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Feedback regulation of NEUROG2 activity by MTGR1 is required for progression of neurogenesis

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Abstract

The sequential steps of neurogenesis are characterized by highly choreographed changes in transcription factor activity. In contrast to the well-studied mechanisms of transcription factor activation during neurogenesis, much less is understood regarding how such activity is terminated. We previously showed that MTGR1, a member of the MTG family of transcriptional repressors, is strongly induced by a proneural basic helix-loop-helix transcription factor, NEUROG2 in developing nervous system. In this study, we describe a novel feedback regulation of NEUROG2 activity by MTGR1. We show that MTGR1 physically interacts with NEUROG2 and represses transcriptional activity of NEUROG2. MTGR1 also prevents DNA binding of the NEUROG2/E47 complex. In addition, we provide evidence that proper termination of NEUROG2 activity by MTGR1 is necessary for normal progression of neurogenesis in the developing spinal cord. These results highlight the importance of feedback regulation of proneural gene activity in neurodevelopment.

Keywords

Development; Neurogenesis; Embryo; transcription; bHLH; spinal cord; MTGR1; NEUROG2; MTG/ETO proteins; nervy; transcriptional repressor

Introduction

Successive up-regulation and down-regulation of transcription factor expression is a characteristic feature of neurogenesis (Roztocil et al., 1997; Torii et al., 1999). For example, cells at different stages of neurogenesis in the developing spinal cord can be identified based on their mediolateral positions, and the stage of development of the cells correlates with the transcription factors that are active (summarized in Fig. 7). Postmitotic cells migrate through at least three distinct layers of the spinal cord as they progress through neurogenesis: the ventricular zone (VZ), the intermediate zone (IZ), and the mantle zone (MZ). Early proneural transcription factors such as NEUROG1 (previously NGN1, ATH4C), NEUROG2 (previously NGN2, ATH4A) and ASCL1 (previously cASH1) are first expressed in the VZ (Lo et al., 1991; Sommer et al., 1996; Ma et al., 1997; Murciano et al., 2002). Postmitotic cells migrate from the VZ into the IZ as the next group of transcription factors including PROX1, MTGR1

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and NEUROD4 (previously ATH3) reach peak expression, and expression of the early factors begin to fall (Roztocil et al., 1997; Torii et al., 1999; Koyano-Nakagawa and Kintner, 2005). Finally, as cells reach the MZ, late transcription factors such as NEUROD1, MTG8, MTG16, PAX2 and ISL1 are expressed, and the earlier factors are lost. Each wave of transcription factors promotes expression of the next group. In order for expression of the intermediate group of transcription factors to fall as the cells enter the MZ, it is necessary that activity of the early transcription factors be terminated.

The importance of terminating the activity of proneural transcription factors once they have fulfilled their roles was underscored by studies in which expression of such proteins was artificially extended. For example, in the developing brain, prolonged expression of proneural basic helix-loop-helix (bHLH) transcription factors in postmitotic neurons resulted in increased neuronal cell death and malformation of the cytoarchitecture (Isaka et al., 1999; Cai et al., 2000). Because it is estimated that newly born neurons migrate through the VZ and the IZ in a few hours (Koyano-Nakagawa and Kintner, 2005; Gui et al., 2007), cells should successively up- and down-regulate the expression of transcription factors rapidly within this short window of time.

Mechanisms that initiate transcription factor activity have been well investigated, whereas the mechanisms that terminate activity are poorly understood. Termination of transcription factor activity could be accomplished by at least three mechanisms that are not mutually exclusive: 1) ending further production of transcription factors at mRNA or protein level, 2) removal of already produced transcription factors by proteolysis, and 3) inhibiting the activity of transcription factors. While there is evidence indicating the employment of the first two mechanisms (Sriuranpong et al., 2002; Vosper et al., 2007), the third, inhibition of bHLH transcriptional activity, is less well established. Members of the MTG family of transcriptional repressors are strongly induced during neurogenesis, and it has been suggested that they inhibit transcription factor activity (Koyano-Nakagawa and Kintner, 2005).

MTG8 (RUNXT1, ETO), MTG16 (CBFA2T3) and MTGR1 (CBFA2T2) are members of the MTG/ETO/CBFA2T protein family, a small group of transcriptional repressors (for reviews, see Davis et al., 2003; Hug and Lazar, 2004). They are reported to act as a “protein scaffold”, bridging different transcription factors. Several groups including ours have previously reported that MTGR1 is strongly induced in *Xenopus laevis* by various proneural bHLH proteins including X-NGNR-1, Xash3, Xath3 (XNeuroD4), Xath5 (XAtoh7) and XNeuroD (Cao et al., 2002; Koyano-Nakagawa and Kintner, 2005; Logan et al., 2005; Seo et al., 2007). The strong induction by proneural bHLH proteins as well as its highly conserved structure across species suggests that MTGR1 may play an important role in neuronal differentiation. In this report, we show that MTGR1 physically interacts with NEUROG2 and inhibits its transcriptional activity. We provide evidence that this inhibition takes place *in vivo* in the developing spinal cord, and is necessary for normal progression of the neurogenic program. Our results indicate that termination of NEUROG2 activity is ensured by transcriptional repression by MTGR1, in addition to down-regulation of *NEUROG2* expression and degradation of NEUROG2 protein. These three independent mechanisms are used to warrant proper progression of neurogenesis.

Materials and Methods

Cells

Cell lines were maintained according to the protocol by American Type Culture Collection. P19 embryonal carcinoma cells were cultured in alpha-MEM (Cellgro, Herndon, VA) supplemented with 2.5% fetal calf serum (FCS) and 7.5% newborn calf serum with 100 units/ml penicillin, 100µg/ml streptomycin, and 0.24µg/ml of amphotericin B as Funigizone® (Gibco, Grand Island, NY). HEK293T cells were cultured in Dulbecco's Modification of

Eagle's Medium (Cellgro, Herndon, VA) supplemented with 10% FCS with 1g/L glucose, 100 units/ml penicillin, 100µg/ml streptomycin, and 0.24µg/ml of amphotericin B as Funigizone® (Gibco, Grand Island, NY).

Plasmids

Chick and *Xenopus* MTGR1 clones were previously described (Koyano-Nakagawa and Kintner, 2005). All MTG constructs were cloned into the pCS2(+) vectors with or without the myc epitope tag. pCS2(+) cMTGR1-VP16 and pCS2(+) cMTGR1-EnR were constructed by fusing the activation domain of VP16 protein and the repressor domain of engrailed, respectively, to the 3' side of the entire open reading frame of cMTGR1 in-frame. Expression of the fusion proteins was confirmed by analyzing expression of myc epitope-tagged molecules in a parallel reaction (Fig. S3C). Expression vectors for GST fusion molecules, pGEX-cNEUROG2, pGEX-cE47 were constructed by shuttling the entire coding frames of the factors in-frame into the pGEX vectors (GE Healthcare Technologies, Waukesha, WI). The following plasmids were kind gifts from the laboratories listed below. pcDNA3 HA-mNeurog2, pcDNA3 HA-cNEUROG2, pcDNA3 HA-hE47 and *in situ* probe for chicken NEUROD4: Soo-Kyung Lee and Sam Pfaff (Lee et al., 2005); DLL4: Domingos Henrique (Henrique et al., 1995); EMSV-mE12: Atsushi Asakura; pcDNA3 cE47: Klemens Meyer (Conlon and Meyer, 2004). pGL3P(E)7Luc reporter gene was made by shuttling the E-box fragment of pGL2P(E)7Luc (given by Masato Nakafuku) into the pGL3P backbone. The E-box fragment contains seven tandem repeats of sequence, AGGCAGGTGGC (E-box is underlined). pRL-CMV is from Promega (Madison, WI). siRNA vector for MTGR1 (psiRNA-cMTGR1) was constructed by inserting 66 mer oligonucleotide GTA CCT CGC ACT ACA CCC TGG AAG ACA TTC AAG AGA TGT CTT CCA GGG TGT AGT GCT TTT TGG AAA (only the top strand is shown) into the *HindIII*-*Acc65I* fragment of psiRNA-h7SKGFPzeo (InvivoGen, San Diego, CA). This makes a bi-cistronic vector expressing siRNA and GFP. An siRNA vector targeted to a different part of cMTGR1 was constructed using 66 mer, GTA CCT CGC CCG TGG AAG TGA AGA TAC ATC AAG AGT GTA TCT TCA CTT CCA CGG GCT TTT TGG AAA, which yielded essentially the same results. In this paper results with the first siRNA construct are shown. psiRNA-h7SKgz-Scr, a vector expressing siRNA of "scramble" sequence that is not complementary to any genomic sequence in the database was used as a control (InvivoGen, San Diego, CA).

