Neurogenin2 expression together with NeuroM regulates GDNF family neurotrophic factor receptor α1 (GFRα1) expression in the embryonic spinal cord

Takako Shimada a, Hiroyuki Yaginuma a, Noboru Sato b, Shunsaku Homma a, *

a Department of Anatomy, School of Medicine, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan
b Department of Anatomy, School of Medicine, Niigata University, Asahimachi-dori 1, Niigata, Niigata 951-8510, Japan

ARTICLE INFO

Article history:
Received 26 August 2011
Received in revised form 1 August 2012
Accepted 2 August 2012
Available online 10 August 2012

Keywords:
Math3
NeuroD4
Cash1
Ptf1a
p27
Chick
Electroporation
In situ hybridization

ABSTRACT

In many regions of the nervous system, the combinatorial action of transcriptional factors specifies the individual fate of neuronal subtypes. Contrary to this, we report that a single transcriptional factor controls a phenotype shared by different subtypes of neurons, namely the expression of a neurotrophic factor receptor in the spinal cord. Along the dorsoventral axis of the chick embryo spinal cord, the expression pattern of a specific receptor for glial cell line derived-neurotrophic factor (GDNF family of receptors α1: GFRα1) was related to that of two basic helix-loop-helix (bHLH) transcriptional factors (NeuroM and Neurogenin2: Ngn2). In ovo electroporation in the chick embryo revealed that the overexpression of NeuroM alone was sufficient to induce ectopic GFRα1 expression without overt neuronal differentiation, whereas the suppression of NeuroM activity resulted in the specific loss of GFRα1 expression, indicating that NeuroM may act as a differentiation factor for GFRα1 expression. Ngn2 overexpression was also sufficient to induce precocious GFRα1 expression. However, the forced expression of both obligate suppressor and activator forms of Ngn2 also induced aberrant GFRα1 expression. Thus, any deviation from an optimum level of Ngn2 expression resulted in aberrant GFRα1 expression. Consistent with this, manipulation of Ngn2 expression levels by other bHLH factors also resulted in ectopic GFRα1 expression. For example, the downregulation by Ascl1 and the upregulation by Ptf1a induced ectopic GFRα1 expression, irrespective of endogenous expression patterns of Ascl1 and Ptf1a (Ascl1/Ptf1a) in the spinal cord. The suppression of Ascl1/Ptf1a activities abolished Ngn2 and GFRα1 expression, even in Ascl1/Ptf1a-negative regions. These data indicate the presence of a distinct regulatory sequence for a determinant of GFRα1 expression, which occurs in multiple subtypes of spinal neurons. © 2012 Elsevier Inc. All rights reserved.

Introduction

Subsets of developing neurons acquire an appropriate collection of functional proteins such as the surface adhesion molecules, neurotransmitter-synthesizing enzymes and receptors, ion channel molecules, etc. In the developing nervous system, distinct subsets of neurons express unique combinations of neurotrophic factor receptors. This differential expression of neurotrophic factor receptors is critical for the proper development of the nervous system, because neurotrophic factors and cognate receptors regulate many aspects of neural development such as differentiation, survival, process outgrowth, and plasticity. A well-known example of such differential expression is the Trk neurotrophin receptor in spinal dorsal root ganglion cells: TrkA and TrkC are respectively expressed in the small- and large-sized neurons in the spinal ganglia, and selectively mediate the survival of corresponding neurons (Snider, 1994).

The four specific receptors for another neurotrophic factor family, glial cell line-derived neurotrophic factor (GDNF), the GDNF family of receptors α (GFRα1–4), are also differentially expressed in various types of neurons in both the central and peripheral nervous system. GDNF signaling through GFRα1 receptor is constantly required in specific subpopulations of neuronal cells for various aspects of neuronal development (Paratcha and Ledda, 2008). In the initial phases of the neural development, GFRα1 is necessary for the differentiation and migration of a subpopulation of cortical inhibitory interneurons (Pozas and Ibáñez, 2005; Canty et al., 2009). Gene-knockout studies of GFRα1 have also demonstrated their crucial roles in later phases of neural development: GFRα1 is required for the survival of distinct subpopulations of neurons in both the central and peripheral nervous system. 

* Corresponding author. Fax: +81 24 549 8811.E-mail address: shomma@fmu.ac.jp (S. Homma).
subsets of neurons in the CNS and PNS, for example, for the survival of specific subpopulation of motoneurons (Airaksinen et al., 1999; Garcés et al., 2000; Airaksinen and Saarma, 2002; Gould et al., 2008); GDNF and GFRα1 are also necessary to establish the correct innervation pattern between a specific motoneuron group and its peripheral target (Haase et al., 2002; Vrieseling and Arber, 2006); and neuronal cell adhesion induced by GDNF- GFRα1-interaction promotes synapse formation (Ledda et al., 2007). Despite the increased understanding of significant developmental roles of GFRα1, little is known about regulatory mechanisms for the selective expression of neurotrophic factor receptors including GFRα1 in particular subsets of neurons. Originally, GDNF was identified as a potent neurotrophic factor for dopaminergic neurons in the midbrain (DA neurons), which are selectively lost in Parkinson's disease (Lin et al., 1993). Genetic deletion of the GFRα1 gene results in degenerative changes of DA neurons and increasing vulnerability to toxic insult during aging (Boger et al., 2008; Zaman et al., 2008). Despite the crucial role of GFRα1 for the survival of DA neurons, the mechanism for regulation and maintenance of GFRα1 expression in the nervous system is unknown. Understanding the embryonic regulatory mechanism for GFRα1 expression could provide new insights into the etiology of Parkinson's disease.

