Temporal control of a dendritogenesis-linked gene via REST-dependent regulation of nuclear factor I occupancy

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ABSTRACT **Developing neurons undergo a series of maturational stages, and the timing of these events is critical for formation of synaptic circuitry. Here we addressed temporal regulation of the *Gabra6* gene, which is expressed in a delayed manner during dendritogenesis in maturing cerebellar granule neurons (CGNs). Developmental up-regulation of Gabra6 transcription required a binding site for nuclear factor I (NFI) proteins. The amounts and DNA binding activities of NFI proteins were similar in immature and mature CGNs; however, NFI occupancy of the Gabra6 promoter in native chromatin was temporally delayed in parallel with Gabra6 gene expression, both in vivo and in culture. The *trans*-repressor RE1 silencing transcription factor (REST) occupied the Gabra6 proximal promoter in CGN progenitors and early postmitotic CGNs, and its departure mirrored the initial onset of NFI binding as CGNs differentiated. Furthermore constitutive REST expression blocked both Gabra6 expression and NFI occupancy in mature CGNs, whereas REST knockdown in immature CGNs accelerated the initiation of both events. These studies identify a novel mechanism for controlling the timing of dendritogenesis-associated gene expression in maturing neurons through delayed binding of NFI proteins to chromatin. They also establish a temporal function for REST in preventing premature promoter occupancy by NFI proteins in early-stage postmitotic neurons.**

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INTRODUCTION

Development of postmitotic neurons occurs in specific stages, including axon extension, migration, dendritogenesis, and synaptogenesis. Elaboration of these events requires that numerous genes be turned on and off with precise timing and sequence. Disrupted temporal function can impact synaptic connectivity (Campbell *et al.*, 2007; Di Cristo *et al.*, 2007; Petrovic and Hummel, 2008), and altered timing of neuronal development and synaptic maturation have been implicated in autism (Geschwind and Levitt, 2007) and anxiety (Ansorge *et al.*, 2004). Temporal disruption of neurodevelopment may therefore contribute to multiple CNS disorders. How temporal programming of gene expression in postmitotic maturing neurons is coordinated remains largely unexplored.

Cerebellar granule neurons (CGNs) play an important role in the flow of information between cerebellar inputs and outputs via synaptic interactions with mossy fibers and Purkinje neurons, respectively (Medina *et al.*, 2000; D'Angelo and De Zeeuw, 2009). They undergo a well-defined program of differentiation in which specific gene subsets are expressed with distinct temporal patterns (Goldowitz and Hamre, 1998). Postnatally, CGN progenitors (CGNPs) proliferate within the outer portion of the external germinal layer (EGL). They then exit the cell cycle and initiate differentiation in its inner portion (premigratory zone [PMZ]). Within the PMZ, immature CGNs extend bipolar axons (parallel fibers), and subsequently the CGN soma migrates radially to form the internal granule cell layer

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Abbreviations used: CGN, cerebellar granule neuron; CGNP, cerebellar granule neuron progenitor; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; DIV, days in vitro; EGL, external germinal layer; GABAergic, γ-aminobutyric-acid–releasing; IGL, internal granule cell layer; NFI, nuclear factor I; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; Pol II, RNA polymerase II; PMZ, premigratory zone; REST, RE1 silencing transcription factor.

(IGL). The final phase of CGN maturation then ensues with the formation of dendrites and synaptic connections with excitatory mossy fibers and inhibitory, γ-aminobutyric-acid–releasing (GABAergic) terminals from Golgi type II neurons.

Within the cerebellum, the $GABA_A$ receptor α6 subunit (Gabra6) is selectively expressed in granule neurons (Kato, 1990; Laurie *et al.*, 1992a). Gabra6-containing receptors are preferentially localized to extrasynaptic sites (Nusser *et al.*, 1998) where they mediate GABA-induced tonic regulation of CGN excitability (Rossi and Hamann, 1998). Gabra6 is developmentally expressed in postmigratory CGNs in the IGL with a temporal delay, increasing during the second through fourth postnatal weeks in the mouse (Zheng *et al.*, 1993; Varecka *et al.*, 1994; Mellor *et al.*, 1998). Its expression is coincident with the appearance of GABAergic synapses and tonic inhibition of CGNs within the IGL (Brickley *et al.*, 2001; Takayama, 2005). Gabra6 temporal expression appears to be largely intrinsically determined (Gao and Fritschy, 1995; Lin and Bulleit, 1996), suggesting the operation of an internal timing mechanism (Mellor *et al.*, 1998; Jones *et al.*, 2000).

The nuclear factor I (NFI) family of transcription factors (*NFIa, b, c, x*) are important for CNS development in mice and humans (das Neves *et al.*, 1999; Shu *et al.*, 2003; Steele Perkins *et al.*, 2005; Deneen *et al.*, 2006; Driller *et al.*, 2007; Wong *et al.*, 2007; Campbell *et al.*, 2008; Kumbasar *et al.*, 2009; Piper *et al.*, 2009). NFI proteins are expressed throughout CGN postmitotic development, where they control axon extension, migration, and dendritogenesis (Wang *et al.*, 2004, 2007; Kilpatrick *et al.*, 2010). They also are required for expression of the *Gabra6* gene via direct binding to its proximal promoter (Wang *et al.*, 2004). Here we have examined the role of NFI proteins in the timing of Gabra6 expression in maturing CGNs.

RESULTS

NFI is involved in Gabra6 temporal regulation

Purified CGNs isolated from the early postnatal mouse cerebellum are selectively derived from the EGL/PMZ and consist of a mixture of CGNPs as well as postmitotic CGNs expressing early differentiation markers (Raetzman and Siegel, 1999; Solecki *et al.*, 2001; Diaz *et al.*, 2002; Manzini *et al.*, 2006). On plating, dissociated cultures rapidly become postmitotic and then express an intrinsic differentiation program over ~1 wk (Lin and Bulleit, 1996; Powell *et al.*, 1997; Raetzman and Siegel, 1999; Manzini *et al.*, 2006). Consistent with previous results (Lin and Bulleit, 1996), purified CGN cultures exhibited a temporal delay in Gabra6 gene expression similar to that observed in vivo, reaching a peak at ~6 d in vitro (DIV) (Figure 1A). This finding indicated that the timing of Gabra6 expression was subject to intrinsic control in CGNs, as previously suggested (Mellor *et al.*, 1998; Jones *et al.*, 2000).

A 6-kb mouse Gabra6 promoter region was previously shown to reproduce temporally delayed expression of Gabra6 when delivered by lentivirus into CGN cultures (Wang *et al.*, 2005). In this case, the promoter is integrated into host cell DNA and thus is sensitive to chromatin-dependent mechanisms. NFI proteins bind in vivo to a consensus site located within the Gabra6 proximal promoter, and this site is required for expression of the 6-kb Gabra6 promoter in mature CGNs (Wang *et al.*, 2004). We therefore tested the importance of the proximal NFI site by its mutation within the 6-kb promoter lentivirus. This mutation abolished temporal up-regulation of Gabra6 promoter activity in differentiating CGN cultures after either 4 or 7 DIV (Figure 1B). This finding suggested the potential involvement of NFI proteins in the Gabra6 timing mechanism.

