

ORIGINAL ARTICLE

The adhesion-GPCR *BAI3*, a gene linked to psychiatric disorders, regulates dendrite morphogenesis in neuronsV Lanoue^{1,2,3,4,5,10}, A Usardi^{1,2,3,4,10}, SM Sigoillot^{1,2,3,4,10}, M Talleur^{1,2,3,4}, K Iyer^{1,2,3,4}, J Mariani^{5,6}, P Isope⁷, G Vodjdani⁸, N Heintz⁹ and F Selimi^{1,2,3,4}

Adhesion-G protein-coupled receptors (GPCRs) are a poorly studied subgroup of the GPCRs, which have diverse biological roles and are major targets for therapeutic intervention. Among them, the Brain Angiogenesis Inhibitor (BAI) family has been linked to several psychiatric disorders, but despite their very high neuronal expression, the function of these receptors in the central nervous system has barely been analyzed. Our results, obtained using expression knockdown and overexpression experiments, reveal that the *BAI3* receptor controls dendritic arborization growth and branching in cultured neurons. This role is confirmed in Purkinje cells *in vivo* using specific expression of a deficient *BAI3* protein in transgenic mice, as well as lentivirus driven knockdown of *BAI3* expression. Regulation of dendrite morphogenesis by *BAI3* involves activation of the RhoGTPase Rac1 and the binding to a functional ELMO1, a critical Rac1 regulator. Thus, activation of the *BAI3* signaling pathway could lead to direct reorganization of the actin cytoskeleton through RhoGTPase signaling in neurons. Given the direct link between RhoGTPase/actin signaling pathways, neuronal morphogenesis and psychiatric disorders, our mechanistic data show the importance of further studying the role of the BAI adhesion-GPCRs to understand the pathophysiology of such brain diseases.

Molecular Psychiatry advance online publication, 30 April 2013; doi:10.1038/mp.2013.46

Keywords: adhesion-GPCR; dendrite; morphogenesis; ELMO1; *BAI3*; Purkinje cell

INTRODUCTION

The increased complexity of behaviors that appeared during evolution has been correlated with an increased molecular complexity of upstream signaling membrane proteins.¹ Genetic studies have linked mutations and copy number variations in genes coding for these upstream signaling pathways with many psychiatric and neurodevelopmental diseases, such as autism and schizophrenia. Deciphering these signaling pathways and how they regulate the formation of a functional neuronal network in mammals will thus help understand their contribution to brain diseases.

The Brain Angiogenesis Inhibitor (BAI) family is part of the poorly understood family of adhesion-G protein-coupled receptors (GPCRs).² Adhesion-GPCRs are unique in that they contain a very long extracellular domain with multiple modules potentially conferring adhesive and recognition properties. The few studies of those receptors have shown their roles in physiology, including in the central nervous system, and pathology. For example, CELSR3 deficiency leads to abnormal neuronal migration, defects in tract development and reduced dendritic development.^{1,3–5} Mutations in the adhesion-GPCR GPR56 lead to deficits in neuronal migration in patients with bilateral frontoparietal polymicrogyria.⁶ The BAI proteins are highly expressed in the brain and have been identified at post-synaptic densities in the forebrain⁷ and in the cerebellum.⁸ These proteins have several structural features that suggest their potential involvement in the development of functional neuronal networks: their extracellular domain contains

several thrombospondin type 1 repeats domains that could provide adhesive and recognition properties. A PDZ-binding domain in their C-terminus could enable their association with synaptic scaffolding proteins such as PSD95. Sequence analysis shows that this subfamily of adhesion-GPCRs has homologs only in vertebrates, and that these homologs are extremely well conserved (Supplementary Figure 1). The BAI proteins may thus have important functions in controlling the development of complex cognitive abilities that are specific to vertebrates. This is highlighted by the fact that BAI proteins could contribute to behaviors defective in psychiatric disorders: single nucleotide polymorphisms and copy number variations in the *BAI3* gene have been associated with schizophrenia,^{9–11} bipolar disorder¹² and addiction,¹³ and the *Bai2* knockout mouse has an anti-depressant phenotype.¹⁴

The regulation of dendrite morphogenesis in neurons is key to the formation of functional neuronal networks and is deficient in several neurodevelopmental disorders, such as autism, Fragile X syndrome or schizophrenia.^{15–18} This process involves stabilization of dynamic filopodia through regulation of the actin cytoskeleton,¹⁹ in particular by the modulation of RhoGTPases.^{20,21} Direct interference with the activity of RhoGTPases, such as RAC1, or their guanylate exchange factor activators, such as Tiam1, betaPIX, kalirin and the ELMO1/DOCK180 complex, leads to defects in dendrite morphogenesis.^{22–24} However, which upstream pathways coordinate RhoGTPases activation by integrating extracellular cues during dendrite morphogenesis is

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Received 16 May 2012; revised 21 February 2013; accepted 18 March 2013

not well understood. The BAI1 receptor regulates phagocytosis through the modulation of the ELMO1/DOCK180/RAC1 signaling pathway.²⁵ BAI1 interacts with ELMO1 through a motif conserved in BAI2 and BAI3, suggesting that the control of the small GTPase RAC1 through the ELMO1/DOCK180 module is a general feature of the BAI receptors and might be important for their role in the central nervous system. Here we show that the BAI3 protein controls dendritic arborization growth and complexity in neurons, partially through its interaction with ELMO1.

MATERIALS AND METHODS

BAI3 constructs, knockdown and transgenic mice

The BAI3-wild-type (WT) construct was cloned into the pEGFP-C2 vector from mouse cDNA clone no. BC099951. The Quikchange Site-Directed Mutagenesis kit (Agilent technologies, Santa Clara, CA, USA) was used to change the RKR sequence to AAA (residues 1431–1433) for the BAI3-WT-A construct. The BAI3-FLT construct codes for the entire BAI3 protein with an insertion of green fluorescent protein (GFP) after amino acid 1349. In BAI3-EMT, the cytoplasmic tail is replaced by GFP after amino acid 1174. The BAI3-SCT construct is a fusion between GFP and the cytoplasmic tail of BAI3 starting at amino acid 1166. The cDNA coding for BAI3-EMT was subcloned in the BamHI site of the L7/pcp2 promoter.²⁶ A HindIII fragment was then purified for microinjection in the male pronucleus of C57BL/6N oocytes (Institut Clinique de la Souris, Strasbourg, France). The small hairpin RNA (shRNA) sequence for BAI3 was: 5'-ggtgaaggagtcattat-3', and was subcloned under the H1 promoter in either pSUPER vector for transfection in cultured hippocampal neurons or in a lentiviral vector that also drives GFP expression.²⁷

RESULTS

The adhesion-GPCR BAI3 modulates dendrite morphogenesis in neurons

The BAI3 receptor was found to localize to actin-rich cell protrusions, such as filopodia and lamellipodia in HEK-293H cells, and dendrites and filopodia in cultured DIV5 hippocampal neurons (Supplementary Figure 2). Moreover, quantitative reverse transcription PCR (qRT-PCR) analysis shows expression of the endogenous BAI3 in developing hippocampal neurons in culture (Supplementary Figure 3). Given these data and the fact that BAI1 regulates RAC1, a major modulator of actin function, and dendrite and spine morphogenesis, we hypothesized that the BAI3 receptor has a role in the regulation of the actin cytoskeleton and dendrite morphogenesis in neurons. We first used a RNA interference strategy to knockdown the expression of the BAI3 protein in cultured hippocampal neurons, a classical model for the study of signaling pathways controlling dendrite morphogenesis (Supplementary Figure 3). Our quantitative analysis showed a significant increase in total dendrite length per neuron after BAI3 knockdown compared with control conditions (Figure 1a). We also observed a tendency for an increased total number of dendrites per neuron following BAI3 knockdown due to a significant increase in the number of dendrites of order 2 and more. As BAI3 is highly expressed in cerebellar Purkinje cells *in vivo*, a neuronal type of exquisite complexity in terms of dendritic arborization, we tested BAI3's role in this neuronal type by transducing cerebellar mixed cultures with a lentivirus driving the expression of a shRNA directed against BAI3 or the corresponding controls. Knockdown of BAI3 also increased dendrite length in Purkinje cells significantly (Figure 1b). Hence, the role of the BAI3 protein in dendrite morphogenesis is a general feature of this adhesion-GPCR that can be found in multiple neuronal types.