EMSA and *in vitro* degradation assay

The following probe was synthesized for EMSA. E-box motifs are underlined; (Ebox)₃: GATCCAGGCAGGTGGCAGGCAGGTGGCAGGCAGGTGGCAGATC. Unlabeled transcription factors were synthesized using TNT® coupled Reticulocyte Lysate Systems (Promega, Madison, WI). Parallel reactions including ³⁵S-methionine were set up to monitor protein synthesis. Fifteen µl of binding reaction containing 30% glycerol, 40 mM HEPES (pH 7.9), 10 mM MgCl₂, 0.2 mM DTT, 0.02% TritonX-100, 4 µl *in vitro* translated protein, 200 ng sheared salmon sperm DNA and 1 ng of labeled probe was set up. Competition experiments included 20 ng of unlabeled oligonucleotide. Reactions were incubated at 23°C and separated on a 1.5 mm thick 4% acrylamide gel (acrylamide:bisacrylamide=80:1, 6.75mM Tris pH7.9, 3.3mM NaOAc, 1mM EDTA, 2.5% glycerol). The gel was run at 4°C with buffer circulation. For *in vitro* degradation assay, EMSA reactions were set up with unlabeled oligonucleotide. Ten µl of reaction was removed periodically and boiled immediately in 1X sample buffer. All gels were dried after electrophoresis and imaged on Storm Phosphorimager (GE Healthcare Technologies, Waukesha, WI).

GST Pull-down and immunoprecipitation assays

GST fusion proteins induced in BL21 cells were purified using glutathione sepharose beads (GE Healthcare Technologies, Waukesha, WI). Proteins bound to beads were analyzed by SDS-PAGE and similar amounts of protein based on Coomassie staining were used for binding reactions. In a typical reaction, 25–50 μl of ^{35}S -labeled transcription factors were diluted in 800 μl of binding buffer (Zhang et al., 2004) and mixed with GST fusion proteins preadsorbed to glutathione beads. After three washes in binding buffer, proteins remaining on the beads were separated by SDS-PAGE and autoradiographed. For immunoprecipitation, HEK293T cells were transfected with expression vectors of transcription factors using Fugene 6 (Roche) and cultured for 24 hrs. Proteasomal inhibitors MG132 (23 μM) and lactacystine (4.7 μM) were added to the culture and further incubated for 6 hrs before harvest. The treatment with proteasomal inhibitors was necessary because NEUROG2 protein has a short half-life and it was not possible to detect the protein without the treatment. The samples were then immunoprecipitated with either 2 μg of anti-HA antibody (Santa Cruz SC 805) or 0.2 μg of anti-myc tag antibody (Upstate 4A6).

In ovo electroporation, immunohistochemistry and *in situ* hybridization

In ovo electroporation and immunohistochemistry were performed as described previously (Koyano-Nakagawa and Kintner, 2005). Embryos were electroporated at Hamburger-Hamilton (HH) stage 13 (day 2 of incubation) and harvested at stage 16 (24 hours after electroporation) or stage 22 (48 hours after electroporation). Antibodies and dilutions were as follows: rabbit anti-GFP (Molecular Probes, 1:400), mouse anti-myc epitope (Upstate 4A6, 1:5000), rat anti-HA (Roche 3F10, 1:2000), rabbit anti-Pax2 (Zymed, 1:200), mouse anti-Isl1 (Developmental Studies Hybridoma Bank, 1:10), mouse anti-HuC/D (Molecular Probes, 1:100), TuJ1 (Covance, 1:500), rat anti-BrdU (Harlan, 1:500), rat anti-NEUROD4 (a gift from Thomas Jessell, 1:2000), rabbit anti-cNEUROG2 (a gift from David Anderson, 1:500) and rabbit anti-hMTGR1 (a gift from Issay Kitabayashi, 1:5000). *In situ* hybridization on 20 μm thick cryosections were done following standard protocol (Tuttle et al., 1999). For double fluorescent *in situ* hybridization, digoxigenin- and fluorescein-labeled RNA probes were used for hybridization and the hybridized probes were detected as previously described (Fior and Henrique, 2005).

Quantification of *in situ* hybridization signals

Areas positive for *NEUROD4* and *DLL1* were measured using Image J (NIH) following the instruction to the software. Briefly, TIFF images were converted to 8-bit and smoothed for median, with a radius of 2 pixels. Multithresholder plugin was used to set a threshold with maximum entropy. Pixels of the positive areas were counted and converted to square microns. The boundaries of the VZ, the IZ, and the MZ were morphologically identified by the arrangement of nuclei as visualized by DAPI nuclear staining.

Luciferase assay

P19 cells were plated at 7×10^4 per well (24-well cluster) and transfected in triplicates with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with pRL-CMV as an internal control. In all experiments, 0.2 μg of the reporter genes, 0.3 μg of bHLH expression vectors, 0.1 μg of E12 expression vector was used. Total amount of DNA transfected per well was adjusted with an empty vector, so that the DNA:Lipofectamine ratio is constant for all samples. Twenty-four hours later, cells were harvested and luciferase activity was measured using Dual Luciferase Assay Reagent (Promega, Madison, WI). A relative luciferase unit (RLU) was calculated by dividing the luciferase activity by Renilla luciferase activity and averaged. Data were normalized to the mean control value to calculate fold change.

Western blot

Ten microliter each of the lysate used for luciferase assay was separated on a SDS polyacrylamide gel. Western blotting and development was done as described (Ausubel, 1994). Mouse anti-myc epitope antibody (Upstate 4A6) was used at 1:20000 dilution.

Results

MTGR1 is transiently expressed in differentiating neurons

MTG proteins are known to interact with sequence specific transcription factors and repress transcription. We aimed to identify the target transcription factor whose activity is repressed by MTGR1 in the developing nervous system. Since MTGR1 is induced during neurogenesis, MTGR1 is likely to interact with transcription factors that are expressed in differentiating neurons. A report showing the interaction of MTG8 with HEB, a bHLH protein related to E2A (Zhang et al., 2004), prompted us to investigate whether MTGR1 similarly targets a bHLH protein, *NEUROG2*. We first compared the temporal pattern of *MTGR1* gene expression with that of bHLH transcription factors, *NEUROG2* and *NEUROD4*, in chick embryos (Fig. 1). Two color fluorescent *in situ* hybridization combined with immunohistochemistry showed that *NEUROG2* is induced the earliest among these three genes in the VZ and continues to be expressed in the medial half of the IZ (Fig. 1A, C). *MTGR1* follows *NEUROG2* expression and peaks in the IZ (Fig. 1A). Its expression level decreases when cells reach the MZ and begin to express TuJ1. *NEUROD4* shows similar pattern of overlap with *NEUROG2*, induced after *NEUROG2* and peaking in the IZ (Fig. 1C). Double-labeling experiments show that both mRNA and proteins of *MTGR1* are co-localized with those of *NEUROD4* in the VZ, the IZ and the MZ, particularly in the ventral spinal cord (Fig. 1B, D). These results indicate the temporal sequence of gene expression in the differentiating neurons; *NEUROG2* is expressed first, followed by *MTGR1* and *NEUROD4*, and later by a neuronal marker TuJ1. The same sequence of gene induction was observed throughout the neurogenic stages from embryonic day (E)2 to E7 (Fig. S1). Thus, we conclude that MTGR1 is expressed in differentiating neurons, closely following *NEUROG2* expression. This temporal sequence of gene expression was consistent with that observed in development of the mouse central nervous system (Alishahi et al., 2009). Because over-expression of MTGR1 in the chick spinal cord at E2 by *in vivo* electroporation did not suppress *NEUROG2* expression (Fig. S2), we hypothesized that MTGR1 acts at the protein level to negatively regulate the *NEUROG2* activity during neurogenesis.