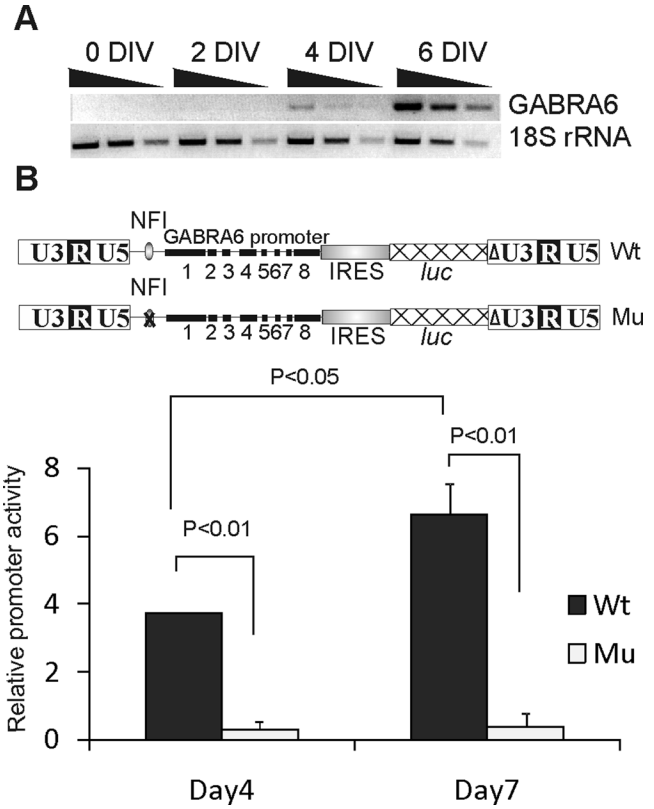

FIGURE 1: Developmental up-regulation of Gabra6 gene expression requires NFI transactivation. (A) Time course for Gabra6 mRNA in differentiating CGN cultures using semiquantitative RT-PCR. (B) Top panel, lentiviral constructs expressing wild-type (Wt) and NFI site-mutant (Mu) Gabra6 promoters. Bottom panel, luciferase activities of lentiviral promoter vectors following transduction of CGN cultures on 0 DIV.

Previous work showed that NFI proteins are expressed in CGNs throughout their development and that they regulate genes expressed both in early and late postmitotic stages (Wang *et al.*, 2007, 2010). Western analysis showed that the amounts of NFI proteins in nuclear extracts were similar during CGN maturation in culture and in the mouse cerebellum at early (day 6 [P6]) and later (P21) postnatal ages (Figure 2A), when in vivo Gabra6 expression is low or robust, respectively (Varecka *et al.*, 1994; Lin and Bulleit, 1996). Furthermore gel shift analysis showed similar amounts of DNA binding activity for the Gabra6 NFI site during development in both CGN cultures and the postnatal mouse cerebellum (Figure 2B). Thus the in vitro DNA binding activities and amounts of NFI proteins were not in temporal accord with Gabra6 expression. Phosphorylation of NFI proteins has been linked to decreased expression of NFI target genes but unaltered in vitro DNA binding activity (Yang *et al.*, 1993; Bisgrove *et al.*, 2000). Phosphatase treatment, however, did not alter the migration of NFI proteins in freshly prepared CGN nuclear extracts (unpublished data).

Transcription factor binding and activity also are sensitive to changes in chromatin interactions and structure in vivo. We therefore examined NFI association with the Gabra6 proximal promoter during CGN differentiation. As previously shown (Wang *et al.*, 2004), chromatin immunoprecipitation (ChIP) assays detected strong NFI occupancy of the Gabra6 proximal promoter region in mature (6-DIV) CGN cultures (Figure 2C). Although NFI DNA binding activity is robust in immature CGN nuclei, NFI binding to the Gabra6 proximal

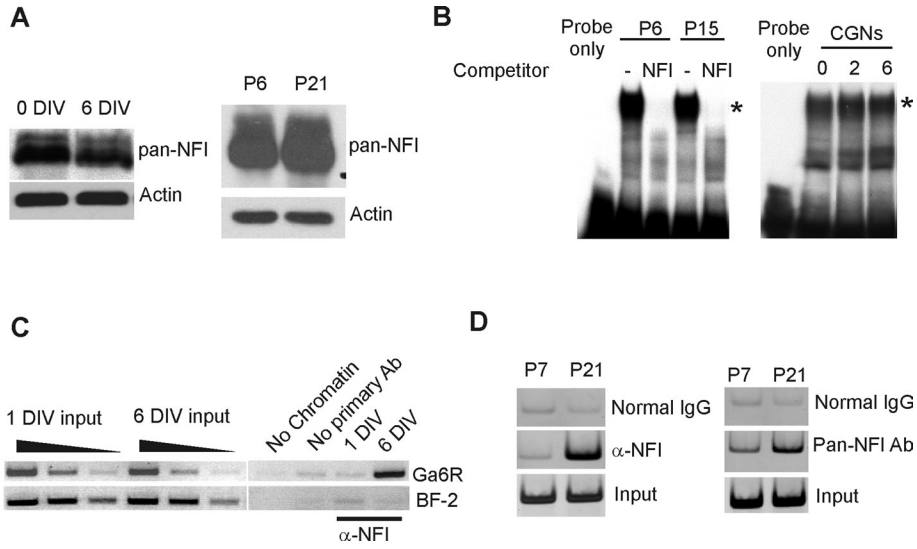

FIGURE 2: Temporal patterns of expression for NFI proteins and their DNA binding in CGNs maturing in culture and in vivo. (A) Western analysis of nuclear NFI proteins in immature (0 DIV) and mature (6 DIV) CGN cultures and P6 and P21 mouse cerebellum. Nuclear β-actin served as a loading control. (B) Gel shift assays of NFI binding to the Gabra6 promoter site in P6/P15 cerebella and in maturing CGN cultures (0, 2, and 6 DIV). *NFI–DNA complexes. (C) ChIP assay of NFI binding to the proximal Gabra6 promoter in chromatin from 1- and 6-DIV CGN cultures. Triangles indicate threefold serial dilution of input DNA. BF2 genomic sequences served as a genomic sequence negative control. (D) ChIP assay of NFI occupancy in P7 and P21 cerebellar nuclei using two different NFI antibodies.

region was undetectable in immature CGNs on 1 DIV (Figure 2C). Thus NFI occupancy of the Gabra6 promoter was subject to a temporal delay in maturing CGNs.

Because Gabra6 expression is restricted to granule neurons in the developing mouse cerebellum, we also performed ChIP assays using chromatin from postnatal cerebellar nuclei that had been fixed in situ and then purified using Percoll gradients. We initially characterized in situ–fixed nuclei using immunostaining for NeuN, a marker for postmitotic granule neurons in the developing mouse cerebellum (Weyer and Schilling, 2003). Percoll preparations were highly enriched in CGN nuclei at each age (~80% at P7; >95% at P21). Furthermore the majority of NeuN(+) nuclei in the postnatal mouse cerebellum derive from CGNs within the IGL (Weyer and Schilling, 2003). Thus NeuN(+) CGNs from the PMZ presumably contribute only a small fraction to chromatin from P7 cerebellar nuclei (the EGL/PMZ is largely absent by P15–P21).

We further confirmed the minor contribution by PMZ-derived CGNs to cerebellar nuclei using RNA polymerase II (Pol II) occupancy studies. Nhlh1 is highly expressed in immature CGNs within the mouse PMZ (Duncan *et al.*, 1997) and is transiently detected at lower levels in the early postnatal IGL (Uittenbogaard *et al.*, 1999; Kruger and Braun, 2002). In contrast, F3 is predominantly expressed by mouse CGNs within the IGL but not in the postnatal EGL/PMZ (Virgintino *et al.*, 1999; Bizzoca *et al.*, 2003). Pol II occupancy of the *Nhlh1* gene promoter was robust in freshly isolated CGNPs/CGNs which derive from the EGL/PMZ but was barely detectable in P7 mouse cerebellar nuclei (Supplemental Figure S1). The inverse pattern was observed for Pol II ChIP of the IGL marker *Cntn1*/F3 gene (Figure S1). Thus Percoll-purified mouse cerebellar nuclei at P7 and later ages derive mainly from postmigratory CGNs at varying stages of maturation within the IGL.