BAI3 interacts with ELMO1, a regulator of RAC1 activity

Next we were interested in determining the signaling pathway used by the BAI3 receptor to control dendrite morphogenesis. The BAI1 receptor interacts with the N-terminal part of ELMO1, through an RKR motif present in its cytoplasmic tail²⁵ and

conserved throughout the BAI family. To test whether BAI3 also binds ELMO1 through the same motif, we performed coimmunoprecipitation experiments using extracts of HEK-293H cells cotransfected with several tagged mutants of BAI3 and ELMO1 (Figure 2a). Using an anti-GFP antibody, we were able to affinity purify the wild-type form of BAI3 (BAI3-WT) from cells expressing ELMO1-GFP, but not from cells expressing soluble GFP, showing the specific interaction of BAI3 with ELMO1 (Figure 2a). This result was further confirmed by performing the reverse experiment in which we affinity purified different GFP-tagged forms of the BAI3 receptor and checked for the copurification of myc-tagged ELMO1. ELMO1-myc was coimmunopurified with the cytoplasmic tail of BAI3 (BAI3-SCT, soluble cytoplasmic tagged), but not with the mutant BAI3 receptor lacking the whole cytoplasmic domain (BAI3-EMT, extracellular membrane tagged). The interaction was reduced when using the full-length BAI3 with a GFP inserted in its cytoplasmic tail close to the RKR motif (BAI3-FLT, full length tagged), and totally abolished by mutagenesis of the RKR motif in the BAI3 protein (BAI3-WT-A). These results were further confirmed by immunofluorescence analysis and quantification, which showed that the colocalization of BAI3 with ELMO1 in actin-rich filopodia of transfected HEK-293H cells was dependent on the presence of the cytoplasmic tail of BAI3, and more particularly of the RKR motif (Figure 2b). Overall, these results show that ELMO1 interacts with the RKR motif located in the cytoplasmic tail of the BAI3 receptor.

The BAI3 protein regulates cell morphogenesis, partly through binding of ELMO1

ELMO1 is part of the RAC1 guanylate exchange factor ELMO1/DOCK180²⁸ and BAI1 has been shown to regulate RAC1 activity through its binding to the ELMO1/DOCK180 module.²⁵ The modulation of dendrite morphogenesis mediated by BAI3 could thus be a result of the regulation of Rac1 activity. We tested this hypothesis using a classical *in vitro* assay, the cell-spreading assay,²⁸ which consists in measuring the spreading of transfected HEK-293H cells at different time points after plating on fibronectin (Figure 2c). BAI3-expressing cells showed a significant reduction in their spreading both at 30 min (BAI3-WT: $140 \pm 3 \mu\text{m}^2$; GFP: $192 \pm 5 \mu\text{m}^2$, respectively) and at 5 min (BAI3-WT: $118 \pm 3 \mu\text{m}^2$; GFP: $139 \pm 6 \mu\text{m}^2$) when compared with control GFP-expressing cells. This effect on cell spreading was totally absent when the cytoplasmic tail of BAI3 was deleted (BAI3-EMT), and partially abolished when the RKR motif was mutated (BAI3-WT-A). Thus overexpression of the BAI3 receptor inhibits cell spreading through its cytoplasmic tail, partially through ELMO1 binding, suggesting that BAI3 signaling could indeed regulate dendrite morphogenesis through RAC1 modulation.

The BAI3/ELMO1 interaction is involved in the regulation of dendrite morphogenesis

Our data suggested the implication of a new BAI3/ELMO1 signaling pathway controlling neuronal morphogenesis. We first confirmed that the BAI3 receptor could colocalize with ELMO1-myc in DIV5 hippocampal neurons, in particular in developing dendrites (Supplementary Figure 2). We then transfected hippocampal neurons with either a construct coding for BAI3 WT or BAI3 mutant constructs deficient for ELMO1 interaction (BAI3-WT-A, BAI3-EMT, see Figure 2). As shown in Figure 3, overexpression of the BAI3 protein results in a 55% increase in the total dendritic length per neuron when compared with GFP-expressing neurons, as well as in an increase in the number of branches of order 2 and more per neuron (Figure 3a). These effects were partially abolished when mutant BAI3 constructs unable to bind ELMO1 were used (see Figure 3a) or when BAI3-WT was cotransfected with a truncated ELMO1 unable to bind DOCK180 (see Figure 3b). Finally cotransfecting a dominant-negative RAC1 with BAI3-WT

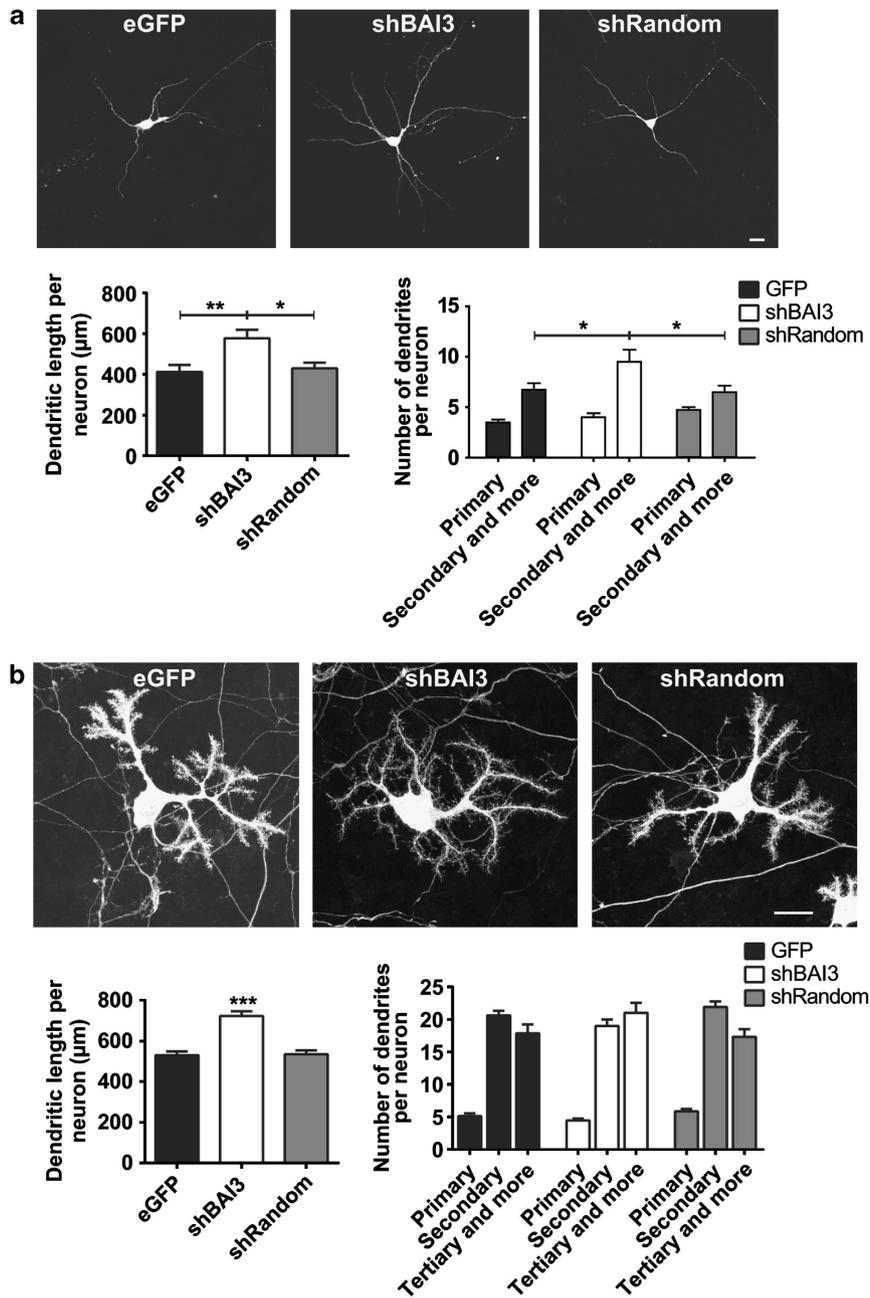


Figure 1. Knockdown of BAI3 promotes growth and branching of dendrites in several neuronal populations. **(a)** DIV5 hippocampal neurons were fixed two days after transfection with mCherry, and either a vector driving the expression of eGFP or a small hairpin RNA against BAI3 (shBAI3) or a control small hairpin RNA (shRandom). $N = 35\text{--}40$ neurons per condition, four independent experiments. **(b)** DIV7 cerebellar cultures infected at DIV4 with a lentivirus driving either GFP alone, shBAI3 or shRandom were immunostained for calbindin, a Purkinje cell-specific marker. $N = 40\text{--}50$ neurons per condition, three independent experiments. Scale bars: $20\ \mu\text{m}$. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

totally prevented BAI3's induced promotion of dendritogenesis (see Figure 3c). Taken together, our data show that the BAI3 protein regulates dendrite morphogenesis by regulating RAC1 activity, partially through binding to the ELMO1/DOCK180 complex. They also suggest another, yet to be found, BAI3 signaling pathway associated with domains other than its C-terminus.

