BDNF activates an NFI-dependent neurodevelopmental timing program by sequestering NFATc4

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ABSTRACT How intrinsic and extrinsic signals are coordinated to regulate synaptic maturation and its timing is an important question for neurodevelopment and its disorders. We investigated the influence of the neurotrophin BDNF on the developmental timing of a dendrite/synapse-related gene program controlled by nuclear factor I (NFI) in maturing cerebellar granule neurons (CGNs). BDNF accelerated the onset of NFI-regulated late-gene expression and NFI temporal occupancy in CGN cultures in a MEK5/ERK5-dependent manner. BDNF and NFI occupancy were mutually regulating, with BDNF enhancing the temporal binding of NFI to the *Bdnf4* promoter itself. Moreover, BDNF induced phosphorylation and accelerated the departure of the *trans*-repressor NFATc4 from NFI late-gene promoters, including *Bdnf4*, which is permissive for NFI binding. BDNF dismissal of NFATc4 from late genes was linked to MEK5/ERK5-dependent sequestration of NFATc4 in the *cis*–Golgi, an event mirrored in CGNs developing in vivo. These studies reveal an expanded autoregulatory gene network for NFI temporal occupancy involving BDNF and NFATc4 extranuclear sequestration. Based on these and earlier findings, NFATc4 integrates intrinsic developmental signaling from membrane potential/calcineurin and autocrine/paracrine BDNF/TrkB to control initiation of NFI occupancy in maturing CGNs. We also identify a local *Bdnf/Etv1* gene circuit within the larger NFI autoregulatory network.

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INTRODUCTION

Neuronal development involves an intricate interplay between extrinsic and intrinsic mechanisms (Russ and Kaltschmidt, 2014; Dong *et al.*, 2015). Cerebellar granule neurons (CGNs) have provided a valuable system for defining such interactions (Goldowitz and Hamre, 1998; Kilpatrick *et al.*, 2012). These neurons are born postnatally and undergo a well-defined series of differentiation events including axon extension and tangential migration within the external germinal layer (EGL) and premigratory zone (PMZ), radial migration from the PMZ, and subsequent dendrito-synaptic maturation in the internal granule cell layer (IGL). Within the IGL, CGN dendrites form synaptic glomerular structures involving excitatory (glutamatergic) mossy fiber axons arising from deep cerebellar nuclei, as well as inhibitory (GABAergic) axons from Golgi interneurons.

Disruption of neuronal development during critical time periods of synaptogenesis has been linked to neurodevelopmental disorders (NDs) (Meredith *et al.*, 2012). The mechanisms coordinating appropriate timing of synaptic maturation thus can provide important insight into events underlying the causes of these disorders. Chromatin dynamics that regulates transcription factor accessibility

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Abbreviations used: BFA, brefeldin A; CaN, calcineurin; Cb, cerebellum; CGNs, cerebellar granule neurons; ChiP, chromatin immunoprecipitation; DIV, days in vitro; IGL, internal granule cell layer; NDs, neurodevelopmental disorders; NFI, nuclear factor 1.

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and binding is also implicated in NDs (Bourgeron, 2015; Lopez and Wood, 2015). Recent work has identified an intrinsic transcriptional program in CGNs that drives the developmental timing and expression of numerous genes required for dendrite and synapse formation involving the nuclear factor I (NFI) transcription factors (Ding *et al.*, 2013, 2016). This temporal program is controlled by the developmentally delayed binding of NFI to consensus sites within late-expressed target gene promoters. Two key intrinsic *trans*-factors have been identified so far that govern the timing of NFI binding to chromatin sites. NFATc4 functions as a repressor that prevents premature NFI binding to target gene chromatin in immature CGNs (Ding *et al.*, 2013). NFATc4 activity is sustained in these immature cells via elevated calcineurin (CaN) activity, which promotes NFATc4 nuclear localization. Declining CaN activity as the cerebellum matures releases this repressive "brake," providing a permissive state for NFI occupancy to proceed. More recently, a second, intrinsic activating mechanism for NFI binding was identified involving the *trans*-activator ETV1 (Ding *et al.*, 2016). ETV1 expression is temporally up-regulated in maturing CGNs and it binds to, coactivates, and promotes NFI occupancy of, a subset of ETV1/NFI coregulated genes, including the *Etv1* gene itself. In addition to these intrinsic mechanisms, extracellular factors also likely influence the expression and timing of the NFI temporal program.

Acting through its receptor, kinase TrkB, BDNF is a secreted neurotrophic factor required for proper cerebellar development. *Bdnf*–null mice exhibit significant disruption of CGN development, including delayed migration and poor survival (Schwartz *et al.*, 1997). BDNF exerts multiple effects on CGN maturation, including enhanced onset of differentiation, without affecting cell cycle exit (Choi *et al.*, 2005) as well as promoting radial migration away from the EGL (Borghesani *et al.*, 2002). This migratory effect involves a BDNF chemotactic concentration gradient generated by postmigratory CGNs within the IGL. Further, BDNF released from migrating CGNs acts in an autocrine manner to promote radial migration toward the IGL (Zhou *et al.*, 2007). BDNF also controls later CGN development through the activation of genes involved in neuronal differentiation and dendrite/synapse formation expressed in postmigratory CGNs in the IGL (Lin *et al.*, 1998; Bulleit and Hsieh, 2000; Nakanishi and Okazawa, 2006; Sato *et al.*, 2006; Abe *et al.*, 2012).

Previous studies reported that BDNF enhanced the early expression of the NFI late gene *Gabra6* and its protein product in CGNs developing in culture and in vivo (Lin *et al.*, 1998; Bao *et al.*, 1999). These observations raised the possibility that BDNF functions as a temporal regulator of the broader NFI temporal occupancy program. Here, we address the mechanisms by which BDNF signaling regulates the timing of late CGN differentiation and its connection to the NFI temporal regulon.

RESULTS

BDNF controls the timing of maturation-dependent CGN gene expression via the MEK-ERK pathway

To address potential timing effects on the NFI late-gene program, we added BDNF to freshly prepared CGNPs and immature postmitotic CGNs derived from the mouse EGL starting on day 0 in vitro (0 DIV). This treatment strongly up-regulated the expression of numerous late genes (*Gabra6, Rps6ka1, Gabra1, Grin2c, Tiam1*) on 2 DIV, an early time point in CGN maturation (Figure 1A). BDNF stimulation of late genes at 2 DIV was inhibited by the Trk inhibitor K252a, consistent with signaling through the TrkB receptor. This stimulatory effect of BDNF treatment observed between 0 and 2 DIV was greatly reduced when examined in more mature CGNs (0–6 DIV) (Figure 1B), indicating a mainly early accelerating effect of BDNF on NFI late gene expression. At least one NFI late gene (*Tesc*) was insensitive to BDNF at both 2 and 6 DIV (Figure 1, A and B), revealing selectivity in the actions of this neurotrophin on the NFI temporal regulon.

BDNF-TrkB signaling is known to act upstream of the MEK-ERK pathway in CGNs (Bulleit and Hsieh, 2000; Abe *et al.*, 2012). Consistent with this, U0126, which inhibits MEK-ERK1/2/5, suppressed BDNF stimulation of NFI-late genes (Figure 1A). Previous work implicated the MEK5/ERK5 pathway in BDNF action (Cavanaugh *et al.*, 2001; Tatake *et al.*, 2008). We found that BIX2189, a selective inhibitor of MEK5/ERK5 (Tatake *et al.*, 2008), also effectively repressed BDNF stimulation of NFI-late genes (Figure 1A). Further, lentiviral expression of a dominant negative form of MEK5 (MEK5-DN) in immature CGNs inhibited BDNF regulation of affected NFI Late genes, with no significant effect on *Tesc* (Figure 1C). Thus, MEK5/ERK5 signaling is important for BDNF-TrkB signaling to the NFI temporal program.

BDNF promotes NFI temporal occupancy and MAP2(+) neurite formation

A central mechanism controlling the NFI temporal program is delayed timing of NFI binding to late genes (Ding *et al.*, 2013). We therefore addressed whether BDNF also enhanced NFI occupancy of relevant NFI late genes. We selected five genes previously shown to exhibit NFI temporal binding (*Gabra6, Rps6ka1, Tiam1, Grin2c, Tesc*) (Ding *et al.*, 2013, 2016). Treatment with BDNF from 0 to 2 DIV up-regulated the NFI occupancy of each gene, but not of the BDNF-insensitive *Tesc* gene (Figure 1D). Thus, BDNF-stimulated late-gene expression is linked to enhanced NFI temporal binding to target-gene chromatin sites.

The NFI switch program is directly implicated in CGN dendrite formation (Ding *et al.*, 2013), and loss of the NFI family member *Nfia* interferes with both NFI temporal program gene expression (Ding *et al.*, 2013) and CGN dendritic development in the postnatal cerebellum (Wang *et al.*, 2007). We therefore determined whether BDNF stimulated dendritelike process growth in immature CGNs. Treatment with BDNF significantly increased the total length of MAP2(+) processes in maturing CGN cultures at 2 DIV (Figure 1E). Further, BDNF stimulation was inhibited by the Trk inhibitor K252a, the MEK/ERK1/2/5 inhibitor U0126, and the MEK5/ERK5 inhibitor BIX2189, as well as by lentiviral expression of NFI dominant repressor (NFI/EnR) (Figure 1E). We were unable to determine the impact of MEK5-DN lentivirus on MAP2(+) neurites because of significant cell death with this treatment, either with or without BDNF. This effect, which was not observed in RNA studies, may reflect greater sensitivity to viral expression of MEK5-DN at the >4-fold lower cell densities required for neurite measurements. Collectively, these findings indicate a link between BDNF, MEK5/ERK5 signaling, NFI temporal occupancy, and dendritelike neurite formation in maturing CGNs.

