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ORIGINAL ARTICLE

Pea3 Transcription Factors, Etv4 and Etv5, Are Required for Proper Hippocampal Dendrite Development and Plasticity

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Abstract

The proper formation and morphogenesis of dendrites is essential to the establishment of neuronal connectivity. We report that 2 members of the Pea3 family of transcription factors, Etv4 and Etv5, are expressed in hippocampal neurons during the main period of dendritogenesis, suggesting that they have a function in dendrite development. Here, we show that these transcription factors are physiological regulators of growth and arborization of pyramidal cell dendrites in the developing hippocampus. Gain and loss of function assays indicate that Etv4 and Etv5 are required for proper development of hippocampal dendritic arbors and spines. We have found that in vivo deletion of either Etv4 or Etv5 in hippocampal neurons causes deficits in dendrite size and complexity, which are associated with impaired cognitive function. Additionally, our data support the idea that Etv4 and Etv5 are part of a brain-derived neurotrophic factor–mediated transcriptional program required for proper hippocampal dendrite connectivity and plasticity.

Key words: BDNF, dendrite architecture, dendritic spine development, functional plasticity, hippocampal development

Introduction

The development of a complex, type-specific dendrite morphology determines the functional properties of neurons and neural circuits (Gulledge et al. 2005; Spruston 2008). Many neurodevelopmental disorders are due to structural abnormalities of dendrites and their connections (Kaufmann and Moser 2000; Penzes et al. 2011). The size and shape of dendritic arbors result from the interplay of intrinsic genetic programs and extrinsic signals. Extrinsic cues including growth factors, adhesion molecules, and neuronal activity modulate the design of dendritic arbors via specific proteins, which regulate intracellular signaling molecules that influence cytoskeletal elements and transcriptional programs. However, accumulating evidence highlights the importance of cell-intrinsic mechanisms in the control of dendrite morphogenesis and connectivity, governing entire developmental programs as well as orchestrating neuronal responses to extrinsic cues. Identifying transcriptional programs and signaling pathways triggered by extracellular cues that control neuronal circuit formation will be of great importance to be able to decipher and understand the functioning of mature nervous system. Transcriptional control of gene expression represents a major mode of cell-intrinsic regulation of neuronal development (de la Torre-Ubieta and Bonni 2011). In recent years, a number of transcription factors have been described to control distinct stages of dendrite development. Among others, the transcription factor CREB, the brain-enriched protein FOXO6, the bHLH protein Neuro D, and the transcription factor MEF2A play important roles in different stages of dendrite morphogenesis (de la Torre-Ubieta and Bonni 2011). An interesting feature of the transcription factors

in the regulation of dendrite development is that their functions are controlled by extrinsic factors such as neuronal activity and neurotrophic factors (Whitford et al. 2002). In particular, brain-derived neurotrophic factor (BDNF) has been described to be required for dendritic growth and spine formation in different brain regions including the cortex (Chakravarthy et al. 2006; English et al. 2012; Vigers et al. 2012), hippocampus (Tyler and Pozzo-Miller2003; Luikart et al. 2005), and striatum (Rauskolb et al. 2010). Thus, BDNF activation of its receptor TrkB triggers the PI3K and Ras/MAPK signaling pathway, resulting in the direct phosphorylation of the transcription factor CREB (Reichardt 2006), which plays an essential role in dendrite development.

Pea3 (Polyoma enhancer activator 3) subfamily of ETS transcription factors comprise 3 members namely Etv1, Etv4, and Etv5 (also named Er81, Pea3, and Erm, respectively), and many reports propose the different Etvs proteins as effectors of the Ras/ERK (MAPK) pathway (Yordy and Muise-Helmericks 2000). Recently, we have described that Etv4 and Etv5 are expressed in NGF-responsive DRG neurons and mediate retrograde signaling and axonal growth of sensory neurons in response to this neurotrophin (Fontanet et al. 2013). More recently, both factors have been described to mediate axonal growth in response to BDNF in a different subpopulation of sensory neurons (Liu et al. 2016). The third member of the family, Er81/Etv1, mediates the formation of DRG proprioceptive afferent-motor neuron connections in response to neurotrophin-3 (NT3) (Arber et al. 2000; Patel et al. 2003). In the central nervous system (CNS), the induction of Etv4 by the neurotrophic factor GDNF in specific motor neuron pools is essential for the control of cell body positioning, axonal trajectory patterns, and proper sensory-motor connections in the spinal cord (Livet et al. 2002; Vrieseling and Arber 2006).

The expression pattern of the 3 members of the Pea3 family has been described at different rostrocaudal levels of the mouse telencephalon at early embryonic stages, and the suppression of the activities mediated by Pea3 transcription factors disrupted neuronal radial migration (Hasegawa et al. 2004; Zimmer et al. 2010).

These observations led us to examine the expression pattern of Etv4 and Etv5 transcription factors in the developing hippocampus. The expression of Etv4 and Etv5 at the moment that hippocampal dendrite/synapse development takes place prompted us to investigate whether these factors might be involved in the control of the mechanisms that underlie hippocampal circuit formation such as dendrite arborization and spine morphogenesis. Here, we show that Etv4 and Etv5 are both positively regulated by BDNF signaling, and that these factors are required for proper hippocampal development. Our studies suggest that Etv4 and Etv5 are key components of a gene network downstream of BDNF/TrkB that promotes and controls hippocampal dendrite morphology. These findings show for the first time a physiological requirement of Etv4 and Etv5 in the establishment of the hippocampal connectivity and plasticity.

Materials and Methods

Cell Lines, Recombinant Proteins, and Inhibitors

PC12 cells were grown in DMEM supplemented with 5% horse serum and 10% FBS (Invitrogen) as previously described (Fontanet et al. 2013). BDNF was purchased from R&D systems. The inhibitor PD98059 was from Sigma-Aldrich.

Primary Neuron Cell Cultures

To obtain hippocampal neurons from mice, dissections of P0 mouse were performed as previously described (Otero et al. 2014). Rat hippocampal neurons were prepared from E17.5 Wistar rat embryos as previously described (Ledda et al. 2007). Dissociated hippocampal neurons were transfected using Lipofectamin (Invitrogen) according to the manufacturer´s suggestions. For details, see Supplementary Information.

DNA Constructs, Cell Transfection, and Neurite Outgrowth Assays

Details of DNA constructs, cell transfection, and neurite outgrowth can be found in Supplementary Information.

PCR and Real-Time PCR

PCR and real-time PCR were performed using standard methodologies. See Supplementary Information for details.

Pharmacological Treatment, Nuclear Extract Preparation, and Western Blot Analysis

Hippocampal cell cultures were treated with the specific MEK inhibitor PD98059 (50 μM) for 30 min at 37°C before BDNF stimulation (25 ng/mL) for 2 h. The RNA was extracted with RNA-easy columns (Quiagen) and analyzed by semiquantitative PCR using the indicated primers. Nuclear extract preparation was based in the protocol described by Blokzijl et al. (Blokzijl et al. 2002), and western blot analysis was performed as previously described (Paratcha et al. 2003). For details, see Supplementary Information.

Mouse Strains

Etv4$^{-/-}$ and Etv5 flox/flox mice were generously provided by Dr Olivia Bermingham-McDonogh (University of Washington, Seattle, USA). The Etv4-mutant mice were described in detail in Livet J et al. (2002). The *Etv*5-conditional mutant mice were described (Zhang et al. 2009). Etv5$^{flox/flox}$ mice were mated with Nestin-Cre mice (Tronche et al. 1999) to generate Etv5-conditional animals Etv5^{cKO}. All transgenic strains were genotyped using PCR-based strategy. PCR primer sequences are available upon request. The use of animals was approved by the Animal Care and Use Committee of the School of Medicine, University of Buenos Aires.

Image Analysis and Quantification

For dissociated neurons and cryostat sections, images were obtained using an Olympus confocal microscope, using 60× objective with sequential acquisition settings. For dendritic spine assays, a Z-series projection of each neuron was made. To determine spine density, the number of spines on segments of at least 100 μm of dendritic length/neuron was counted. Among 15–25 transfected neurons were chosen randomly for quantification per experiment and 2–3 independent experiments were done for each construct. Statistical significance was calculated using Student's *t* test or analysis of variance (ANOVA) as indicated. For the analysis of morphometric parameters in dissociated neurons, pictures were acquired using an Olympus IX-81 microscope, using 20× or 40× objective, and

measurements of dendritic complexity were done as previously described (Sholl 1953) using Neuro J plugin Image J software.

Immunostaining and Microscopy

For immunofluorescence of hippocampal cultures, the cells were fixed and incubated with the indicated antibodies. For details, see Supplementary Information.

Golgi Silver Impregnation Method

The staining was performed following the protocol described by Alsina et al (Alsina et al. 2016). Briefly, mice brains were placed in a fixative solution (formalin 10%) and stored in darkness for 24 h at room temperature (RT). Then, the brains were transferred into a 3% potassium dichromate water solution and stored at RT for 4 days. Thereafter, the brains were transfer into a 2% silver nitrate water solution for 24 h. The brains were cut in sections of 100 μm thickness using a vibratome. Brain coronal sections were collected on a 0.3% gelatin water solution, dried at RT, and dehydrated in ethanol/xylene series. Finally, they were mounted on 0.3% gelatinized slides. Proximal neuronal complexity and primary dendrites number were evaluated in isolated Golgi silver impregnated hippocampal CA1 neurons. Bright field images were taken on an Olympus IX-81 inverted microscope.

Behavioral Studies

For behavioral testing, we used male mice. The behavioral tests performed in the studies are described in detail in Supplementary Information.

Statistical Analysis

Statistical analysis was performed in GraphPad Prism 5.0. In the indicated cases, Student's *t* test or ANOVA analysis followed by Dunnett, Bonferroni, Newman–Keuls, or Tukey post-test was performed.

Results

Etv4 and Etv5 Are Expressed in the Developing Hippocampus

To investigate a potential role of Etv4 and Etv5 transcription factors in hippocampal development, we used real-time PCR to quantify the expression of these genes in the developing rat hippocampus during the main period of dendrite elongation and synapse formation. A substantial increase in the expression levels of *Etv4* and *Etv5* mRNA was detected after birth during the first 2 weeks of postnatal development (Fig. 1*A*).