ChIP analysis of cerebellar nuclei fixed in situ revealed that NFI occupancy of the proximal Gabra6 promoter increased developmentally (Figure 2D), in accordance with Gabra6 gene expression. Similar temporal patterns were observed using two different NFI antibodies. In summary, at both P6 and P7 NFI occupancy of the Gabra6 promoter is low in both CGNPs/CGNs from the EGL/PMZ as well as in immature postmigratory CGNs in the IGL. It then increases as CGNs further differentiate within the IGL during the next 2 wk along with Gabra6 expression. These results directly implicated NFI in developmental control of Gabra6 expression in CGNs maturing in vivo through its delayed binding to chromatin. Furthermore this temporal process was accurately mirrored in differentiating primary CGN cultures.

Epigenetic changes within the Gabra6 proximal promoter

Several mechanisms could account for temporally delayed NFI occupancy, including binding site occlusion or inhibition involving chromatin structure, DNA methylation, and/or locally bound repressors. We therefore examined temporal changes in epigenetic events within the Gabra6 promoter region containing the NFI site. DNA accessibility assays were performed using the restriction enzyme *Hin*fI, which cuts adjacent to the proximal NFI site (Figure 3A). This genomic segment was largely resistant to restriction enzyme cleavage in EGL/PMZ-derived CGNPs/CGNs (Figure 3A). This proximal region was highly accessible, however, in differentiated (6-DIV) neurons (Figure 3A). Furthermore several histone marks linked to gene activation were up-regulated in maturing CGN cultures, including acetylated histone H3 and H4 and trimethylated H3K4 (Figure 3B). In contrast, the repression-associated mark methyl-H3K9 did not vary significantly with CGN differentiation (Figure 3B). H4 histone acetylation within the Gabra6 proximal promoter region also increased between P7 and P21 in the mouse cerebellum (Figure 3C).

A more detailed temporal analysis revealed a close correlation between the onset of H4 acetylation and NFI occupancy in maturing CGNs. Both events markedly increased between 3 and 6 DIV (Figure 3D), in a pattern that mirrored the timing of Gabra6 expression (see Figure 1A). Together these findings indicated that access to the NFI binding region increased as CGNs matured and chromatin remodeling closely paralleled the gradual onset of NFI occupancy and Gabra6 gene activation. In contrast, methylation-sensitive single nucleotide primer extension revealed low methyl-C at two CpGs adjacent to the Gabra6 NFI site that did not vary significantly during CGN differentiation (Supplemental Figure S2). Thus methylation of local CpG sites does not apparently contribute to temporal binding of NFI to the *Gabra6* locus.

Identification of a potential repressor of NFI binding

Locally acting repressors and/or activators can regulate transcription factor binding to chromatin, either directly or via regulation of chromatin structure. We therefore used gel shift assays to determine the binding of nuclear proteins to a ~200-base-pair segment of the Gabra6 proximal promoter region that spans the NFI site (A region; Figure 4A). Lower percentage gels were used to provide better resolution of complexes bound to this larger DNA probe. Three sets of complexes were detected in nuclear extracts of freshly prepared

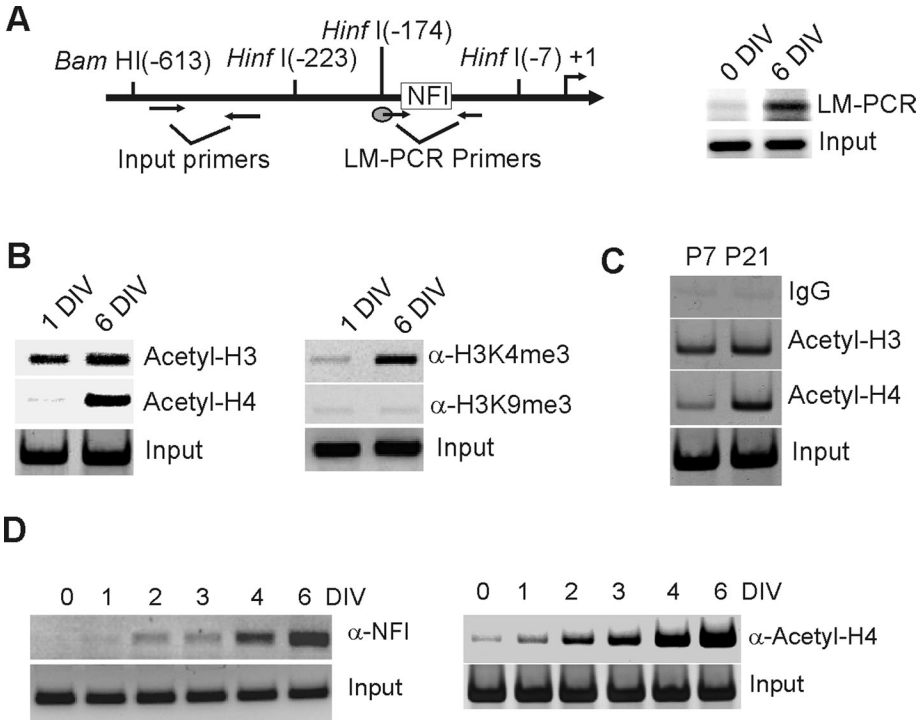

FIGURE 3: Epigenetic changes associated with Gabra6 temporal expression. (A) DNA accessibility of the Gabra6 NFI site in 0- and 6-DIV CGNs. Nuclei were digested with *Hin*fI and then assayed using LM-PCR primers. Input primers were used for normalization. (B) H3 and H4 histone acetylation (left panels) and H3K4 and H3K9 trimethylation (right panels) within the Gabra6 A region in immature and mature CGN cultures. (C) Changes in Gabra6 histone acetylation in the developing postnatal cerebellum. (D) Temporal coordination of NFI occupancy and H4 acetylation of the Gabra6 A region in maturing CGNs.

CGNPs/CGNs: an intermediate set corresponding to NFI complexes as well as those with slower or faster mobility (Figure 4B). These complexes were specifically depleted by an excess of unlabeled A region sequences. Significantly, binding activity for the slowly migrating doublet set of complexes was temporally down-regulated as CGNs matured (Figure 4C). The binding time course was the inverse of that for onset of NFI binding and activation of Gabra6 expression (see Figures 1A and 3D). This finding suggested that these slower complexes might function as temporal repressors of Gabra6 activation and possibly NFI occupancy. They were tentatively termed putative repressor complexes.

Attempts to localize binding sites for the putative repressor complexes using gel shift competition with smaller fragments of the A region proved unsuccessful (unpublished data) possibly because of cooperative protein binding to multiple sites. We therefore took a candidate approach for identifying the nature of putative repressor complex protein(s). The RE1 silencing transcription factor (REST or NRSF) is a *trans*-repressor of neuronal genes (Chong *et al.*, 1995; Schoenherr *et al.*, 1996) that is expressed in certain neural progenitors and undergoes subsequent down-regulation as neurons mature (Schoenherr and Anderson, 1995; Ballas *et al.*, 2005). It is also a relatively large protein that migrates as a slowly moving DNA–protein complex in gel shifts, sometimes as a doublet (Shimojo *et al.*, 2001). Supershift assays were therefore performed to determine whether REST was present in the putative repressor complexes. Inclusion of REST antibodies disrupted these complexes in supershift assays of CGNPs/CGNs (Figure 4D). Furthermore ChIP analysis using two different antibodies showed that REST occupied the Gabra6 proximal promoter in these cells, and this association was specifically localized to the A region (Figure 4E). Thus REST appeared to be a component of the putative repressor complexes and it occupied the Gabra6 A region in CGNPs/CGNs derived from the EGL/PMZ.