The BAI3 protein regulates dendrite morphogenesis *in vivo* Katoh *et al.*²⁹ have shown by *in situ* hybridization that ELMO1 is expressed in multiple neuronal populations in the mouse brain,

including Purkinje cells. Double immunolabeling of mouse cerebellar sections using an antibody against the ELMO1 protein and an antibody against calbindin, a Purkinje cell marker (Figure 4a), showed the presence of ELMO1 in the growing tips of Purkinje cell dendrites at postnatal day 3. By P10, it was filling the whole Purkinje cell dendritic arborization. This pattern, together with the expression of BAI3 in Purkinje cells^{8,30} and in the cerebellum during development (Figure 4b), is in agreement with a potential role of the BAI3/ELMO1 signaling pathway in the morphogenesis of Purkinje cells, whose elaborate dendritic arborization is the result of extensive reorganization between P0

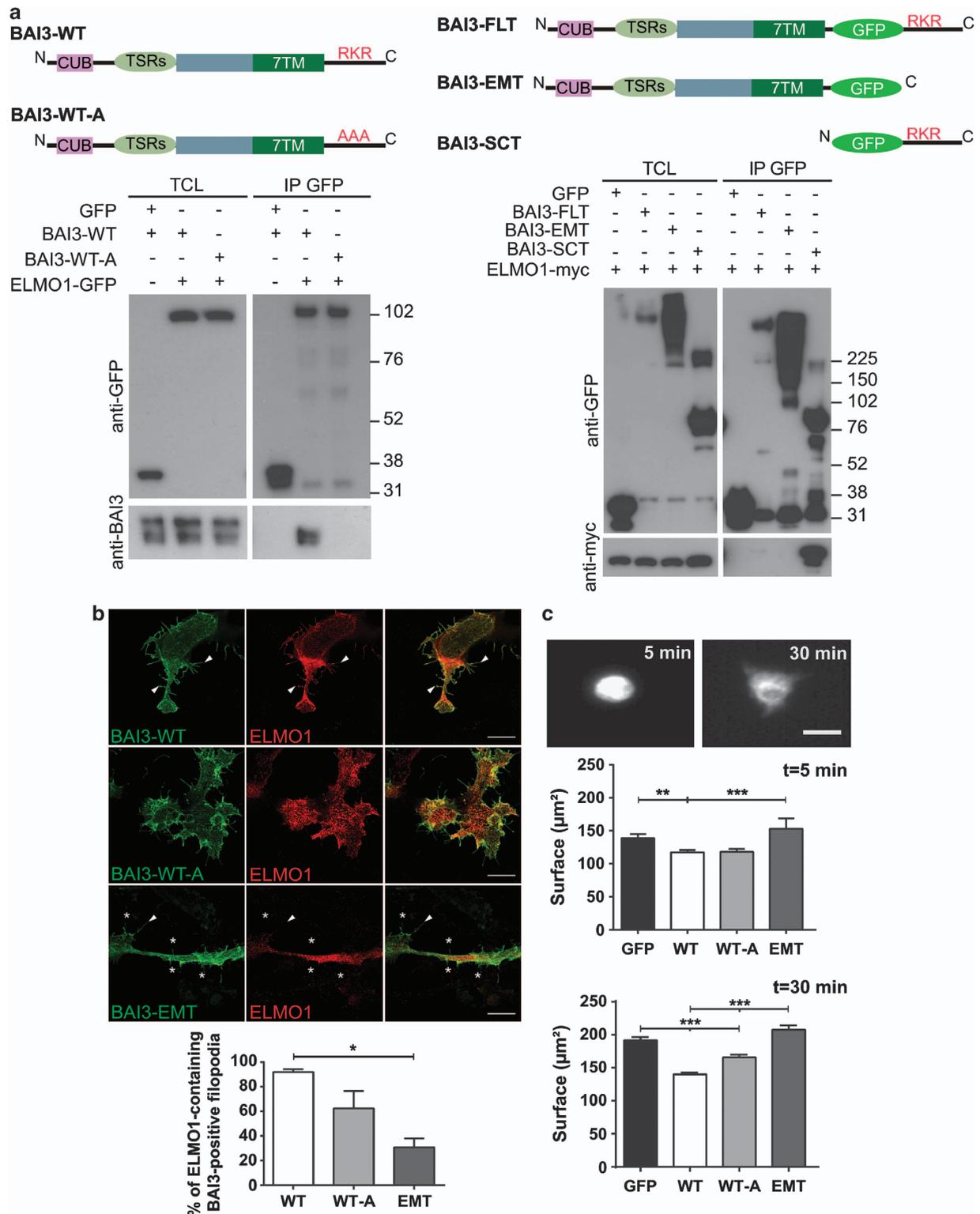


Figure 2. BAI3 regulates cell morphogenesis, partially through its interaction with ELMO1, a critical regulator of Rac1 signaling. **(a)** Top: schematic representations of the BAI3 constructs. Bottom: affinity-purified proteins from transfected HEK-293H cells were detected by immunoblot analysis with anti-GFP, anti-BAI3 antibodies and anti-myc antibodies. ELMO1 was either tagged with GFP (expected molecular weight 112 kDa, left) or with myc (expected molecular weight 85 kDa, right). Control experiments were performed in parallel on HEK-293H cells expressing a soluble GFP instead of the GFP-tagged constructs. TCL: total cell lysate. IP GFP: samples affinity purified using an anti-GFP antibody. **(b)** Top: immunostaining of HEK-293H cells cotransfected with ELMO1-myc (detected with an anti-myc antibody) and either BAI3-WT, BAI3-WT-A (detected with an antibody against the N-terminus of the receptor) or BAI3-EMT (detected with an antibody against GFP). Arrows denote filopodia colabelled for BAI3 and ELMO1; asterisks denote filopodia missing ELMO1. Scale bar: 10 μm. Bottom: quantification of BAI3/ELMO1 colocalization in filopodia. Mean ± s.e.m., $n = 40$ cells per condition, 4 independent experiments. **(c)** Top: example of HEK293 cell shapes after 5 or 30 min of spreading on fibronectin. Scale bar: 20 μm. Middle and bottom: quantification of the cell surface area at 5 or 30 min of spreading. Mean ± s.e.m., $n = 600$ –900 cells per condition, six independent experiments. ^{*} $P < 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$.