To determinate the distribution of Etv4 and Etv5 proteins in the hippocampus, we analyzed the expression pattern of these transcription factors by immunofluorescence using specific antibodies. A prominent labeling of Etv4 and Etv5 was evident in the nuclei of pyramidal neurons from CA1, CA3, and the granule cell layer of the dentate gyrus (Fig. 1*B*), as well as in certain populations of inhibitory neurons (data not shown). Expression of Etv4 and Etv5 was observed in cells positive for the neuronal markers MAP-2 and βIII-tubulin, indicating that these transcription factors are mainly expressed in neurons (Supplementary Fig. 1), and overlapping expression of Etv4 and Etv5 was observed in the majority of hippocampal neurons (Fig. 1*B*). Moreover, in dissociated cultures of hippocampal neurons, Etv4 and Etv5 immunoreactivity was evident in the nuclei of the majority of cells positive for neuronal markers Tau or βIII-tubulin (Fig. 1*C*) as well as for the nuclear marker specific for post-mitotic neurons, NeuN (Fig. 1*D*).

Altogether, our data indicate that Etv4 and Etv5 are expressed in the developing hippocampus during the main period of dendrite growth and synapse formation suggesting that they play a key role in the establishment of hippocampal connectivity.

Etv4 and Etv5 Are Required for Proper Hippocampal Dendrite Development

To analyze a potential role of Etv4 and Etv5 transcription factors in hippocampal neuron development, we evaluated the consequences of reducing either Etv4 or Etv5 protein levels in rat hippocampal neurons. We used small-hairpin RNAs (shRNAs) directed against rat *Etv4* (*Etv4*-shRNA) or *Etv5* (*Etv5*-shRNA) mRNAs, which have been previously tested for specific downregulation of the targeted molecules (Fontanet et al. 2013). Hippocampal neurons were cultured and transfected at 15 days in vitro (DIV) with specific *Etvs*-shRNA or control-shRNA expressing enhanced green fluorescence protein (GFP) vectors. Three days after transfection, the neurons were analyzed for neuronal tree complexity measured by Sholl analysis, which quantifies the number of neurites intersecting concentric circles of increasing radius centered on the cell body (Sholl 1953). Dendrites were identified by morphology and/or staining with MAP-2 antibodies. Downregulation of Etv4 and Etv5 resulted in a significant decrease in hippocampal neurite complexity and total extension compared with neurons transfected with control plasmids (Fig. 2*A–C*). To exclude the possibility that the effects seen with *Etv4* and *Etv5*-shRNA were due to apoptosis, the morphology of the nuclei was assessed using the nuclear staining DAPI. Survival was similar in control-shRNA and in *Etvs*-shRNA-transfected neurons (Data not shown). These findings indicate that reducing the levels of Etv4 or Etv5 in cultured hippocampal neurons resulted in a simplification of the dendritic complexity.

The data indicating that downregulation of Etv4 or Etv5 restricts neurite outgrowth, prompted us to test whether overexpression of these factors was sufficient to promote dendrite growth and/or complexity of hippocampal neurons. To this end, hippocampal cells were transfected with control, HA-tagged-Etv4 (HA-Etv4), or Flag-tagged-Etv5 (Flag-Etv5) plasmids in combination with GFP-expression vector. Our data indicated that either Etv4 or Etv5 overexpression was sufficient to induce a marked increase in dendrite length and complexity compared with neurons transfected with control plasmids (Fig. 2*D–F*), similar to the increase in these parameters induced by the neurotrophin BDNF (Fig. 2*D–F*).

Knockout of Etv4 or Etv5 Impairs Dendrite Development In Vitro and In Vivo

To evaluate the physiological relevance of Etv4 and Etv5 in dendrite development, we analyzed *Etv4*-deficient mice ($Etv4^{-/-}$) (Livet et al. 2002) and mice in which the function of Etv5 can be conditionally inactivated using Cre-lox-P system ($Etv5^{flox/flox}$) (Zhang et al. 2009). We generated conditional *Etv5*-mutant mice by breeding *Nestin-Cre* (Tronche et al. 1999) with mice carrying loxP-flanked *Etv5* alleles. Because nestin is expressed in neuronal progenitors, *Nestin-Cre*:$Etv5^{flox/flox}$ mice (conditional *Etv5* mutants, $Etv5^{cKO}$ for brevity) represent a model in which *Etv5* is deleted from neurons. We confirmed that Etv4 and Etv5 were

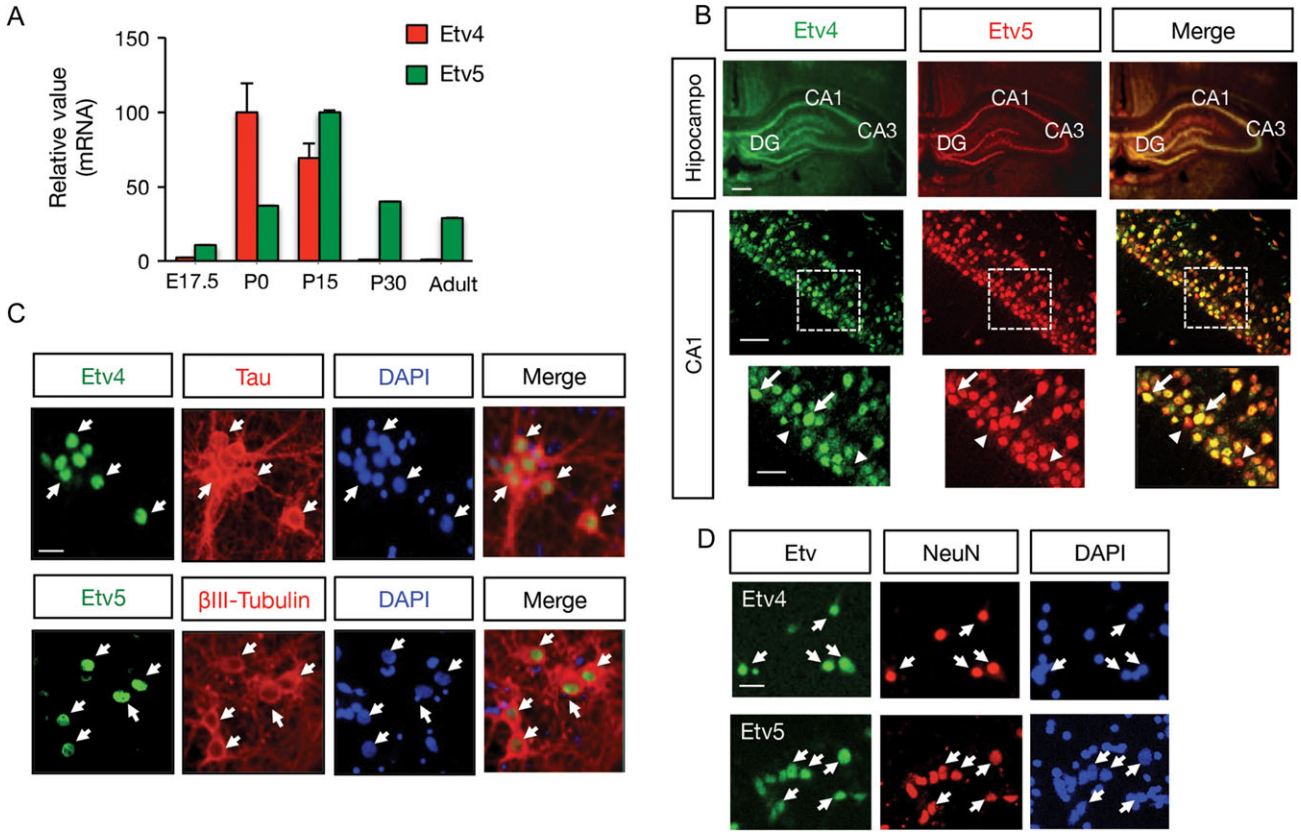

Figure 1. Developmental expression and localization of Etv4 and Etv5 in hippocampus. (*A*) Quantitative analysis of *Etv4* and *Etv5* mRNA developmental expression by real-time PCR in hippocampus at different developmental stages. Embryonic (E) day 17.5; postnatal (P) days 0, 15, 30, and adult are indicated. Expression at each age was normalized to that of the housekeeping gene *Tbp* and is stated relative to the highest levels reached by each of them. Shown are averages ± SD of triplicate determinations. (*B*) Localization of Etv4 (green) and Etv5 (red) in coronal sections of P15 rat hippocampus. In the last panel, the overlapping of Etv4 and Etv5 staining is shown. Images of CA1 area stained with Etv4 and Etv5 are shown in lower panels. High magnification images of boxed areas are also shown. Arrows indicate individual cells showing Etv4 and Etv5 coexpression. Arrowheads indicate cells expressing Etv5 in the absence of Etv4. Upper panel: Scale bar, 500 μm. Middle panel: Scale bar, 100 μm. Bottom panel: Scale bar, 50 μm. (*C*) Expression of Etv4 (top) or Etv5 (bottom) (green) with neuronal markers, total Tau or βIII-tubulin (red), respectively, by immunofluorescence in dissociated rat hippocampal neurons after 12 DIV. Nuclear staining with DAPI is also shown. Last panel showed merged images. Cells expressing Etv4 and Tau or Etv5 and βIII-tubulin are indicated. Scale bar, 15 μm. (*D*) Expression of Etv4 or Etv5 (green) with the nuclear post-mitotic neuronal marker NeuN (red). Cells expressing Etvs and NeuN are indicated. Scale bar, 20 μm. (See also Figures S1 and S2).

absent in the neurons of these mice by immunostaining using specific antibodies (Supplementary Fig. 2).

We analyzed hippocampal neurons from control, *Etv4*, or *Etv5*-deficient mice grown for different days in vitro and stained with the dendritic marker MAP-2. We found that cultured hippocampal neurons from either *Etv4*- or *Etv5*-deficient mice showed reduced dendritic arbor length and complexity compared with neurons obtained from control mice at different developmental stages (Fig. 2*G–I*, and 2*K–M*). This dendritic complexity resulted from a significant reduction in the number of primary dendrites and dendritic length, confirming our results from knockdown experiments (Fig. 2*A–C*). Interestingly, this effect could be reverted by overexpression of HA-Etv4 in cultured neurons derived from *Etv4*-mutant mice or by transfecting Flag-Etv5 in cultured neurons derived from *Etv5*-deficient mice (Fig. 2*J*,*N*).

