the primary downstream effector of ABR in regulation of axon branching. Taken together, these results indicate that ARHGEF18 and ABR employ different downstream effectors for axon branch regulation.

Endogenous ARHGEF18 and ABR are both necessary to form horizontal axon branches

Role of endogenous ARHGEF18 and ABR in horizontal axon branching was further examined by RNA interference. For this purpose, I constructed the plasmid vectors that encode short hairpin RNA (shRNA) targeting rat Arhgef18 (shArhgef18) or Abr (shAbr), and the knockdown effects were examined by qRT-PCR. When these each vector was transfected into the cortical neurons, shArhgef18 suppressed expression of endogenous *Arhgef18* by about 90% (Figure 8A, relative *Arhgef18* expression 0.09± 0.31, n = 3) and shAbr suppressed the endogenous *Abr* expression by about 40% (Figure 9B, relative *Abr* expression 0.55±0.29, n =3). Each vector together with the EGFP vector was transfected into upper layer neurons of cultured slices, and horizontal axon morphology was examined at 14 DIV.

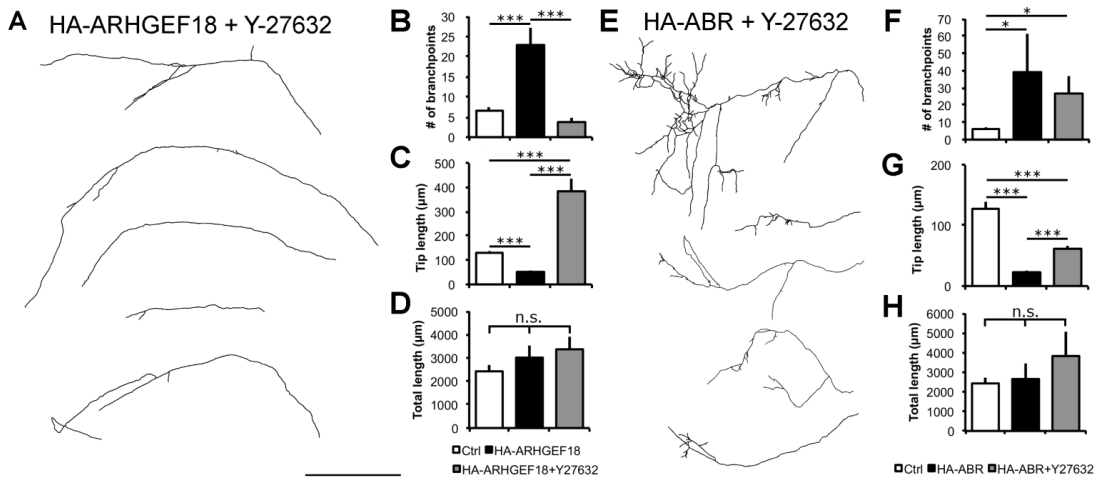

Figure 7. Involvement of RhoA signaling in the action of the two RhoA-GEFs. (A) Representative drawing images of horizontal axons at 14DIV. Cultured cortical slices were subjected to transfection with HA-ARHGEF18 expression vectors at 4-5DIV and were treated with Y-27632 during the second week. Scale bar = 500 μm (also applied to panel F). (B-E) Quantitative analysis of the number of branch points, tip length, total length, and distance from the origin to the tip of horizontal axons. *** $p < 0.001$, n.s. not significant, Steel-Dwass test in panel B, and Tukey test in panels C-E. (F) Representative drawing images of horizontal axons at 14DIV. Cultured cortical slices were transfected with HA-ABR expression vectors at 4-5DIV and were treated with Y-27632 during the second week. (G-J) Quantitative analysis of the number of branch points, tip length, total length, and distance from the origin to the tip of horizontal axons, respectively. * $p < 0.05$, *** $p < 0.001$, n.s. not significant, Steel-Dwass test in panel G, and Tukey test in panels H-J.