BDNF and NFI occupancy are mutually regulating

BDNF is most highly expressed in the IGL of the mature mouse Cb (Hofer *et al.*, 1990), and its gene expression is temporally up-regulated in mouse CGNs maturing both in culture and within the IGL between P14 and P21 (Qiao *et al.*, 1996; Koibuchi *et al.*, 2001; Zhou *et al.*, 2007). This up-regulation of BDNF in the IGL is linked to formation of a migratory chemotactic gradient between the IGL and EGL (Zhou *et al.*, 2007). Mouse BDNF is expressed from as many as nine separate promoters, with each noncoding exon being spliced to a common coding exon (Aid *et al.*, 2007). *Bdnf4* is predominantly

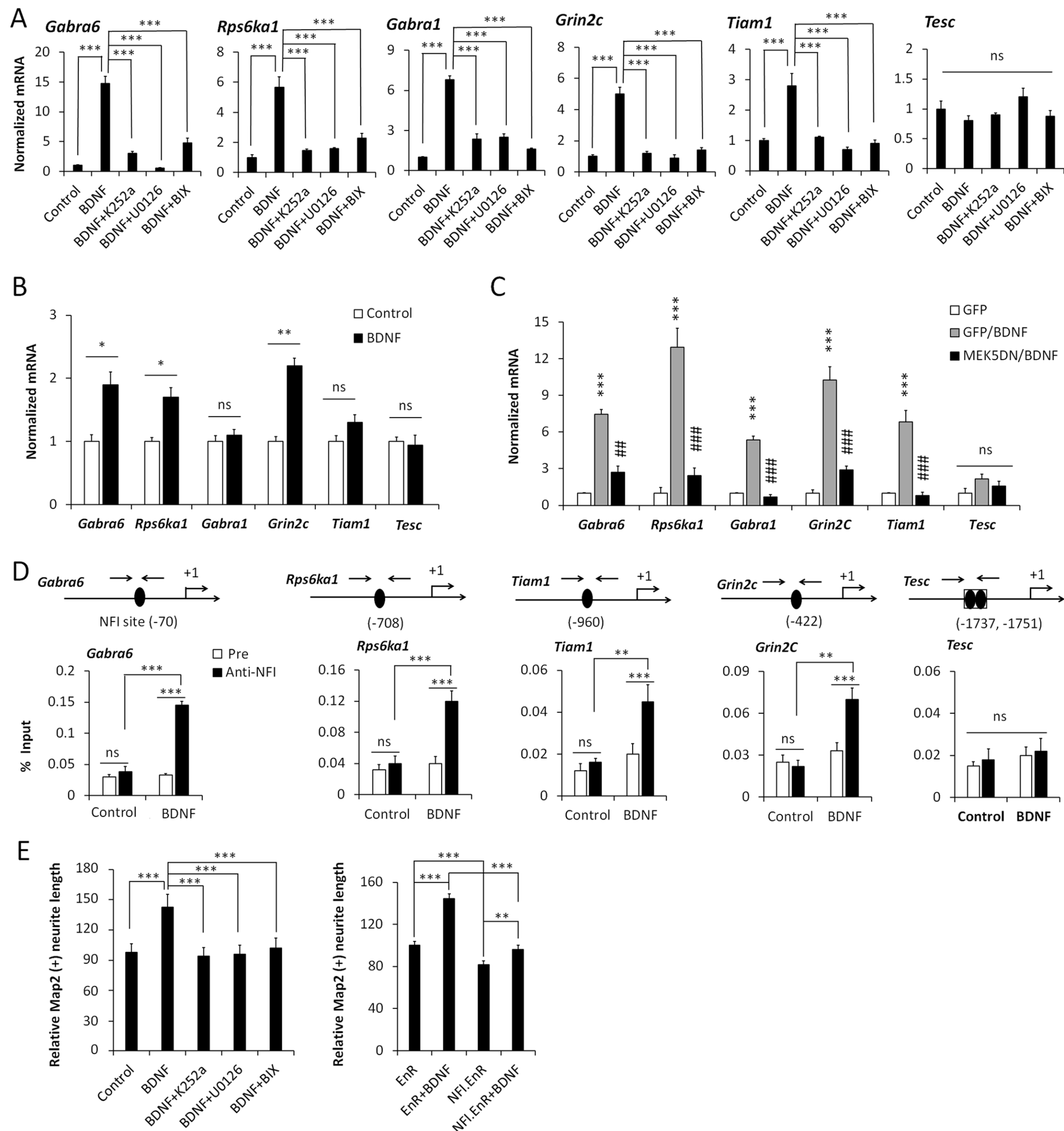

FIGURE 1: BDNF accelerates the developmental timing of late-gene expression and NFI occupancy in immature CGNs. (A) RT-qPCR assays of late-gene transcripts in 2-DIV CGN cultures treated upon plating with DMSO and water (control), DMSO + BDNF (BDNF), or BDNF with various inhibitors. No significant differences were observed between values for water or DMSO alone. (B) CGNs were treated as in A and then cultured until 3 DIV, when fresh reagents and medium were added. Cultures were extracted for RNA analysis on 6 DIV. (C) CGNs were treated upon plating with lentiviruses expressing GFP or MEK5-DN, and fresh medium supplemented with either BDNF or water vehicle was substituted the next day, as indicated. Late-gene transcripts were assayed in extracts harvested on 3 DIV. (D) NFI ChIP assays were performed for previously identified NFI binding sites within each promoter (locations are shown above panels for each gene). CGNs were treated from 0 to 2 DIV with BDNF or water (control). Antibodies raised to xNFIB (Anti-NFI), which recognizes NFIA and NFIB (Ding *et al.*, 2013), or preimmune serum (PRE) were used for ChIP assays. (E) BDNF regulation of MAP2(+) neurite outgrowth in maturing CGNs. *Left panel*, cells were cultured as described in panel A and fixed on 2 DIV. Right panel, cells were transduced with EnR and NFI/EnR lentiviral vectors upon plating and cultured as in (C) above until 2 DIV. Slides were processed for MAP2 staining as outlined in *Materials and Methods*. Means were determined using three biological replicates. ***, ###$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, no significant difference. In C, *** are comparisons with GFP controls while ### are relative to GFP + BDNF values.

expressed in mouse Cb (Liu *et al.*, 2006; Aid *et al.*, 2007), and NFI consensus binding sites are present upstream of its promoter (Figure 2A), suggesting that this promoter was a possible NFI target. RT-qPCR confirmed that both total *Bdnf* and *Bdnf4* expression are temporally up-regulated in maturing CGN cultures and in the developing cerebellum (Figure 2B). Further, expression of both total *Bdnf* and *Bdnf4* was down-regulated at 6 DIV in CGN cultures transduced on 0 DIV with an NFI dominant repressor, as well as in the P15 cerebellum of *Nfia*-null mice (Figure 2B). Chromatin immunoprecipitation (ChIP) assays also demonstrated NFI temporal binding to a

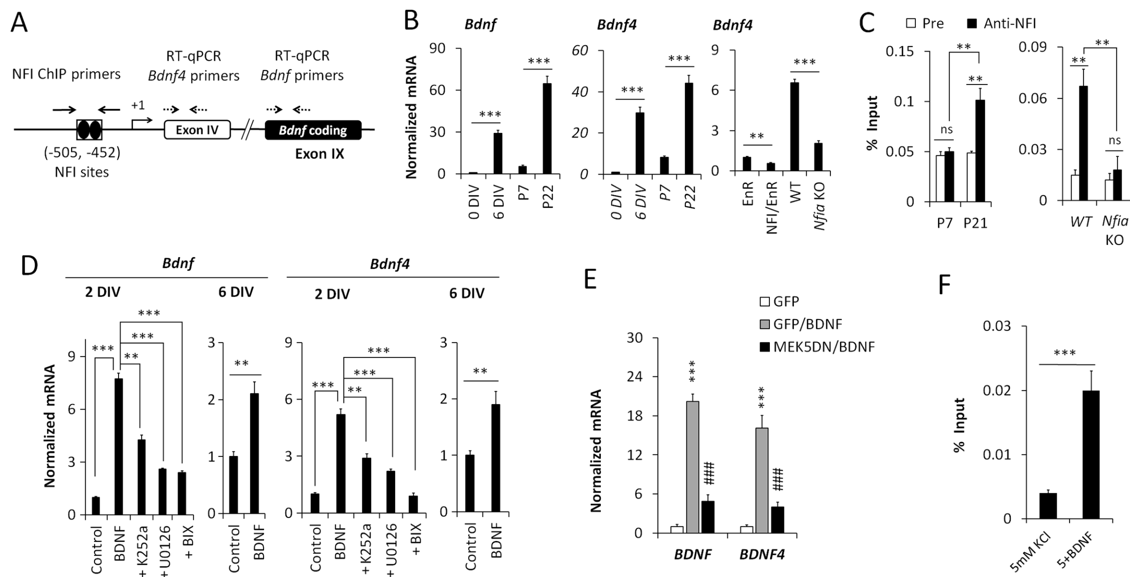

FIGURE 2: *Bdnf* is an autoregulated NFI late gene in maturing CGNs. (A) Locations of primers used for RT-qPCR of *Bdnf4* (Exon IV) and protein-coding (Exon IX) sequences as well as NFI sites and related primers for ChIP analysis. (B) Left, middle panels, RT-qPCR assays of *Bdnf4* and total *Bdnf* transcripts in differentiating CGN cultures (0 and 6 DIV) and P7, P22 mouse cerebellum. Right panel, *Bdnf4* transcripts in CGN cultures treated on 1 DIV with lentivirus expressing an NFI dominant repressor (NFI/EnR) or its control (EnR) and then extracted on 6 DIV, as well as in P15 *Nfia* knockout (*Nfia* KO) and wild-type (WT) mouse cerebellum. (C) NFI ChIP assays of NFI sites upstream of the *Bdnf4* promoter (see panel A). Left panel, temporal binding of NFI to the *Bdnf4* promoter in the developing cerebellum. Right panel, NFI binding to *Bdnf4* in *Nfia* null and wild-type mouse cerebellum at P15. (D) Comparison of the effects of BDNF on total *Bdnf* and *Bdnf4* mRNAs in the presence or absence of various inhibitors at 2 DIV, and at 6 DIV. (E) CGNs were treated with GFP or MEK5-DN lentiviruses and cultured as in Figure 1C until 3 DIV. (F) NFI occupancy of the *Bdnf4* promoter in CGN cultures treated with BDNF between 0 and 2 DIV. ***, $^{###}P < 0.001$, $^{**}P < 0.01$; ns, no significant difference. See Figure 1C for explanation of *P* values in E. xNFIB antibody was used for ChIP assays in C and F.

proximal NFI site in the *Bdnf4* promoter in the developing Cb (P7 and P22) (Figure 2C). Thus, *Bdnf4* is an NFI-dependent late-gene promoter that undergoes NFI temporal binding in postmigratory CGNs maturing with the IGL.