To analyze the contribution of these transcription factors to dendrite development in vivo, we performed Golgi staining to visualize the dendritic arbors of pyramidal cells in the CA1 area of the hippocampus of $Etv4^{-/-}$ and $Etv5^{cKO}$ mice at P15, the developmental stage at which these transcription factors are highly expressed in hippocampus (Fig. 1). This analysis revealed that pyramidal dendrite arbors of $Etv4^{-/-}$ mice were dramatically reduced in size and complexity compared with those of control animals (Fig. 3*A–D*). A similar reduction in total dendrite size and complexity was observed in *Etv5*-conditional mice ($Etv5^{cKO}$) compared with control mice (Fig. 3*F–I*). The same analysis performed on hippocampal neurons from control and *Etvs*-deficient adult mice showed similar results (Supplementary Fig. 3 and data not shown). There were no evident differences in gross hippocampal histological organization between control animals and *Etv4*- or *Etv5*-deficient mice. No substantial change in neuronal density measured by NeuN staining was observed, indicating that none of these two transcription factors are required for hippocampal neuron survival (Supplementary Fig. 4).

The similar abnormalities in dendrite growth observed on pyramidal neurons of *Etv4*- and *Etv5*-deficient mice indicate that these factors are not functionally redundant because they cannot compensate each other. In agreement with these findings, we detected by immunoblotting a reduction of Etv5 expression levels in hippocampal nuclear extracts obtained from P15 $Etv4^{-/-}$ mice and a downregulation of Etv4 protein in extracts prepared from $Etv5^{cKO}$ mice (Fig. 3*E*,*J*).

In order to analyze whether combined loss of Etv4 and Etv5 results in greater impairment in neuronal differentiation than does loss of any single gene alone, we expressed *Etv5*-shRNA or control vector in hippocampal neurons from $Etv4^{+/+}$ and

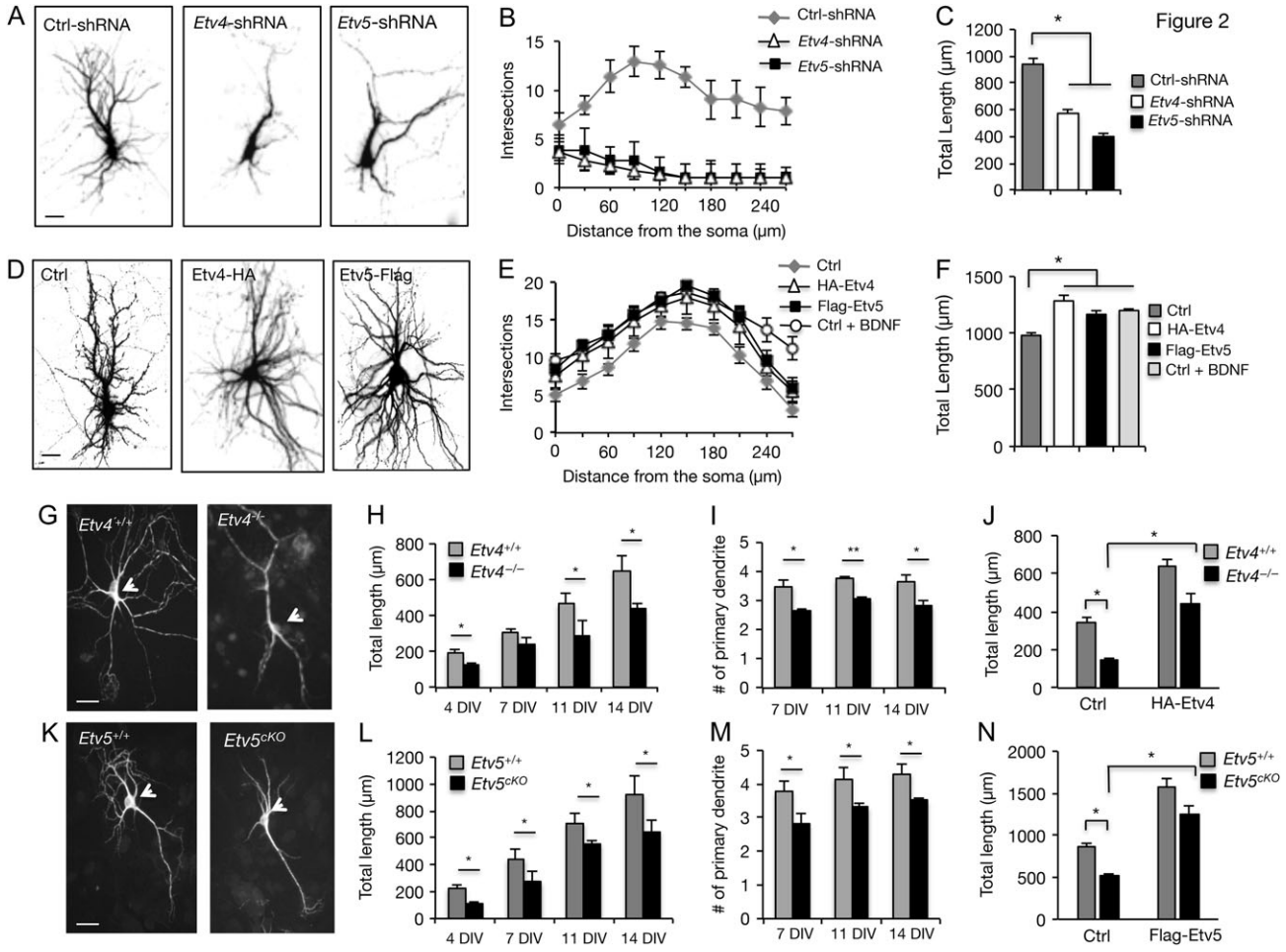

Figure 2. Etv4 and Etv5 mediate dendritic growth and branching of hippocampal neurons. (*A*) Representative inverted images of rat hippocampal neurons transfected with either GFP-expressing control-shRNA (Ctrl-shRNA), *Etv4*-shRNA, or *Etv5*-shRNA at 15 DIV, maintained for 3 additional days in vitro, fixed, stained with anti-GFP, and analyzed for dendrite development. Scale bar, 20 μm. (*B*) Quantification of dendritic complexity by Sholl analysis of hippocampal neurons expressing control (Ctrl-shRNA) or specific *Etvs*-shRNA. Data represent mean ± SD. (*C*) Bar graph shows the quantification of total dendritic length from neurons transfected with the indicated plasmids. Data (mean ± SEM) are representative of $n = 3$ independent experiments. *$P < 0.001$ (two-way ANOVA followed by Bonferroni's test). (*D*) Representative inverted images of P0 mouse hippocampal neurons cotransfected with either GFP-expression vector and HA-Etv4 or Flag-Etv5 or control (Ctrl) vector, at 10 DIV and maintained for 3 additional days in vitro, fixed, and stained with anti-GFP. Scale bar: 20 μm. (*E*) Sholl analysis of hippocampal dendritic arbor on GFP-positive neurons expressing the indicated plasmids. Neurons transfected with control plasmid and treated with BDNF (25 ng/ml) were included in the analysis. Data represent mean ± SD. (*F*) Bar graph shows the quantification of total dendritic length from neurons transfected with the indicated plasmids or cultured in the presence of BDNF. Data (mean ± SEM) are representative of $n = 3$ independent experiments. *$P < 0.001$ (ANOVA followed by Bonferroni's test). (*G* and *K*) Representative images of MAP-2 immunostained hippocampal neurons obtained from $Etv4^{+/+}$ and $Etv4^{-/-}$ (*G*) or $Etv5^{+/+}$ and $Etv5^{cKO}$ (*K*) cultured for 11 DIVs. Arrowheads indicate neuron cell body. Scale bar, 20 μm. (*H* and *L*) Bar graphs showing the quantification of total MAP-2-positive neurite length of neurons coming from $Etv4^{+/+}$ and $Etv4^{-/-}$ (*H*) or $Etv5^{+/+}$ and $Etv5^{cKO}$ (*L*) cultured for 4, 7, 11, and 14 DIVs. (*I* and *M*) Bar graphs showing the quantification of the number (#) of primary dendrites of neurons isolated from control and $Etv4^{-/-}$ (*I*) or $Etv5^{cKO}$ (*M*) mice and cultured for the indicated days in vitro. Data (mean ± SD) are representative of $n = 3$ independent experiments. *$P < 0.05$, **$P < 0.001$ by Student's *t* test. (*J* and *N*) Bar graphs showing the dendritic length of neurons coming from $Etv4^{+/+}$ and $Etv4^{-/-}$ (*J*) or $Etv5^{+/+}$ and $Etv5^{cKO}$ (*N*) mice transfected at 4 DIV with GFP and empty vector (Ctrl) or HA-Etv4 (*J*) containing plasmid or Flag-Etv5 (*N*) containing plasmid as indicated. The cells were fixed and analyzed at DIV7. *$P < 0.05$ (ANOVA, followed by Newman–Keuls test). The results are shown as mean ± SEM of a representative experiment performed in triplicate. Two experiments with similar results were done.