Interestingly, bioinformatic analysis did not detect RE1-related REST binding sequences (Johnson *et al.*, 2007; Otto *et al.*, 2007), including half-sites, within or closely adjacent to the A region. Consistent with this finding, gel shift assays revealed that RE1 sequences did not compete for binding of the putative repressor complexes to an A region probe (Figure 4F). Furthermore REST protein that was overexpressed in K293 cells bound to an RE1 probe but not to the A region (unpublished data). Thus REST occupancy of the A region apparently involves interactions other than direct binding to RE1-related sequences. Previous studies have demonstrated REST occupancy of genomic regions lacking RE1-like sites (Otto *et al.*, 2007), which may reflect REST interactions with other DNA binding proteins.

REST occupancy declines as CGNs differentiate

We next performed ChIP studies to examine the involvement of REST in Gabra6 temporal control. REST occupancy of the Gabra6 A region declined markedly in 6-DIV CGN cultures relative to CGNPs/CGNs derived from the EGL/PMZ (Figure 4G). More detailed analysis revealed that REST gradually departed the A region during early maturation of CGNs (Figure 4H). Substantial REST binding was still evident in 1- and 2-DIV cultures, when most CGNs are postmitotic (Wang *et al.*, 2005; Manzini *et al.*, 2006). Importantly, REST departure closely mirrored the temporal pattern for decreased putative repressor complex DNA binding activity and preceded the onset of NFI occupancy and Gabra6 expression (Figures 4H, 1A, and 3D). This result suggested the involvement of REST occupancy in Gabra6 temporal control.

Further studies were performed to address the timing of REST occupancy of the Gabra6 proximal promoter in vivo, in particular its persistence and decline in postmitotic CGNs. REST association with chromatin was determined in premigratory, postmitotic CGNs purified by cytometric sorting of NeuN(+) EGL/PMZ cells from P6 mice. ChIP analysis showed that REST occupancy of the proximal Gabra6 promoter persisted in these premigratory CGNs (Figure 5). In contrast, the amount of specific REST occupancy of the Gabra6 proximal promoter was markedly lower in P7 mouse cerebellar nuclei (Figure 5), which derive mainly from CGNs within the IGL (see earlier in the text). Thus REST is substantially dismissed from the proximal A region in CGNs within the IGL at P7, when onset of NFI occupancy and Gabra6 expression begin to increase (see Figure 2E).

REST regulates Gabra6 expression, NFI binding, and chromatin structure

To directly test REST function in Gabra6 gene regulation, we transduced CGN cultures with a REST lentiviral expression vector. Constitutive REST expression strongly inhibited Gabra6 expression in mature (6-DIV) CGNs at both the mRNA and protein levels (Figure 6, A

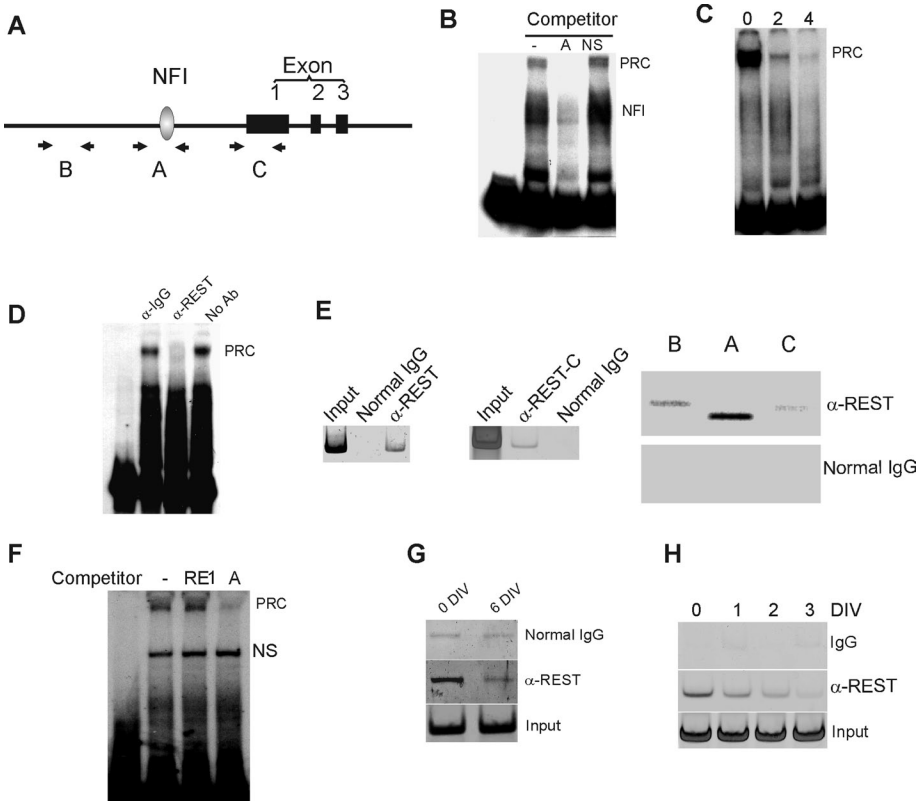

FIGURE 4: REST associates with the Gabra6 proximal promoter in a temporal manner. (A) Location of the A region spanning the NFI site within the Gabra6 proximal promoter. PCR primers used for REST ChIP assays in (E) are shown by arrows. (B) Gel shifts of 0-DIV CGN nuclear extracts using the 200-base-pair A region as probe. Competitors (100×) used were A region (A) and unrelated 200-base-pair genomic sequences generated by PCR (NS); (–) no competitor. Positions of relevant complexes are indicated. (C) Gel shift assay of the putative repressor complexes in maturing CGNs (0, 2, and 4 DIV). (D) Supershift using REST antibodies showing disruption of the putative repressor complexes in 0-DIV CGN extracts. (E) ChIP assay for REST occupancy of the Gabra6 A region in chromatin from 0-DIV CGNs using mouse monoclonal (left) and rabbit polyclonal (middle) anti-REST antibodies. ChIP tiling confirmed that REST occupancy was enriched within the A region (right panel; see panel A for PCR primer locations). Equal amounts of the same input DNA were used for each primer set. (F) Competitive gel shifts using the A region probe and 100× excess of either unlabeled A region or RE1 sequences. NS, nonspecific complex. (G) ChIP assay of REST occupancy of the A region in 0- and 6-DIV CGNs. (H) ChIP time course showing that REST gradually departs the A region as CGNs mature. Monoclonal antibodies were used in G and H.

and B). This effect was not due to a generalized repression of differentiation-related gene expression as REST had no significant effect on transcripts for Gabra1 (unpublished data), another $GABA_A$ receptor subunit that is up-regulated in maturing CGNs (Laurie *et al.*, 1992b). To address whether REST regulates Gabra6 transcription, we performed promoter cotransduction studies using Gabra6 promoter lentiviruses in CGN cultures. Coexpression of REST strongly repressed reporter activity from the 6-kb Gabra6 promoter lentiviral vector (see Figure 1B) in 6-DIV CGNs relative to a GFP expression vector (Figure 6C).

Previous studies showed that Gabra6 proximal promoter sequences spanning the A region are expressed well in transiently transfected CGNs (McLean *et al.*, 2000). We therefore generated a lentiviral vector containing A region sequences from the mouse Gabra6 promoter to test REST repression of this specific promoter segment. Promoter activity was assayed 48 h following transduction to better assess the direct effects of REST. REST strongly inhibited expression from the Gabra6 proximal promoter but had negligible effect on transcription driven by a cytomegalovirus (CMV)–luciferase promoter vector (Figure 6D and unpublished data). Thus the proximal A promoter region is sufficient to mediate REST repression of Gabra6 transcription.

On the basis of REST occupancy of the Gabra6 proximal promoter in P6 CGNPs/CGNs, we next examined its impact on NFI binding to this genomic region. Constitutive REST expression in differentiated 6-DIV CGNs inhibited NFI occupancy of A region chromatin (Figure 6E). This result was not an indirect effect on the expression of NFI proteins in transduced CGNs (Supplemental Figure S3). Furthermore expressed REST occupied the A region in transduced CGNs on 6 DIV (Figure 6F), when endogenous REST occupancy is normally low (see Figure 4G). Constitutive REST expression also markedly inhibited H4 acetylation of the Gabra6 proximal promoter region in mature CGNs (Figure 6G). Thus REST occupancy of the A region is linked to inhibition of Gabra6 promoter activity and endogenous gene expression, NFI binding, and changes in promoter chromatin structure.