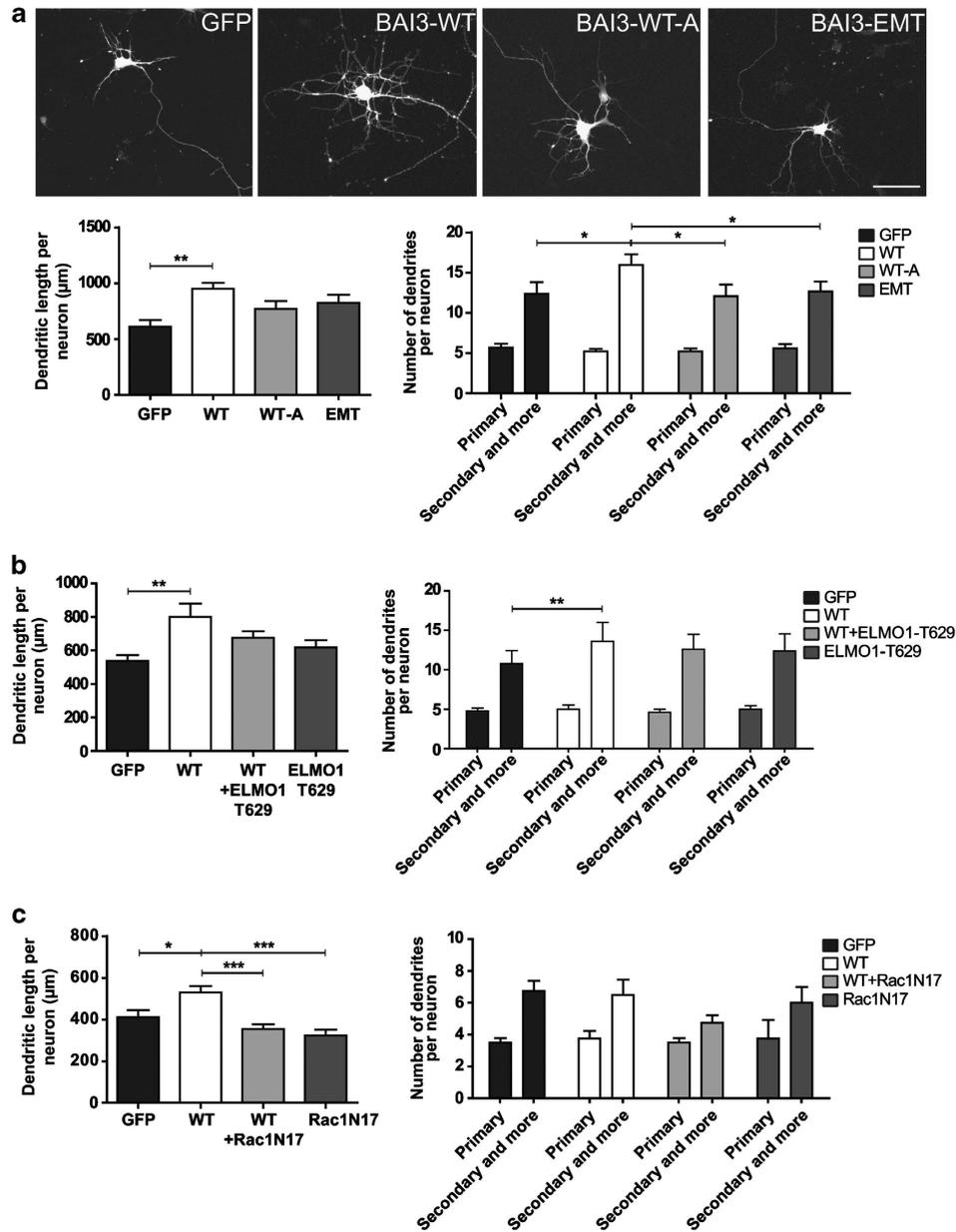


Figure 3. Overexpression of BAI3 promotes dendritic arborization growth and complexity through interaction with ELMO1 and Rac1 activity. (a) Top: confocal images of representative neurons transfected with the indicated constructs at DIV3 and fixed at DIV5. Scale bar: 50 μm. Bottom: the total dendrite length and the number of dendrites per neuron were quantified on DIV5 hippocampal neurons transfected with mCherry and either a GFP control construct, wild-type BAI3 (WT) or BAI3 mutants (WT-A, EMT). Mean ± s.e.m., $n = 30$ neurons per construct, four independent experiments. (b) Cotransfection of BAI3-WT and a mutant ELMO1 protein unable to bind DOCK180 (ELMO1T629) partially abolishes BAI3's promotion of dendrite morphogenesis in DIV5 hippocampal neurons. Mean ± s.e.m., $n = 55$ neurons per construct, five independent experiments. (c) Cotransfection of BAI3-WT and a dominant-negative Rac1 (Rac1N17) totally prevents BAI3's effect on dendrite morphogenesis in DIV5 hippocampal neurons. Mean ± s.e.m., $n = 40$ neurons per construct, four independent experiments. '*' denotes $P < 0.05$, '**' denotes $P < 0.01$, '***' denotes $P < 0.001$.

and P15 in the mouse cerebellum. To test this role *in vivo*, we generated transgenic mice expressing the BAI3-EMT mutant protein, which lacks the entire cytoplasmic domain and the ability to regulate neuronal morphogenesis (see Figures 2 and 3). In addition, the BAI3-EMT construct reduces by 44% the effect of the BAI3-WT receptor on dendritogenesis in hippocampal neurons (Supplementary Figure 4), and can thus act partially as a dominant-negative form. We used the *Pcp2* promoter to specifically drive the expression of BAI3-EMT in Purkinje cells in the cerebellum.²⁶ This specific expression was confirmed by immunoblot and was detected as early as P3 by GFP

immunohistochemistry (Supplementary Figure 5). It was estimated by qRT-PCR to be equivalent to 60% of the endogenous *Bai3* gene. Calbindin immunostaining of cerebellar sections and morphometric measurements did not reveal any gross reorganization as a consequence of the expression of the mutant construct (Supplementary Figure 5). However, quantitative analysis of single-labeled Purkinje cells from *Pcp2*/BAI3-EMT mice revealed important changes in their dendritic morphology: increased dendritic length, number of junctions and terminal dendrites when compared with wild-type Purkinje cells (Figure 5a). Scholl analysis shows that the complexity of Purkinje cell dendritic arborization is

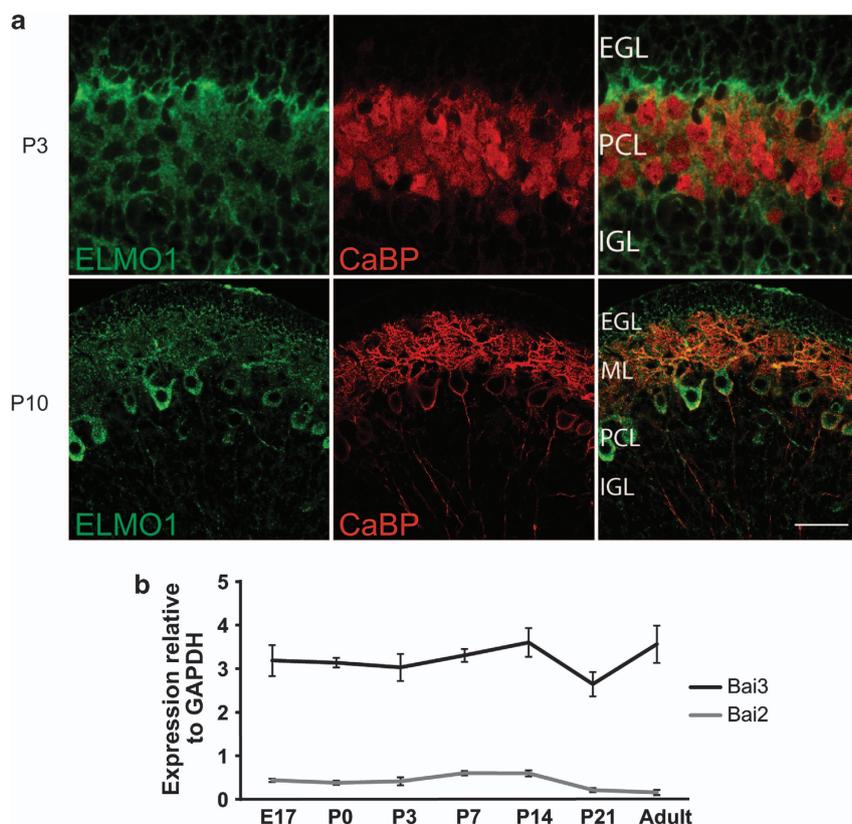


Figure 4. The BAI3 receptor and ELMO1 are expressed in the developing cerebellum. **(a)** Cerebellar sections from wild-type mice were immunolabelled for the endogenous ELMO1 protein and for calbindin (CaBP), a marker of Purkinje cells, at P3 and P10. EGL: external granular layer, ML: molecular layer, PCL: Purkinje cell layer, IGL: internal granular layer. Scale bar: 50 μ m. **(b)** qRT-PCR shows a high expression of the *Bai3* gene during postnatal development in the mouse cerebellum relative to the *Gapdh* gene.

particularly increased in distal parts of the cells relative to the soma. Finally, we analyzed the effects of BAI3 knockdown *in vivo* by injecting the lentiviral vectors in the cerebellar cortex of P6 pups (Figure 5b), and imaging after 4 days of infection. Defects in dendritic arborization were clearly visible as dendrites were longer, thinner and misoriented in Purkinje cells transduced with the shRNA against BAI3 compared with the control shRNA. Altogether, these results show a major role for BAI3 in regulating Purkinje cell dendritic arbor formation *in vivo*.