$Etv4^{-/-}$ mice. We observed that *Etv5*-shRNA delivery in neurons coming from Etv4-deficient mice resulted in a similar reduction in dendrite growth and number of primary dendrites compared with $Etv4^{-/-}$-deficient neurons transfected with control vector, indicating that downregulation of Etv4 was enough to impair the correct dendritic development. A similar experiment was performed in neurons derived from $Etv5^{cKO}$ mice transfected with *Etv4*-shRNA or control-shRNA and similar results were obtained (Fig. 4*A*). Together, these results are in agreement with a cross-regulation between these 2 transcription factors. Based on this, we asked whether cross-overexpression of these factors could recover the morphological dendritic defects detected in neurons coming from the 2 deficient mice. Cultured hippocampal neurons derived from either Etv4- or Etv5-deficient mice were transfected with Etv4-HA and Etv5-Flag constructs separately, and the effect on dendrite development was analyzed. When Etv4-HA was transfected in neurons isolated from $Etv4^{-/-}$ mice, the normal dendrite phenotype was recovered. However overexpression of Etv5-Flag was not able to recover the normal neuronal morphology. Similarly, overexpression of Etv5-Flag, but not Etv4-HA, could recover dendritic defects observed in neurons derived from Etv5-deficient mice (Fig. 4*B*,*C*). Altogether, these results are in agreement with a cross-regulation and functional interaction between these 2

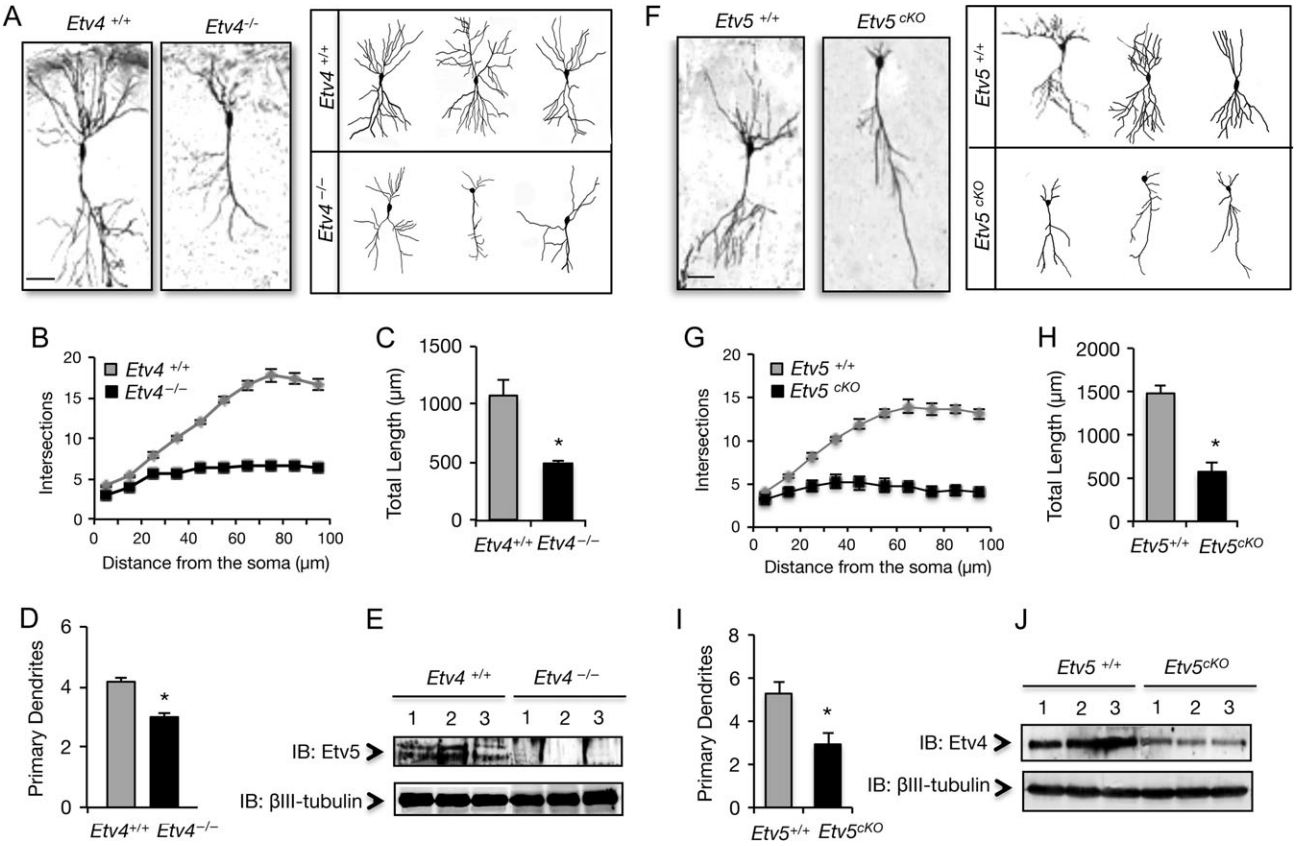

Figure 3. Etv4 and Etv5 deletion results in a reduction of hippocampal dendrite complexity in vivo (*A* and *F*) Representative images (left) and representative tracing (right) of Golgi silver impregnated hippocampal CA1 pyramidal neurons from 2 weeks-old $Etv4^{+/+}$ and $Etv4^{-/-}$ (*A*) or $Etv5^{+/+}$ and $Etv5^{cKO}$ (*F*) littermate mice. Scale bar, 50 μm. (*B* and *G*) Proximal Sholl analysis of total dendritic arbor of CA1 pyramidal hippocampal neurons from 2-week-old $Etv4^{+/+}$ versus $Etv4^{-/-}$ mice (*B*) or $Etv5^{+/+}$ versus $Etv5^{cKO}$ mice (*G*). (*C* and *H*) Quantification of total dendritic length of hippocampal CA1 pyramidal neurons from $Etv4^{+/+}$ versus $Etv4^{-/-}$ mice (*C*) and $Etv5^{+/+}$ versus $Etv5^{cKO}$ (*H*). (*D* and *I*) Quantification of the number of primary dendrites of hippocampal CA1 pyramidal neurons from $Etv4^{+/+}$ versus $Etv4^{-/-}$ (*D*) and $Etv5^{+/+}$ versus $Etv5^{cKO}$ mice (*I*). The results shown are mean ± SEM, *$P < 0.05$ (Student's *t* test). Quantifications showed were performed in ~40 neurons from $n = 4$ $Etv4^{+/+}$ and $n = 4$ $Etv4^{-/-}$ littermate mice or $n = 4$ $Etv5^{+/+}$ and $n = 4$ $Etv5^{cKO}$ mice. (*E* and *J*) Analysis by immunoblotting of Etv5 and Etv4 expression in nuclear extracts of hippocampal tissue obtained from P15 $Etv4^{-/-}$ (*E*) and $Etv5^{cKO}$ mice (*J*), respectively. Immunoblot of the cytosolic fractions was done with βIII-tubulin. Three animals from different genotypes were analyzed ($n = 3$) (See also Figures S2, S3 and S4).

transcription factors necessary for the correct development of hippocampal dendrites.

Behavioral Deficits in Etv4 and Etv5-Mutant Mice

The size and complexity of the hippocampal dendritic arbors determine the properties of neurons, and many neurodevelopmental and neuropsychiatric diseases are mainly due to structural abnormalities of the dendrites and their connections (Kaufmann and Moser 2000). To evaluate the functional consequences of the reported defects, we carried out behavioral analyses. First, we performed an open field test and found that *Etv4*- and *Etv5*-deficient mutant mice display similar locomotor activity and similar anxiety levels than control littermates (Fig. 5A–B). As it has been reported that lesions of the hippocampus results in impaired nesting behavior (Antonawich et al. 1997; Deacon et al. 2001), we examined this ability in *Etv4*- and *Etv5*-mutant mice. Compared with controls, neither *Etv4*- nor *Etv5*-deficient mice were able to form a nest properly within 16 h and had a tendency to scatter nesting material, a feature associated with poor capacity to have an organized behavior and social withdrawal (Del Pino et al. 2013) (Fig. 5C). Finally, to determine whether Etv4 and Etv5 deficits leads to cognitive dysfunction, we used the novel object location memory task, which is heavily dependent on hippocampal function. This test is based on the spontaneous tendency of rodents to spend more time exploring an object that has been relocated, than a similar object that remains in a familiar position. During the training session, control, and both *Etv*-deficient mice were exposed to 2 equal objects (A_1 and A_2, Fig. 5D), and they did not show a preference for any of 2 identical objects. Twenty-four hours later, mice were presented with the same objects, but during this phase (testing phase), object A_1 was placed in a familiar location (the same position as in the training phase), object A_2 was placed in a novel location, and exploration time was measured. Although control animals display the expected novel position object preference, *Etv4*- and *Etv5*-deficient mice showed no preference at all, demonstrating impaired recognition memory.

Altogether, these results indicate that *Etv4*- and *Etv5*-mutant mice behaviorally display social and cognitive deficits, which are consistent with the concept that Etv4 and Etv5 regulate hippocampal dendrite morphology

Etv4 and Etv5 Are Induced by BDNF and Mediate BDNF/TrkB-induced Neurite Outgrowth in Neuronal Cells

Previous work indicates that Pea3 transcription factors mediate the effect of neurotrophic factors on different neuronal