MTGR1 inhibits transcriptional activity of *NEUROG2*

We first tested the hypothesis that MTGR1 inhibits the function of *NEUROG2* by using a transient transfection assay in P19 cells (Fig. 2A, B). As a reporter, we used the plasmid, pGL3(E)7-Luc, which contains seven repeats of an E-box sequence, a recognition sequence of bHLH proteins, linked to the *luciferase* reporter gene (Lee et al., 2005). *NEUROG2* activates this reporter by heterodimerizing with its binding partner E12 or E47, which are splice variants produced from the *E2A* gene. This reporter gene alone had very low activity that is indistinguishable from the background luciferase activity from mock transfection control (Fig. 2A, B; lane 1). *NEUROG2* alone activated the pGL3(E)7-Luc reporter significantly, presumably by acting together with the endogenous E proteins (Fig. 2A, lane 2; 2B, lane 4). Adding E47 augmented *NEUROG2* activity by three fold (Fig. 2A, lane 4; 2B, lane 2), whereas E47 alone did not activate the reporter (Fig. 2A, lane 3). Transcriptional activity by the combination of *NEUROG2* and E47 was inhibited by MTGR1 in a dose-dependent manner (Fig. 2A lanes 4–7). Transcriptional activity by *NEUROG2* alone was also inhibited dose-dependently (Fig. 2B, lanes 4–7). MTGR1 alone without *NEUROG2* and E47 did not change transcriptional activity (Fig. 2A, lane 8). Similarly, MTGR1 did not affect transcription activity of a control reporter without E-box motifs either with or without co-transfection of vectors

encoding *NEUROG2* and *E47* (data not shown). These results suggest that *MTGR1* inhibits E-box dependent *NEUROG2*-*E47* activity by interacting with this protein complex. Similar inhibitory activity of *MTGR1* was observed using combinations of mouse *Neurog2* (m*Neurog2*), mouse *E12* (m*E12*) and *Xenopus* *MTGR1* (X*MTGR1*) (Fig. S3A), or m*Neurog2*, human *E47* (h*E47*) and chicken *MTGR1* (Fig. S3B). Thus inhibition of *NEUROG2*-*E47* activity by *MTGR1* occurs across species, and it is likely the protein-protein interaction interfaces are conserved.

To examine if *MTGR1* represses transcription of the *NEUROG2*-*E47* complex bound to DNA or by other mechanisms such as competitively inhibiting binding to DNA or to co-factors, we made fusion proteins of *MTGR1* with the repressor domain of engrailed (*EnR*) or the activation domain of *VP16* (Fig. 2C). If *MTGR1* interacts with the *NEUROG2*-*E47* complex bound to DNA, a forced fusion with *EnR* should repress, and fusion with *VP16* should augment the transcriptional activity of *NEUROG2*-*E47*. As shown in Figure 2D, lanes 4 and 5, *VP16* fusion enhanced and *EnR* fusion of *MTGR1* inhibited the activity of *NEUROG2*-*E47*, respectively. Either construct did not affect transcriptional activity in the absence of *NEUROG2*, *E47*, or the target E-box sequence (data not shown). Thus, these results indicate that *MTGR1* inhibits transcriptional activity at least in part by interacting with the *NEUROG2*-*E47* complex bound to the E-box sequence. These results, however, do not eliminate the possibility that *MTGR1* also inhibits the *NEUROG2*-*E47* complex by other mechanisms such as inhibition of DNA binding.

MTGR1 physically interacts with NEUROG2 and E47

The reporter gene assays described above suggested that *MTGR1* might act by interacting with the *NEUROG2*-*E47* complex. Thus, we used a GST pull-down assay to test if *MTGR1* directly interacts with *NEUROG2* and/or *E47*. We found that *MTGR1*, *MTGR1*-*EnR*, and *MTGR1*-*VP16* co-purified with GST-*NEUROG2* but not with the control GST protein (Fig. 3A, lanes 1–4). The efficiency of co-purification was comparable to *E47* protein purified with GST-*NEUROG2* (lane 5). *MTGR1* and *MTGR1*-*EnR* also co-purified with GST-*E47*, but the recovery was not as efficient as with GST-*NEUROG2*, and the interaction between *MTGR1*-*VP16* and *E47* was very weak compared to other interactions (lanes 6–8). These results indicated that *MTGR1* physically interacts with *NEUROG2*, and less efficiently with *E47* *in vitro*.

Next, we tested if this interaction takes place in cultured cells by immunoprecipitation assays. Expression vectors of *MTGR1* tagged with the myc epitope and *NEUROG2* or *E47* tagged with the haemagglutinin epitope (HA) were transfected into HEK293T cells. When anti-HA antibody was used for immunoprecipitation, *MTGR1* co-precipitated with *NEUROG2*, but not with *E47* (Fig. 3B lanes 3, 4). In contrast, myc epitope-tagged *MTG8*, another *MTG* family member, did not co-purify with either *NEUROG2* or *E47* under the same condition (lanes 1, 2). A reverse experiment was done by immunoprecipitating the cell extracts with anti-myc epitope antibody (Fig. 3C). As expected from the results in Fig. 3B, *NEUROG2* but not *E47* co-purified with *MTGR1* (lanes 3, 4), and neither of the proteins co-purified with *MTG8* (lanes 1, 2). Thus, our results showed that the interaction between *NEUROG2* and *MTGR1* is specific and takes place in a cellular environment. Interaction between *E47* and *MTGR1* was not detected under this condition. We attempted to detect interaction of endogenous proteins using nuclear extracts of embryonic day 14.5 mouse cortex, in which both *NEUROG2* and *MTGR1* are abundantly expressed (Alishahi et al., 2009), but were not able to carry out the experiment due to the low sensitivity of anti *NEUROG2* and anti *MTGR1* antibodies.

It is common that protein association changes the affinity of those proteins to a third molecule. Thus, we tested whether heterodimerization of *NEUROG2* and *E47* changes their ability to interact with *MTGR1* by pulling down co-synthesized *NEUROG2* and *E47* with *MTGR1*-GST

fusion proteins. The recovery rate was the same as separately synthesized proteins, indicating that heterodimerization of NEUROG2 and E47 did not change the affinity to MTGR1 (data not shown).

Taken together, our immunoprecipitation assay showed specific interaction of MTGR1 and NEUROG2 both *in vitro* and in cultured cells. MTGR1 also interacted with E47 as a synthetic protein *in vitro*, but this interaction was much weaker than the interaction between MTGR1 and NEUROG2. This interaction was not detectable in HEK293T cells, suggesting that the interaction is weak, if any happens *in vivo*.

MTGR1 inhibits DNA binding of the NEUROG2-E47 complex, but does not dissociate the preformed complex from DNA

Next, we examined if transcriptional inhibition by MTGR1 involves a change in DNA binding activity of the NEUROG2-E47 complex. To examine the DNA binding activity, electrophoretic mobility shift assay (EMSA) was performed with proteins synthesized *in vitro*. In our experimental condition, E47 but not NEUROG2 bound to the E-box sequence as a homodimer (Fig 4A, lanes 1, 3). When NEUROG2 and E47 were co-translated, they preferentially formed a heterodimer, and a homodimer of E47 was not detected (Fig 4A, lane 5). Since the combination of NEUROG2 and E47 activates transcription strongly (Fig. 2), we used co-translated proteins of NEUROG2 and E47 in the following assays. First, we asked if MTGR1 prevents DNA association of the NEUROG2-E47 complex. When we pre-incubated NEUROG2, E47 and MTGR1 before adding the probe, the DNA binding activity of the NEUROG-E47 complex was reduced (Fig. 4B, C lanes 2, 3). To examine if MTGR1 forms a higher-order complex with NEUROG2 and E47 that binds to DNA, we titrated down the amount of the radiolabeled DNA probe and made it the limiting reagent. Even after a long exposure to the film, we did not observe formation of additional bands that show different mobility from the one formed by NEUROG2-E47 alone (Fig. 4C and data not shown). Therefore, when MTGR1 complexes with NEUROG2-E47 before DNA binding, it primarily inhibits the association of the NEUROG2-E47 complex with DNA. It should be noted, however, that EMSA requires the protein-DNA complex to stay associated throughout electrophoresis, and does not detect a complex that has short half-life. Thus, it remains possible that MTGR forms a minor population of unstable DNA binding complexes with NEUROG2-E47. The inhibition of DNA binding activity was also observed using combinations of X-NGNR-1 and chicken or *Xenopus* MTGR1 (Fig. 4D lanes 1–3), and mouse Neurog2 and chicken or *Xenopus* MTGR1 (lanes 4–6), indicating that the protein interaction can take place across species.

It is possible that association of the NEUROG2-E47 complex with MTGR1 induced degradation of NEUROG2 or E47 and that resulted in reduction of the DNA binding activity. To examine this possibility, we radiolabeled the three proteins and monitored the protein levels in a reaction identical to EMSA. The levels of NEUROG2 and E47 proteins remained constant throughout the experiment, either in the presence or absence of MTGR1, indicating that inhibition of DNA binding was not due to degradation of NEUROG2 or E47 (Fig. S4A).

To determine whether MTGR1 disrupted the heterodimer of NEUROG2 and E47 and inhibited its DNA binding ability, we carried out an immunoprecipitation assay (Fig. S4B). NEUROG2 and E47 were co-synthesized and immunoprecipitated with the antibody against NEUROG2. E47 co-purified with NEUROG2 regardless of the presence or absence of MTGR1, indicating that binding to MTGR1 did not inhibit heterodimerization of NEUROG2 and E47.