Identification of *Bdnf* as an NFI late gene raised the interesting possibility that BDNF regulates its own expression in maturing CGNs. This was confirmed using RT-qPCR. Both total *Bdnf* and *Bdnf4* expression were stimulated by BDNF treatment of immature CGNs (Figure 2D). This gene activation was inhibited by K252a as well as the MEK/ERK inhibitors U0126 and BIX2189 and MEK5-DN lentivirus (Figure 2, D and E). BDNF stimulation of its own gene expression was also substantially diminished at 6 DIV (Figure 2D), consistent with a temporal accelerating effect. Importantly, BDNF also increased NFI occupancy of the *Bdnf4* promoter at 2 DIV (Figure 2F).

Collectively, these findings showed that BDNF accelerates the timing of its own expression and NFI occupancy of the *Bdnf4* promoter in immature CGNs. Thus, *Bdnf* autoregulation and control of its NFI temporal occupancy are directly implicated in the developmental timing and formation of the BDNF autocrine gradient within the IGL.

BDNF regulates a transcriptional repressor in maturing CGNs

The foregoing findings indicated that BDNF activates early steps in the NFI late-gene program and in CGN differentiation. Departure of NFATc4 from late-gene promoters is an initiating step required for activation of NFI temporal programming and occupancy (Ding *et al.*, 2013). BDNF was previously shown to control NFATc4 activity in neurons (Groth and Mermelstein, 2003). We therefore examined whether the NFATc4 pathway was regulated by BDNF in immature CGNs. Remarkably, NFATc4 occupancy of several NFI-regulated late genes was strongly down-regulated on 2 DIV by BDNF exposure (Figure 3A). These results directly implicated accelerated NFATc4 departure from its late-gene binding sites in the BDNF-induced activation of the NFI temporal program in immature CGNs.

Calcineurin (CaN) dephosphorylation of NFATc4 promotes its occupancy and repression of late-gene promoters in maturing CGNs (Benedito *et al.*, 2005; Ding *et al.*, 2013). We therefore tested whether CaN antagonizes BDNF stimulation of late-gene expression in immature CGNs. CGNs were transduced on 0 DIV with lentivirus expressing either GFP or constitutively active CaN, and cultures were then treated with BDNF for 48 h starting on 1 DIV. CaN transduction markedly inhibited BDNF stimulation of late-gene expression (Figure 3B). Similarly, expression of a constitutively active form of NFATc4 (NFATc4-Ala) also repressed late-gene expression in the presence of BDNF (Figure 3C). Further, both constitutively active CaN and NFATc4-Ala inhibited MAP2(+) neurite formation by CGNs in response to BDNF (Figure 3D). Thus, conditions that promote NFATc4 transcriptional activity antagonize the ability of BDNF to accelerate late-gene expression and promote dendritelike neurite extension in immature CGNs.

On the basis of the above findings, we examined whether NFATc4 was involved in *Bdnf* temporal gene expression in immature CGNs. Transcripts for total *Bdnf* and *Bdnf4*, as well as NFI occupancy of the *Bdnf4* promoter, were markedly elevated in the early postnatal cerebellum of *Nfatc4* knockout mice (Figure 4, A and B), consistent with derepression of NFI late-genes (Ding *et al.*, 2013). Conversely, total *Bdnf* and *Bdnf4* transcripts were highly repressed in more mature

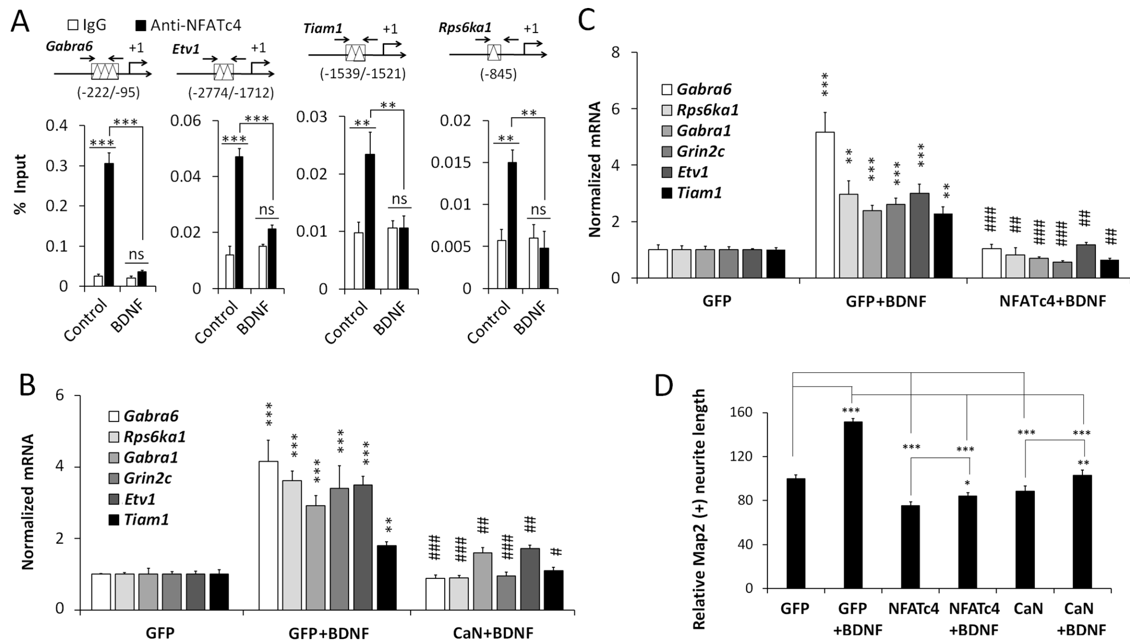

FIGURE 3: BDNF and CaN/NFATc4 interactions in maturing CGNs. (A) CGN cultures were exposed to BDNF from 0 to 2 DIV and then fixed and assayed for NFATc4 ChIP. IgG was used as a negative control. (B) CGNs were treated with lentiviruses expressing either GFP or constitutively active CaN on 0 DIV. They were then exposed to BDNF or water on 1 DIV, as indicated. Late-gene transcripts were assayed in extracts harvested on 3 DIV. (C) Effects of constitutively active NFATc4 on BDNF-stimulated acceleration of late-gene mRNAs. Experiments were performed as in B using GFP and NFATc4-Ala lentiviruses. (D) CGNs were transduced with GFP, constitutively active NFATc4, or constitutively active CaN lentiviruses and assayed for MAP2(+) neurite extension as in Figure 1E. ***, $^{###}P < 0.001$; **, $^{##}P < 0.01$; *, $^{#}P < 0.05$; ns, no significant difference. See Figure 1C for explanation of *P* values in B and C.

(6 DIV) CGN cultures transduced on 0 DIV with constitutively active NFATc4 (Figure 4C). Sequence analysis identified a cluster of NFAT sites ~1 kb upstream of the *Bdnf4* promoter, and ChIP confirmed NFATc4 occupancy of this region in the immature (P7) cerebellum that was developmentally down-regulated at P15 (Figure 4D). Collectively, these results indicated that, similarly to other NFI late genes, expression and NFI occupancy of the *Bdnf4* gene are repressed by NFATc4 in immature CGNs, and declining NFATc4 binding leads to NFI occupancy and onset of *Bdnf4* expression.

We next addressed whether NFATc4 played a role in *Bdnf* gene autoregulation. Significantly, BDNF treatment of CGN cultures enhanced NFATc4 departure from the *Bdnf4* gene at 2 DIV (Figure 4E). Further, BDNF stimulation of *Bdnf* and *Bdnf4* mRNAs was inhibited by lentiviral expression of constitutively active forms of either CaN or NFATc4 in CGN cultures (Figure 4F). These findings strongly support a direct role for NFATc4 dismissal and derepression in BDNF-enhanced NFI occupancy and autoregulation of the *Bdnf4* promoter.

BDNF stimulates phosphorylation and extranuclear localization of NFATc4

BDNF was previously shown to regulate *Nfatc4* gene expression as well as NFATc4 subcellular localization in neurons (Groth and Mermelstein, 2003; Groth *et al.*, 2007). BDNF did not alter *Nfatc4* mRNA or total NFATc4 protein in maturing CGNs at 2 DIV (Figure 5, A and B). However, BDNF markedly increased the amount of phosphorylated NFATc4 at this developmental stage (Figure 5B). BDNF-induced phosphorylation was inhibited by BIX2189 (Figure 5B), mirroring the effects on MEK5/ERK5 inhibition on BDNF-stimulated late-gene expression.

ERK5-dependent phosphorylation has been linked to cytoplasmic localization of NFATc4 (Yang *et al.*, 2008). Consistent with this, BDNF stimulated the appearance of extranuclear NFATc4 in maturing CGN cultures, an effect that was inhibited by U0126 and BIX2189 (Figure 5C, D). Further, NFATc4 was localized to the nucleus in the presence of MEK/ERK inhibitors (Figure 5D), congruent with their blocking effects on BDNF-stimulated late-gene expression. These results supported a central role for MEK5/ERK5-dependent NFATc4 phosphorylation and its subsequent extranuclear shuttling in the accelerating effects of BDNF on the NFI late-gene program and BDNF autoregulation.

A unique feature of the shift in NFATc4 localization in response to BDNF was its localization into more intensely labeled "foci" outside CGN nuclei (Figure 5C). At higher magnification, this clustering of NFATc4 closely abutted the nucleus (Figure 5, C and E). Although CGNs possess a relatively small cytoplasmic space, these observations suggested that extranuclear NFATc4 was localized within a specific subcellular structure located near the nucleus, such as the Golgi apparatus. To assess this, we used the *cis*-Golgi marker GM130 and confocal microscopy to examine colocalization with NFATc4. GM130 staining was present in puncta that substantially colocalized with juxtanuclear NFATc4 foci in BDNF-treated cultures (Figure 6, A and B). In addition, short-term treatment with the Golgi disrupting agent brefeldin A (BFA) dispersed both GM130 and NFATc4 staining more uniformly within the cytoplasmic space (Figure 6, A and B). Collectively, these findings indicated that BDNF stimulates extranuclear NFATc4 sequestration to the *cis*-Golgi compartment that is sensitive to MEK5/ERK5 inhibitors.