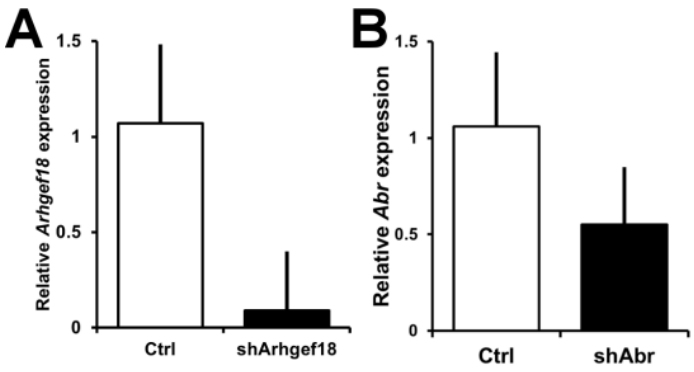

Figure 8. Knockdown efficiency of the shRNAs for rat Arhgef18 and Abr. The expression levels of endogenous *Arhgef18* and *Abr* in transfected cortical neurons were analyzed by qRT-PCR. (A) relative quantitiy of endogenous *Arhgef18*. (B) relative quantity of *Abr*.

As shown in Figure 9A, the cortical neurons transfected with shArhgef18 formed a few horizontal axon branches. The number of branch points was significantly smaller than the control (Figure 9B, Ctrl, 6.4±0.9, n = 31; shArhgef18, 2.5±0.5, n = 19, p = 0.034, Tukey test). The decrease in the number of branch points followed by ARHGEF18 knockdown was rescued by co-transfection of shRNA-resistant ARHGEF18 (Figure 9A and B, shARHGEF18 + HA-ARHGEF18, 8.0±2.2, n = 14, p = 0.016 against shArhgef18, Tukey test). In association with the decrease in the number of branches, the total length on average was decreased by about 40% compared to control (Figure 9C, Ctrl, 2429±256 μm, n = 31; shArhgef18, 1548±224 μm, n = 19; shArhgef18+HA-ARHGEF18, 2115±635 μm, n = 14, p > 0.05, Tukey test). Furthermore, the tip lengths were roughly two times longer in shArhgef18 transfected axons than the control, and this reduction was rescued (Figure 9D, Ctrl, 127.8±11.7 μm, tip number = 210; shArhgef18, 267.4±36.1 μm, tip number = 66, p < 0.001 against

Ctrl; shArhgef18+HA-ARHGEF18, 105.5±13.4 μm, tip number = 124, $p < 0.001$ against shArhgef18, Tukey test).

Knockdown of ABR expression also decreased the number of horizontal axon branches, and this effect was rescued by shRNA-resistant ABR (Figure 10A and B, Ctrl, 6.4±0.9, n = 31; shAbr, 2.8±0.8, n = 13, $p < 0.05$ against control, shAbr+HA-ABR, 17.3±2.5, n = 6, $p < 0.01$ against shABR, Tukey test), but the total length did not change significantly (Figure 10C, Ctrl, 2429±256 μm, n = 31; shAbr, 1943±304 μm, n = 13, 2299±219 μm, n = 6).