Finally, we asked if MTGR1 promotes dissociation of the NEUROG2-E47 complex already bound to DNA (Fig. 4E, F). In this experiment, the NEUROG2-E47 complex was mixed with the probe first and then MTGR1 was added. The same volumes were removed from the reaction

every 30 minutes and immediately applied to a running non-denaturing gel. In contrast to our experiments above, we did not observe a decrease in DNA binding activity even after 120 min of incubation. No additional DNA binding complex was detected in this assay (data not shown). Therefore, if NEUROG2-E47 was already bound to DNA, MTGR1 does not dissociate the complex from DNA.

Collectively, our results show that MTGR1 can inhibit DNA association of the NEUROG2-E47 complex, but it cannot induce dissociation of an already formed complex on DNA.

NEUROG2 activity is inhibited by MTGR1 *in vivo*

We next asked if suppression of NEUROG2-E47 activity by MTGR1 takes place *in vivo*. To address this question, we chose to use *NEUROD4* and *DLL1* as a readout of NEUROG2 activity. Xath-3, the *Xenopus laevis* counterpart of *NEUROD4* is demonstrated as a direct target of neurogenin (Logan et al., 2005; Seo et al., 2007). In the mouse telencephalon, *Neurod4* gene expression is completely lost in *Neurog2* null animals (Mattar et al., 2008). In the chick and mouse nervous system, *NEUROD4* closely follows *NEUROG2* expression throughout the developmental period (Fig. 1, S1 and Alishahi et al., 2009), and *NEUROD4* expression is induced by ectopic expression of NEUROG2 (Fig. S5C–d). Furthermore, we noticed that the 5' upstream region of the *NEUROD4* gene contains 16 putative E-box sequences within the 4 kb region upstream of the transcription initiation site (data not shown). In particular, E-box sequences were 12 fold overrepresented compared to random distribution of nucleotides between –3.3 and –2.8 kb relative to the transcription start site. EMSA analysis showed that the NEUROG2-E47 complex binds efficiently to the E-box sequence found in this promoter region (data not shown). Thus, evidences suggest that, similar to other species, chicken *NEUROD4* is induced by NEUROG2 and can be used as readout of NEUROG2 activity. Similarly, *DLL1* is a known direct target of Neurog2 in mouse and frog (Castro et al., 2006; Seo et al., 2007), and the conserved expression patterns of these genes in chick embryo show that it is highly likely that chicken *DLL1* is also directly regulated by NEUROG2. Thus, *NEUROD4* and *DLL1* expression was used as an indicator of NEUROG2 activity.

We previously reported that over-expression of MTGR1 alone at day 2 of incubation does not significantly affect neuronal differentiation of chick spinal cord, likely due to regulatory response by endogenous mechanisms or compensation by the growth of the untransfected cells (Koyano-Nakagawa and Kintner, 2005). Thus, to further examine the interaction of NEUROG2 and MTGR1 *in vivo*, we used a gene knockdown approach. When an expression vector for short interference RNA against *cMTGR1* (siRNA-cMTGR1) was electroporated into E2 chick spinal cord, *NEUROD4* was up-regulated after 24 h hours in the electroporated area (Fig. S5 A, a). This up-regulation of *NEUROD4* following knock down of MTGR1, an indication of increased NEUROG2 activity, supports our hypothesis that MTGR1 inhibits NEUROG2 activity *in vivo*. At 48 h, *MTGR1* expression was reduced to 53% of the control side (Fig. S5, B, b), but *NEUROD4* expression did not differ significantly from the control side (Fig. 5, B1, B2, D, E). We reasoned that because endogenous NEUROG2 is expressed transiently *in vivo*, reducing expression level of MTGR1 alone might not dramatically affect the expression pattern of *NEUROD4*. Thus, in the next series of experiments, we reduced the expression level of MTGR1 while over-expressing NEUROG2.

First, we examined the induction profile of *NEUROD4* in response to exogenous NEUROG2. When NEUROG2 alone was introduced into the chick spinal cord, *NEUROD4* expression was up-regulated at 24h (Fig. S5C–d). At this stage, the MZ has not formed been yet and expression of *NEUROD4* that corresponds to the IZ is found at the lateral edge of the spinal cord. In contrast, after 48 h of incubation, when most electroporated cells have already migrated to the MZ, *NEUROD4* was down-regulated in these MZ cells even though they still expressed high amounts of NEUROG2 (Fig. 5A1, A4, E). These cells were post-mitotic and expressed

neuronal markers (Lamar et al., 2001 and data not shown). To test if the failure of NEUROG2 to activate *NEUROD4* in the MZ is due to the lack of its binding partner E47, which is normally down-regulated in the MZ (Fig. S1), we co-expressed cE47 with NEUROG2. Similar to the experiments with NEUROG2 alone, cells migrated to the MZ after 48 hrs but still did not express *NEUROD4* (Fig. 5E, column 7). These observations indicated two things: 1) that neurogenic gene cascade is activated and cells begin to differentiate in response to exogenous NEUROG2 (as shown by early induction of *NEUROD4* at 24 h); and 2) that transcriptional activity of NEUROG2 is suppressed by the time cells reach the MZ (at 48 h post-electroporation) by an endogenous mechanism induced in this gene cascade.

Thus, using this paradigm, we asked if MTGR1 is involved in suppressing the transcriptional activity of NEUROG2. We co-expressed NEUROG2 along with siRNA-MTGR1 in a developing chick spinal cord. (Fig. 5C1–4, D, E). This creates an environment where exogenous NEUROG2 is supplied and MTGR1 expression is reduced. In contrast to individual electroporation of NEUROG2 or siRNA-MTGR1 alone, *NEUROD4* was ectopically induced in the MZ (Fig. 5C1, arrowhead, and E, column 5). This ectopic induction of *NEUROD4* was not observed in control experiments expressing NEUROG2 and scrambled siRNA (siRNA-scramble) (Fig. 5E, column 4), and was partly prevented by co-expression of *Xenopus* MTGR1, which is not affected by siRNA-MTGR1 (Fig. 5E, column 6).

Analysis of neighboring sections by *in situ* hybridization with the *DLL1* probe, another readout of NEUROG2 activity, showed essentially the same results (Fig. 5, A3, B3, C3, F). *DLL1* has restricted expression along the dorso-ventral axis of the spinal cord, but in the levels that have ventricular/intermediate *DLL1* expression, ectopic expression in the MZ was observed when NEUROG2 and siRNA-MTGR1 was co-expressed (Fig. 5C3, arrowheads and F).

These cells expressing ectopic *NEUROD4* or *DLL1* in the MZ have prolonged and/or enhanced NEUROG2 activity, because NEUROG2 is ectopically expressed but MTGR1 level is decreased. To examine the outcome of prolonged NEUROG2 activity, adjacent sections were examined for expression of ISL1, a marker for postmitotic motoneurons (Fig. 6). Electroporation of siRNA-MTGR1 or cNEUROG2 vector alone did not cause change in the overall number of ISL1 positive cells at 48 h post electroporation, however co-electroporation of siRNA-MTGR1 vector with cNEUROG2 vector reduced overall number of ISL1 positive cells (Fig. 6A–C). We quantified the proportion of GFP positive cells that co-labeled with ISL1 (Fig. 6D). In embryos electroporated with siRNA-MTGR1, control siRNA or cNEUROG2 vectors, 60–70% of GFP positive cells co-labeled with ISL1. In contrast, in embryos co-electroporated with siRNA-MTGR1 and cNEUROG2 vectors, only 34% of cells co-labeled with ISL1 (Fig. 6C, arrowheads and Fig. 6D, lane 4). Similar results were obtained when scored for co-expression of GFP with PAX2 or HuC/D (data not shown).

In summary, our data suggest that MTGR1 functions *in vivo* to terminate NEUROG2 activity as neurogenesis progresses. When MTGR1 function was attenuated by siRNA in the presence of ectopic NEUROG2, cells initiated the differentiation program, but continued to express target genes of NEUROG2, *NEUROD4* and *DLL1*, even in the MZ and failed to fully induce postmitotic markers. Migration of the cells to the MZ, in contrast, appeared to happen normally and did not seem to require down-regulation of NEUROG2 activity by MTGR1.