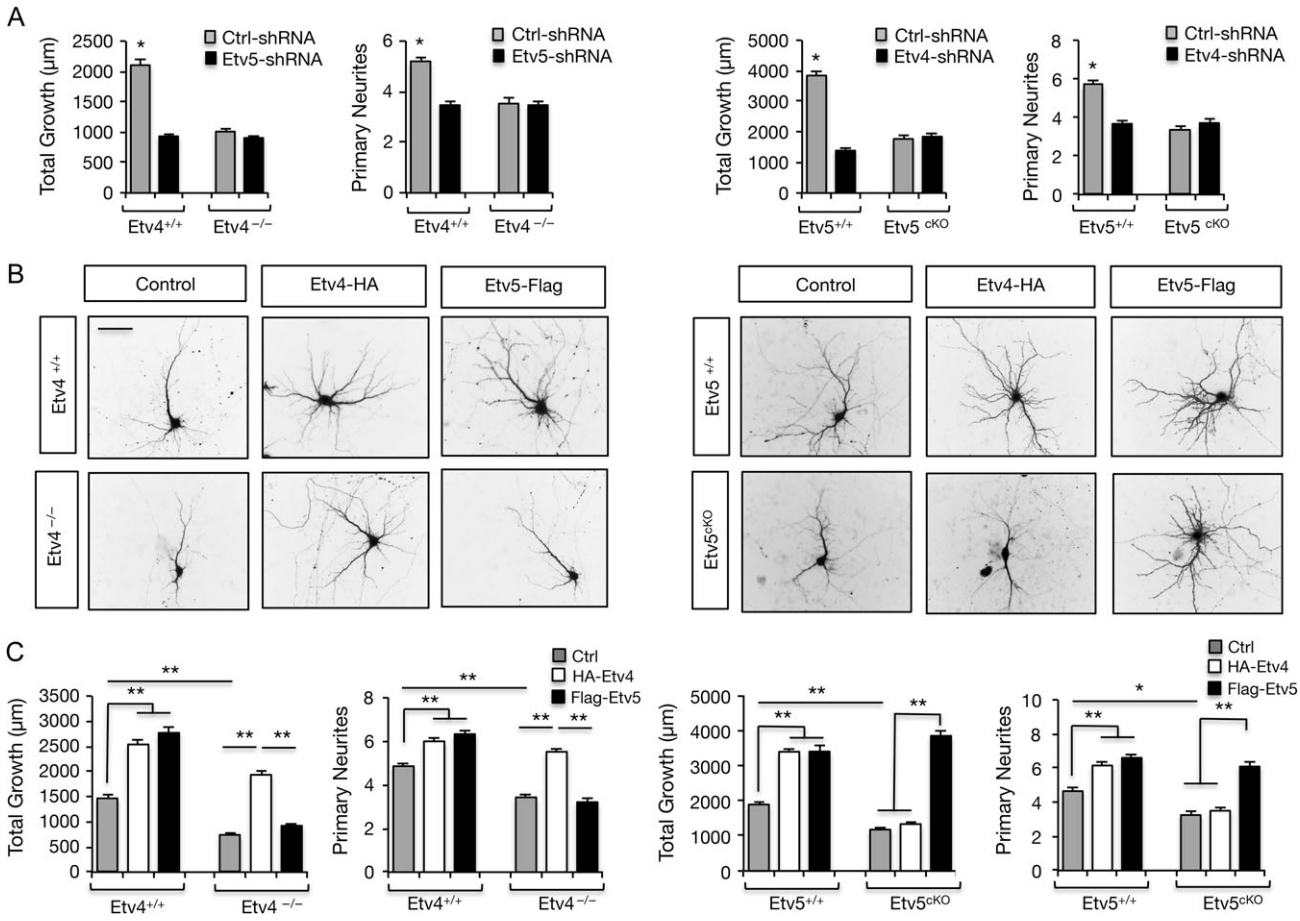

Figure 4. Etv4 and Etv5 are required simultaneously for the correct development of hippocampal pyramidal neurons. (*A*) Cultured hippocampal neurons isolated from P0 control (*Etv4*$^{+/+}$, *Etv5*$^{+/+}$) or Etv-deficient mice (*Etv4*$^{-/-}$, *Etv5*^{cKO}) were cotransfected with Control-shRNA (Ctrl-shRNA, gray bars) or specific *Etv*-shRNA (black bars) expressing GFP, as indicated in the figure. After 3 days, the neurons were and analyzed for neurite outgrowth. The bar graphs show the quantification of total dendritic length (μm) and number of primary dendrites. Data (mean ± SEM) are representative of $n = 3$ independent experiments. *$P < 0.0001$ (ANOVA followed by Tukey's test). (*B*) Representative inverted images of P0 mouse hippocampal neurons from littermates of the indicated genotypes cotransfected with pCDNA3 (Control, Ctrl) Etv4-HA or Etv5-Flag and GFP-expression vector at 10 DIV and maintained 3 additional DIV. Scale bar: 30 μm. (*C*) Bar graphs show the quantification of total dendritic length (μm) and number of primary dendrites from neurons of the different genotypes transfected with the indicated plasmids. Data (mean ± SEM) are representative of $n = 3$ independent experiments. *$P < 0.001$; **$P < 0.0001$ (ANOVA followed by Tukey's test).

populations. Since BDNF is highly expressed in hippocampus and has been observed to stimulate dendrite growth and development of hippocampal neurons (Cheung et al. 2007; Kwon et al. 2011; Lazo et al. 2013), we decided to evaluate whether Etv4 and Etv5 could be involved in hippocampal BDNF signaling. By immunostaining, we observed a clear coexpression of Etv4 and Etv5 with the BDNF receptor, TrkB in hippocampal neurons (Fig. 6A). To investigate whether Etv4 and/or Etv5 have a role in BDNF signaling, we performed a BDNF-dependent neuronal differentiation assay in PC12 cell line. The neuronal cell line was cotransfected with control-shRNA, *Etv4*-shRNA, or *Etv5*-shRNA, and a plasmid expressing the BDNF receptor TrkB. The cells were maintained in the presence of BDNF and then analyzed for neuronal differentiation. In cells transfected with control plasmid, BDNF treatment induced neurite outgrowth, while downregulation of either *Etv4* or *Etv5* by shRNA abrogated BDNF-induced neurite outgrowth in this cell line (Fig. 6B,C). These results indicate that Etv4 and Etv5 independently mediate the BDNF-induced differentiation of PC12 cells.

As both Etv4 and Etv5 expression is regulated by NGF in sensory neurons (Fontanet et al. 2013), we decided to examine whether these genes were regulated by BDNF in hippocampal neurons. Analysis by real-time PCR revealed a significant induction of *Etv4* and *Etv5* mRNA in hippocampal cultures exposed to BDNF compared with untreated controls (Fig. 6D). As we have previously reported that Etv proteins act as effectors of the MEK/ERK (MAPK) pathway in NGF-induced sensory neuronal differentiation, we used a pharmacological approach to investigate whether BDNF-induced Etv4 and Etv5 gene expression was mediated by this signaling. As shown in Figure 6E, the MEK inhibitor PD98059 blocked BDNF-induced gene expression of the *Etv4* and *Etv5* mRNA in hippocampal cultures. The inhibitory activity of PD98059 was confirmed by measuring MAPK activation by immunoblotting of hippocampal total lysates obtained from cells maintained in the presence or absence of PD98059 and stimulated with BDNF (Fig. 6F). Our results indicate that Etv4 and Etv5 are required for BDNF signaling in hippocampal neurons but not exclude the possibility that these transcription factors could also be induced by neural activity. As BDNF is an activity-dependent signal, we analyzed whether induction of Etv4 and Etv5 could be observed in hippocampal cultures depolarized with different concentrations of potassium chloride (KCl) (Fig. 6G). Our results indicated that exposure of hippocampal neurons to elevated extracellular levels of KCl resulted in the

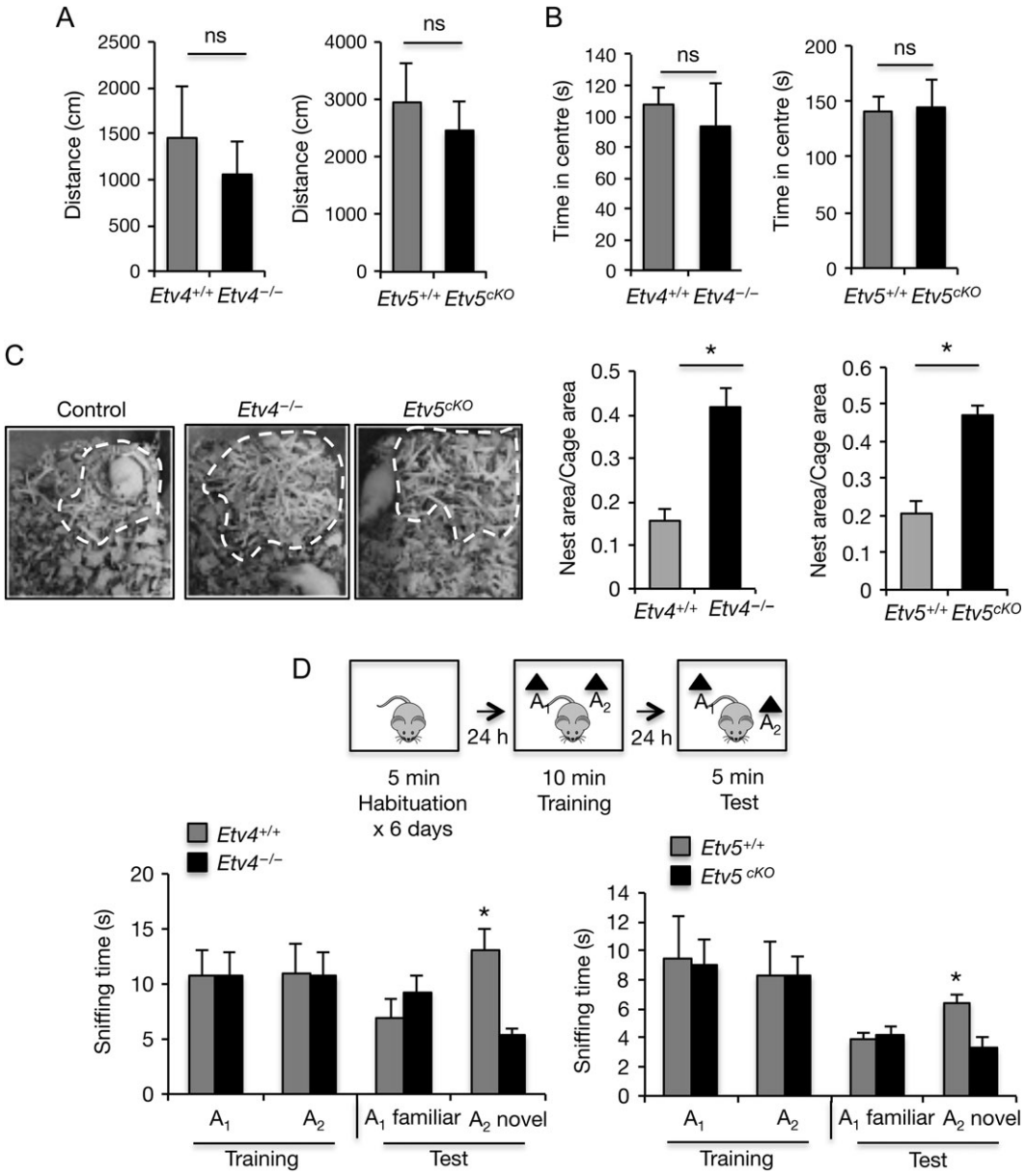

Figure 5. Etv4- and Etv5-deficient mice exhibit behavioral impairments. (*A* and *B*) Locomotor activity of control and mutant mice in a novel environment was assessed by a 15-min session in an open field. (*A*) Bar graphs show the total distance traveled in 15 min. (*B*) Bar graphs show the total time (s) spent in the center area of the open field chamber. ns, not significant (Student's *t* test). Number of animals analyzed of each genotype, $n = 5–7$. Data represent mean ± SEM. (*C*) Nest building test in control and Etv4- and Etv5-mutant mice. Representative images of nests built by control, $Etv4^{-/-}$ and $Etv5^{cKO}$ mice after 16 h are shown. Bar graphs represent the nest area relative to the total cage area. Data represent mean ± SEM. *$P < 0.05$ (Student's *t* test). Number of animals of each genotype, $n = 6–13$. (*D*) Cognitive function in control and mutant Etv mice. Schematic illustration of the novel object location task is shown. Bar graphs show the absolute time (s) spent sniffing the objects in the familiar or novel location. *$P < 0.05$ (ANOVA followed by Student–Newman–Keuls multiple comparison test). Number of animals of each genotype, $n = 7–8$. Data represent mean ± SEM.

induction of Etv4 and Etv5 expression showing that membrane depolarization as well as BDNF treatment modulate the expression of the 2 transcription factors.