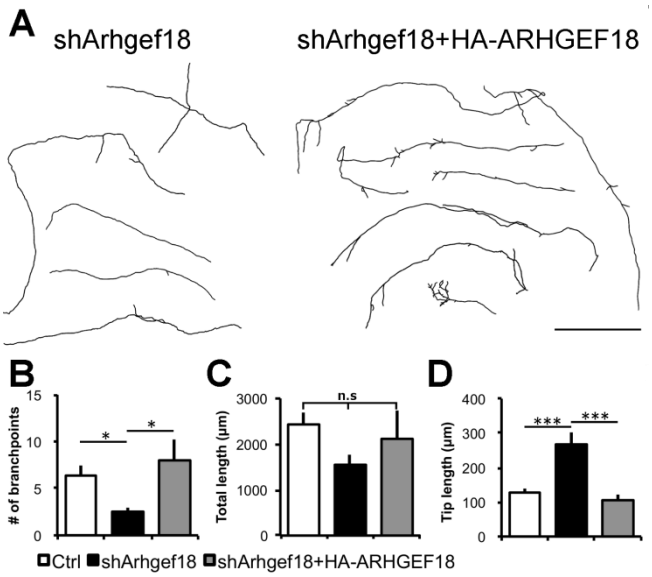

Figure 9. Endogenous Arhgef18 is necessary for horizontal axon branching. (A) Representative drawing images of horizontal axons at 14DIV after transfection of Arhgef18-targetting shRNA construct only (shArhgef18) or together with the shRNA-resistant ARHGEF18 expression vectors (shArhgef18 + HA-ARHGEF18). Scale bar = 500 μm (B) Quantitative analysis of the number of branch points per horizontal axon. * $p < 0.01$, Tukey test. (C-E) Quantitative analysis of the distance from the origin and the tip of horizontal axons, total axon length, and tip length, respectively. ** $p < 0.01$, *** $p < 0.001$, Tukey test.

The tip length became longer in shAbr than Ctrl, and this effect was rescued by the resistant vector (Figure 10D, Ctrl, 127.8±11.7 μm, tip number = 210; shAbr, 206± 29.1 μm, tip number = 46, $p < 0.01$ against Ctrl; shAbr+HA-ABR, 53.5±11.1 μm, tip number = 110, $p < 0.001$ against Ctrl and shAbr, Tukey test). These results indicate that endogenous ARHGEF18 and ABR are required or horizontal axon branching.

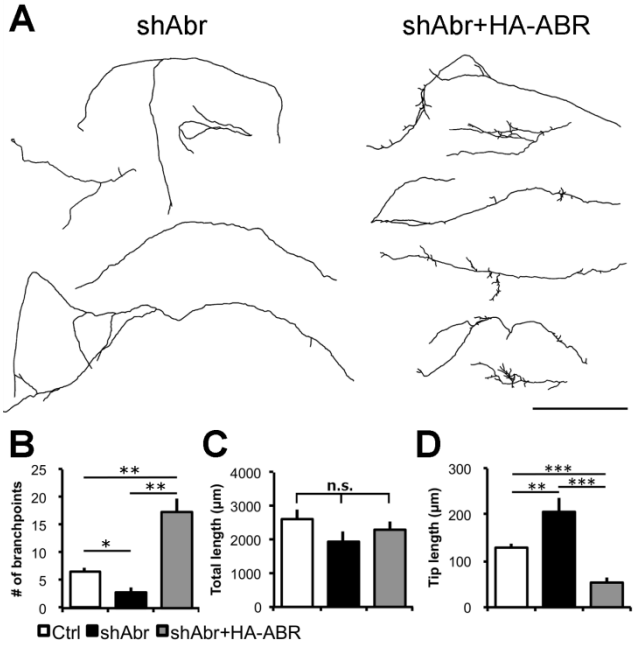

Figure 10. Endogenous Abr is necessary for horizontal axon branching. (A) Representative drawing images of horizontal axons at 14DIV after transfection of Abr-targetting shRNA construct only (shAbr) or together with the shRNA-resistant ABR expression vectors (shAbr + HA-Abr). Scale bar = 500 μm. (B) Quantitative analysis of the number of branch points per horizontal axon. * $p < 0.05$, ** $p < 0.01$, Steel-Dwass test. (C-E) Quantitative analysis of the distance from the origin and the tip of horizontal axons, total axon length, and tip length, respectively. ** $p < 0.01$, *** $p < 0.001$, Tukey test.