REST knockdown accelerates both Gabra6 expression and NFI binding

REST departure from the proximal A region inversely mirrors the onset of Gabra6 expression as well as NFI occupancy of the local promoter (see Figures 1A, 3D, and 4G). This finding suggested that endogenous REST may actively determine the timing of these events in maturing CGNs. To address this possibility, an shrna-expressing lentiviral vector was identified that suppressed mouse REST protein in CGNPs (Figure 7A). Proliferating CGNPs were quantitatively transduced with REST or scrambled shrnas on 0 DIV and

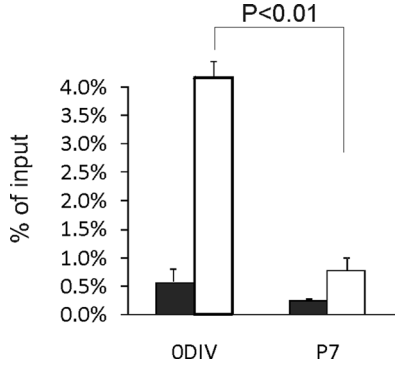

FIGURE 5: REST occupancy of the Gabra6 proximal promoter in CGNs maturing within the cerebellum. REST association with the A region was determined by ChIP/qPCR assays in premigratory CGNs purified by cytometric sorting of NeuN(+) EGL/PMZ-derived P6 cerebellar cells (0 DIV) and in CGN nuclei isolated from the P7 mouse cerebellum that mainly contain postmigratory CGN nuclei (see text). Shaded bars, antibody control; open bars, REST antibody.

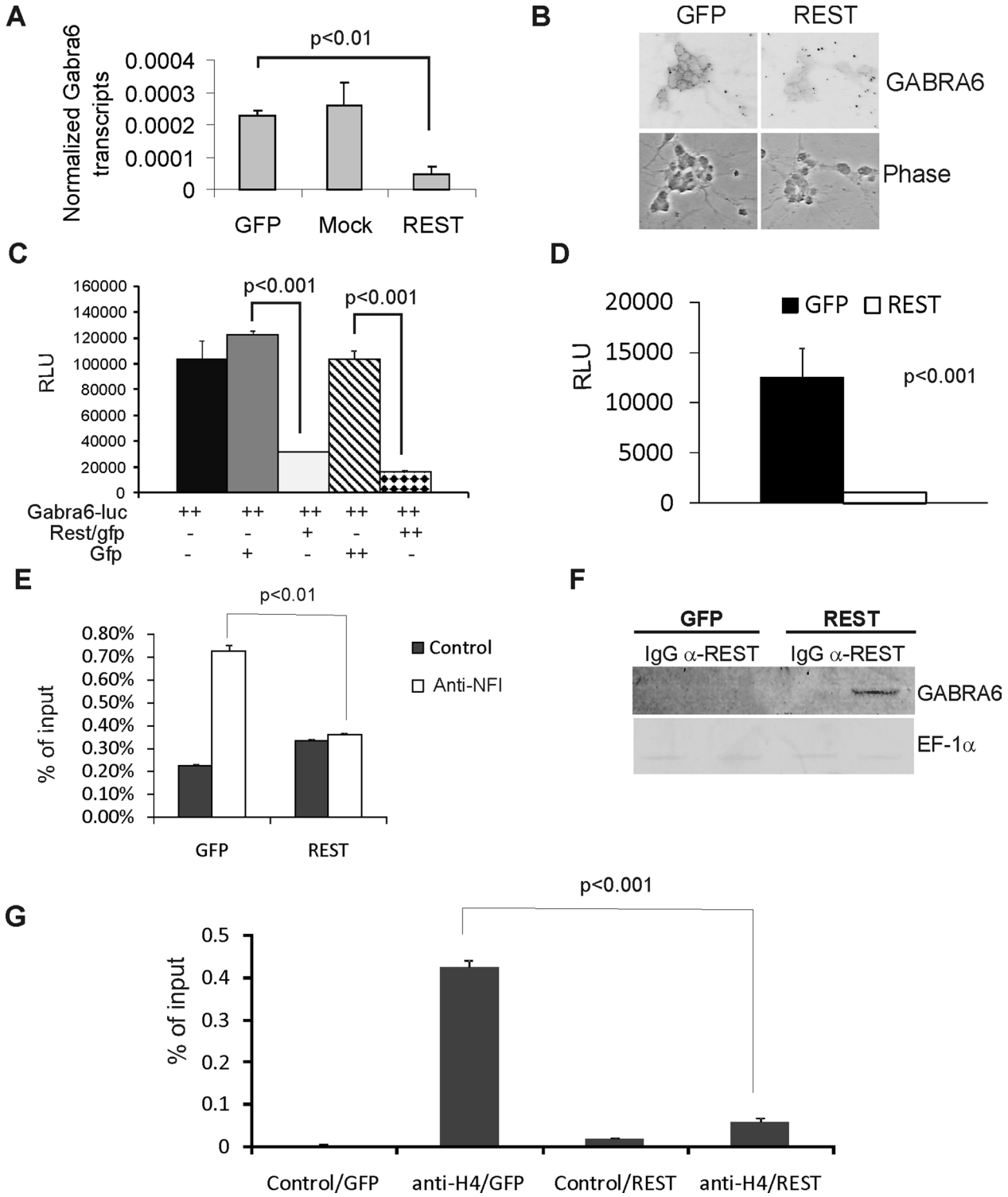

FIGURE 6: Constitutive REST expression inhibits Gabra6 transcription and NFI occupancy. (A and B) CGNs were infected with REST- or GFP-expressing lentiviruses on 0 DIV and examined for Gabra6 expression on 6 DIV by semiquantitative RT-PCR (A) or indirect immunofluorescence (B), respectively. (C) CGNs were coinfected with the 6-kb Gabra6 promoter and either REST or GFP lentiviruses on 0 DIV at protein/promoter lentivirus ratios of 1:3 (+/++) or 1:1 (++/++). Luciferase activity (RLU) was determined on 6 DIV. (D) REST or GFP/promoter coinfections (1:1) were performed as in (C) using a 0.2-kb A region Gabra6 promoter, and luciferase activity was assayed on 2 DIV. (E) Cultures were transduced on 0 DIV with REST or GFP lentivirus, and NFI occupancy of the Gabra6 A region was determined by ChIP/qPCR on 6 DIV. (F) REST ChIP of 6-DIV CGN cultures following infection with REST or GFP lentivirus on 0 DIV. EF1α genomic sequences and anti–mouse IgG served as negative controls. (G) Cultures were transduced with REST or GFP lentiviruses on 0 DIV, and acetylated H4 within the Gabra6 A region was determined by ChIP/qPCR on 6 DIV.