Discussion

A negative feedback mechanism to terminate NEUROG2 activity is required for progression of neurogenesis

The neurogenic gene cascade is characterized by transient expression of transcription factors in a stereotypical order (Fig. 7). In addition to those examined in this paper, many transcription

factors are known to be transiently expressed during neurogenesis. Examples include Neurog1, Prox1, Hes6, Ascl1, NeuroD, PHD1, and MyT1/NZF-2b (Saito et al., 1996; Sommer et al., 1996; Ma et al., 1997; Torii et al., 1999; Koyano-Nakagawa et al., 2000; Matsushita et al., 2002; Misra et al., 2008). After the final mitosis, neuronal progenitors move from the ventricular surface through the IZ to the MZ within 6–8 hrs (Koyano-Nakagawa and Kintner, 2005). Thus, it is estimated that these cells switch the array of transcription factors expressed every 2–3 hours. Although the significance of the changing array of transcription factor expression is not completely understood, it is proposed that the proper termination of gene activity used in early steps of differentiation is necessary for normal progression of neurogenesis (Isaka et al., 1999; Cai et al., 2000).

We show in the study presented here that MTGR1, a transcriptional repressor induced by proneural bHLH proteins, feeds back to terminate transcriptional activity of the NEUROG2-E47 complex. It is already known that *NEUROG2* mRNA is expressed in a narrow window of time during development (Figs. 1, S1), and NEUROG2 protein is degraded quickly by a ubiquitin-mediated mechanism (Vosper et al., 2007). Our current study has identified a third mechanism, termination of NEUROG2 activity by MTGR1, which itself is a transcriptional target of NEUROG2. This third mechanism is likely to further limit the duration of NEUROG2 activity even if the NEUROG2 protein is still present (Fig. 7B). We propose that such a feedback system ensures rapid progress through the sequential steps required for neurogenesis.

It has not been readily recognized that termination of bHLH activity is necessary for normal progression of neurogenesis, partly because over-expression of NEUROG2 in the developing nervous system promoted neurogenesis and did not appear to cause aberrant differentiation (Lamar et al., 2001; Novitsch et al., 2001; Garcia-Dominguez et al., 2003). We demonstrate that, in these experiments, the normal course of the neurogenic gene cascade was activated, and the activity of NEUROG2 was inhibited by endogenous MTGR1. Our results clearly show that if activity of NEUROG2 is not properly terminated, cells continue to express markers of intermediate steps of neurogenesis and do not fully differentiate to express postmitotic markers (Fig. 5, 6). This finding has implications for efforts to induce various types of stem cells to differentiate as neurons for therapeutic use. One approach being investigated is misexpression of transcription factors that induce neurogenesis. Our finding indicates that it will be important to ensure the activity of neurogenic factors is terminated in a timely manner.

In light of sequential induction of transcription factors, it is interesting to consider the seemingly discrepant observations regarding the function of MTGR1. Cao et al. reported that over-expression of MTGR1 in *Xenopus* embryos expanded the neural plate but inhibited neurogenesis (Cao et al., 2002), whereas we reported in chick embryos that MTGR1 is a positive regulator of neurogenesis (Koyano-Nakagawa and Kintner, 2005). In fly, precocious expression of Nvy, a MTG homolog, is inhibitory to sensory organ precursor differentiation but isochronic over-expression is not (Wildonger and Mann, 2005). We think that these discrepant observations can be explained by the relative timing of gene expression: since NEUROG2 function is required to initiate neurogenesis, if MTGR1 is over-expressed before the onset of neurogenesis, then it blocks NEUROG2 from initiating the differentiation program resulting in expansion of the progenitor pool. Over-expression of MTGR1 after the onset of neurogenesis does not block neurogenesis because the NEUROG2-induced differentiation program has already turned on the necessary set of genes. However, as we observed in this study, if NEUROG2 activity is sustained after the onset of neurogenesis by blocking MTGR1, cells fail to terminate NEUROG2 activity and cells do not differentiate properly.

Molecules that control progression of neurogenesis

Two main processes, cell fate specification and differentiation, operate during neurogenesis. The transcription factor code that determines cell fate has been well studied in many systems

(Guillemot, 2007; Dessaud et al., 2008; Ohsawa and Kageyama, 2008). In contrast, only a handful of transcription factors are known to be involved in the control of progression of neurogenesis, especially the transition from proliferating progenitors to differentiated neurons. One emerging concept from these studies, nevertheless, is that exit from the cell cycle, lateral migration and expression of postmitotic genes are regulated by different sets of transcription factors. For example, Ebf1 is required for lateral migration and differentiation of spinal neurons, but not for cell cycle exit (Garcia-Dominguez et al., 2003). Sox4 and Sox11 promote expression of neuronal markers, but do not induce cell cycle withdrawal (Bergsland et al., 2006). In contrast, Prox1 and p57 are required for cell cycle exit, but are not sufficient to induce the expression of a full array of neuronal markers (Gui et al., 2007; Misra et al., 2008). There are still many transcription factors downstream of proneural bHLHs whose target pathways are not fully understood (Roztocil et al., 1997; Sandberg et al., 2005; Schmid et al., 2007; Soustelle and Giangrande, 2007). Attenuation of MTGR1 function in the presence of NEUROG2 inhibited expression of postmitotic markers, caused prolonged expression of progenitor characteristics, but did not significantly affect cell migration. It would be of interest to investigate the molecular interaction of MTGR1 with the transcription factors involved in regulating those aspects of neurogenesis.

Mechanism of inhibiting NEUROG2 activity by MTGR1

MTG family proteins are known to repress transcription either by bridging sequence-specific transcription factors and the HDAC complex (acting as an “active” inhibitor) or by competing for interaction with CBP/p300 (acting as an “passive” or competitive inhibitor) (Hug and Lazar, 2004; Rossetti et al., 2004). In either case, they do not directly bind to DNA but function by interacting with other proteins. We show that MTGR1 inhibits NEUROG2 activity by at least two mechanisms, which are not mutually exclusive.

We propose that one of the mechanisms of inhibition is through recruiting inhibitory co-factors to the NEUROG2-E47 complex already formed on DNA. Our transcription assay showing that repression requires the presence of the NEUROG2-E47 complex and the E-box target sequence supports this model. In addition, MTGR1-EnR fusion protein inhibited, and MTGR1-VP16 augmented the NEUROG2-E47 activity through the E box. This result is consistent with the model that this inhibitory activity takes place by binding to the NEUROG2-E47 complex formed on DNA. Based on the report that mouse MTGR1 interacts with mSin3A, N-CoR and HDAC3 (Amann et al., 2005), we speculate that this repression activity involves recruitment of histone deacetylases. The fact that we did not detect the formation of a higher order complex containing NEUROG2, E47 and MTGR1 in EMSA seemingly contradicts the results of the transcription assay. We speculate this is due to the short half-life of the complex or to the heterogeneity of the higher order complex (Rossetti et al., 2004). Future experiments using crosslinking techniques and new antibodies that can be used for immunoprecipitation are needed to clarify this issue.

In contrast, the EMSA experiment suggests a second mode of inhibiting NEUROG2-E47 activity by MTGR1. This experiment clearly showed inhibition of DNA binding activity of the NEUROG2-E47 complex when MTGR1 is associated with the complex before DNA binding. It should be noted, however, that MTGR1 did not dissociate the NEUROG2-E47 complex that was already bound to DNA. Thus, in this mode of inhibition, MTGR1 represses the activity of only the NEUROG2-E47 species that are not bound to DNA. It is possible, in our transfection assays with VP16 or EnR fusion molecules of MTGR1, that some of the fusion molecules have inhibited the DNA binding of unbound NEUROG2-E47 complexes. However, VP16 fusion molecule of MTGR1 strongly activated the transcriptional activity of NEUROG2-E47 further beyond the normal activity of the NEUROG2-E47 complex. Thus, we think that the activity we observed in this experiment reflects the action of VP16 and EnR fusion proteins

on the NEUROG2-E47 complex that was already bound to DNA before the fusion proteins associated with them.

Because *MTGR1* is induced by NEUROG2 and follows the expression of NEUROG2 with a delay (Figs. 1, S1 and Alishahi et al., 2009), we speculate that these two modes of inhibition may take place sequentially *in vivo*. In the early phase of neuronal differentiation, NEUROG2 and E47 are expressed first, and the expression level of *MTGR1* is low. When *MTGR1* levels becomes high, it may bind to the NEUROG2-E47 complex that is already bound to DNA and inhibit transcription using its repressor activity. In the later phase, when NEUROG2, E47 and *MTGR1* are present, *MTGR1* may bind to new NEUROG2 and E47 proteins being synthesized and prevent them from binding to DNA. We think that these two mechanisms of inhibition will efficiently terminate activity of the NEUROG2-E47 complex (Fig. 7B).