Discussion

The present study demonstrated that several RhoA-GEFs promoted branching of horizontal axons, which originate from upper layer neurons. Interestingly, branching phenotypes were different between overexpressing RhoA-GEFs. In particular, ARHGEF18 increased small branches in the distal portion, while ABR enhanced further smaller branches along the entire axonal shafts. The downstream inhibitor also affected distinct aspects of axonal branching by overexpression of these two RhoA-GEFs. Moreover, knockdown of either ARHGEF18 or ABR suppressed horizontal axon branching. Taking account into the fact that these RhoA-GEFs are expressed both in rat and macaque cortex, multiple RhoA-GEFs differentially contribute to horizontal axon branching beyond species.

Branch-promoting activity of RhoGEFs

To date, it has been shown that Dbl family RhoGEFs are involved in axonal and dendritic development, including dendritic branching, spine development, axon growth and guidance (Hall and Lalli, 2010; Luo, 2000; Van Aelst and Cline, 2004). For example, Trio regulates axon guidance through Rac activation (Awasaki et al., 2000; Liebl et al., 2000; Newsome et al., 2000; Steven et al., 1998), Ephexin, also known as Ngef, regulate axon growth (Shamah et al., 2001), and Karilin regulates dendritic spine morphogenesis (Penzes et al., 2001). However, their involvement in axon branching remains elusive (Rico et al., 2004; Spillane and Gallo, 2014). The present finding is the first to demonstrate RhoGEFs promote axon branching via RhoA-Rock signaling.

Although I examined the 17 RhoA-GEFs in overexpression study, seven of them showed obvious branch promoting activity. Potential roles of the other 10

members in axon branching cannot be ruled out, as the GEF activity is not necessarily increased by simple overexpression (Schmidt and Hall, 2002). The interaction with other regulatory molecules may be necessary for expression of the function. In accordance with this view, ARHGEF25 is known to function with focal adhesion kinase to contribute to axon branching of Purkinje cells (Rico et al., 2004). In other word, the fact that only overexpression of the seven RhoA-GEFs promoted axon branching may represent that some intrinsic factors or up-stream molecules are already present and work with these RhoGEFs.

RhoGEF family is composed of about 70 members in mammals. Those members are known to differ with respects to regulatory mechanisms, subcellular localizations, and/or selectivity in downstream effectors. The seven RhoA-GEFs, especially ARHGEF18 and ABR, show differential actions on horizontal axon branching, suggesting that each RhoGEFs control axon branching in a spatially selective manner. A characteristic morphological feature of horizontal axon observed *in vivo* is clustered terminal branches and this morphological feature is thought to underlie physiological connectivity between functionally related cortical columns (Callaway and Katz, 1990; Lohmann and Rörig, 1994). Given the fact that ARHGEF18 promoted terminal branches, its action may be more important to functional connectivity between cortical upper layer neurons.

Possible mechanism for different axonal branching

An interesting aspect in this study is that overexpression of RhoA-GEFs induces distinct branching patterns. How did they induce such differences? As some RhoA-GEFs have been reported to localize or accumulate into subcellular compartment

(Oh et al., 2010; Terry et al., 2011), one possible mechanism is that distinct distributions of RhoA-GEFs induce axonal branching by locally activating the downstream effector molecules. However, this is unlikely because the immunohistochemical staining using antibody against HA-tag revealed that overexpressed ARHGEF18 was distributed throughout horizontal axon shafts (Figure 4), in spite of locally enhanced axon branching. A plausible mechanism may be due to localization of upstream molecules that activate RhoA-GEFs selectively at the specific subcellular compartment. With this regard, ARHGEF18 is regulated by a Wnt member (Tsuji et al., 2010), which promotes axon branching (Bodmer et al., 2009; Krylova et al., 2002; Lucas and Salinas, 1997), and the receptor, Frizzled is known to be localized in specific axonal portions (Varela-Nallar et al., 2009; Varela-Nallar et al., 2012). In addition, ARHGEF18 interacts with LKB1, a protein kinase which is involved in axon branching via mitochondria immobilization (Courchet et al., 2013; Xu et al., 2013). Such a mechanism may function together with distinct downstream effectors to produce different branching morphology (see below).