DISCUSSION

The brain is composed of thousands of different types of neurons that differ drastically in their morphology, in particular in the shape and complexity of their dendritic arborization. This morphology underlies functional differences between neurons, in particular how they integrate signals coming from different inputs. Its proper development is thus essential for the normal function of the central nervous system, and deficits in neuronal morphogenesis have been correlated to psychiatric disorders such as schizophrenia. We have now provided evidence, both *in vitro* and *in vivo*, for a new signaling pathway regulating dendrite morphogenesis involving the BAI receptor BAI3, a member of the poorly studied family of adhesion-GPCRs, and the protein ELMO1, an important regulator of the RAC1 RhoGTPase.

The regulation of dendrite morphogenesis involves integration of extracellular signals and intrinsic molecular programs in order to control the growth and branching of the actin cytoskeleton. The BAI receptors constitute a new regulator of this process that can sense extracellular signals and signal in the cell through their interaction with effectors such as ELMO1. Another family of

adhesion-GPCRs, the CELSR proteins, has been shown to have a role in dendrite morphogenesis through regulation of intracellular calcium signaling. Knockdown of CELSR2 in organotypic cultures in pyramidal neurons and Purkinje cells induces a simplification of their dendritic arborization,³¹ whereas CELSR3 has an opposite role.³ This function is conserved as the *Drosophila* homolog, Flamingo, is also involved in neuronal morphogenesis and more particularly in regulating dendritic field through repulsion.³² Our results show that control of neuronal morphogenesis could be a property of many adhesion-GPCRs *in vivo*. Given the diversity of domains found in the extracellular part of adhesion-GPCRs, an attractive hypothesis is that each type of adhesion-GPCR might regulate the morphology of particular neuronal populations and thus contributes to the diversity of shape, and of function, in the vertebrate central nervous system.

What is the signaling pathway of BAI3 during neuronal morphogenesis? Our results show that its interaction with the protein ELMO1 is partially involved in this process. Previous results have shown that BAI1 can regulate RAC1 and phagocytosis through an interaction with ELMO1.²⁵ Regulation of RhoGTPases is essential for driving changes in the actin cytoskeleton and cell morphogenesis during development. Moreover modifying RAC1 activity in neurons is known to induce changes in dendrite morphogenesis²² and interferes with BAI3's function as shown by our results. Hence BAI3's interaction with ELMO1 constitutes a direct pathway linking extracellular cues and intracellular modification of the actin cytoskeleton during neuronal development. It will be of interest to analyze the role of other potential intracellular partners of BAI3. In particular, IRSp53/BAIAP2 was originally identified as a partner for BAI1,³³ and has since been shown to regulate actin morphogenesis through

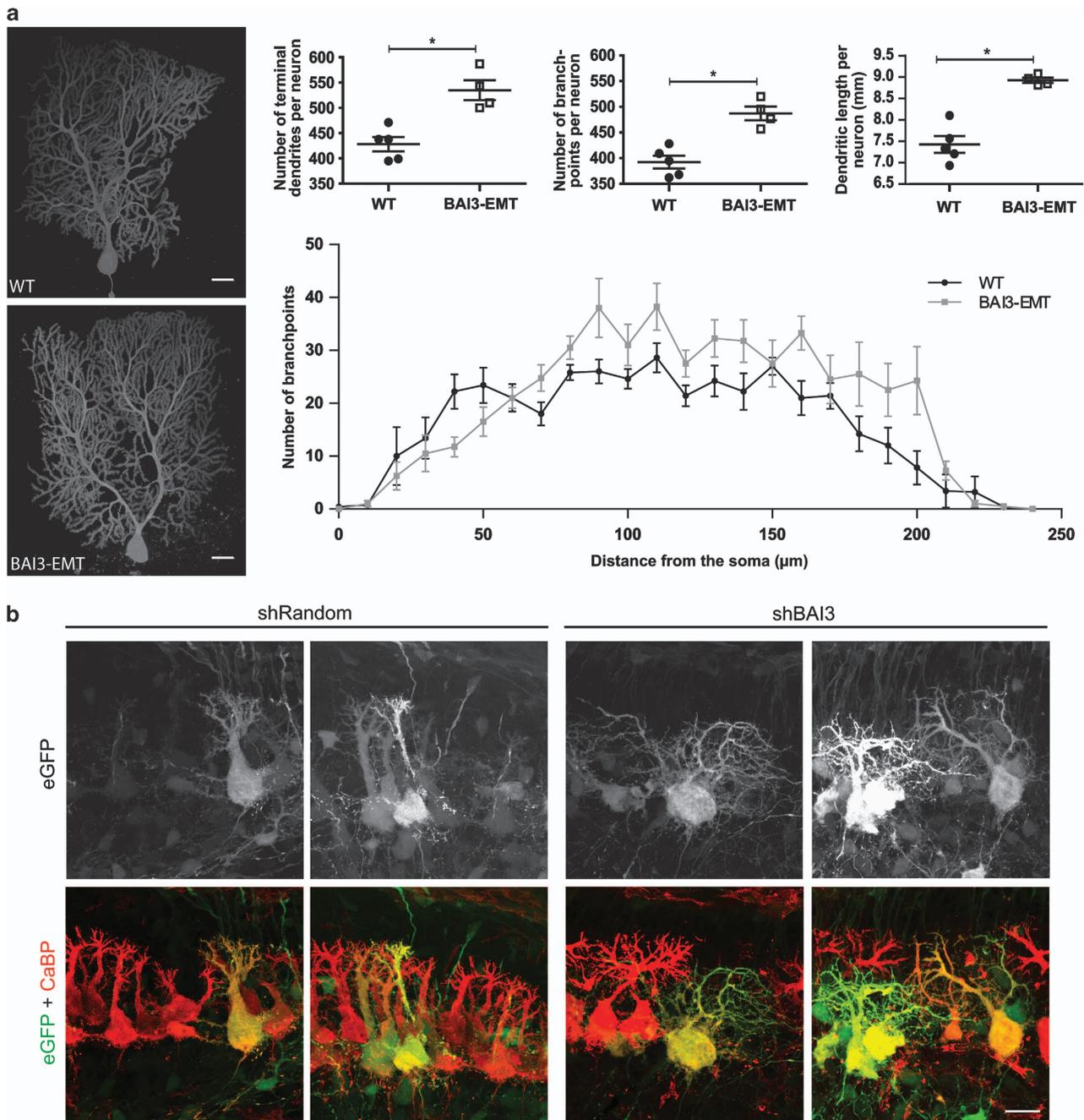


Figure 5. The BAI3 protein controls neuronal development and dendrite morphogenesis *in vivo*. **(a)** Left: images of representative reconstructed Purkinje cells in 1-month-old Pcp2/BAI3-EMT transgenic mice and age-matched wild-type (WT) mice. Right: quantification of total dendritic length, number of junctions and terminal branches per Purkinje cell as well as Scholl analysis. Mean \pm s.e.m., $n = 4-5$ Purkinje cells per genotype. Student's *t*-test followed by Mann-Whitney, ^{*,**} denotes $P < 0.05$. **(b)** Purkinje cells transduced at P6 with lentivirus particles driving either shBAI3 or control shRandom (eGFP positive) were analyzed at P10 using calbindin immunostaining (CaBP). Massive defects in dendritic arborization were observed after BAI3 knockdown when compared with non transduced adjacent cells or cells transduced with the control virus. Scale bars: 20 μ m.

binding of small RhoGTPases and the WAVE complex.³⁴ Small G protein binding, although yet to be demonstrated for BAI proteins, could also have an important role through binding of the third intracytoplasmic loop. Extracellularly, binding of the secreted protein C1QL1 has recently been shown to promote synapse elimination *in vitro*, but no effect was demonstrated on dendrite morphogenesis.³⁵ These results were obtained in mature hippocampal neurons and, taken together with our data, suggest that the function of BAI proteins in the regulation of

dendrite morphogenesis is critical at early stages of neuronal development. Alternatively, other unknown ligands of BAI3 might be critical for this function.