Up to now, E proteins have been the only bHLH transcription factors reported to interact with MTG proteins (Plevin et al., 2006; Wei et al., 2007). We provide evidence that activity of NEUROG2-E47 or NEUROG2 alone is inhibited by *MTGR1*, showing that NEUROG2 is a novel target of *MTGR1*-mediated repression. This does not eliminate the possibility that E proteins are also targeted by *MTGR1*, because endogenous E proteins expressed in P19 cells could potentially contribute to the transcriptional activity we observed. We think, however, the main target of *MTGR1* is NEUROG2, because immunoprecipitation assays of over-expressed proteins in HEK293T cells showed binding of *MTGR1* to NEUROG2 but not to E47, and interaction of *MTGR1*-E47 was significantly weaker than that of *MTGR1*-NEUROG2 in the GST pull-down assay.

The interaction between *MTG8* and HEB takes place through the N-terminal activation domain (AD1) of HEB (Zhang et al., 2004). Since there is no sequence in the NEUROG2 protein that resembles AD1, it is likely that there is a distinct *MTGR1* interaction surface within the NEUROG2 protein. This is not surprising, since MTG proteins are known to function as a protein “scaffold”, interacting with various transcription factors (Hug and Lazar, 2004; Rossetti et al., 2004). It would be of interest to map the interaction domains of *MTGR1* and NEUROG2. Since MTG family proteins are highly conserved, there may be similar but specific interactions between other MTG family members and other proneural bHLH proteins. Indeed, our data showed that NEUROG2 binds *MTGR1* but not *MTG8*. Whether such specific interactions contribute to functional difference of bHLH proteins is under investigation.

Modulators of bHLH protein activity during development

Transcriptional activity of proneural bHLH proteins is modulated by many mechanisms. For example, X-NGNR-1 interacts with CBP/p300, members of histone acetyltransferases (Koyano-Nakagawa et al., 1999). Brg1, a component of the SWI/SNF chromatin remodeling complex, has also been reported as a necessary co-factor of X-NGNR-1 and NeuroD activity in *Xenopus* (Seo et al., 2005). Id and Hes family proteins are known inhibitors of proneural bHLH transcription factors (Kageyama et al., 1997; Yokota, 2001). They heterodimerize with bHLH proteins and inhibit transcription by forming non-DNA binding complexes. We report for the first time that *MTGR1* associates with NEUROG2 and inhibits its function. This adds a new group of repressor proteins that could potentially interact with proneural bHLH proteins. We speculate that proneural bHLH proteins interact with different sets of transcriptional co-factors at different stages of development to rapidly switch on or off their transcriptional activity. This also predicts that specific timing of co-factor expression is important to regulate proneural protein activity.

Recent studies have shown that Neurogenins have late functions after the initial phase of neurogenesis. Such activities do not involve their transactivation capacities and include control of cell fate, cell migration, axon projections and dendrite morphologies (Sun et al., 2001; Seibt

et al., 2003; Schuurmans et al., 2004; Hand et al., 2005; Hand and Polleux, 2006). It will be of interest to know if inhibition of transcriptional activity by MTG proteins is required for neurogenins to exert such late functions. In addition to the spinal cord, *MTGR1* is induced by bHLH proteins in other systems such as retina and small intestine (Amann et al., 2005; Logan et al., 2005). Thus, it is likely that feedback regulation of bHLH protein by *MTGR1* is a widely used mechanism to promote cellular differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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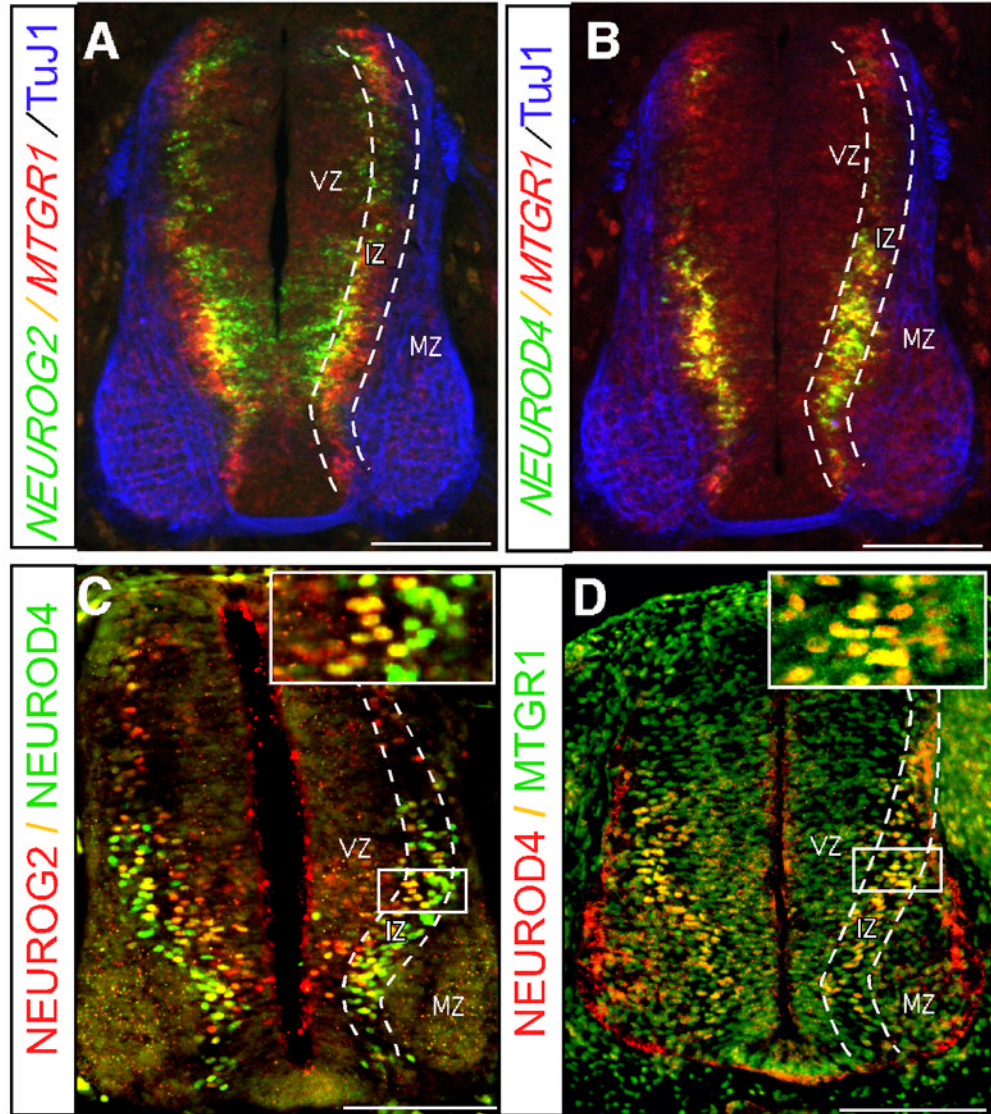


Figure 1. Expression of NEUROG2, MTGR1, and NEUROD4 in the chick embryo spinal cord
 Cross sections of embryos at HH stage 24 at the brachial level are shown. A, B: Fluorescent *in situ* hybridization of *NEUROG2*, *NEUROD4* and *MTGR1* combined with immunohistochemistry of TuJ1. C, D: Double immunohistochemistry of *NEUROG2*, *NEUROD4* and *MTGR1*. Colors of the probes are indicated next to figures. Boundaries of the VZ, the IZ, and the MZ are indicated by dotted lines. Boxed areas are enlarged in the insets of C and D. Bars: 100 μ m. Note that *NEUROG2* is expressed most medially, closest to the ventricle, followed by *MTGR1* and *NEUROD4*, appearing at the same medio-lateral position. Both genes are down-regulated in the MZ.