Downstream effector molecules of RhoA-GEFs

Some RhoA-GEFs activate not only RhoA but also other Rho small GTPases. ARHGEF18 has been reported to act as GEF for RhoA when it regulates actin stress fibers, but for Rac1 when it contributes to production of reactive oxygen species (Blomquist et al., 2000; Herder et al., 2013; Nagata and Inagaki, 2005; Niu et al., 2003; Terry et al., 2011). On the other hand, ABR can act as GEF for Cdc42 and Rac1 as well as RhoA. ABR also function as GAP for Rac1 and Cdc42 (Heisterkamp et al., 1993; Kaartinen et al., 2001; Tan et al., 1993). Regarding ARHGEF18, the Rock inhibitor

treatment completely suppressed the branch-regulating activity. Together with the previous study (Ohnami et al., 2008), the present result suggests the RhoA-Rock signaling is the major downstream pathway of ARHGEF18 in regulation of horizontal axon branching. In contrast, the effect of the Rock inhibitor on ABR overexpression was partial, suggesting that not only RhoA but also other downstream molecules are involved in branch-regulating activity of ABR. Indeed, it has been reported that overexpression of ABR suppressed Rac1 and Cdc42 activity, and vice versa (Kaartinen et al., 2001; Oh et al., 2010). The branch regulatory function of RhoA-GEFs is achieved in part by employing different downstream effectors.

Formation of horizontal axon branching is a dynamic process compared to collateralizations of primary axon, and is closely associated with a generation of neuronal activity and a stopping of axon elongation (Harris et al., 1987; Malyshevskaya et al., 2013; Uesaka et al., 2005; Yamamoto et al., 1997). RhoA has been implicated to be involved in activity-dependent axonal branching. The activity of RhoA is shown to be affected by neuronal activity and active RhoA increased dynamics of the branch addition and elimination. Although current study did not challenge the activity dependence of the function of RhoA-GEFs, it is tantalizing to speculate that some of the branch-promoting RhoA-GEFs contribute to activity dependent aspects of axon branching.

Evolutional aspects

The seven RhoA-GEFs that exhibit promoting activity of horizontal axon branching were expressed in not only the rodent but also primate sensory cortices, suggesting that the gene regulation mechanism is conserved evolutionally. In addition, the fact that

human RhoA-GEFs have branch-promoting activity for rat cortical neurons suggests that the functional role is fundamentally conserved, and may contribute to horizontal axon branching in the mammalian cortex.

On the other hand, horizontal axon branching appears to become more complex during evolution of mammalian species (Callaway and Katz, 1990; Gilbert and Wiesel, 1979; van Brederode et al., 2000). Such alteration can be accounted for by evolutionary changes of gene expression regulation, such as promoter and/or enhancer activity with transcription factors, epigenetic regulation and gene duplication or deletion. A previous study has shown that duplication of the specific domains during evolution in SR-GAP undergo different spine morphology (Fossati et al., 2016). Although such a drastic evolutional change in the genome has not been found in RhoGEFs, even subtle difference in expression level of each RhoGEF might contribute to branching complexity of horizontal axons via combinatory actions of multiple RhoGEFs. A large number of members and the functional diversity of this molecular species, which is true for RhoGAPs, could contribute to different aspects of horizontal axon branching. Thus, it might be quite interesting to investigate the relationship between the diversity of RhoGEF and the evolutionally aspects of axon branching.