Etv4 and Etv5 Mediate BDNF-induced Dendrite Growth in Hippocampal Cells

Our findings in PC12 cells prompted us to examine the physiological significance of Etv4 and Etv5 as mediators of BDNF signaling in hippocampal primary neurons. As it is known that hippocampal cells respond to BDNF inducing dendritic growth and spine formation, we examined the effect of decreasing the expression of these transcription factors in hippocampal neurons transfected with *Etv4*-shRNA, *Etv5*-shRNA, or control-shRNA GFP-expressing vectors. The cells were maintained in the presence of BDNF and analyzed for dendritic complexity. As it has been previously described, we observed an increase in the number of primary dendrites in neurons treated with BDNF (Segal et al. 1995; Horch et al. 1999), while hippocampal cells transfected with *Etv4*- or *Etv5*-shRNA showed a significant decrease in dendritic complexity (Fig. 7*A*-*C*). These results indicated that these transcription factors mediate BDNF signaling

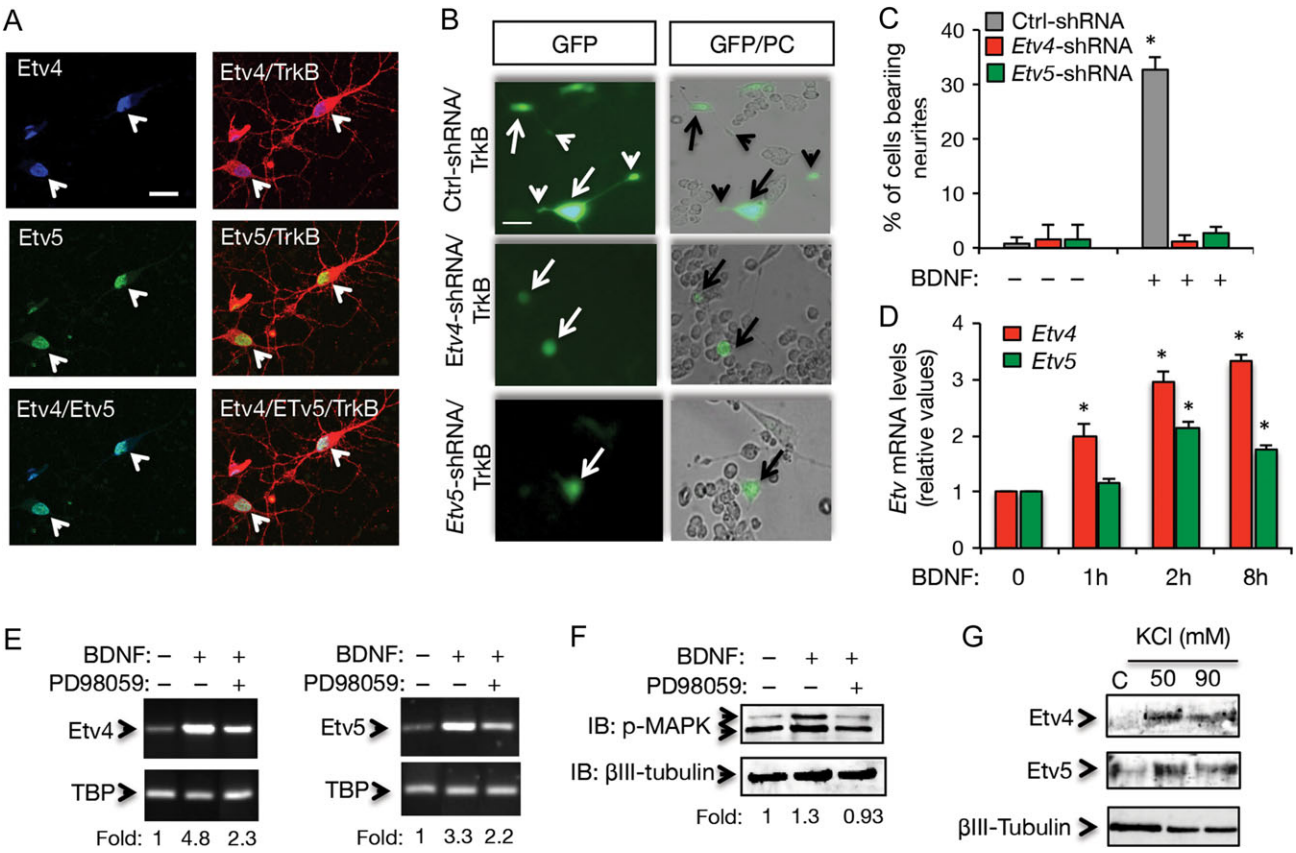

Figure 6. Etv4 and Etv5 are independently required in BDNF-induced neuronal differentiation. (*A*) Localization by immunofluorescence of Etv4 (blue), Etv5 (green) and TrkB (red) in rat hippocampal-dissociated neurons. Arrowheads indicate cells positive for TrkB and the different Etvs transcription factors. Scale bar, 20 μm. (*B*) Photomicrographs show PC12 cells cotransfected with control-shRNA (Ctrl-shRNA), *Etv4*-shRNA, or *Etv5*-shRNA-GFP expression vectors, and the expression plasmid for the BDNF receptor, TrkB. After 72 h of treatment with or without BDNF (25 ng/ml), cells were fixed. Arrows indicate the cell bodies and arrowheads neurite tips. PC, Phase Contrast. Scale bar, 20 μm. (*C*) Bar graph shows the quantification of neurite outgrowth as the number of cells with neurite processes longer than a cell body diameter after 72 h of treatment with BDNF or not. The results are representative of $n = 2$ independent experiments performed in triplicate. The results are shown as mean ± SEM. In each assay, a total number of 10 fields per well were analyzed for the different experimental groups. *$P < 0.001$ (ANOVA followed by a Student–Newman–Keuls multiple comparison test). (*D*) Analysis of *Etv4* and *Etv5* mRNA expression by real-time PCR in hippocampal-derived neuronal cultures treated with BDNF (25 ng/ml) for the indicated times. The levels of *Etv4* and *Etv5* mRNA were normalized using the expression of the housekeeping gene *Tbp*. Shown are averages ± SD of triplicate determinations. *$P < 0.01$. BDNF-treated versus control (non-stimulated) group (ANOVA followed by Dunnetts' test). (*E*) Analysis of *Etvs* mRNA expression examined by semiquantitative RT-PCR (27 cycles) in hippocampal neurons treated or not with the specific MEK inhibitor PD98059 (50 μM) for 30 min, and then stimulated with BDNF (25 ng/ml) for 4 h. (*F*) The inhibition of PD98059 was tested measuring MAPK activation (p-MAPK) after 20 min of BDNF stimulation by immunoblotting (IB). (*G*) Activity-dependent induction of Etv4 and Etv5. Hippocampal cultures were depolarized with two different concentration of KCl and the levels of Etv4 and Etv5 expression in nuclear extracts was analyzed by western blot. Immunoblot of the cytosolic fractions was done with βIII-tubulin.

in hippocampal cells. In agreement with this, overexpression of Etv4 and Etv5 in primary hippocampal neurons was enough to reach levels of dendrite complexity similar to the arborization observed in control neurons cultured in the presence of BDNF (Fig. 7*D–F*, and Fig. 2*E*,*F*). Altogether, these results indicate that Etv4 and Etv5 mediate the effect of BDNF on dendritic growth.

Etv4 and Etv5 Mediate BDNF-induced Dendrite Spine Formation in Hippocampal Neurons

Another physiological effect of BDNF on hippocampal neurons is the induction of dendritic spines, which are the postsynaptic sites of most excitatory synapses in the CNS (Harris and Kater 1994; Hiester et al. 2013). Although many different molecular signals have been proposed to underlie the formation of dendritic spines (Lin and Koleske 2010), our knowledge about the mechanisms involved in this process is incomplete. To investigate whether Etv4 and Etv5 mediated spine formation induced by BDNF in hippocampal neurons, we performed loss of function and gain of function assays. Cultured hippocampal neurons were transfected with control-shRNA, *Etv4*-shRNA, or *Etv5*-shRNA expressing GFP vectors and maintained in the presence or absence of BDNF. Neurons transfected with control plasmids and cultured in the presence of BDNF showed a significant increase in the density of dendritic spines (Fig. 8). Conversely, knockdown of *Etv4* or *Etv5* independently caused a significant decrease of total dendritic protrusion density in control conditions and blocked the increase in spine density caused by BDNF (Fig. 8*A*,*B*). Morphological classification of dendritic protrusions revealed that the different types of dendritic spines were affected (Data not shown). These data suggest that Etv4 and Etv5 are necessary for BDNF-induced dendritic spine formation. We next analyzed spine density in hippocampal neurons overexpressing Etv4 or Etv5 to assess if their expression was sufficient to increase dendritic spine density. Our results show that in the absence, but not in the presence, of BDNF, the overexpression of each transcription factor resulted in an increase in dendritic spine density (Fig. 8*C*,*D*). These