We next addressed whether NFATc4 extranuclear sequestration also occurred in CGNs as they matured in vivo. We focused on the

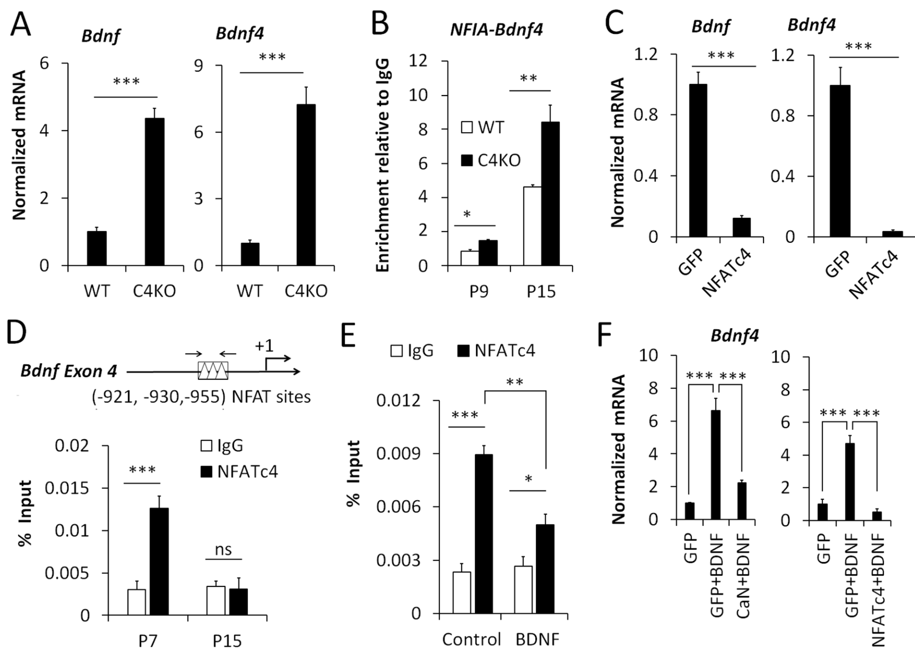

FIGURE 4: (A) *Bdnf* and *Bdn4* transcripts in P10 *Nfatc4*-null (C4KO) and wild-type (WT) mouse cerebellum. (B) NFIA occupancy of the *Bdnf4* promoter in the developing *Nfatc4* knockout mouse cerebellum. ChIP assays were performed using NFIA antibody and IgG control. To simplify the graphics, data are expressed relative to IgG control values. (C) Constitutively active NFATc4 (NFATc4-Ala) represses *Bdnf* and *Bdn4* gene expression in 3-DIV CGNs. (D) ChIP assays of NFATc4 occupancy of the *Bdnf4* promoter in the developing mouse cerebellum. Consensus NFAT binding sites within the *Bdnf4* promoter region and ChIP primer locations are shown above. (E) BDNF effects on NFATc4 binding to the *Bdnf4* promoter. BDNF or water was added to CGN cultures from 0 to 2 DIV and extracts were then assayed as in panel D. (F) Lentivirus-expressed activators of the CaN/NFATc4 pathway suppress *Bdnf* autoregulation in maturing CGNs. Treatments and assays were as described in Figure 3, B and C. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, no significant difference.

CGNs. We found that BDNF addition on 0 DIV markedly up-regulated the expression of both *Camkk2* and *Camk4* transcripts at 2 DIV; this effect was no longer apparent by 6 DIV (Figure 8A). As observed for other NFI late genes, this temporal acceleration was suppressed by the MEK/ERK inhibitors U0126 and BIX2189, as well as by expression of MEK5-DN (Figure 8A). Previous studies showed that expression of both *Camkk2* and *Camk4* is repressed under conditions that up-regulate NFATc4 nuclear activity in maturing CGNs (Ding *et al.*, 2013). Similarly, we found that constitutively active CaN or NFATc4-Ala also repressed BDNF stimulation of the *Camkk2* and *Camk4* genes in immature CGNs (Figure 8B).

Thus, BDNF increases the expression of a broader autoregulatory signaling circuit involving CAM kinases as both upstream and downstream regulators as CGNs differentiate. We were unable to reproducibly detect significant NFI temporal occupancy of NFI sites within the *Camkk2* and *Camk4* proximal promoters (unpublished data); however, this does not preclude temporal binding to more distal sites. This possibility was not further examined here.

We also examined whether the downstream component of the BDNF signaling pathway, *Ntrk2* encoding the BDNF receptor TrkB, was developmentally regulated by NFI in CGNs. *Ntrk2* transcripts were temporally up-regulated both in CGN cultures and in the postnatal cerebellum (Figure 8C), consistent with previous in situ hybridization data showing developmental up-regulation of *Ntrk2* expression specifically in rodent IGL (Masana *et al.*, 1993). Further, *Ntrk2* expression was reduced by NFI dominant repression in CGN cultures and in P15 *Nfia* knockout mouse cerebellum (Figure 8C). Thus, temporally delayed expression of the TrkB receptor gene is also dependent on NFI. Interestingly, BDNF did not significantly regulate *Ntrk2* gene expression in CGN cultures (Figure 8D). Thus, this component of the BDNF signaling pathway was not subject to autoregulation, at least in immature CGN cultures.

P6 cerebellum when NFATc4 protein levels are relatively abundant (Ding *et al.*, 2013). NFATc4 immunostaining was detected in CGNs within the EGL and IGL, as well as in the Purkinje cell layer (Figure 7). Staining within the EGL was largely diffuse; however, NFATc4 staining in the IGL was present in more intense, punctatelike extranuclear structures, along with varying degrees of more diffuse nuclear staining (Figure 7). As seen in CGN cultures, NFATc4 puncta showed significant overlap with GM130 immunostaining. The lower frequency of this costaining in premigratory CGNs within the EGL suggested that extranuclear colocalization was largely a postmigratory feature at P6. Purkinje cells also exhibited NFATc4 staining that was both nuclear and colocalized with extranuclear GM130 (Figure 7).

NFI controls a BDNF regulatory gene circuit in maturing CGNs

Developmental expression of the *Bdnf* gene in CGNs maturing in vivo is dependent on the Ca^{2+}/calmodulin-dependent kinases CAMKK2 and CAMK4 (Kokubo *et al.*, 2009), and CAMK4 is a downstream mediator of BDNF in neurons (Spencer *et al.*, 2008). *Camkk2* mRNA and CAMK4 protein are also highly enriched in postmigratory CGNs in the cerebellum (Ohmstede *et al.*, 1989; Sato *et al.*, 2005). Interestingly, both *Camkk2* and *Camk4* were previously identified as NFI late genes in maturing CGNs (Ding *et al.*, 2013), suggesting that NFI may regulate a *Camkk2-Camk4-Bdnf* signaling cascade. BDNF up-regulation of *Camkk2* was previously reported (Sato *et al.*, 2006), raising the possibility that BDNF controls a broader NFI-dependent temporal autoregulatory loop in maturing

BDNF–ETV1 interactions in early maturing CGNs

The ETV1 transcription factor is selectively expressed in CGNs as they mature within the postnatal IGL (Ding *et al.*, 2016). ETV1 was recently found to promote NFI occupancy and activate several NFI late genes via an "autoregulation-first" mechanism in which ETV1 binding to its own promoter precedes that for other, downstream late genes (Ding *et al.*, 2016). ETV1 and BDNF coregulate strongly overlapping subsets of NFI temporal program genes (Ding *et al.*, 2016). Further, the effects of BDNF on gene expression in CGNs were previously linked to its ability to activate the transcription factor ETV1 by enhancing its MEK/ERK-dependent phosphorylation (Abe *et al.*, 2011, 2012). ETV1 is therefore a potential downstream mediator of BDNF-regulated gene transcription, acting subsequent to NFATc4 dismissal from NFI late-gene promoters (Ding *et al.*, 2016).

Because *Etv1* is also an NFI late gene (Ding *et al.*, 2016), we first examined the effect of BDNF on the temporal onset of this gene in immature CGNs. As for other late genes, BDNF treatment markedly