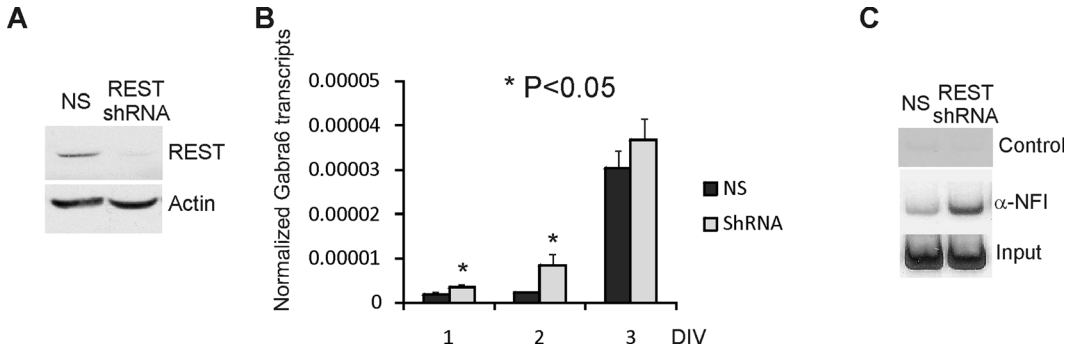

FIGURE 7: Knockdown of REST accelerates the onset of Gabra6 expression and promoter occupancy by NFI. (A) Western analysis of REST protein in CGNPs that were transduced with lentiviruses expressing scrambled (NS) or REST shrnas and cultured with sonic hedgehog for 2 d. (B) Sonic hedgehog–treated CGNPs were transduced with control (NS) or REST shrnas as in (A). Gabra6 mRNA was determined on subsequent days following removal of sonic hedgehog (DIV post–sonic hedgehog) by qRT-PCR. Data are from a representative experiment that was performed independently three times. (C) Following transduction of CGNPs with nonspecific control (NS) and REST shrnas and culturing with sonic hedgehog for 2 d, sonic hedgehog was removed and NFI occupancy of the Gabra6 A region was determined 2 d later.

maintained for 2 d in the presence of the mitogen sonic hedgehog (Wechsler-Reya and Scott, 1999) to allow for efficient REST knockdown prior to differentiation. REST shrna did not alter CGNP proliferation (bromodeoxyuridine incorporation) or onset of the differentiation marker NeuN (Supplemental Figure S4).

Following withdrawal of sonic hedgehog and initiation of differentiation, Gabra6 was expressed with a typical delay between 2 and 6 DIV in CGNs transduced with scrambled shrna (Figure 7B). In contrast, REST knockdown induced a more rapid onset of Gabra6 expression that was already evident 1 d after sonic hedgehog removal and was elevated fourfold relative to control shrna at 2 d (Figure 7B). This effect of REST knockdown resulted in a temporal shift in Gabra6 expression from a strongly delayed pattern to one with a greatly reduced lag. This stimulation was largely over by 3 DIV (Figure 7B) and at later stages (unpublished data). Thus endogenous REST appears to preferentially inhibit the onset of Gabra6 up-regulation, providing an initial "brake" to prevent premature expression in early differentiating CGNs.

Because local chromatin occupancy by NFI was directly implicated in the timing of Gabra6 expression, we asked whether REST knockdown would also enhance this process in immature CGNs. REST shrna markedly elevated NFI binding to the A region in CGNs relative to scrambled shrna-treated cultures 2 d after removal of sonic hedgehog (Figure 7C). Thus endogenous REST is directly implicated in the temporal onset of Gabra6 expression by delaying NFI binding to chromatin. These findings also further support a central role for local NFI occupancy in controlling the timing of Gabra6 expression.

Temporal expression of REST in maturing CGNs

Because REST departure was a critical factor in the temporal control of NFI occupancy and Gabra6 expression, we examined the developmental expression of this *trans*-repressor. REST transcripts were down-regulated in differentiating CGN cultures (Figure 8A), with full-length REST mRNA predominating in CGNPs and maturing CGNs and REST4 mRNA present at only low levels (unpublished data). Also, full-length REST protein (~190–200 kDa) was detected in freshly isolated P6 CGNPs/CGNs and dramatically declined in differentiated CGNs (Figure 8B). Immunodetection of REST protein in fixed postnatal cerebellar tissue gave inconsistent results using available antibodies. We therefore confirmed REST protein expression in immature CGNs freshly prepared from P6 mouse cerebellum by immunocytochemistry, using NeuN as a marker for postmitotic CGNs. REST protein was detected in ~90% of freshly isolated P6 CGNPs/CGNs, and >90% of NeuN(+) cells were also REST(+) (Supplemental Figure S5). Thus REST expression persists in early postmitotic CGNs derived from the PMZ. Down-regulation of REST expression presumably contributes to its gradual departure from the Gabra6 promoter during later CGN differentiation.

DISCUSSION

Gabra6-containing receptors predominate in mature CGNs (Quirk *et al.*, 1994) and play an important role in cerebellar information processing and storage via GABA-induced tonic inhibition (Rossi and Hamann, 1998; Hamann *et al.*, 2002). Furthermore temporal up-regulation of Gabra6 expression within the IGL coincides with the development of GABAergic synapse formation, tonic $GABA_A$ receptor–mediated conductance, and motor control and learning (Brickley *et al.*, 1996; Wall and Usowicz, 1997; Takayama, 2005). Thus its proper temporal expression is important for the development of mature $GABA_A$ responses of CGNs and, in turn, for information processing within the developing and adult cerebellum. Our findings directly implicate delayed promoter occupancy by NFI proteins in the timing of Gabra6 expression in developing CGNs.

Onset of Gabra6 expression within the IGL has been attributed to a cell-intrinsic "timer" that is initiated by cell cycle exit of CGNPs within the EGL (Mellor *et al.*, 1998; Jones *et al.*, 2000). This hypothesis is supported by our present findings. REST is expressed in CGNPs and immature CGNs and is an important regulator of the timing of NFI occupancy and Gabra6 expression. In particular, REST departure appears to be a critical determinant of the initial onset of NFI binding

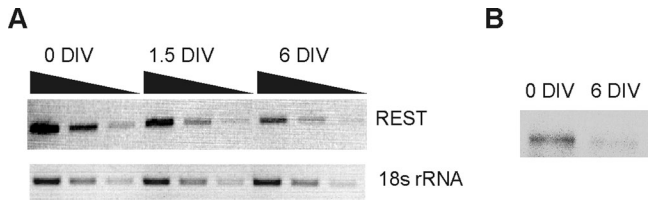

FIGURE 8: REST expression is down-regulated in maturing CGNs. (A) Time course for REST mRNA in CGN cultures using RT-PCR. Triangles: threefold serial dilutions. (B) REST Western analysis in immature and mature CGNs.

to chromatin and of Gabra6 expression: Persistent REST expression inhibits these events, and REST knockdown accelerates them in immature CGNs. Thus REST appears to function as a brake to prevent premature NFI occupancy and Gabra6 gene activation, with the timing of its departure determining the initial onset of NFI binding.

REST binding can repress neuronal target genes through the recruitment of histone deacetylases (Huang *et al.*, 1999; Naruse *et al.*, 1999). Consistent with this activity, constitutive REST expression blocked H4 acetylation of the Gabra6 promoter region in maturing CGNs. Thus, as for several other neuronal genes, REST departure appears to be a prerequisite for enhanced histone acetylation of the Gabra6 promoter. H4 acetylation remained relatively low in the P7 cerebellum when REST was largely dismissed from the Gabra6 promoter. Thus this histone modification occurred after REST departure in vivo. Subsequent mechanisms therefore appear to promote H4 acetylation. The timing of NFI occupancy and H4 acetylation at the Gabra6 proximal promoter in differentiating CGNs are tightly linked. Our attempts to address whether histone acetylation is required for NFI occupancy of the Gabra6 promoter using histone deacetylase inhibitors proved unsuccessful, as both trichostatin A and sodium butyrate *inhibited* Gabra6 expression instead of accelerating it (unpublished data).

On the basis of these findings, we suggest the following model for the Gabra6 intrinsic timer (Figure 9): REST occupies the Gabra6 promoter in CGNPs and in recently postmitotic, premigratory CGNs derived from the EGL/PMZ, ensuring that NFI occupancy and Gabra6 expression are not initiated prematurely. REST then gradually departs the promoter as CGNs continue to differentiate, leading to onset of NFI binding, H4 acetylation, and H3K4 trimethylation. Additional temporal mechanisms may operate in maturing CGNs to promote NFI occupancy, because its binding gradually increases following REST departure. In vivo, these postREST events appear to occur largely within the IGL: REST occupancy is already low in immature, postmigratory CGNs at P7, after which NFI binding increases during the next 2 wk along with chromatin remodeling and Gabra6 gene activation.