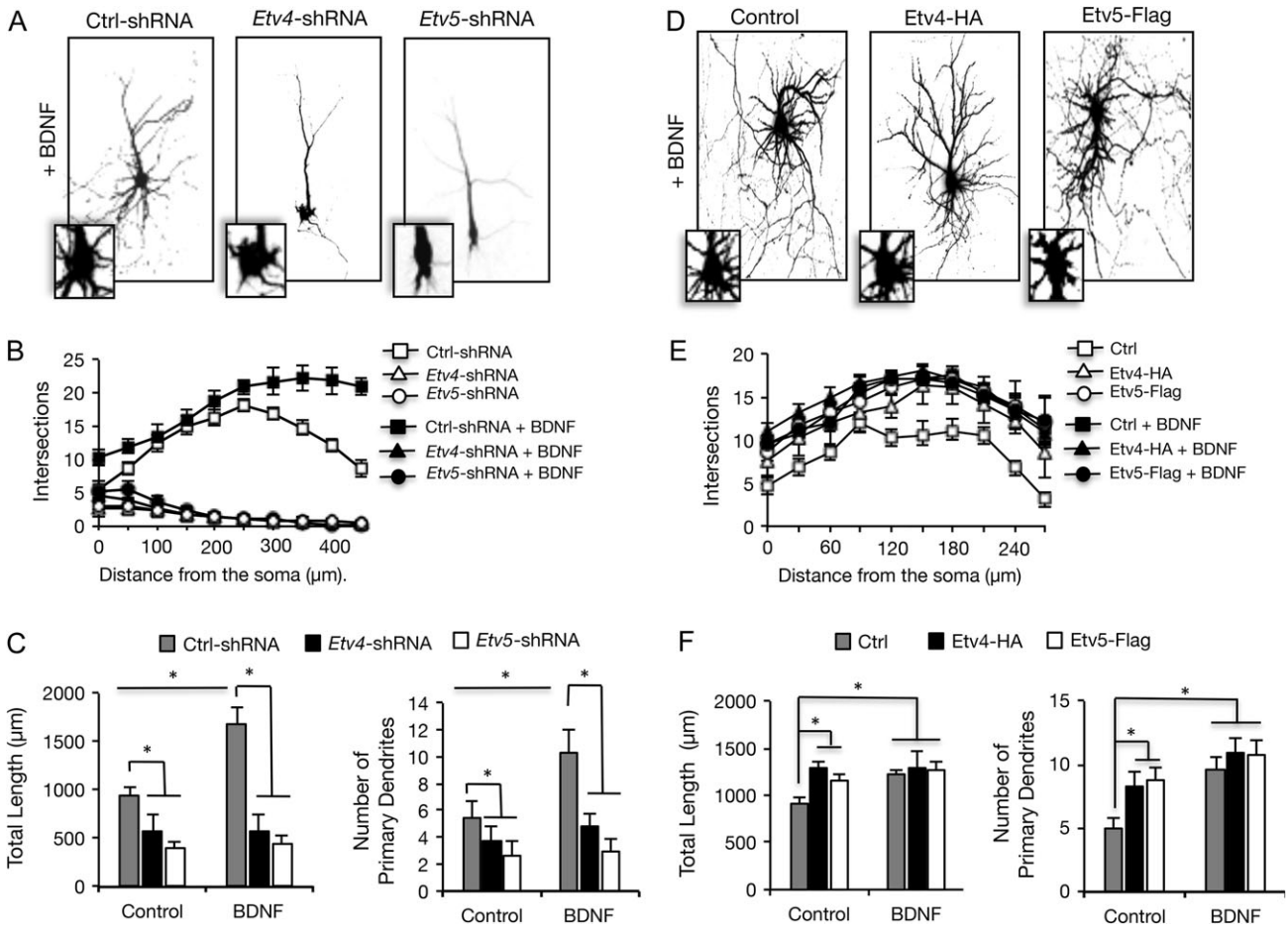

Figure 7. Etv4 and Etv5 mediate BDNF-induced hippocampal dendrite growth. (*A–C*) *Etv4* or *Etv5* downregulation reduces BDNF-induced dendritic growth in hippocampal neurons. (*A*) Representative inverted images of E17.5 rat hippocampal neurons transfected with either GFP-expressing control-shRNA (Ctrl-shRNA), *Etv4*-shRNA, or *Etv5*-shRNA at 15 DIV, maintained for 3 additional days in vitro treated or not with BDNF (25 ng/ml). Boxed area represents a higher-magnification image showing the primary dendrites of the transfected neurons. (*B*) Sholl analysis of hippocampal dendritic arbor of GFP-positive neurons described in (*A*). (*C*) Left panel: Bar graph showing the quantification of total dendritic length; Right panel: Bar graph showing the number of primary dendrites of transfected hippocampal neurons described in (*A*). Data (mean ± SD) are representative of $n = 3$ independent experiments. *$P < 0.001$ (ANOVA followed by Bonferroni's test). (*D–F*) Etv4 and Etv5 overexpression independently stimulates dendritic growth of P0 mouse hippocampal neurons to similar levels reached by BDNF (25 ng/ml) treated neurons. (*D*) Representative inverted images of P0 mouse hippocampal neurons cotransfected with either GFP-expression vector and control (Ctrl), HA-Etv4 or Flag-Etv5 vector at 10 DIV and maintained for 3 additional days in vitro treated or not with BDNF (25 ng/ml). Boxed area represents a higher-magnification image showing the primary dendrites of the transfected neurons. (*E*) Sholl analysis of hippocampal dendritic arbors on GFP-positive neurons of the experiment described in (*D*). (*F*) Left panel: Bar graph showing the quantification of total dendritic length; Right panel: Bar graph showing the number of primary dendrites of transfected hippocampal neurons described in (*D*). *$P < 0.001$ (ANOVA followed by Bonferroni's test). Data (mean ± SD) are representative of $n = 3$ independent experiments. *$P < 0.001$ (ANOVA followed by Bonferroni's test).

results indicate that Etv4 and Etv5 are necessary and sufficient to mediate BDNF-induced dendritic spine formation in hippocampal neurons.

Discussion

In this study, we characterized Etv4 and Etv5 transcription factors, as new molecular determinants underlying hippocampal connectivity. Our results indicate that these 2 molecules are jointly expressed in hippocampal neurons, reaching their highest levels during early postnatal weeks, when dendritogenesis and synaptogenesis occur (Ben-Ari et al. 2007). In this work, we demonstrated by gain and loss of function assays a crucial role of Etv4 and Etv5 in the control of dendrite growth and complexity as well as in spine dendrite formation of hippocampal neurons. Developmental loss of *Etv4* and *Etv5* in hippocampal neurons resulted in significant reduction in the size and complexity of hippocampal pyramidal cell dendrites, as well as in deficits in nesting behavior and cognitive functions. In this study, we also report that Etv4 and Etv5 are transcriptionally regulated by BDNF and their expression requires activation of MEK/ERK (MAPK) signaling pathway. Finally, we provide evidence that Etv4 and Etv5 are critical mediators of BDNF signaling in hippocampal neurons and required for BDNF-induced dendrite development and spine formation. Our findings lead us to propose a model in which neurotrophic factors, such as BDNF, acting through their receptors and MEK/ERK (MAPK) signaling pathway induces the expression of Etv4 and Etv5, which are simultaneously required to promote the expression of a non-yet identified transcriptional program that is involved in dendrite growth and maturation of hippocampal pyramidal neurons (Fig. 8*E*).

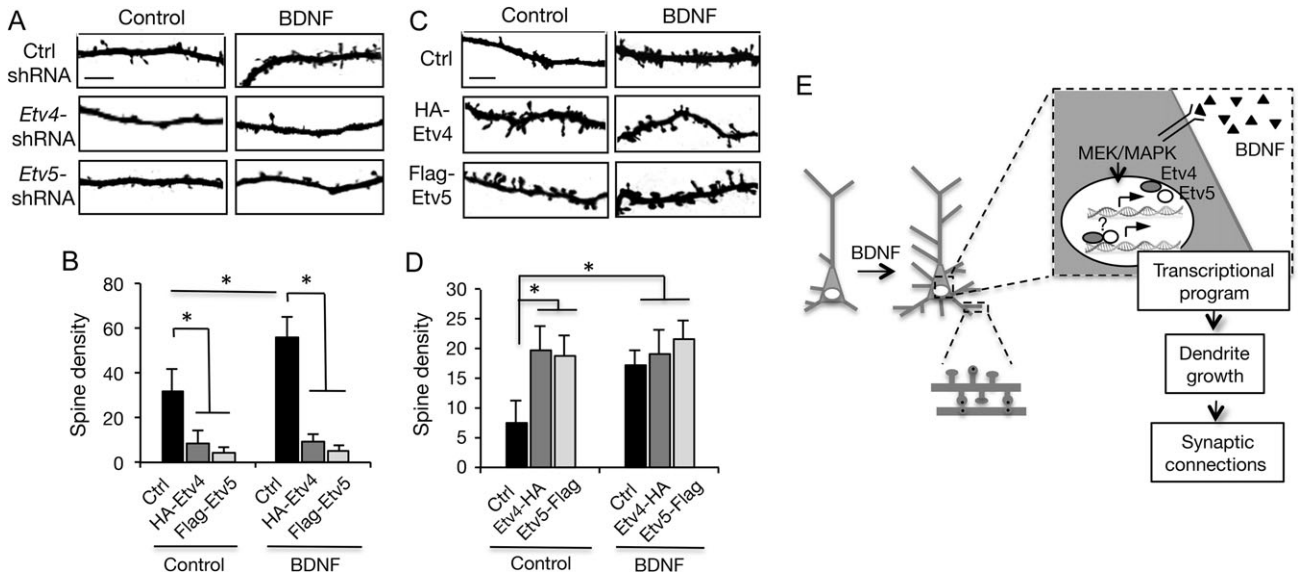

Figure 8. Etv4 and Etv5 mediate BDNF-induced hippocampal spine development. (*A*) Representative inverted confocal images showing dendritic spines from rat hippocampal neurons transfected with GFP-expressing control-shRNA (Ctrl-shRNA), *Etv4*-shRNA, or *Etv5*-shRNA, treated or not with BDNF (25 ng /ml). Scale bar, 10 μm. (*B*) Quantification of the effect of decreased Etv4 or Etv5 expression on dendritic spine density. Bar graph shows the number of total dendritic spines along 100 μm of dendritic length of neurons treated as indicated. (*C*) Representative inverted confocal images showing dendritic spines from mouse hippocampal neurons cotransfected with GFP-expressing vector and empty vector (Ctrl), HA-Etv4, or Flag-Etv5, treated or not with BDNF (25 ng /ml). Scale bar, 10 μm. (*D*) Quantification of the effect of Etv4 or Etv5 overexpression on dendritic spine density. Bar graph shows the number of total dendritic spines along 100 μm of dendritic length of neurons treated as indicated. Data (mean ± SD) are representative of $n = 3$ independent experiments. *$P < 0.001$ (ANOVA followed by Bonferroni's test). (*E*) Model describing Etv4 and Etv5 as mediators of BDNF-dependent signaling in hippocampal neurons. BDNF binds TrkB on dendrites, activates MEK/MAPK signaling pathway, and induces the expression of Etv4 and Etv5 transcription factors. These factors are components of a transcriptional program involved in the control of dendrite arborization and spine formation, which are required for the proper establishment of hippocampal connections.