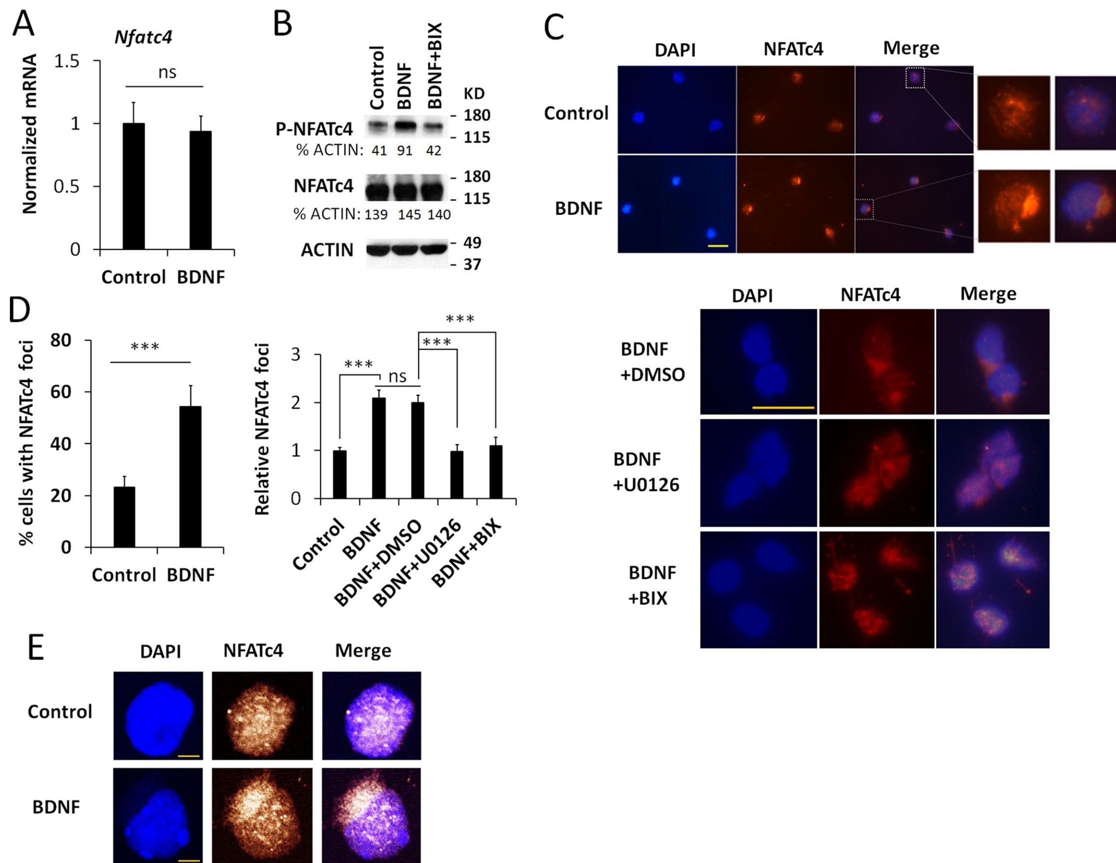

FIGURE 5: BDNF regulates NFATc4 localization in immature CGNs. (A) RT-qPCR assays of *Nfatc4* gene expression in CGNs treated with BDNF from 0 to 2 DIV. (B) CGN cultures were treated with or without BDNF + DMSO or BDNF + BIX2189, harvested on 2 DIV, and analyzed by Western analysis for phosphorylated NFATc4 (P-NFATc4), total NFATc4, and ACTIN proteins. Data were quantified and are expressed relative to ACTIN, as shown below each band. (C) Top panel, NFATc4 immunostaining following exposure of CGNs to BDNF for ~16 h. Bottom panel, the effect of MEK/ERK inhibitors on NFATc4 foci in response to BDNF. (D) Quantitation of BDNF's effect on extranuclear localization of NFATc4 and its inhibition by MEK/ERK inhibitors. (E) Confocal micrographs of individual CGNs in control and BDNF-treated cultures showing nuclear and extranuclear NFATc4 localization, respectively. In C and E, DAPI was used for nuclear staining. Scale bars represent 20 μM (C) or 5 μM (E). No primary antibody control yielded undetectable signal. For D ****P* < 0.001; ns, no significant difference.

enhanced expression of the *Etv1* gene at 2 DIV but not at 6 DIV, and also increased its early occupancy by NFI (Figure 9A).

Etv1 knockdown was reported to inhibit the effects of BDNF on *Tiam1* and *Grin2c* expression in maturing (5 DIV) CGN cultures (Abe *et al.*, 2012). To further address the potential role of ETV1 in BDNF-stimulated gene expression, we examined whether BDNF treatment increased ETV1 occupancy of NFI late genes. Consistent with this, ChIP analysis revealed that ETV1 occupancy of the *Etv1* gene was enhanced by BDNF at 2 DIV (Figure 9B). Interestingly, analysis of *Gabra6*, an NFI/ETV1 coregulated late gene (Ding *et al.*, 2016), showed no significant increase in ETV1 occupancy at 2 DIV (Figure 9B). This observation suggested an ETV1 autoregulation-first mechanism as an early response to BDNF treatment, with acceleration of the downstream *Gabra6* gene being independent of direct activation by ETV1 at 2 DIV. The contrasting dependence of downstream late-gene expression on ETV1 observed here and in studies by Abe *et al.* likely reflects the difference in CGN maturation status in the two studies (2 vs. 5 DIV) and the correspondingly higher levels of *Etv1* gene expression in 5-DIV cultures (Abe *et al.*, 2012).

Finally, we examined whether ETV1 was part of a broader autoregulatory loop involving its reciprocal regulation of BDNF circuit genes during CGN maturation in vivo. In *Etv1*-null mice, *Bdnf4* and *Ntrk2* expression were actually *up*-regulated relative to wild-type littermates in P10 cerebellum (Figure 9C). In contrast, expression of the *Bdnf* regulatory genes *Camkk2* and *Camk4* was down-regulated in the absence of ETV1, while *Tesc* was unregulated (Figure 9C). Thus, *Etv1*-deficiency exerts opposing effects on BDNF regulatory and signaling genes in the developing cerebellum. These effects may reflect homeostatic feedback interactions within the BDNF regulatory circuit mediated by ETV1 in developing CGNs, with ETV1 activating upstream regulatory genes (*Camkk2*, *Camk4*) and repressing BDNF-TrkB signaling. These actions by ETV1 may be direct, indirect, or both.

DISCUSSION

The NFI temporal occupancy program controls numerous genes in postmigratory CGNs maturing in the IGL that are linked to dendrite/synapse formation and function (Ding *et al.*, 2013). The present studies identify a mutually regulating interface between BDNF-TrkB signaling and the NFI late temporal program in maturing CGNs: 1) BDNF promotes MAP2(+) process formation in maturing CGNs; 2) BDNF accelerates the temporal expression of several

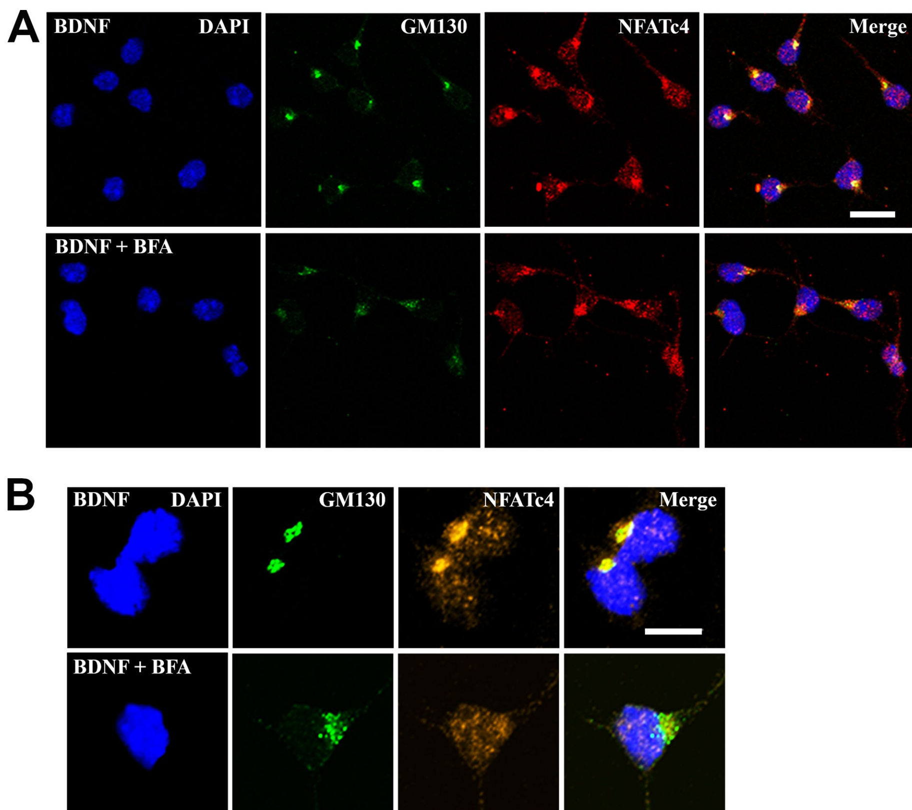

FIGURE 6: Juxtanuclear NFATc4 colocalizes with the *cis*-Golgi. (A) CGNs were cultured for ~18 h with BDNF, and then either brefeldin A (BFA; 5 µg/ml) or DMSO was added for an additional 30 min. Immunostaining was performed for GM130 and NFATc4. Scale bar = 50 µm. (B) Higher-magnification confocal images of CGNs treated with either BDNF or BDNF+BFA as in A.

NFI-regulated late genes, and this involves enhanced temporal onset of NFI occupancy; 3) a central action of BDNF on late-gene expression involves extranuclear sequestration of NFATc4; 4) reciprocally, the *Bdnf* gene is itself a target of NFATc4-sensitive NFI temporal occupancy; and 5) NFI proteins regulate a BDNF regulatory and signaling gene circuit. Collectively, these results indicate that the NFI temporal program is an important link between BDNF-TrkB signaling and dendrite–synapse gene expression and facilitates *Bdnf* gene and signaling autoregulation in maturing CGNs.

BDNF function is critical for normal CGN migration, differentiation, and survival (Schwartz *et al.*, 1997; Borghesani *et al.*, 2002; Zhou *et al.*, 2007). CGN maturation is delayed during postnatal week 2 in *Bdnf*-null mice (Borghesani *et al.*, 2002), when *Bdnf* and other NFI late genes are normally up-regulated in the IGL. Further, *Nfia* and *Bdnf* knockout cerebella exhibit several shared phenotypes during the early postnatal period, including thickened EGL/PMZ, retarded radial migration, and diminished Purkinje cell dendrite arborization (Schwartz *et al.*, 1997; Borghesani *et al.*, 2002; Wang *et al.*, 2007). These similarities suggest to us that reduced BDNF expression contributes to defective CGN maturation and other alterations observed in *Nfia* null mice. Developmental up-regulation of *Bdnf* expression in the IGL is directly implicated in CGN radial migration from the EGL/PMZ by forming a chemoattractant gradient (Borghesani *et al.*, 2002). Thus, *Bdnf* developmental up-regulation and NFI temporal occupancy of the *Bdnf4* promoter are likely to be key for generating this BDNF gradient, as well as for autocrine/paracrine development of postmigratory CGNs. Finally, BDNF promotes CGN dendrite formation at least in part through up-regulation of the NFI temporal occupancy program in CGNs within the IGL.