Several candidate genes linked with neurodevelopmental disorders are proteins regulating neuronal morphogenesis.³⁶ For example, mutations in SHANK3 associated with autism induce defects in spine morphogenesis and in actin polymerization.³⁷ Neuregulin1's mutation at valine 321, previously linked to schizophrenia, has been shown to prevent neuregulin's

control of dendritic arborization growth and complexity.³⁶ Given the evidence associating BAI genes with psychiatric disorders,^{9,13,14,10,12} our data reveals a new pathway involved in the etiology of these brain diseases through regulation of dendrite morphogenesis, and highlights the potential of the BAI signaling pathway for therapeutic intervention.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We would like to thank Dr Sophie Vriza and Dr Jean-Louis Bessereau for critical reading of the manuscript. The ELMO1-GFP and ELMO1T629-GFP constructs were a kind gift of Dr Lorraine Santy (PennState, USA). We would like to thank R Schwartzman and S Bolte at the IFR83 imaging facility (UPMC, Paris, France), and J Teillon at the CIRB imaging facility (Collège de France, Paris, France). We would like to thank J Bakouche and V Gautheron for helping us with cloning and mouse colonies. This work was supported by the CNRS (PEPS, PICS), ATIP-AVENIR, Association Française du Syndrome de Rett, UPMC (Emergence), Ecole des Neurosciences de Paris. VL was supported by the ED3C (UPMC) and the Ministère de la Recherche et de l'Enseignement Supérieur, AU by UPMC and AVENIR, SMS by NERF and LABEX MEMOLIFE, and MT by FRM, PI by ANR-09-MNPS-038 and ANR-2010-JCJC-1403-1.

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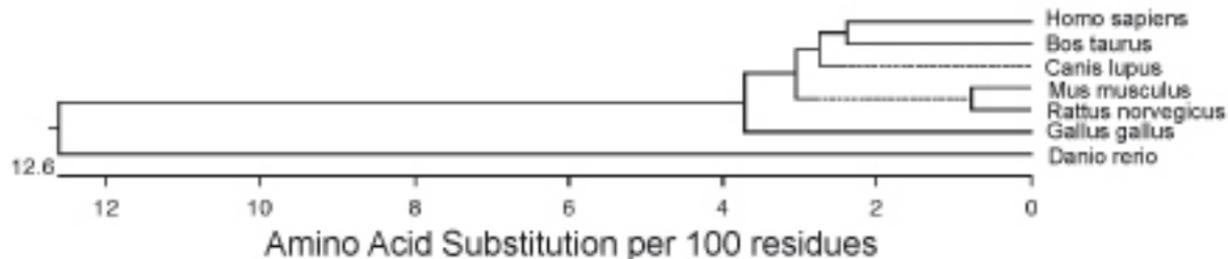
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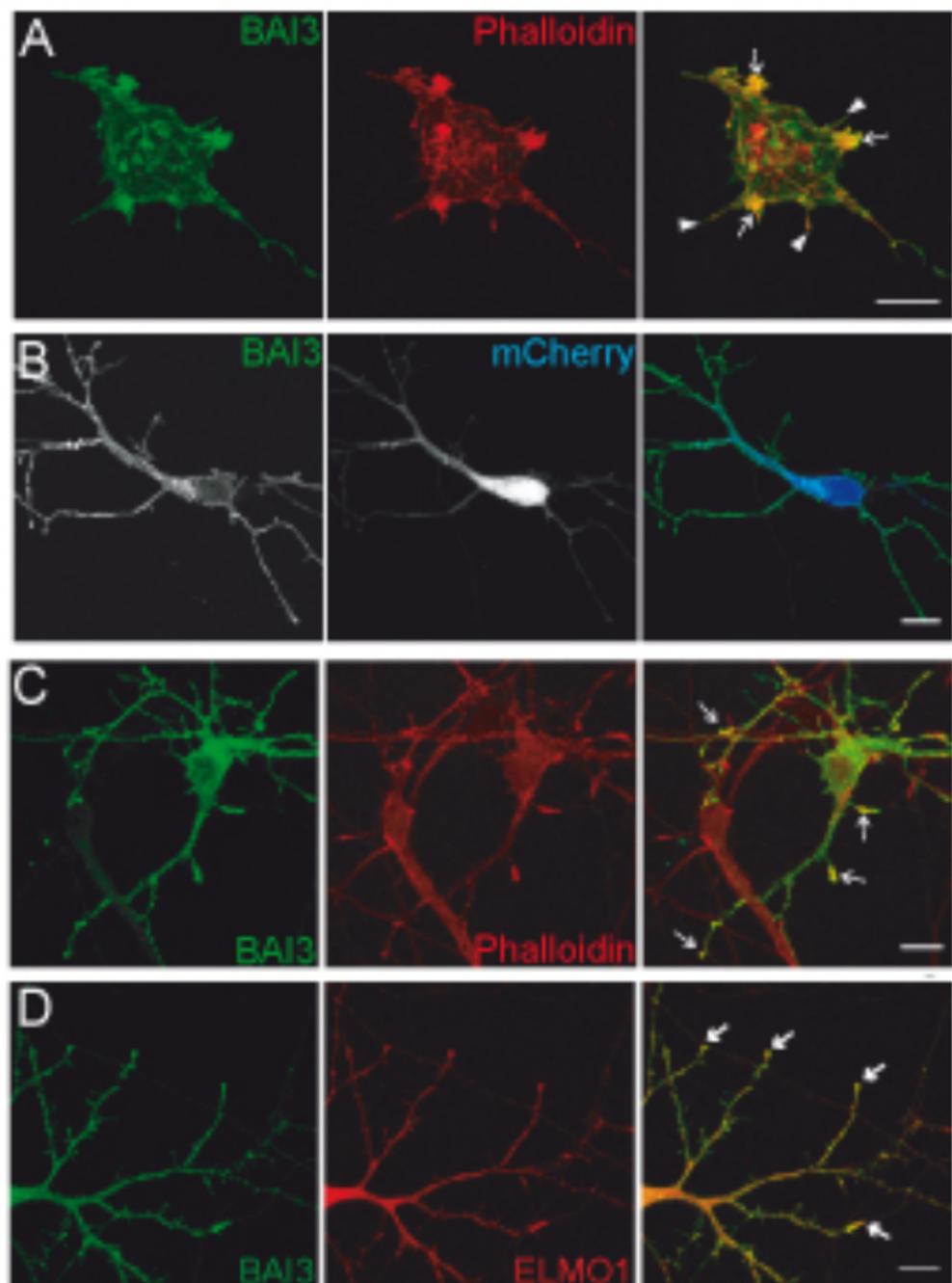
Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)



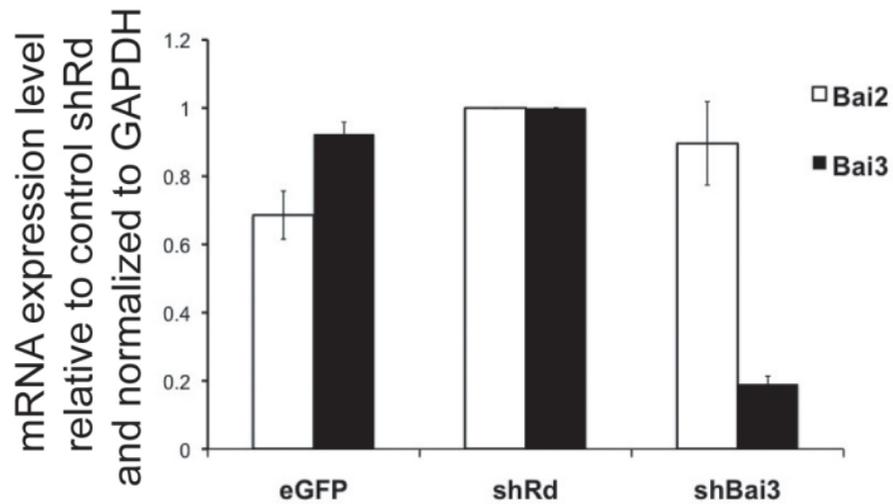
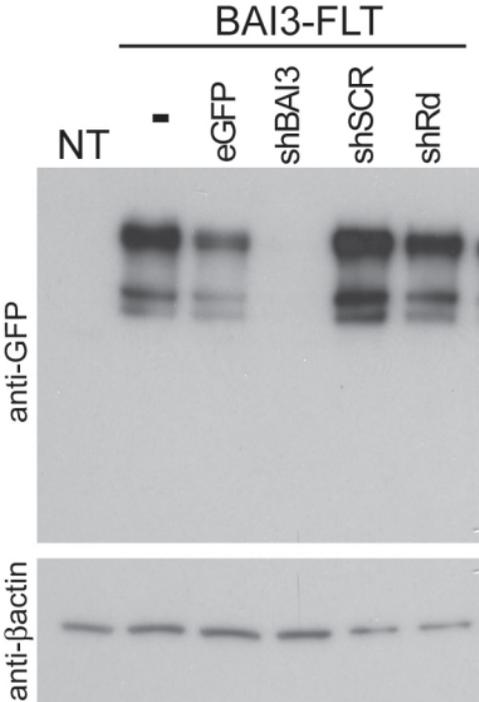
Percent Identity