Although in this work we show evidences indicating that Etv4 and Etv5 have complementary roles in hippocampal pyramidal neurons, the discrepancies observed in the expression levels detected along development indicate that these factors can also have independent roles in other hippocampal cells, such as GABAergic neurons.

Etv4 and Etv5 in Nervous System Development

Analysis of *Etv1*, *Etv4* and *Etv5* mRNA distribution indicated that they are expressed in diverse organs and tissues during embryonic development as well as in adults, in processes that involve remodeling of extracellular matrix (Lu et al. 2009; Zhang et al. 2009; Oh et al. 2012). Although, Pea3 transcription factors have a similar expression pattern in many tissues, this can be drastically different in others indicating that these Pea3 members are not regulated in the same fashion and could have distinct physiological functions (Oh et al. 2012). In general, Etv4 and Etv5 perform similar roles during kidney, breast, and limb bud morphogenesis, whereas Etv1 seems to have different functions (Chotteau-Lelievre et al. 2003; Lu et al. 2009; Mao et al. 2009; Zhang et al. 2009). Moreover *Etv1*-knockout mice display a phenotype different from *Etv4*- and *Etv5*-deficient mice. While *Etv1*-knockout mice die approximately 1 month after birth (Arber et al. 2000; Kucera et al. 2002), Etv4- and Etv5-deficient ones survive and have defects in mating behavior and in reproductive organs, respectively (Laing et al. 2000; Chen et al. 2005; Eo et al. 2011).

The different members of the Pea3 transcription factor subfamily are expressed in different populations of neurons from the peripheral nervous system (PNS) and CNS. In the PNS, Etv1 has been reported in proprioceptive sensory neurons involved in the formation of proprioceptive sensory-motor connection (Arber et al. 2000; Patel et al. 2003). Our group has described an essential role of Etv4 and Etv5 as key factors required for the growth of nociceptive sensory axons in response to NGF (Fontanet et al. 2013) and a very recent work showed that Etv4 and Etv5 are also involved in axonal growth of another subpopulation of DRG neurons expressing the BDNF receptor, TrkB (Liu et al. 2016). Regarding to the CNS, previous studies showed that Etv1 and Etv4 are expressed in non-overlapping pools of spinal motor neurons (Lin et al. 1998; Arber et al. 2000) and Etv4 plays an important role in the control of the dendritic orientation and neuronal positioning of spinal cord motor neuron subpopulations (Livet et al. 2002; Vrieseling and Arber 2006). During the last years, the expression of the 3 members of the Pea3 subfamily have been described in the forebrain at early developmental stages. While Etv4 and Etv5 are expressed in the region of the rostral forebrain, Etv1 is expressed in the marginal zone of the cortex (Zimmer et al. 2010). In the neocortex of postnatal mice, expression of Etv1 has been ascribed only to layer V pyramidal cells (Hevner et al. 2003) and in scatter PV^+ interneurons (Dehorter et al. 2015). However, nothing was known about the role of Etv4 and Etv5 during late-embryonic and postnatal hippocampal development.

The ETS transcription factors of the Pea3 group shares a highly conserved DNA binding ETS domain and two conserved transactivating domains. These transcription factors regulate gene expression of different genes through protein–protein interactions and through specific posttranslational modifications such as phosphorylation, acetylation, and sumoylation (Li et al. 2000; Verger and Duterque-Coquillaud 2002; Bojovic and Hassell 2008). Our findings, indicating that downregulation of Etv4 and Etv5 independently restricts dendritic growth and

complexity of hippocampal pyramidal neurons, together with the observation that the two transcription factors cannot compensate each other, are in agreement with the requirement of a functional complex between the two molecules. Furthermore, our data showed that Etv4- and Etv5-deficient mice evidenced decrease levels of Etv5 and Etv4, respectively, suggesting that they are mutually regulated. An important question for future studies is how these transcription factors promote the differentiation and maturation of hippocampal pyramidal neurons. Thus, it will be important to identify protein partners, specific posttranslational modification of Etvs molecules in neuronal cells, as well as to identify the transcriptional downstream targets of Etv4 and Etv5 that participate in these developmental processes.

Etv4 and Etv5 as Mediators of BDNF Signaling in Hippocampal Dendrite Development

Dendrites play a critical role in the integration of the information in the nervous system. Transcription factors represent cell-intrinsic cues that do not strictly depend on extrinsic factors to operate within neurons, but can be influenced by environmental signals (de la Torre-Ubieta and Bonni 2011; Valnegri et al. 2015). Neurotrophins represent a family of secreted factors, which have essential roles in dendrite morphology in different types of neurons. In particular, BDNF through its receptor TrkB has been involved in hippocampal and cortical dendrite development, maturation, and plasticity. Interestingly, deletion of TrkB in cortical pyramidal neurons reduces dendrite complexity (Xu et al. 2000). Moreover, BDNF has emerged as a major regulator of activity-dependent plasticity at excitatory synapses in the mammalian central nervous system (Bramham and Messaoudi 2005; Minichiello 2009; Edelmann et al. 2014). Our results show that BDNF as well as neural activity induced by KCl depolarization resulted in the induction of Etv4 and Etv5 expression. These findings suggest that the induction of Etv4 and Etv5 in response to depolarization might be mediated by BDNF release.

Although BDNF has essential roles in dendrite patterning, the precise downstream mechanisms remain to be clarified. In this paper, our data showed that BDNF induces the expression of Etv4 and Etv5 in hippocampal neurons. Downregulation of Etv4 and Etv5 were able to restrict BDNF-induced dendrite growth and spine formation, indicating that these factors mediate the effects of BDNF on hippocampal neurons. We did not observe differences between neurons overexpressing Etv4 or Etv5 when they were cultured in the presence or absence of BDNF, suggesting that these factors are sufficient to promote dendrite growth and spines. Here, we identify Etv4 and Etv5 as new transcriptional targets of BDNF in hippocampal neurons

There is wide evidence indicating that the different Pea3 transcription factors are downstream effectors of RTK activated by neurotrophins as well as other growth factors (Haase et al. 2002; Patel et al. 2003; Vrieseling and Arber 2006; Abe et al. 2012; Fontanet et al. 2013; Liu et al. 2016).

Physiological Relevance of Etv4 and Etv5 Transcription Factors in Hippocampal Development

In this work, we have assessed the physiological requirement of Etv4 and Etv5 for proper hippocampal development. We found no abnormalities in the gross morphology of the hippocampal structure neither in *Etv4*- nor in *Etv5*-deficient mice versus control mice. The density of neurons and their arrangement in the hippocampus were indistinguishable in *Etv4*- or *Etv5*-deficient versus control mice. However, the examination of the dendritic structure of CA1 pyramidal neurons revealed by Golgi staining showed that dendritic arborization of these neurons in *Etvs*-knockout mice were drastically reduced in size and complexity compared with those of control animals. These data were in agreement with the in vitro observations indicating that downregulation of Etv4 or Etv5 in hippocampal neurons resulted in stunted dendritic arbors.

Dendrite architecture strongly influences the processing of information across the brain, and defects in dendrite morphology have been associated to neurodevelopmental and neurological disorders (Kaufmann and Moser 2000). Thus, understanding the precise regulation of dendritic growth and patterning could provide useful insight for the development of new and better therapies for these pathological conditions. In accordance with the very stunted dendritic arbors of hippocampal pyramidal cells observed in both *Etv4*- and *Etv5*-deficient mice, we found that these animals present deficits in behaviors that involve hippocampal circuitry. Our current findings reveal abnormal hippocampal connectivity in both *Etv4*- and *Etv5*-deficient mice, which resulted in a decline in cognitive function including deficits in novel object location paradigm. Beyond cognition, deficits in Etv4 or Etv5 also have consequences in nesting behavior that have been previously associated with hippocampal function (Antonawich et al. 1997). Our results indicate that *Etv4*- and *Etv5*-mutant mice behaviorally display social and cognitive deficits, which are consistent with the notion that Etv4 and Etv5 regulates hippocampal dendrite morphology in response to neural activity and BDNF. Recent work, has described that synaptic activity may control Etv1 expression to modulate the intrinsic properties of fast spiking basket cells by regulating the expression of Kv1.1 (Dehorter et al. 2015). Thus, neural activity and neurotrophin control the expression of Etvs genes in different neuronal populations, modulating the intrinsic properties these neurons.

Summary and Conclusions

This study demonstrates that Etv4 and Etv5 transcription factors are expressed in hippocampal pyramidal neurons during the main period of dendritogenesis. Our experiments reveal a novel role for Etv4 and Etv5 transcription factors for hippocampal circuit development and the coordination of synaptic maturation. Additionally, our data indicates that both factors are essential molecules of the transcriptional program linking BDNF signaling to dendrite development. It will be important to analyze whether mutations in Etv4 and/or Etv5 could have implications for human brain development and disorders of the nervous system characterized by cognitive and social alterations.

Author contributions

Conceived and designed the experiments: P.A.F., G.P., and F.L. Performed the experiments and statistical analysis: P.A.F., A.S.R., and F.C.A. Analysis and interpretation of the data: P.A.F., A.S.R., F.C.A., F.L., and G.P. Wrote the paper: F.L.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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