Our findings indicate that BDNF acts selectively at an early step to *accelerate* onset of the NFI temporal regulon, since its actions on late-gene expression were greater in immature CGNs. This is consistent with previous findings showing BDNF preferentially affects early CGN development (Segal *et al.*, 1995). This early regulation of the NFI temporal program is mediated at least in part through reduced NFATc4 *trans*-repression associated with its extranuclear sequestration, which is necessary and permissive for NFI temporal occupancy (Ding *et al.*, 2013). CaN opposes this effect in immature CGNs by promoting NFATc4 dephosphorylation and nuclear localization (Ding *et al.*, 2013). In contrast, in rat hippocampal and dorsal root ganglia neurons BDNF acts through intracellular Ca^{2+} release and CaN to *stimulate* NFATc4 *trans*-activation, including activation of the *Bdnf* gene itself (Groth and Mermelstein, 2003; Groth *et al.*, 2007; Vashishta *et al.*, 2009). These divergent NFATc4 actions suggest important cell-specific differences in signaling pathways governing gene expression, which may include MEK5/ERK5. Consistent with our findings, ERK5 also stimulates NFATc4 nuclear export in nonneural cells (Yang *et al.*, 2008; Zhu *et al.*, 2014).

The Golgi apparatus has a central role in regulating CGN polarity and migration (Zmuda and Rivas, 1998; Bellion *et al.*, 2005). Previous work (Barbato *et al.*, 2007) described *cis*-Golgi localization of the RNAse III endonuclease Dicer in late-maturing CGNs, although its regulatory significance was unclear. The thyroid hormone receptor coactivator TRIP230/GMAP210 undergoes phosphorylation-dependent *cis*-Golgi→nucleus translocation in nonneural cells (Chen *et al.*, 1999), the functional impact of which was also not determined. The present findings suggest a novel role for the *cis*-Golgi in BDNF regulation of transcription factor localization in maturing neurons. A presumptive consequence of BDNF-induced sequestration in the *cis*-Golgi is reduced NFATc4 cytoplasmic–nuclear shuttling, at least in part. Consistent with this, MEK/ERK inhibition of BDNF signaling resulted in NFATc4 retention in the nucleus and blocked the effects of BDNF on gene regulation (see Figures 1, A and C, and 5, C and D).

NFATc4 colocalization with the *cis*-Golgi marker GM130 was less frequent in the EGL than in the IGL in developing mouse cerebellum, suggesting that NFATc4 localization in the *cis*-Golgi in vivo is contemporaneous with postmigratory differentiation of CGNs. This is consistent with reduced NFATc4 repression and activation of the NFI late-gene program in CGNs maturing within IGL (Ding *et al.*, 2013).

Autoregulatory gene circuits are involved in developmental and activity-dependent mechanisms in neurons (Shibata *et al.*, 2015; Mullins *et al.*, 2016), and self-regulating feedback loops are a key feature of the NFI temporal occupancy program controlling dendrite/synapse formation in CGNs (Ding *et al.*, 2013, 2016). Based on our findings, BDNF accelerates NFATc4 dismissal and onset of NFI occupancy and expression of its own gene (e.g., *Bdnf4*

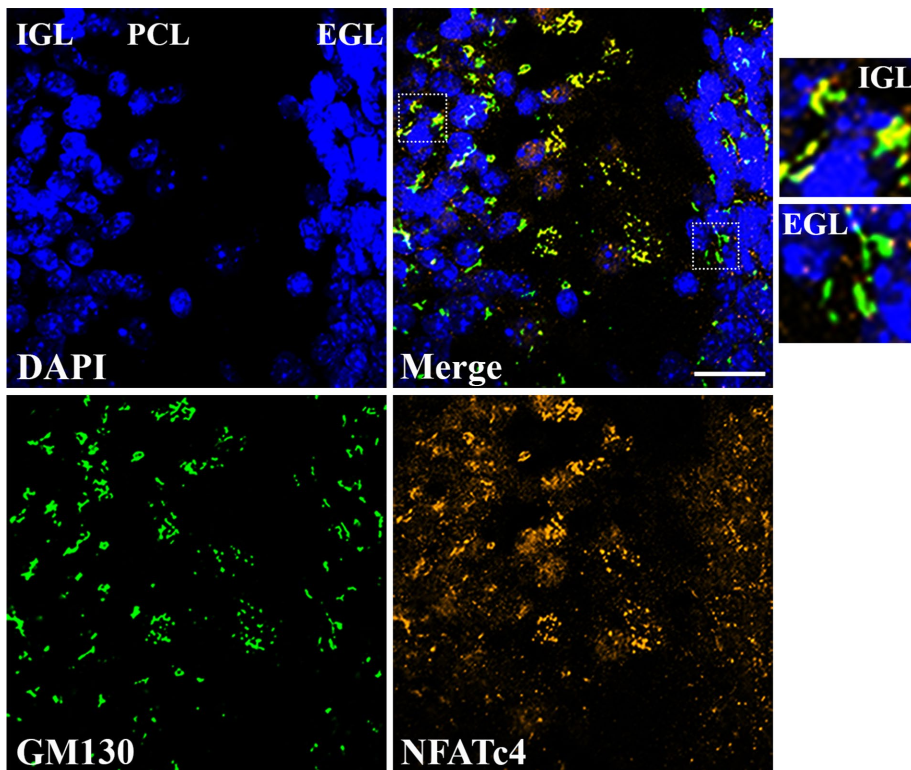

FIGURE 7: Representative confocal micrographs demonstrating extensive costaining of NFATc4 and GM130 in CGNs and Purkinje neurons. Coronal sections of P6 cerebellum were stained for NFATc4 (orange), GM130 (green), and nuclei (DAPI; blue). The EGL, IGL and Purkinje cell layers (PCL) are indicated. Inserts shows regions of colocalization, or the lack thereof, within the upper margin of the IGL and within the EGL, respectively, at higher magnification. Scale bar = 20 µm.

promoter) via stimulation of MEK5/ERK5-dependent phosphorylation of NFATc4 (Figure 10A). This BDNF autoregulatory pathway therefore links NFI temporal occupancy to what was originally identified as an independent upstream temporal regulatory pathway: CaN/NFATc4-mediated repression driven by a more depolarized resting membrane potential in immature CGNs (Ding *et al.*, 2013; Figure 10A). This suggests that BDNF feedback and NFATc4 *cis*–Golgi sequestration may form part of a larger autoregulatory loop within the NFI temporal program that derepresses NFI occupancy as CGNs mature. Thus, NFATc4 serves as a central integrator of opposing signaling mechanisms that control the timing of onset for NFI occupancy as the relative activities of membrane potential/CaN and BDNF/TrkB signaling shift with CGN maturation (Figure 10A).

Excitability and membrane potential in CGNs are governed by developmental expression of $GABA_A$ receptors as well as ion channels and transporters, which in turn affects CGN development and gene expression (Smeyne and Goldowitz, 1989; Semyanov *et al.*, 2004; Nakanishi and Okazawa, 2006; Ding *et al.*, 2013). Similar to NFI (Ding *et al.*, 2013), BDNF regulates the expression of genes that control membrane potential, including ion channels (e.g., *Kcnq3*) and GABA receptor subunits (Lin *et al.*, 1998; Sato *et al.*, 2006; also Figure 1, A and C, here). Correspondingly, BDNF enhances the maturation of GABAergic responses in CGNs developing in vivo that promote tonic hyperpolarization (Brickley *et al.*, 1996; Bao *et al.*, 1999). We hypothesize that this hyperpolarization contributes to reduced CaN activity, further augmenting the BDNF-induced accumulation of phosphorylated NFATc4, its *cis*-Golgi sequestration, and the resulting increase in expression of NFI target genes that further drive membrane hyperpolarization, *Bdnf4* expression, and CGN maturation itself (Figure 10A). While speculative, this scenario suggests that these opposing BDNF/CaN signaling pathways can directly influence each other's (as well as their own) activities by regulating NFATc4 phosphorylation status and *cis*-Golgi sequestration, forming a higher level of mutually interacting autoregulatory loops within the NFI temporal occupancy program (Figure 10A).

ETV1 is both a transcriptional target and a regulator of NFI temporal occupancy in maturing CGNs (Ding *et al.*, 2016). ETV1 binding and activation of its own gene precedes that for other ETV1/NFI coregulated genes, suggesting that ETV1 autoregulation-first provides an intrinsic timing mechanism for NFI temporal binding (Ding *et al.*, 2016). Autoregulation of NFI temporal occupancy thus involves two phases: early extracellular/autocrine–paracrine (BDNF) and later, nuclear/transcriptional (ETV1-dependent) steps. BDNF stimulation of MEK/ERK signaling increases phosphorylation of both ETV1 (Abe *et al.*, 2012) and NFATc4, thereby increasing *Etv1* gene expression through the dismissal of NFATc4 from the *Etv1* promoter and by enhancing ETV1 *trans*–activity (Abe *et al.*, 2012), which likely promotes *Etv1* autoregulation (Ding *et al.*, 2016).

Further, these interactions appear to form a local *Bdnf-Etv1* regulatory circuit within the larger NFI temporal regulon (Figure 10B). Acting through TrkB and MEK/ERK, BDNF accelerates expression of its own gene as well as *Etv1* via enhanced NFATc4 departure and NFI occupancy, with *Etv1* also driving its own autoregulation. ETV1 is also required for optimal expression of the *Camkk2* and *Camk4* genes, which through their protein products activate *Bdnf* expression via phosphorylation of CREB (Kokubo *et al.*, 2009; Figure 10B). Whether NFI and CREB interact to regulate *Bdnf* expression remains unclear. TrkB and TIAM1 drive endosome-dependent signaling by BDNF in maturing CGNs (Zhou *et al.*, 2007), and NFI regulation of both of their genes forms yet another facet of *Bdnf-Etv1* NFI circuitry: ETV1 binds to and up-regulates the *Tiam1* gene (Abe *et al.*, 2011; Ding *et al.*, 2016), and *Ntrk2* and *Bdnf* are under direct or indirect negative control by ETV1 (Figures 9C and 10B), perhaps reflecting ETV1-mediated homeostatic feedback control of BDNF/TrkB expression/signaling. These results thus suggest a highly integrated autoregulatory local gene circuit controlling BDNF-pathway gene expression as well as signaling, which includes CGN promigratory mechanisms that are local (autocrine–endosomal) as well as more distal between the IGL and EGL.