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | | |
|------------|---|------|------|------|------|------|------|------|---|-------------------|
| Divergence | 1 | ████ | 98.3 | 98.0 | 94.9 | 98.6 | 95.4 | 79.0 | 1 | Homo sapiens |
| | 2 | 1.7 | ████ | 98.5 | 94.7 | 98.1 | 94.8 | 79.4 | 2 | Mus musculus |
| | 3 | 2.0 | 1.5 | ████ | 94.3 | 97.6 | 94.3 | 79.0 | 3 | Rattus norvegicus |
| | 4 | 5.3 | 5.5 | 6.0 | ████ | 95.1 | 91.9 | 79.4 | 4 | Gallus gallus |
| | 5 | 1.5 | 1.9 | 2.5 | 5.1 | ████ | 95.4 | 79.5 | 5 | Canis lupus |
| | 6 | 4.8 | 5.4 | 5.9 | 8.6 | 4.8 | ████ | 76.8 | 6 | Bos taurus |
| | 7 | 24.7 | 24.1 | 24.7 | 24.1 | 24.0 | 27.8 | ████ | 7 | Danio rerio |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | | |

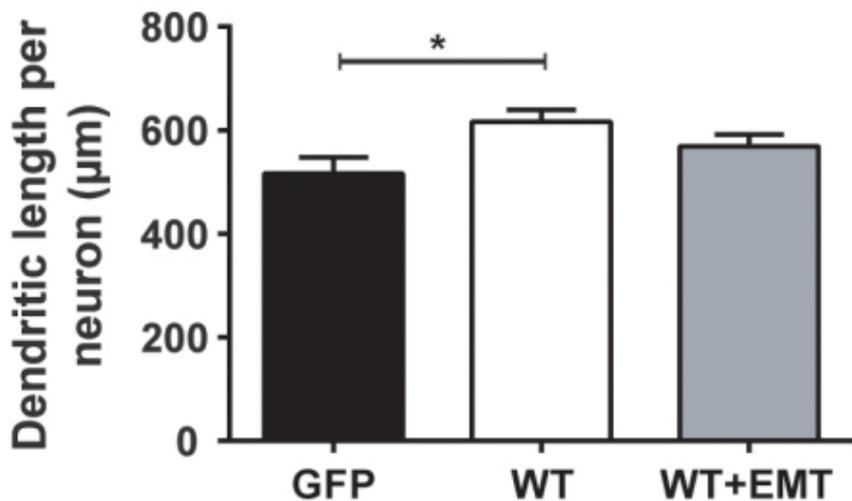
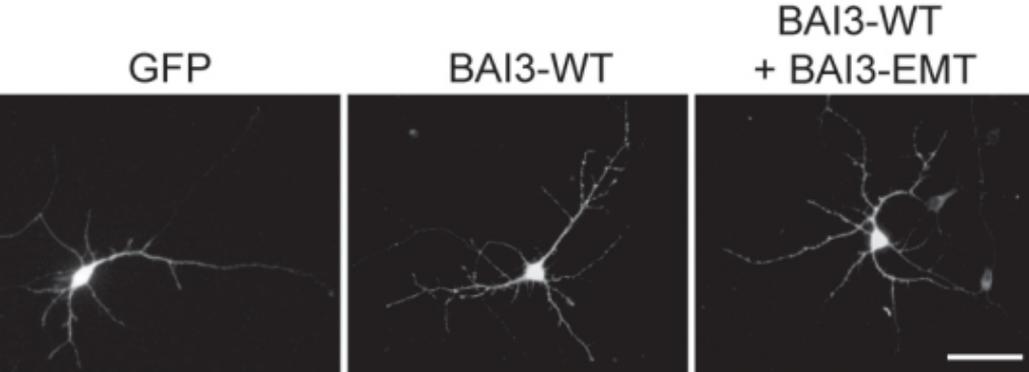
Supplemental figure 1
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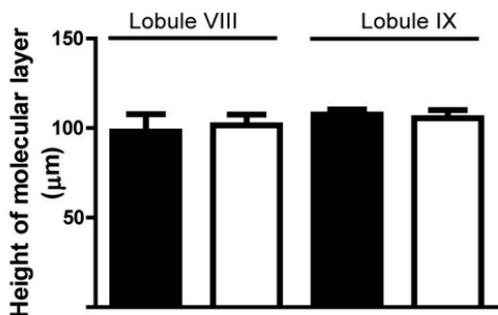
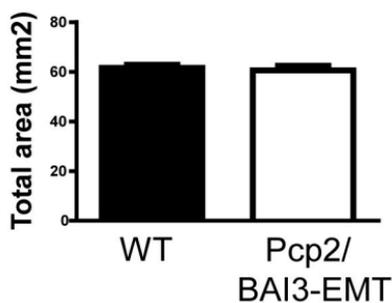
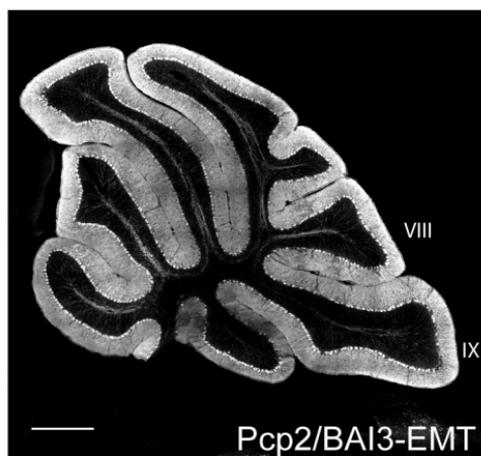
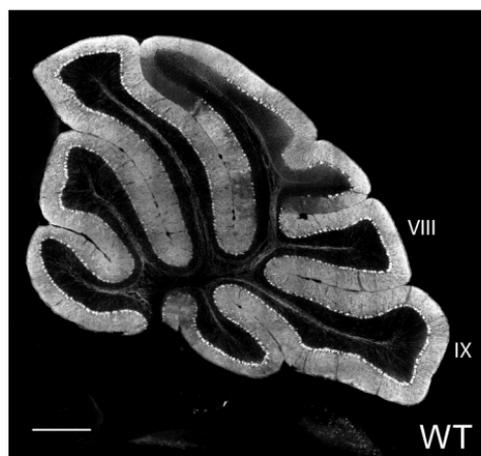
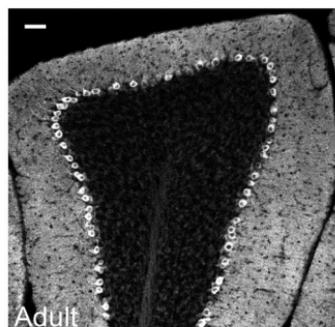
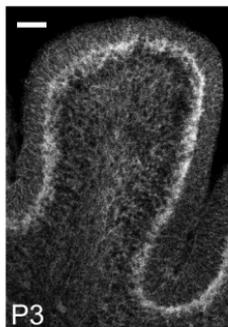
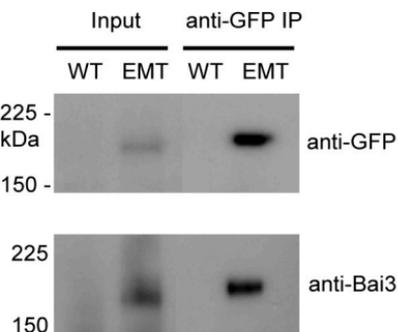
Supplemental figure 2
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Supplemental figure 3
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Supplemental figure 4
Lanoue et al.