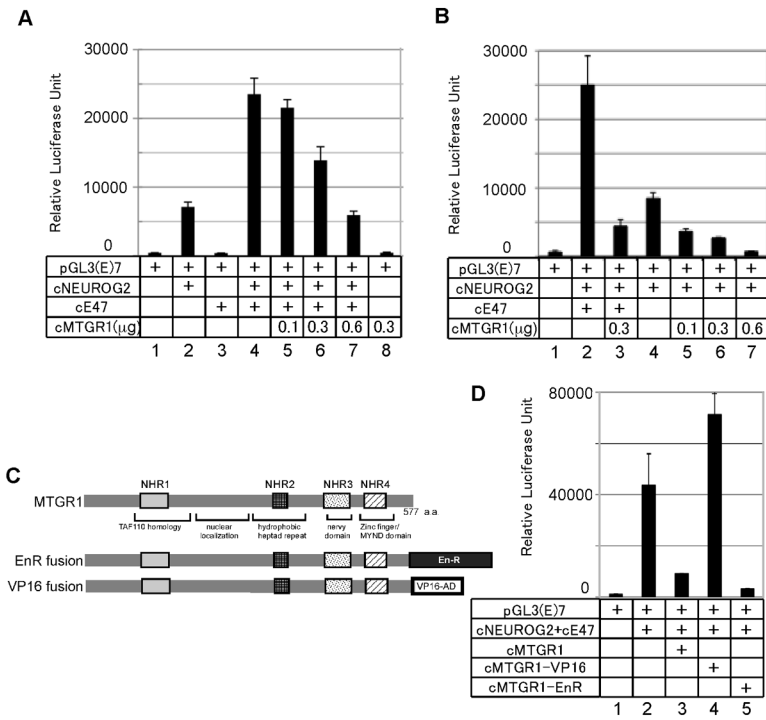


Figure 2. MTGR1 represses transcriptional activity of the NEUROG2-E47 complex
 A, B, D: Transient transfection assay. C: Diagram of the fusion molecules of chick MTGR1. A, B: P19 cells were transfected with the indicated vectors and increasing amounts of the MTGR1 expression vector (0.1, 0.3 and 0.6 μ g). MTGR1 repressed transcription activity of NEUROG2-E47 (A) or NEUROG2 alone (B) dose dependently. Total amount of DNA transfected per sample was adjusted with an empty vector. D: Transcription activity of MTGR1 fusion proteins. P19 cells were transfected with the indicated vectors and 0.3 μ g of vectors encoding MTGR1 fusion molecules. Bars show SD for all pael. Essentially the same results were obtained from P19 and HEK293T cells for all assays, and the results from P19 cells are shown.

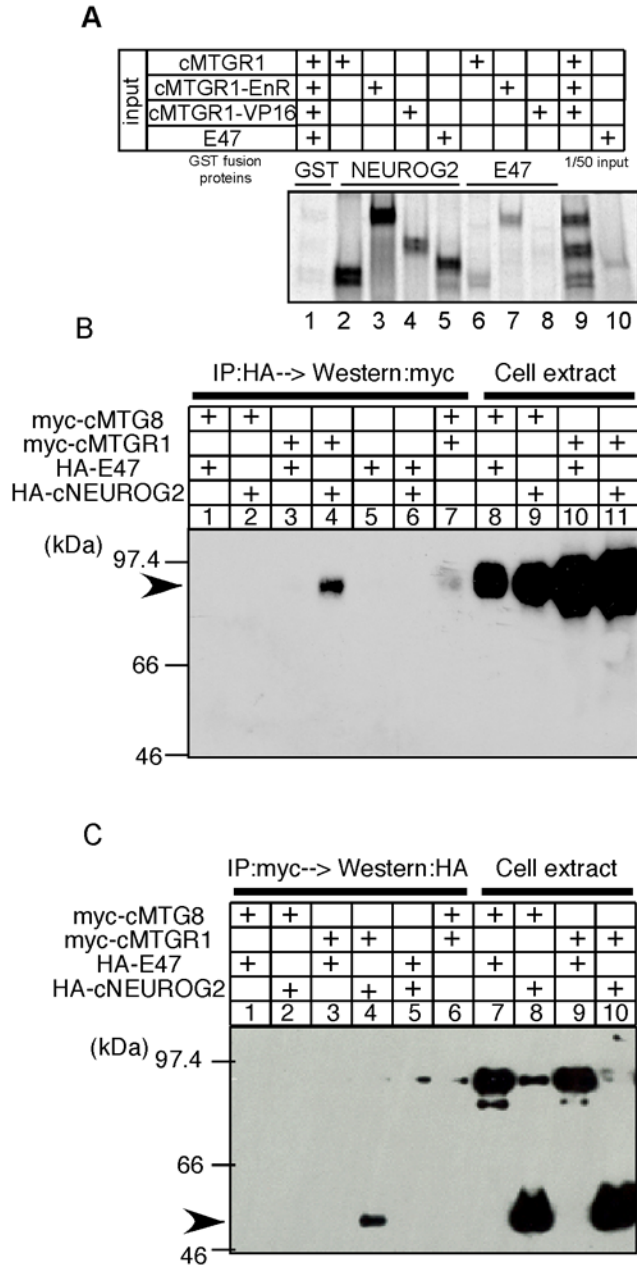


Figure 3. MTGR1 directly interacts with NEUROG2

A: GST pull-down assay. Radiolabeled MTG proteins or E47 were pulled down with glutathione sepharose beads coated with GST molecule alone (lane 1), GST-NEUROG2 fusion (lanes 2–5) or GST-E47 fusion molecules (lanes 6–8). Lanes 9–10 contain 1/50 of input proteins. B, C: Immunoprecipitation assays. HEK293T cells were transfected with expression vectors of the indicated proteins. Cell lysates were immunoprecipitated with antibodies against the HA (B) or the myc epitopes (C), and recovered proteins were analyzed by Western blot using anti-myc (B) or anti-HA (C) antibodies. Whole cell extracts were analyzed in lanes 8–11 of B and lanes 7–10 of C to confirm protein expression.

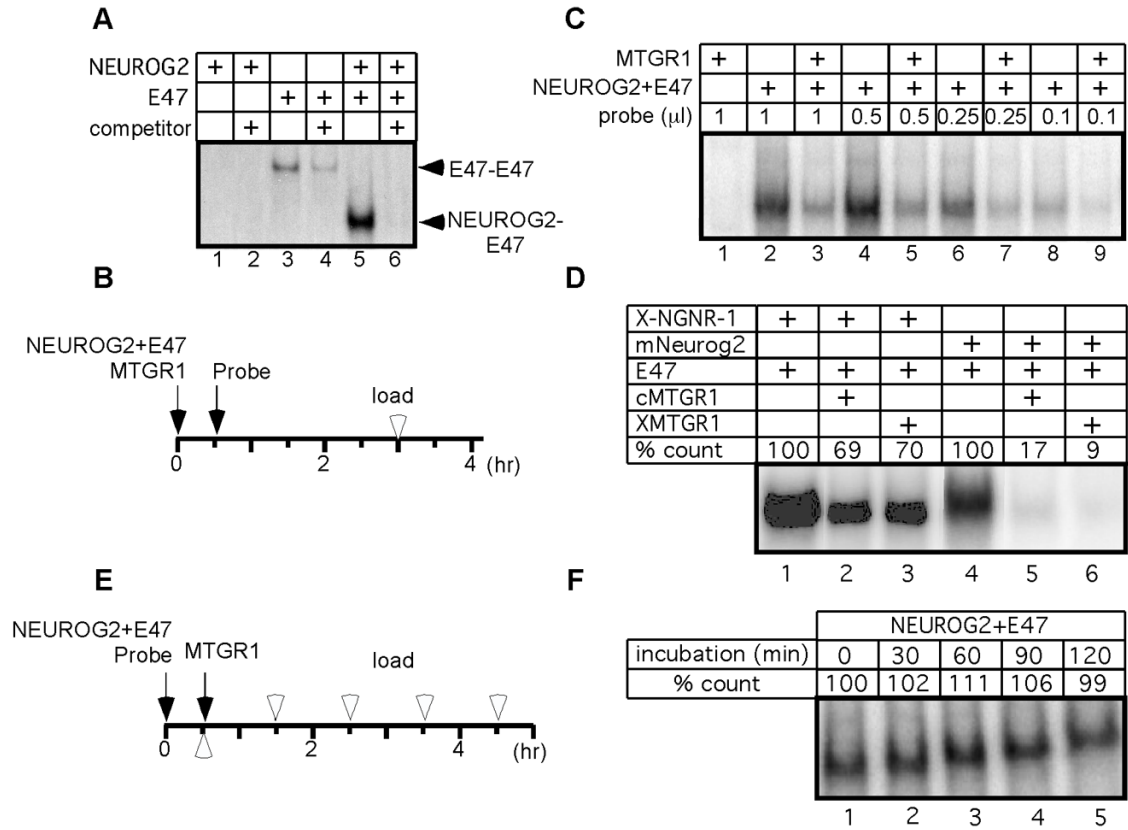


Figure 4. MTGR1 blocks DNA binding activity of the NEUROG2-E47 complex

A: EMSA of NEUROG2 and E47, either synthesized individually (lanes 1–4) or in the same reaction (lanes 5–6). Twenty-fold excess of cold competitor was added to test the specificity (lanes 2, 4, 6). Arrowheads point to bands containing E47 homodimer (E47-E47) and NEUROG2-E47 heterodimer (NEUROG2-E47). B, E: Experimental schedule testing the effect of MTGR1 on association (B) and dissociation (E) of NEUROG2-E47 complex to/from DNA, respectively. Black arrows and white arrowheads indicate the times when proteins and probes were mixed and loaded on a gel, respectively. C: EMSA was carried out using schedule shown in B with varying amounts of probes. D: Various MTG molecules were examined following the schedule shown in B. Intensity of each band was quantified on a phosphorimager and shown as percentage of the band without MTGR1. F: EMSA was done as diagrammed in E. Relative radioactivity compared to lane 1 is shown. Since samples were applied every 30 min on a continuously running gel, migration distances appear different from each other.

