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 Bdnf 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.

INTRODUCTION

Neuronal development involves an intricate interplay between extrinsic and intrinsic mechanisms (Russ and Kalschmidt, 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...
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. NFIAC4 functions as a repressor that prevents premature NFI binding to target gene chromatin in immature CGNs (Ding et al., 2013). NFIAC4 activity is sustained in these immature cells via elevated calcineurin (CaN) activity, which promotes NFIAC4 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 CGNP s 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; Tatak e et al., 2008). We found that BIX2189, a selective inhibitor of MEK5/ERK5 (Tatak e 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
BDNF promotes CGN maturation via NFATc4

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
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...
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.

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 and 2 DIV (Figure 4E). Further, BDNF stimulation of Bdnf and Bdnf4 mRNAs was inhibited by lentiviral expression of constitutively active 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 Meremelstein, 2003; Groth et al., 2007). BDNF did not alter Nfat4 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.

(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 was localized 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
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 postmitotic 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 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 reproduce the significant NFI temporal occupancy of NFATc4 nuclear activity in CGNs within the cerebellum. We then examined the effect of BDNF on the temporal occupancy of NFATc4 in postnatal cerebellum, focusing on NFATc4 nuclear activity in CGNs at the time of NFATc4 dismissal from NFI late-gene promoters (Ding et al., 2013). 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 4:** (A) Bdnf and Bdnf4 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 Bdnf4 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.
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 NF1 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 NF1/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 auto-regulatory 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
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 MEKs/ERKs. 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 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.](image-url)
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 CNS development and gene expression (Smyrne and Goldowitz, 1989; Semyanov et al., 2004; Nakashima and Okawara, 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, Bdnf 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/autoocrine–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 Camk2 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 TIA1M drive endosome-dependent signaling by BDNF in maturing CGNs (Zhou et al., 2007), and NFI regulation of both of their gene 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 Ntkr2 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 (autoocrine–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/autoocrine, 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...
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), CS7B1/6 for Nfatc4(–/–) (Ding et al., 2013), and CS7B1/6NTac 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 H2O). 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). Lentiviruses were prepared via cotransfection of 293T cells with self-inactivating 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^(-ΔΔCT) method and mean values were determined using three biological replicates. 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. (C) Effects of constitutively active CaN and NFATc4 on BDNF stimulation of Ntrk2 transcripts in CGN cultures expressing the NFI dominant repressor (NFI/EnR) or EnR control between 1 and 6 DIV, and in P15 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. procedures involving the handling of mice were in compliance with and approved by the relevant IACUC institutional guidelines and committees.

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). *** and ***, ***, * are 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

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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.

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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...
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. (39036; Active Motif), and NFATc4 (sc-1153; Santa Cruz Biotechnology). The term NFI is used to distinguish ChIP assays employing the Xenopus NFB1 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 then 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 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.

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**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. xNFB1 antibody was used for ChIP assays. (B) ChIP analysis of ET1 binding to its own 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.
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 AC-TIN (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.

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