Supplemental figure 5
Lanoue et al.

Supplemental figure legends

Supplemental figure 1: Phylogenetic analysis of the BAI3 protein in vertebrates using ClustalW

Supplemental figure 2: The BAI3 receptor is localized in actin-rich structures, in particular in dendrites of neurons, and colocalizes with ELMO1.

A. In HEK-293H cells, transfected wild-type BAI3 (BAI3) colocalizes with F-actin (labeled with phalloidin-TRITC) in cell protrusions such as lamellipodia (arrows) and filopodia (arrowheads). Scale bar: 10 μ m.

B. The BAI3 protein (BAI3) is detected in dendrites and protrusions of DIV5 hippocampal neurons transfected with mCherry and wild-type BAI3. Scale bar: 10 μ m

C. In dendrites of DIV5 hippocampal neurons, clear colocalization is detected between transfected BAI3 (BAI3) and F-actin (stained with phalloidin-TRITC), in particular in protrusions (arrows). Scale bar: 10 μ m.

D. Immunostaining for transfected BAI3 and ELMO1-myc (ELMO1) in DIV5 hippocampal neurons shows partial colocalization with the BAI3 protein (arrows). Scale bar: 10 μ m

Supplemental figure 3: Characterization of the specificity of BAI3 knockdown

Left: Immunoblot analysis of total protein extracts from HEK-293H cells co-transfected with a vector coding for GFP-tagged full length BAI3 (FLT, cf. figure 2) and with a vector coding for either eGFP, a small hairpin RNA against BAI3 (shBAI3), or a non targeting control small hairpin RNA (shRandom). Actin immunoblotting was used to confirm analysis of equal amounts of proteins between samples.

Right: *Bai3* and *Bai2* mRNA expression quantified by RTqPCR on cDNA extracts from hippocampal neurons transduced with the corresponding lentivirus particles. Data are presented normalized to expression of the housekeeping gene GAPDH and relative to control

shRandom values. Note that only the expression of Bai3 is significantly reduced by shBAI3. Mean \pm SEM of 3 independent experiments.

Supplemental figure 4: The BAI3-EMT construct has a partial dominant-negative effect on wild-type BAI3 function

Top: Confocal images of representative neurons transfected with the indicated constructs at DIV3 and fixed at DIV5. Scale bar: 50 μ m. Bottom: The total dendrite length per neuron was quantified on DIV5 hippocampal neurons transfected with mCherry and either a GFP control construct, wild-type BAI3 (WT) or wild-type BAI3 and BAI3-EMT mutant (WT+EMT) at equimolar ratio. Mean \pm SEM, n= 60 neurons per condition, 3 independent experiments. * p<0.05.

Supplemental figure 5: Characterization of the pcp2/BAI3-EMT transgenic mice

Copy number was estimated at about 21 using quantitative PCR on genomic DNA extracts (6 samples per genotype). Relative expression levels were quantified using quantitative RT-PCR performed on cDNA from cerebella of transgenic and wild-type mice: the transgene was expressed at about 60% of the BAI3 wild-type level (mean from 3 different animals per genotype).

A. Left: Affinity-purification using an anti-GFP antibody (anti-GFP IP) on total cerebellar protein extracts (Input) and immunoblot analysis showed the expression of the BAI3-EMT transgene in cerebella from adult transgenic mice at the expected size (detected either using the GFP antibody or the BAI3 antibody).

Right: Anti-GFP immunohistochemistry on parasagittal cerebellar sections from P3 and 4 weeks-old Pcp2/BAI3-EMT mice. Scale bar= 50 μ m.

B. Cerebellar sagittal sections from 4 weeks-old transgenic mice expressing the mutant BAI3-EMT protein specifically in Purkinje cells (Pcp2/BAI3-EMT) and from wild-type mice (WT) immunostained for calbindin. Scale bar= 500 μ m. Morphometric measurements using ImageJ

showed no significant changes in total cerebellar area and mean molecular height. N=3 animals per genotype.

Supplemental material and methods

All animal protocols were approved by the Comité Régional d’Ethique en Expérimentation Animale.

Additional constructs

A myc tag (ELMO-myc) or a GFP tag (ELMO1-GFP) was fused to the C-terminus of the ELMO1 cDNA in the pEBB vector. ELMO1GFP and ELMO1T629GFP were a kind gift of Dr. LC Santy. The dominant-negative Rac1 construct was pcDNA3-EGFP-Rac1(T17N) (Invitrogen, ¹).

Neuronal cultures and image analysis

Hippocampal cultures were prepared from E18 Swiss mouse embryos as previously published with minor modifications ². Neurons were transfected at DIV3 using Lipofectamine-2000 (Life technologies, Carlsbad, USA) according to the manufacturer’s protocol and fixed after 48 hours. Cerebellar mixed cultures were prepared from P0 mouse cerebella were dissected and dissociated according to previously published protocol ³. Neurons were seeded at a density 5×10^6 cells/ml.

Neurons were imaged using a Leica SP5 confocal microscope at 20X (for hippocampal neurons) or at 63X (for Purkinje cells). Dendrites were analyzed using the NeuronJ plugin of ImageJ. Statistical analysis was performed using for dendrite length: One-way ANOVA followed by Tukey’s or Dunn’s *posthoc* test; for dendrite number: Two-way ANOVA followed by Bonferroni *posthoc* test.

Immunocytochemistry, immunohistochemistry and antibodies

Actin staining was performed on fixed cells using 100 nM phalloidin-TRITC (Sigma-Aldrich, St-Louis, USA). For immunohistochemistry, thirty micrometers sagittal cerebellar sections were obtained from 4% paraformaldehyde perfused mice using a freezing microtome. Immunohistochemistry for ELMO1 was performed using the TSA amplification kit (Perkin Elmer, Waltham, USA). Antibodies used were: anti-ELMO1, 1/1000 (Abcam, Cambridge, UK, #ab2239); anti-BAI3, 1/500 (Sigma, #HPA015963); anti-GFP, 1/100 (Abcam, #6556); anti-CaBP, 1/5000 (Swant, Marly, Switzerland, #300); anti-myc, 1/500 (Sigma, #M4439).

Cell spreading assay

Transfected HEK-293H cells were incubated for 30 min in 2.5 μM Cell Tracker (Invitrogen) diluted in DMEM, washed for 30 min in DMEM/10% FBS, and mechanically detached in DMEM/10% FBS. After 30 min of recovery, cells were plated at a concentration of 5×10^4 cells/ml on coverslips coated with 10 $\mu\text{g} \cdot \text{mL}^{-1}$ fibronectin. Cells were fixed after 5 or 30 min of spreading and imaged under epifluorescence at 20X magnification with a Qicam camera. Quantification of the cell surface area was performed using the ImageJ software by an investigator without knowledge of the condition.

qPCR and RT-qPCR

Copy number was estimated using genomic DNA purified from mouse tails followed by quantitative PCR using the LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, Meylan, France) and the following primers for the Bai3 gene: forward: 5'ctggccatgacagataaacg3' and reverse 5'ctctccgaagaatgcagtgg3'.

Relative expression levels were quantified for the BAI3EMT transgene using RT-qPCR. RNA samples were obtained from cerebella using the RNeasy Mini kit (QIAGEN, Hilden, Germany), cDNA were amplified using the SuperScript® VILO™ cDNA Synthesis kit (Life technologies, Paisley, UK) according to manufacturer's instructions. Quantitative PCRs were performed as for the copy number assessment.

Single Purkinje cell reconstruction

Single Purkinje cells were filled with biocytin in cerebellar slices obtained from 4 weeks old-animals as described in ⁴, slices were fixed with 4% paraformaldehyde and biocytin labelling was revealed using the Vectastain ABC kit coupled with the TSA-FITC substrate (tyramide signal amplification kit, Perkin Elmer). Purkinje cells were imaged using a SP5 Leica confocal microscope, a 300 nm z-step and a x63 objective with a zoom of 1.7. Images were deconvolved using the Huygens Essential software (Scientific Volume Imaging B.V., Hilversum, Netherlands) and dendritic arbors were reconstructed using the Neuron Studio software (CNIC, Mount Sinai School of Medicine, New York, USA).

Supplemental references

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