Disruption of neurodevelopmental timing mechanisms and of chromatin remodeling is directly implicated in NDs (Bourgeron, 2015; Meredith, 2015), and autoregulatory transcriptional circuits appear to play a key role in autism and related disorders (Mullins *et al.*, 2016). Collectively, our findings reveal how paracrine/autocrine, intrinsic signaling, and chromatin interactions are integrated into an autoregulatory network to control the timing of late-gene expression and ultimately dendritogenesis–synaptogenesis in a major population of differentiating CNS neurons. These findings

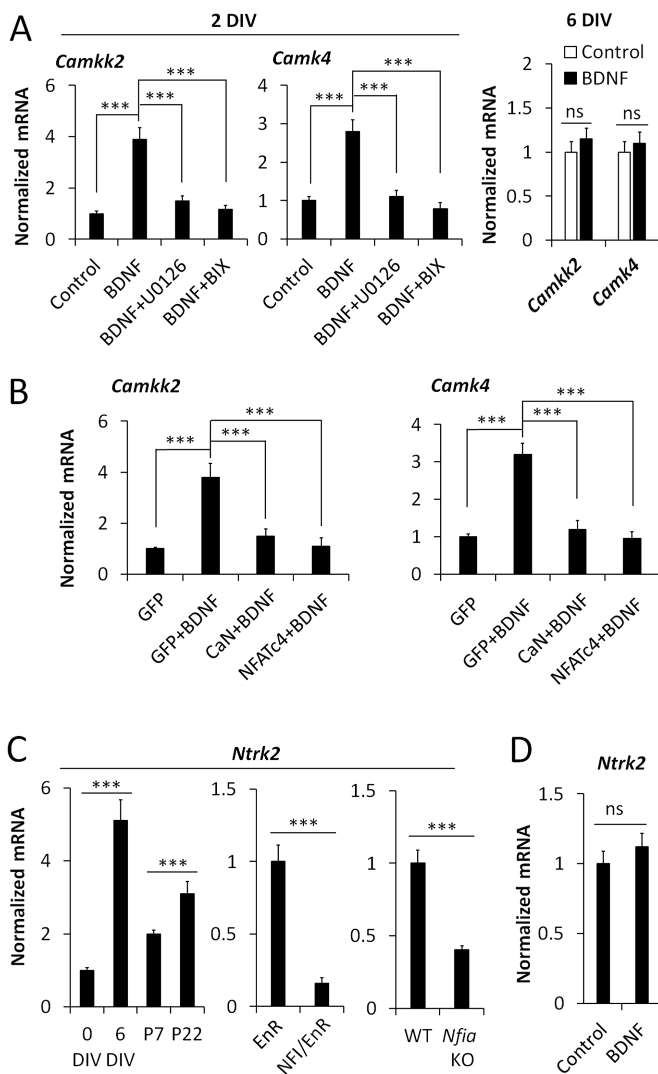

FIGURE 8: Identification of a BDNF autoregulatory circuit in maturing CGNs. (A) Left, CGNs were treated with BDNF between 0 and 2 DIV with and without MEK/ERK inhibitors and assayed for expression of the upstream regulatory genes *Camkk2* and *Camk4*. Middle, effect of BDNF treatment of CGNs from 0–6 DIV. Right, effect of MEK5-DN lentivirus on BDNF stimulation of *Camkk2* and *Camk4* expression. (B) Effects of constitutively active CaN and NFATc4 on BDNF stimulation of *Camkk2* and *Camk4* expression. (C) Left panel, developmental changes in *Ntrk2* mRNA in maturing CGN cultures and in the developing mouse cerebellum. Middle and right panels, *Ntrk2* transcripts in CGN cultures expressing the NFI dominant repressor (NFI/EnR) or EnR control between 1 and 6 DIV, and in P15 *Nfia* knockout (*Nfia* KO) and wild-type (WT) mice. (D) *Ntrk2* transcripts in immature CGNs cultured with BDNF (0–2 DIV). ***, $^{###}P < 0.001$; ns, no significant difference. *** denotes comparisons with GFP controls, while ### is relative to GFP+BDNF values.

may prove relevant for neurodevelopmental disorders involving movement, speech, and other cognitive functions in which the cerebellum is increasingly implicated (Wang *et al.*, 2014).

MATERIALS AND METHODS

Animals and primary cultures

Cerebellar tissues were isolated from postnatal knockout mice and wild-type littermates with the following genetic backgrounds: 129/Sv and C57B/6J hybrids for *Etv1*(–/–) (Arber *et al.*, 2000), C57Bl/6 for *Nfatc4*(–/–) (Ding *et al.*, 2013), and C57Bl/6NTac for *Nfia*(–/–) (Shu *et al.*, 2003). Mouse CGNs were generated from postnatal day 6 (P6) CD1 mouse pups of either sex and cultured as previously described (Ding *et al.*, 2013, 2016; Selvakumar and Kilpatrick, 2013). Cells were plated at a density of 5×10^4 cells/cm^2 onto chamber slides or cell culture dishes coated with poly-D-lysine/laminin (Invitrogen) in Neurobasal medium (Invitrogen) containing B-27 serum-free supplement (50×; Invitrogen). The following reagents were added to culture medium and were refreshed on 3 DIV, where relevant: 50 ng/ml BDNF (EMD Millipore), 50 nm K252a (Alomone Labs, Jerusalem, Israel), 20 µM U0126 (Selleckchem), 10 µM BIX2189 (Selleckchem), brefeldin A (BFA) (Cell Signaling Technology), and vehicle control (dimethylsulfoxide [DMSO] or sterilized distilled H_2O). All experiments were performed using at least three biological replicates (cell preparations or cerebellar tissues obtained from distinct mice of a given strain/genotype). Procedures involving the handling of mice were in compliance with and approved by the relevant IACUC institutional guidelines and committees.

Plasmids and cell lines

Self-inactivating lentiviruses expressing green fluorescent protein (GFP), hemagglutinin (HA)-tagged NFI dominant repressor (NFI/EnR), or *Drosophila* engrailed repressor domain alone (EnR), constitutively active FLAG-tagged NFATc4 (NFATc4-Ala), or constitutively active calcineurin (CaN) were described previously (Wang *et al.*, 2004; Ding *et al.*, 2013, 2016). These plasmids are available upon request. MEK5-DN lentivirus was also described previously (Doebele *et al.*, 2009). Human embryonic kidney 293T cells (American Type Culture Collection) were grown in DMEM (Invitrogen) containing 10% heat-inactivated fetal bovine serum (Invitrogen).

Lentivirus preparation and transduction of primary CGNs

Lentiviruses were prepared via cotransfection of 293T cells with lentiviral expression plasmid, psPAX2, and pCMV-VSVG and were concentrated and titrated as before (Wang *et al.*, 2005; Ding and Kilpatrick, 2013). Titers were determined as described previously (Ding and Kilpatrick, 2013). CGN cultures were transduced on 0 DIV using a multiplicity of infection of ~3, yielding 80–90% cell transduction.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from tissues or cultured cells and cDNAs were prepared as described previously (Ding *et al.*, 2013, 2016). Real-time quantitative PCR (RT-qPCR) was performed in triplicate using primers, SYBR Green PCR master mix (Qiagen), and a StepOnePlus or ViiA 7 Real-Time PCR System (Applied Biosystems). Gene expression data were analyzed using the $2^{-\Delta Ct}$ method and then normalized to 18S rRNA as before (Ding *et al.*, 2013, 2016). Two or three technical replicates were used for each biological replicate value, and means were determined using three biological replicates. PCR primer sequences are available upon request.

ChIP assays

Nuclei were isolated from fixed tissues and cell cultures using either Percoll gradient centrifugation (Wang *et al.*, 2011; Ding and Kilpatrick, 2013) or differential centrifugation (1400 × *g* for 3 min, 4°C) in 0.25 M sucrose (Ding *et al.*, 2013, 2016). Chromatin was prepared as described previously (Ding *et al.*, 2013, 2016), and samples were assayed by real-time PCR as for RT-qPCR analyses. Results from triplicate biological replicates were expressed as the fold enrichment relative to antibody controls. ChIP antibodies were used for ETV1 (sc-28681; Santa Cruz Biotechnology), *Xenopus* NFIB1, which recognizes mammalian NFIA and NFIB (Ding *et al.*, 2013, 2016), NFIA

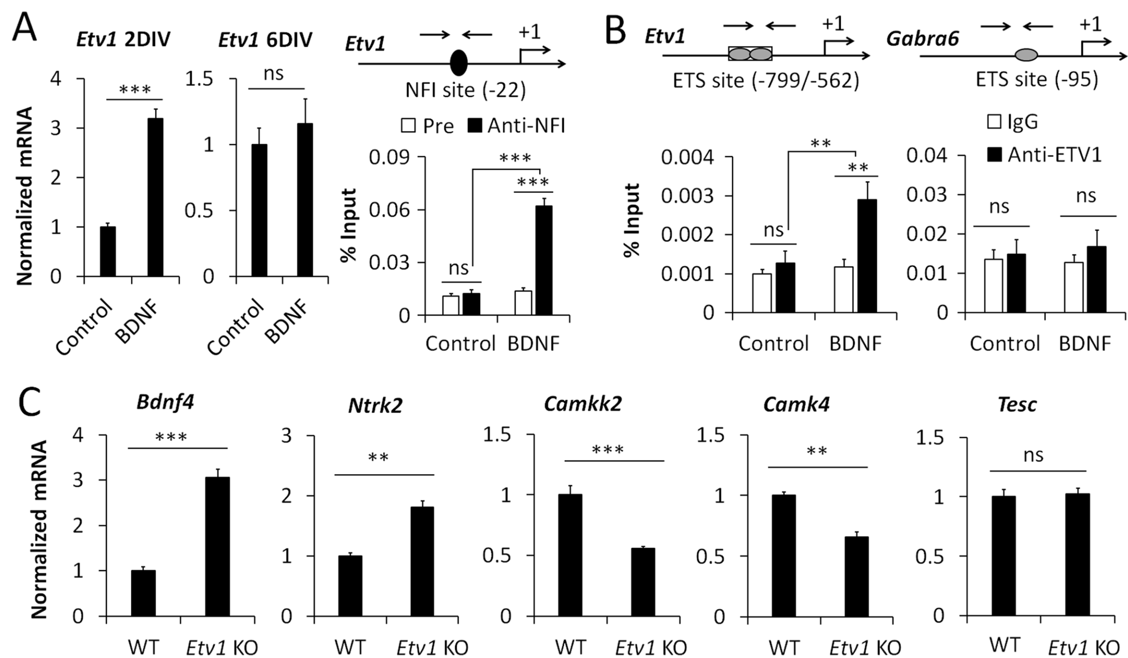

FIGURE 9: BDNF–*Etv1* Interactions in immature CGNs. (A) Left panel, *Etv1* gene expression in CGN cultures treated with BDNF from 0 to 2 DIV and from 0 to 6 DIV. Right panel, effect of BDNF on NFI occupancy of the *Etv1* promoter in immature (2-DIV) CGN cultures. xNFIB antibody was used for ChIP assays. (B) ChIP analysis of ETV1 binding to its own gene promoter and the *Gabra6* gene in immature CGN cultures treated with BDNF from 0 to 2 DIV. (C) Expression of BDNF signaling genes (*Bdnf4*, *Ntrk2*, *Camkk2*, *Camk4*) in P10 *Etv1* knockout (KO) and wild-type (WT) mouse cerebellum. *Tesc* was used as a negative control. ***$P < 0.001$; **$P < 0.01$; ns, no significance.