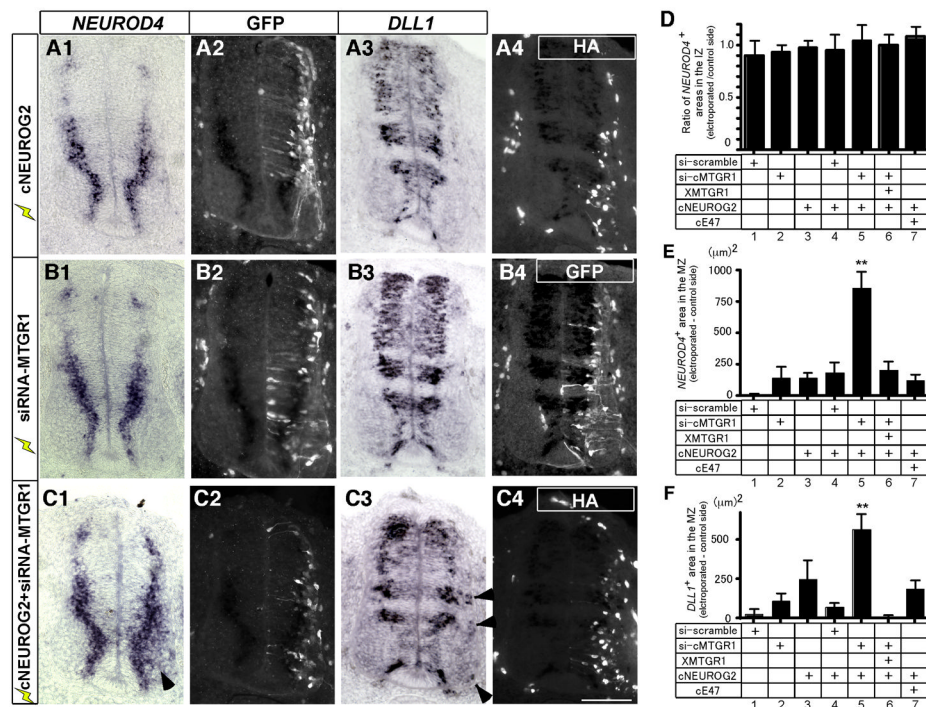


Figure 5. NEUROG2 activity is inhibited by MTGR1 *in vivo*

A–C: Chick spinal cord was electroporated with expression vectors of HA-tagged cNEUROG2 (A1–4), siRNA-MTGR1 (B1–4) or combination of both (C1–4) at E2 and harvested at E4. The siRNA-MTGR1 vector has a GFP gene that allows identification of the electroporated cells. For A1–4, an expression vector for GFP was co-electroporated as a tracer. Sections at the brachial level were analyzed for *NEUROD4* expression by *in situ* hybridization (A1, B1, C1), anti-GFP immunohistochemistry (A2, B2, B4, C2), *DLL1* *in situ* hybridization (A3, B3, C3), and immunohistochemistry against the HA tag (A4, C4). The left two and the right two pictures of each line are from the same section, and the two pairs of pictures are from the neighboring sections. *In situ* hybridization signal is visible in the immunofluorescent channels as dark areas due to absorption of light by color pigments. The electroporated sides are oriented to the right in all figures. Arrowheads in C1 and C3 point to ectopically expressed *NEUROD4* and *DLL1* in the MZ. Scale bar: 100 μ m. D–F: Quantification of the areas expressing *NEUROD4* in the IZ (D), *NEUROD4* in the MZ (E) and *DLL1* in the MZ (F). D. Pixels of *NEUROD4* positive areas within the IZ were counted and the ratios of electroporated and un-electroporated sides were calculated. E, F. Pixels of positive areas for each marker in the MZ was quantified and the difference between electroporated and control sides is shown. Error bars: SEM.

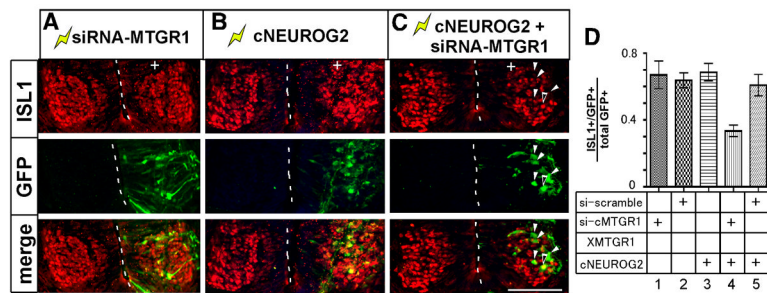


Figure 6. Cells with elevated NEUROG2 activity in the MZ show impaired neurogenesis
 A–C: Electroporated embryos were analyzed for expression of ISL1 (red) and GFP (green). Electroporated sides are oriented to the right and indicated by (+). The midlines of the spinal cord are demarcated by dotted lines. White arrowheads in C point to GFP positive, ISL1 negative cells. Black arrowhead points to a double positive cell. Bar: 100 μ m. D: Quantification of the ratio of cells co-labeled with ISL1 and GFP. GFP positive cells within the postmitotic motorneuron domain were counted. Each bar represents average of 10–15 sections. Error bars: SEM.

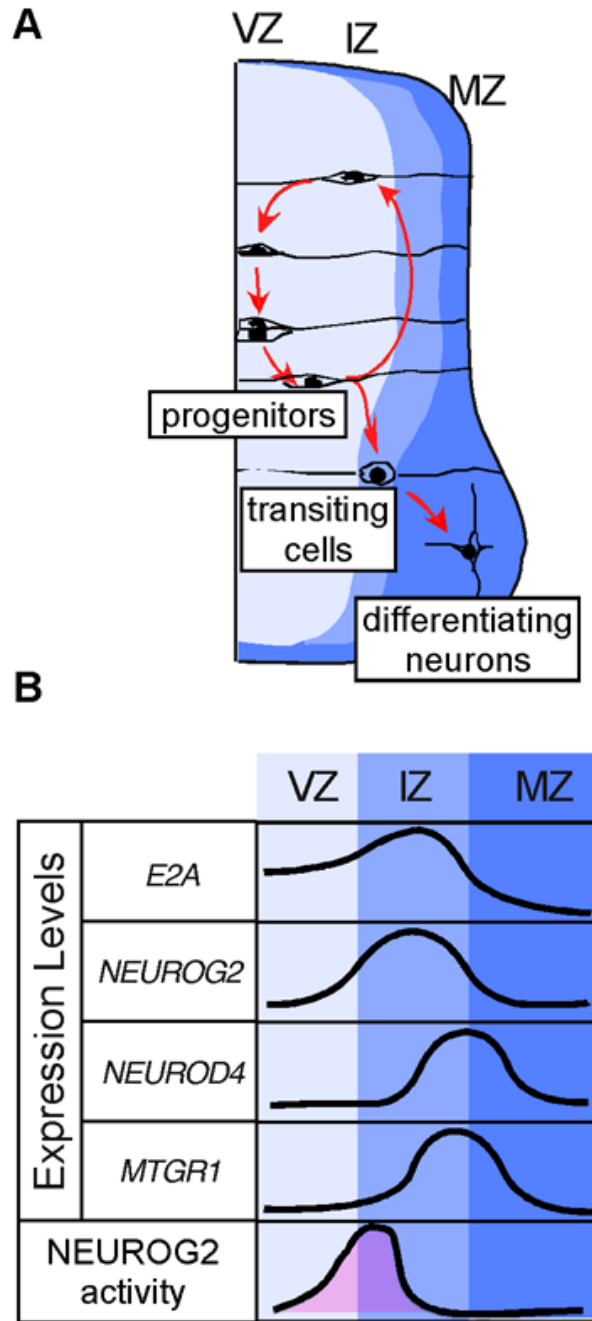


Figure 7. Model: NEUROG2 activity is limited to a narrower area compared to its expression domain by the function of MTGR1

A: Diagram of cell movement during neurogenesis. Progenitors are located in the VZ. After the last mitosis, committed neuronal progenitors migrate to the IZ, and delaminate to the MZ. Note that similar gene expression pattern is observed in development of the mouse central nervous system (Alishahi et al., 2009). B: Relative expression levels of genes studied in this paper. The area shaded in pink represents conceptual transcriptional activity of NEUROG2 based on our findings. Note that the activity of NEUROG2 is limited to narrower window of time compared to the period of its gene expression.