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Table 1. A list of ORF clones used in slice culture

Gene symbol	Product ID
ABR	FXC01324
ARHGEF11	FXC00522
ARHGEF12	FXC11597
ARHGEF15	FXC00681
ARHGEF18	FXC01977
ARHGEF2	FXC01109
ARHGEF25	FXC00977
ARHGEF3	FXC02839
ARHGEF40	FXC01651
BCR	FXC00975
FARP1	FXC01837
MCF2	FXC01849
MCF2L	FXC00517
NET1	FXC11951
NGEF	FXC09378M
TRIO	FXC22132
VAV2	FXC05989

Table 2. Primers used for cloning ISH probe synthesis in rat

Target gene	Forward (5'-3')	Reverse (5'-3')	NCBI Accession #
Abr	CCTCTCTTGGATCGACACCCTCT	CGGGAGCTCTCAGACATTTCCAC	NM_001105814
Akap13	GACACAGTCACTTCTGACACTGC	TCTGTACTCGAGGTTTTCTGCTC	NM_001106271
Arhgef1	GGAGATTGGTGATGTGCTACTGG	AGGCTCAACAGGTTTTCCTCAAC	NM_021694
Arhgef11	ACCAGGTCTCTTGAGAACCCAAC	TCTTGTCTTTGCTGATCCTCCAG	NM_023982
Arhgef12	CACCTGAGAGTTTCCGTCCCC	TCCCTGGACACTCGCTGATA	NM_001003246
Arhgef15	CGTATCAACCCTCCAGTCATCAG	GCGGCTATACGTCTCCTCTTGAT	NM_001105789
Arhgef17	CCGACGTCTCCAAGAACCTCTAT	GTGCACCCATACGCTGTATCTTC	XM_003748932
Arhgef18	AGGAGATCGTAGCCAAGATGGAC	CGATGACTGCCTGGAGACTAAGA	NM_001107115
Arhgef19	CGAGGAGCTCATTCACCTGAGTA	CGATTTTCTCCTGAGACCCAGAT	NM_001108692
Arhgef2	GGGAACCAAGCCATAGGTATCAG	AGGTAGTGACAGCCCCTTTTCAG	NM_001012079
Arhgef25	GAATGTACCGAGGAGGATCAGAC	GCAGTCAGTTTTCCCTCAAATCC	NM_199395
Arhgef28	AGGATCTTCCCCTGCTTAGATG	GTCCTTCCTCCTTCTTCTTCTGG	NM_001108542
Arhgef3	ACTCGACCTCTGGAATTTCCTTG	AAAGTGCCTCTCCAAACTGTTCC	NM_001106061
Arhgef40	GATCTGCAATTATTGGGGCTGAC	CTGAATCCTCCTTTCCAGCTCTT	NM_001271313
Arhgef5	GGCTCTCTGATCCCAAGAAGAAA	AACCTGTTCACTGCTACTCTCTG	XM_001073085
Bcr	TTACATTGTACGCCAGTGTGTGG	CATTAGGAAGCAGCAGCAGTGTT	XM_001079915
Ect2	TCCTCTTCAATGACTGCCTTGAG	AGCCACTGGTCTTCTGATGTGAG	XM_003749256
Farp1	GGAGATTACCAAAGAATCGGGGA	GTCCTTGTGGATGTTCTCAGACT	NM_001107287
Mcf2	GAAGAGATTCTGGCAGGTCGTGCT	TGGCTATCCTGAGTGGCTTGGACT	XM_229172
Mcf2l	CATGACCTATCTCACCAGCATCC	CTGGACTTCTCCTCAAAGGTGGT	NM_053951
Net1	CGACAAACTGGAGTACCTGGATG	GCACTCCACCTGAGCTCTAACAA	NM_001039023
Ngef	AGTCATCCTCCACCCCTGGAAAT	TGACAATGTTCTCCTCCATGCGG	NM_001136241
Obscn	TCCTCTTCAATGACTGCCTTGAG	AGCCACTGGTCTTCTGATGTGAG	XM_008767886
Plekhg5	GAAGGATTCCAAGTCCCTCAGTT	TACAGTATCGGATGTACGGCTTG	NM_201272
Trio	AGGAGAGGTCGTTCAAATTCTGG	CCAAACTCTGATCCACCAAGATG	XM_003749226
Vav1	TCAAGATTACCTCAGTGGAACGG	CTTGTAAGGAAACTGCAAGGTGG	NM_012759
Vav2	GAGAGTACTGTAGCCACATGGAG	CTATCACATCCCCTGTCTGGAAG	NM_001106563
Vav3	GCATCTGGACAGAGGGGTAATAG	CTGTAAACACCTCTACCTCTGGG	NM_001191714