(39036; Active Motif), and NFATc4 (sc-1153; Santa Cruz Biotechnology). The term NFI is used to distinguish ChIP assays employing the *Xenopus* NFIB1 antibody from those specifically assaying NFIA occupancy. Note that the NFI and NFIA antibodies both detect binding to the same genomic regions in ChIP assays (Ding *et al.*, 2013, 2016). Preimmune serum or normal rabbit immunoglobulin G (IgG [PP64]; EMD Millipore) was used as a negative control antibody. PCR primer sequences for ChIP assays are available upon request.

Immunostaining

Immunofluorescence of neuronal cultures was carried out as in previous studies (Ding *et al.*, 2013, 2016). Briefly, neurons were cultured on chamber slides coated with poly-D-lysine/laminin (Invitrogen) and then fixed on 2 DIV with 4% paraformaldehyde in phosphate-buffered saline (PBS). For endogenous NFATc4 nuclear localization assays, fixed samples were heated at 95°C for 10 min in antigen retrieval buffer (100 mM Tris, 5% [wt/vol] urea, pH 9.5) before further processing. Cells were permeabilized with 1% Triton X-100 solution and incubated with 5% normal goat serum followed by primary antibodies at 4°C overnight and then incubated with conjugated secondary antibodies. Slides were coverslipped using either Antifade Gold (Invitrogen) or Fluor-Gel with TES buffer (Electron Microscopy Sciences). Primary antibodies used were anti-MAP2 antibody (AB5622; EMD Millipore), anti-NFATc4 (Santa Cruz Biotechnology, sc-13036), and anti-GM130 (BD Biosciences). 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI,1 μg/ml; Sigma-Aldrich) was used to stain nuclei. Fluorescence imaging was performed by either wide-field or confocal fluorescence microscopy using Leica DM IRE2 wide-field or Leica SP-5 and Nikon A1 confocal microscopes. Neurite length was measured with Image Pro Plus 6.0 software as previously described (Ding *et al.*, 2016). Extranuclear NFATc4 was quantified by counting immunostained foci, and data were expressed as percentages of counted cells. Lentiviral titrations were determined in infected K293T cells using antibodies against hemagglutinin (HA) (NFI/EnR and EnR; C29F4, Cell Signaling Technology), FLAG (NFATc4 proteins; M2 antibody, Sigma) or CaN (GeneScript), or by detection of GFP-expressing cells.

For tissue immunostaining, cerebella were dissected from P6 mouse pups and fixed, and sections were mounted onto slides (Gold plus) as described (Ding *et al.*, 2016). Slides were incubated in ~50 ml antigen retrieval buffer in a Coplan jar (10 mM citric acid, 2 mM EDTA, 0.05% Tween 20, pH 6.2; preheated to 95°C) and then cooled to room temperature and incubated in blocking buffer (5% normal goat serum, 1% bovine serum albumin [BSA], 0.1% Triton X-100, 0.02% sodium azide, 1× PBS). Sections were incubated with rabbit anti-NFATc4 (1:200) and/or mouse anti-GM130 (1:400) antibodies diluted in antibody dilution buffer (2% BSA, 0.1% Triton X-100, 0.02% sodium azide, 1× PBS), or the same buffer without added antibody, and incubated overnight at 4°C. Following washing, slides were incubated with goat anti-rabbit IgG-Cy3 (EMD Millipore) and goat anti-mouse IgGF(ab′)$_2$-alexa-fluor 488 secondary antibodies (Thermo Scientific; 1:1000). Slides were coverslipped using Fluoro-Gel with TES buffer and confocal fluorescence microscopy was performed using a Nikon A1 confocal microscope.

Means for quantitative immunostaining (neurite assays and extranuclear NFATc4) used three different biological replicates with an average of 120 individual cells counted for each replicate. Colocalization analyses are representative of two (Figure 7) or four (Figure 6) biological replicates. Experiments were blinded, with one individual setting up the experiments and another processing and analyzing the samples.

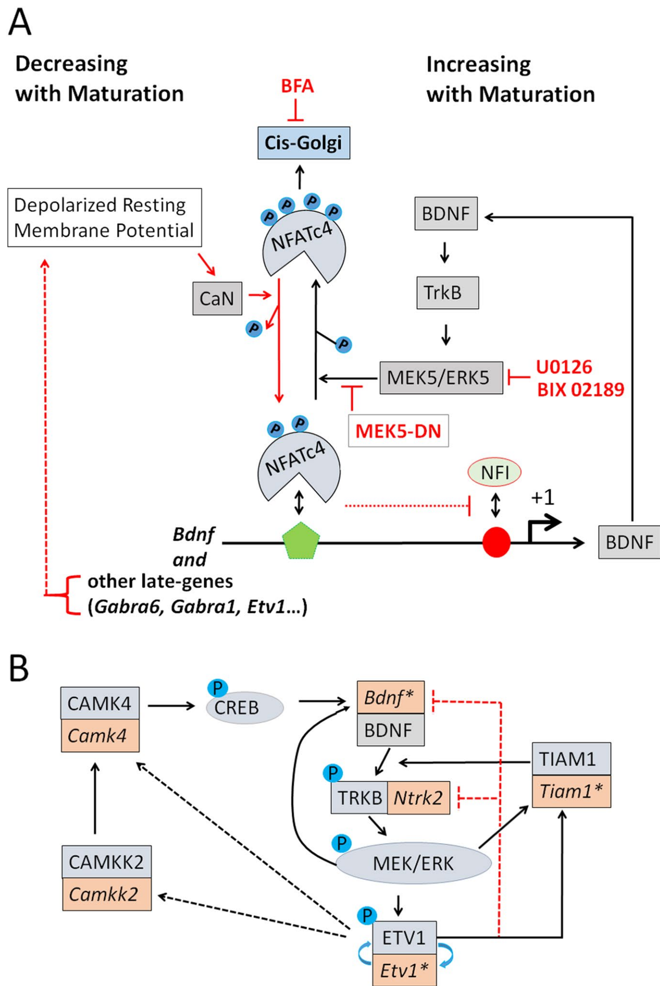

FIGURE 10: Proposed models for autoregulatory interactions within the NFI temporal occupancy program in maturing CGNs. (A) NFATc4 integrates signals from divergent pathways to control the timing of NFI occupancy of late genes in maturing postmigratory CGNs. A more depolarized resting membrane potential in immature CGNs promotes CaN activation and NFATc4 dephosphorylation, nuclear residence, and repression of late-gene promoters, including *Bdnf4*. BDNF/TrkB signaling counters this effect via MEK5/ERK5-dependent phosphorylation and nuclear exclusion of NFATc4 in the *cis*-Golgi. Maturation shifts the balance between these two pathways toward enhanced BDNF/TrkB and decreasing CaN activities. BDNF also may accelerate the reduction in CaN/NFATc4 activation by stimulating NFI late genes controlling CGN membrane potential and excitability. Red lines indicate inhibitory interactions. (B) A BDNF/ETV1/NFI-regulated local gene circuit in maturing CGNs. Genes are shown as open boxes, and their corresponding proteins are shaded in gray. Each gene is regulated by NFI (directly or indirectly), and asterisks highlight genes for which NFI temporal occupancy has been shown. Black lines indicate activating interactions within the gene circuit, and inhibition is shown with red lines. *Etv1* autoregulation is indicated by blue arrows. Dashed lines highlight ETV1 regulation which may be direct or indirect. Blue circles (P) indicate phosphorylated proteins.

Western analysis

Proteins from cultured CGNs were prepared with RIPA buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) containing protease inhibitors (454 Life Sciences). Samples were separated on 6% or 8% SDS–polyacrylamide gels and transferred onto pure nitrocellulose membranes (GE Water & Process Technologies) as previously described (Ding *et al.*, 2013, 2016). After blocking for 1 h, blots were incubated with primary antibodies at 4°C overnight and then with horseradish peroxidase–conjugated secondary antibody at room temperature for 1 h. Bound antibodies were detected with a chemiluminescent substrate (Thermo Scientific). Primary antibodies used were as follows: total NFATc4 (1:200; sc-130306; Santa Cruz Biotechnology), p-NFATc4 (Ser168/170; 1:100; sc-32630; Santa Cruz Biotechnology), and ACTIN (pan antibody-5; 1:3000; Thermo Scientific). Signal densities for different bands were measured using ImageJ software (National Institutes of Health) and data for NFATc4 proteins were expressed as percentages of ACTIN loading controls. Western data are representative of two biological replicates.

Statistics

Unpaired two-tailed Student's *t* tests were used to compare one experimental sample with its control. One-way analysis of variance was used when comparing multiple samples, with either Tukey (when comparing samples with each other) or Dunnett (when comparing samples to a control) post hoc tests. Results are expressed as mean ± SD of three biological replicates, and $P < 0.05$ was treated as significant.

ACKNOWLEDGMENTS

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