REST is expressed in several neuronal progenitor types, and it is typically down-regulated as progenitors are born, leading to derepression and subsequent activation of REST target genes and neuronal differentiation (Schoenherr and Anderson, 1995; Kuwabara *et al.*, 2004; Ballas *et al.*, 2005). Thus REST is an important regulator of the transition from dividing progenitor to differentiating neuron. Our results identify a continuing, postmitotic role for REST in immature neurons: Following cell cycle exit of neuronal progenitors, REST functions as a temporal regulator of dendritogenesis-related gene expression via its gradual promoter departure. Several factors may control REST's temporal function by affecting the timing of its departure from the *Gabra6* locus. In particular, the time course for decreased nuclear levels of REST (Ballas *et al.*, 2005) in immature neurons is likely to be an important determinant of its temporal activity. Other mechanisms also may influence REST's departure, including its interaction with coregulators that may actively promote or hinder its dismissal.

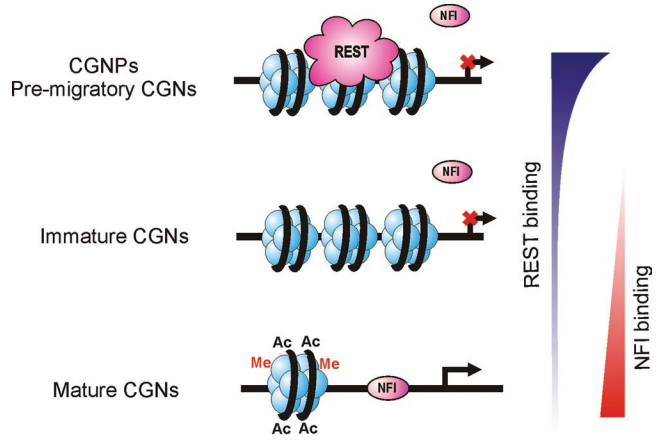

FIGURE 9: Proposed model for the intrinsic "Gabra6 timer" in maturing CGNs. REST occupies the proximal Gabra6 promoter, and NFI binding is inhibited in CGNPs and premigratory CGNs within the EGL/PMZ. REST occupancy then declines in immature CGNs (e.g., postmigratory cells within the IGL at P7), becoming permissive for NFI binding. NFI occupancy, histone acetylation, and H3K4 trimethylation of the proximal promoter gradually ensues as CGNs within the IGL continue to mature at later ages, leading to Gabra6 gene activation.

Global ChIP studies previously identified REST occupancy of genomic regions that lack RE1-related sequences (Otto *et al.*, 2007). The present findings provide direct evidence that such chromatin interactions mediate important REST-dependent transcriptional events. The mechanisms underlying REST occupancy of the Gabra6 proximal promoter remain to be determined. Based on preliminary gel shifts, REST localization may involve multisite interactions by a larger protein complex that may promote or modulate REST occupancy. The possibility that nonlocal REST actions (e.g., involving effects on other *trans*-factors) also contribute to regulation of Gabra6 transcription cannot be excluded. REST's temporal and functional interactions with the proximal A region and its effects on NFI occupancy and Gabra6 expression strongly argue, however, that this is a primary mechanism for its temporal effects.

Synapse formation involves an interplay between pre- and postsynaptic events, and coordinated temporal expression of dendrite- and synapse-related proteins is critical for this process (Lu *et al.*, 2009; Margeta and Shen, 2010). Defects in the timing of these and related neurodevelopmental processes may lead to functional deficits involving changes in the numbers and/or efficacies of synaptic connections. The question of timing is also likely to be important in other CNS events, including learning-associated adult neurogenesis and plasticity-related synapse formation in the mature brain. Disrupted timing of protein function and neurobehavioral events have been linked to several CNS diseases, including Fragile-X syndrome, autism, anxiety, schizophrenia, epilepsy, and Rett syndrome (Schwartzkroin and Walsh, 2000; Gross *et al.*, 2002; Johnston *et al.*, 2003; Ridler *et al.*, 2006; Amaral *et al.*, 2008; Gatto and Broadie, 2008). Dysregulated REST expression and/or function also have been reported in several neurological diseases, including Down syndrome, Huntington's, Fragile-X syndrome, and depression (Zuccato *et al.*, 2003; Canzonetta *et al.*, 2008; Lepagnol-Bestel *et al.*, 2009; Otsuki *et al.*, 2009). It is therefore possible that postmitotic timing mechanisms regulated by REST and other *trans*-factors may be altered and contribute to the phenotypes in multiple CNS diseases.

MATERIALS AND METHODS

Cell culture

CGN cultures were prepared from 6-d-old mouse pups as previously described (Wang *et al.*, 2004). Briefly, cerebella were dissected, meninges were removed, and tissue was digested with 1% trypsin and DNase at 1 mg/ml (Sigma-Aldrich, St. Louis, MO) at room temperature for 3 min. Digests were dissociated by mechanical trituration into single-cell preparations that consist of a mixture of CGNPs and newly postmitotic CGNs derived from the outer EGL and PMZ (EGL/PMZ cells) (Hatten, 1985; Raetzman and Siegel, 1999; Wang *et al.*, 2005; Manzini *et al.*, 2006). CGNPs/CGNs were

further enriched by Percoll (Sigma) gradient centrifugation followed by preplating on poly-D-lysine–coated Petri dishes at 35°C for 1 h. Cells were plated at a density of 5×10^4 cells/cm^2 onto poly-D-lysine/laminin–treated chamber slides or cell culture dishes in Neurobasal medium containing B-27 serum-free supplement, 2 mM L-glutamine and penicillin at 100 U/ml and streptomycin at 100 μg/ml (pen-strep) (Invitrogen, Grand Island, NY) and 0.45% D-glucose. For dividing CGNP cultures, sonic hedgehog at 3 μg/ml (R & D System, Minneapolis, MN) was added to culture medium at the time of plating.

Human embryonic kidney 293T cells and NIH 3T3 mouse fibroblast cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium (Sigma) containing 10% heat-inactivated fetal bovine serum.

Constructs

In initial REST expression studies, a bicistronic lentiviral vector was used that expresses mouse NRSF/REST (pHR′-NRSF-CITE-GFP; Nadeau and Lester, 2002). Later studies used a lentiviral construct expressing myc-tagged mouse REST. This construct was generated by inserting myc-REST from pCS2-MT-NRSF (provided by David Anderson, Caltech) into pHR′-cPPT-CMV-W-Sin18. For promoter assays, lentiviral constructs were used that express the luciferase reporter under control of the wild type or NFI site-mutant mouse Gabra6 promoter as well as a promoterless luciferase lentiviral vector (Wang *et al.*, 2004). A lentiviral construct containing a 200-base-pair Gabra6 proximal promoter (A region; Wang *et al.*, 2004) was generated as follows. The 200-base-pair A region was amplified by PCR from pGL3-GABRA6-IRES (Wang *et al.*, 2004) using primers that added *Mlu*I and *Hind*III restriction sites to the 5′ and 3′ ends, respectively. The promoter fragment was subcloned into *Mlu*I/*Hind*III-digested pGL3 Basic. The promoter-reporter cassette was then inserted into pHR′-CMV-W-Sin18 using *Mlu*I and *Xho*I sites. MicroRNA-based REST shRNA (Clone ID: V2LMM_83031) and nonsilencing control lentiviral vectors were obtained from Thermo Fisher Scientific (Waltham, MA).