Table 3. Primers used for cloning ISH probe in macaque monkey

Target gene	Forward (5'-3')	Reverse (5'-3')	NCBI Accession #
ABR	TCGACACCCTCTACAGCAACTTC	GCTTGGCACACAGTAGGACATCT	XM_002800235
AKAP13	GGAAACAAGCCATCCTCATCTCT	GGACCGTCTACTGGCAAATATC	XM_001086697
ARHGEF1	CTAAGAGGTTGAAGGCTGCTTCC	CAGGTTCTTGAACTCGCTTAGCA	XM_002801482
ARHGEF11	CCTCTGGCAGCAGAGTTCAAGAG	TCAGCCAGATTCCTGTCCAGTTC	XM_001116843
ARHGEF12	TCTCCTCATTCACCTGGAGCATC	GCCTTTGAACCTCTGGATGGACT	XM_002799867
ARHGEF15	CTGCAGGATGAACCTCTGTACCA	TTAGGCTGAGTGATGAGCAGCAG	XM_001118167
ARHGEF17	GATGGCTATGAACCTCTGTACCA	CTAAAGGCACCACCAACAAACTG	XM_001115376
ARHGEF18	AATGTCGGTATGACGGTCTCTCA	TCTTTAGTTTGCCGGAGGACTTC	XM_001095927
ARHGEF2	CGAGACTTTACCAGAATGCAGGA	AATGGCTGGGGAATAGGTCAGTA	XM_002801845
ARHGEF25	AAATCTATGAGTGGCACCGAGA	TACTCAATGGGTGACTGCAATG	XM_001100987
ARHGEF28	GGAGAGAAGAAAGAGGCTGTTCG	TGTCCACAGTTCGTGACTGACAT	XM_001101425
ARHGEF3	AGGCTACTTTGGCTTTGTCCGTC	GTCGATCAGGGAGTCTTTCTGGC	XM_001101218
ARHGEF40	ATGAGGCCTGTGCCTACCTATTC	GAGTCTGGCTTCCCTGAGTCTTC	XM_001095724
ARHGEF5	GGACTCTCAAGCATCCATCTCCA	GTTTCTCCATGGCTGGACCTCTT	XM_001094223
BCR	CTGGAGTCCACTAAAGCGAGTGA	GCCCTCTTCTCTCTCTGGATGTC	XM_002798291
ECT2	AGAGACAGACGTGTCACCGTTTC	CAGGGGTGCTCACCTAGAGAAAT	XM_001086697
FARP1	GCAGGAAGAGATTCCCACTGAT	GTCCTCAACCCACTTCTCCATCT	XM_001089334
MCF2	TGGGTTATAGAGCGGAGATGGAT	CCCTGTTGCTTTTCATCATTCTG	XM_002808551
MCF2L	TGAAGAAGCGCTTTGCTTACCTG	CACAGACACGCCTGATTTCTCCT	XM_001103476
NET1	CTCACCACCAGAGAAATCAAACG	AAACTGTAGATGCCCTCCTCTGG	XM_002805547
NGEF	GTGCTTGAGATCCTACAGCCTGA	CATCCAACGCTTCATCTCACTCT	XM_001114604
PLEKHG5	GTAGACAAGATGGAGCAGCTGGA	AATTCCTTCAGGAGCTTGTCCAC	XM_001092760
VAV1	TCTACACCCTGTCTGCTCTGTCC	GGAGAGCTGGAAATTGGTGATCT	XM_002801041
VAV2	GAGGAGACCACAGAGAATGACGA	CCACCTTGTCAAACAGGAACAAG	NM_001265633
VAV3	TCTTCGAGACTTGCTTGTGGTTC	TCTCCTGACTCTTTGGTCCTGTG	XM_001094831

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Publications and conference presentations

Publications

Hayano Y, Sasaki K, Ohmura N, Takemoto M, Maeda Y, Yamashita T, Hata Y, Kitada K, Yamamoto N (2014) Netrin-4 regulates thalamocortical axon branching in an activity-dependent fashion. Proc Natl Acad Sci U S A 111:15226-15231.

Matsumoto N, Sasaki K, Yamamoto N (2015) Electroporation Method for Mammalian CNS Neurons in Organotypic Slice Cultures. Electroporation Methods in Neuroscience, Neuromethods 102:159-168.

Conference presentation (oral)

Sasaki K, Arimoto K, Kankawa K, Terada C, Watakabe A, Yamamoto N (2017) Role of ARHGEF18 in axon branching of cortical neurons. In: 第 64 回日本生化学会近畿支部例会 大阪大学（大阪府豊中市）

Conference presentations (poster)

Sasaki K, Hayano Y, Yamamoto N (2013a) Netrin-4-Unc5B シグナルが視床軸索分岐を促進する. In: 第 6 回神経発生討論会 理化学研究所和光キャンパス（埼玉県和光市）

Sasaki K, Hayano Y, Yamamoto N (2013b) Role of Netrin-4-Unc5B Signaling in Thalamocortical Axon Branching. In: 36th Annual Meeting of Japan Neuroscience Society（京都府京都市）

Kankawa K, Arimoto K, Sasaki K, Terada C, Yamamoto N (2014) The Expression of RhoGEFs in the Developing Cortex and Their Actions on Axon Branching of Cortical Neurons. In: 国立遺伝学研究所研究会「哺乳類脳の機能的神経回

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Sasaki K, Arimoto K, Kankawa K, Terada C, Yamamoto N (2015a) Role of RhoGEF ABR in axon branching. In: The 8th Annual Meeting for Japanese Developmental Neuroscientists 九州大学（福岡県福岡市）

Sasaki K, Arimoto K, Kankawa K, Terada C, Yamamoto N (2015b) Rho Guanine Nucleotide Exchange Factor Abr Promotes Axon Branching in Cortical Layer 2/3 Neurons. In: 38th Annual Meeting of Japan Neuroscience Society（兵庫県神戸市）

Sasaki K, Arimoto K, Kankawa K, Terada C, Yamamoto N (2015c) Regulation of Cortical Axon Branching by a RhoGEF Member, ARHGEF18. In: Circuit Construction in the Mammalian Brain 国立遺伝学研究所（静岡県三島市）

Sasaki K, Arimoto K, Kankawa K, Terada C, Yamamoto N (2016a) Rho Guanine Nucleotide Exchange Factor ARHGEF18 Promotes Axon Branching of Upper Layer Cortical Neurons. In: 39th Annual Meeting of Japan Neuroscience Society（神奈川県横浜市）

Sasaki K, Arimoto K, Kankawa K, Terada C, Yamamoto N (2016b) ARHGEF18 regulates axon branching of cortical upper layer neurons. In: 2016 Meeting on Axon Guidance, Synapse Formation & Regeneration CSHL

Sasaki K, Terada C, Arimoto K, Kankawa K, Yamamori T, Watakabe A, and Yamamoto N (2018) Involvement of RhoA-GEFs in axon branching of upper cortical layer neurons. In: Cold Spring Harbor Asia Conference on Latest Advances in Development & Function of Neuronal Circuits（兵庫県淡路市）