Virus generation and infections

Vesicular stomatitis virus G protein–pseudotyped lentiviruses were generated by cotransfection of the vector construct, the packaging construct pCMVΔ8.91 and the pMD.G vesicular stomatitis virus G protein viral envelop expression vector into 293T cells. Viruses were collected at 48 and 72 h after transfection. High-titer viral stocks were prepared by ultracentrifugation as previously described (Wang *et al.*, 2005). The titers of viruses were determined by enzyme-linked immunosorbent assays for p24 (Wang *et al.*, 2005) or by infecting 3T3 cells with a viral serial dilution followed by flow cytometric assays for GFP-positive cells. Multiplicity of infections for CGN cultures were 2.5–3 for protein or shrna expression studies, resulting in 80–90% cell transduction (Wang *et al.*, 2005). Multiplicity of infections for promoter or promoter-protein coinfection studies were as previously described (Wang *et al.*, 2004). Luciferase activity was measured using the Promega Luciferase Assay System. Coinfected CMVLacZ virus was used to normalize for temporal onset of viral integration when comparing promoter activities among different time points, as in earlier studies (Wang *et al.*, 2005).

RNA purification and RT-PCR

RNA was extracted from tissues or cultured cells using Tri reagent (Sigma). First-strand cDNAs were synthesized with random hexamers using the SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Semiquantitative PCR was performed using threefold serial dilutions. Real-time PCR was done in triplicate with a Bio-Rad (Hercules, CA) iCycler system, and the ratio of target transcripts relative to 18S rRNA was determined using the $2^{-}\Delta^{ct}$ method as previously described (Wang *et al.*, 2004).

Immunostaining and Western blotting

Stained cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 15 min at room temperature. Samples were pretreated with 5% normal goat serum (Invitrogen, Grand Island, NY) and 0.3% Triton X-100 in PBS at room temperature for 1 h, followed by incubation with primary antibodies at 4°C overnight and with secondary antibodies at room temperature for 1 h. The following antibody dilutions were used: NeuN 1:800); Gabra6 (1:400); REST (1:1000) (each from Millipore, Billerica, MA). For Western analysis, proteins were separated on 8% SDS–polyacrylamide gels and transferred onto polyvinylidene difluoride transfer membranes (Millipore). Membranes were blocked with 1× PBS buffer with 0.1% Tween-20 and 5% nonfat dry milk followed by incubation with primary and horseradish peroxidase–conjugated secondary antibody. Bound antibodies were detected with a Western Lightning Chemiluminescence kit (PerkinElmer Life Sciences, Waltham, MA) according to the manufacturer's protocol. Equivalence of protein loading was confirmed using PonceauS staining and nuclear β-actin. Antibodies used were as follows: pan-NFI antibody (sc-5567, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), REST (1:300; Millipore), and β-actin (1:2000; BD Transduction Labs, Rockville, MD).

Gel shift assays

Nuclei were isolated from mouse cerebella or CGN cultures, and extracts were prepared in the presence of protease inhibitors as previously described (Wang *et al.*, 2004). Nuclear extracts were examined for NFI DNA binding using a probe to the Gabra6 proximal promoter NFI site or to an ~200-base-pair A region probe, as described (Wang *et al.*, 2004). Antibody 12C11 against NRSF/REST (provided by D. Anderson) or control antibody (5 μg) was added to binding reactions for supershift assays. The oligonucleotide ttcagcaccatggacagcgcc was used as an RE1 competitor in A region gel shifts.

ChIP

ChIP assays were performed as described previously (Wang *et al.*, 2004). Briefly, CGN cultures were cross-linked with 1% formaldehyde for 10 min at room temperature followed by addition of 125 mM glycine. Samples were lysed using SDS buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1× protease inhibitor mixture). Chromatin was sonicated to an average length of ~500 base pairs. For P7 and P21 mouse cerebella, minced tissues were cross-linked as described earlier in the text and nuclei were isolated by hypotonic lysis as previously described (Liu *et al.*, 1997). Fixed cerebellar nuclei were further enriched by fractionation on 5%/60% discontinuous Percoll gradients by centrifugation at 10,000 rpm for 1 h at 4°C. Cerebellar nuclei were washed twice in cold PBS, and the percentage of CGN nuclei was determined by NeuN immunostaining. Nuclei were then lysed and chromatin was processed as described earlier in the text. Lysates were diluted 10-fold in ChIP dilution buffer and incubated with primary antibodies or control immunoglobulin (Ig)G. After overnight incubation at 4°C, immune complexes were collected with protein A-Sepharose (GE Healthcare, Little Chalfont, UK). The precipitated chromatin was treated with proteinase K and DNase-free RNase A, followed by overnight incubation to reverse the cross-linking. DNA was extracted and used as template for PCR assays using polyacrylamide gels (Wang *et al.*, 2004, 2010) or real-time PCR. One-tenth of the ChIP reaction was assayed to measure input

chromatin, and real-time data were expressed as a percentage of input using the $2^{-\Delta Ct}$ method. Two different antibodies were used in ChIP assays for NFI (pan-NFI [sc-5567]; Puzianowska-Kuznicka and Shi, 1996) and for REST (12C11 monoclonal and anti-REST C-terminal polyclonal antibody, provided by Gail Mandel, Vollum Institute, Portland, OR). Anti-acetylated histone H3 and H4 antibodies were from Millipore, and antibodies to trimethylated H3K4 and H3K9 antibodies were purchased from Abcam (Cambridge, MA). ChIP PCR primer sequences are available upon request.

DNA accessibility

Cells (10^7) from each group were washed with cold PBS, resuspended in 1 ml of freshly prepared lysis buffer (25 mM HEPES, pH 7.9, 15 mM $MgCl_2$, 10 mM KCl, 0.1% Nonidet P40, 1 mM dithiothreitol, 1 mM PMSF, 1× proteinase inhibitor cocktail) and incubated on ice for 10 min. Cells were homogenized by 20 strokes with a Dounce B homogenizer. Nuclei were collected by centrifugation at 1000 *g* for 4 min, washed once with lysis buffer, and resuspended in 100 µl of digestion buffer (300 mM sucrose, 1× restriction enzyme digestion buffer, and bovine serum albumin at 100 µg/ml). Nuclei (14 µg) were digested with 14 U or 28 U of *Hin*fI at 37°C for 90 min with periodic agitation. The digestion was stopped by adding an equal volume of stop buffer (20 mM Tris at pH 7.4, 20 mM EDTA, 0.4% SDS, 300 mM NaCl, and proteinase K at 1 mg/ml) and incubating overnight at 50°C. Genomic DNA was purified by phenol-chloroform extraction and ethanol precipitation, then resuspended in Tris-EDTA (pH 8.0), and the DNA concentration was determined by 260-nm absorbance. *Hin*fI ligation-mediated-PCR adaptors were generated by annealing the oligos 5′-aatgaattcagatc-3′ and 5′-gcggtgacccgggagatctgaattc-3′, and adaptor ligation was performed using DNA Ligation Kit (ver. 2.2; TaKaRa, Shiga, Japan). Primers used for LM-PCR were ggtgacccgggagatctgaattctagtc (forward) and caatcacctggagaagtcttccaaatc (reverse), and PCR primers for the input control were cgaggggtacatagaggagggatttca (forward) and tgttgtgaaagccaccccttcatacatt (reverse).

Cell sorting

To prepare chromatin from premigratory NeuN(+) cells, freshly isolated P6 EGL/PMZ cells were fixed with 1% formaldehyde and subjected to cytometric sorting using Alexa488-conjugated anti-NeuN antibody (Millipore). Purities of NeuN(+) fractions were >90% based on reanalysis.

Bioinformatic analysis

Mouse Gabra6 promoter sequences were extracted from GenBank and analyzed for RE-1–related sequences using the TRANSFAC Professional database as well as by direct scanning for RE-1-like half-sites.

Statistics

The Student's *t* test was used to determine the significance of differences between experimental values.

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