Candidate molecules for regulating differential expression of the GFRα1 receptor are basic helix-loop-helix (bHLH) transcriptional factors. In vertebrates, a large number of bHLH transcriptional factors are expressed in distinct populations of neuronal progenitors and induce the differentiation of different subtypes of neurons (Bertrand et al. 2002). For example, two major subtypes of cortical neurons, GABAergic inhibitory interneurons and glutamatergic projection neurons, are respectively produced from ventral and dorsal forebrain, where Ascl1 and Neurogenin2 (Ngn2) are expressed in complementary fashion. Ascl1 deterministically regulates the production of GABAergic interneurons in the ventral telencephalon, whereas Ngn2 permissively regulates that of glutamatergic neurons in the dorsal telencephalon (Fode et al., 2000). However, the ventricular layer in the ventral forebrain encompasses multiple microdomains, which are defined by the combinatorial expression pattern of various homeodomain transcriptional factors (Sussel et al., 1999; Puelles and Rubenstein, 2003). After the GABAergic phenotype is specified by Ascl1, subtypes of cortical interneurons that share the GABAergic phenotype are generated from specific microdomains in the ventral forebrain according to a combinatorial code of homeodomain-containing transcriptional factors (Wonders and Anderson, 2006; Gelman and Marín, 2010). Thus, stepwise specification ensures coupling a shared neurotransmitter subtype, which is controlled by a single bHLH factor, with a region-specific neuronal subtype identity, which is controlled by a combinatorial code of a different class of transcriptional factors (Ma, 2006).

The ventricular layer of early embryonic spinal cord is also subdivided into microdomains, which generate distinct subtypes of neurons along the dorsoventral axis, according to a combinatorial expression pattern of transcriptional factors (Jessell, 2000; Caspary and Anderson, 2003; Guillemot et al., 2007; Sugimori et al., 2007). A variety of bHLH transcriptional factors are expressed in the multiple microdomains of the spinal cord and have been shown to regulate local production of neuronal subtypes, together with regionally expressed co-factors (Mizuguchi et al., 2001; Novitch et al., 2001; Sugimori et al., 2007). However, unlike in the forebrain where a single bHLH transcriptional factor regulates the differentiation of a neurotransmitter phenotype shared by multiple subtypes of neurons, it is unknown whether a bHLH factor may also control the differentiation of a particular neuronal feature shared by the different subclasses of neurons in the spinal cord. This is largely owing to the lack of information as to how unique neuronal phenotypes relate to the overall expression pattern of a particular bHLH factor in the spinal cord. In a previous study of the expression pattern of GFRα1 in the spinal cord at early developmental stages, we noticed a close relationship in the expression patterns between GFRα1 and NeuroM, or between GFRα1 and Ngn2 (Homma et al., 2003). These similarities in expression patterns suggest that NeuroM and Ngn2 may be involved in the regulation of GFRα1 expression. Based on this observation, we have now explored the regulatory role of NeuroM, Ngn2 and related bHLH factors in GFRα1 expression using in vivo electroporation in the chick embryo.

Materials and Methods

Embryos and electroporation

Retrovirus-free fertilized eggs were purchased from Takeuchi Farm (Nara, Japan). The eggs were incubated in the laboratory at 37°C and 60% relative humidity. The Hamburger and Hamilton (1951) stage series (HH stage) was used for age determination of embryos.

Electroporation was carried out in prospective lumbar spinal cord at HH stage 14. The concentration of plasmid was 0.5 μg/μl in saline. Electroporation conditions were 20 mV, 50 msec duration, 150 msec intervals, for 5 times with electrodes 2 mm apart. Trace amounts of pEGFP-N1 vector (Clontech) were sometimes mixed in the plasmid solution to monitor electroporation (0.005 μg/μl at final concentration).

cDNA template

pACGFP1-Nuc vector (Clontech) was used as a template for nuclear-localized GFP (Nuc-GFP) cDNA. Complementary DNA template of NeuroM was generous gift from Dr.Ballivet (Roztocil et al., 1997), Neurogenin2 (Ngn2) cDNA was generous gift from Dr. Anderson (Perez et al., 1999). Ret receptor tyrosine kinase cDNA was kindly provided by Dr. Costantini (Schuchardt et al., 1995). The coding regions in full length of chick GFRα1, GFRα2, GFRα4, achaete-scute homolog 1 (Ascl1), and engrailed1 were isolated by PCR using the first strand cDNA reverse-transcribed from poly(A)−RNA derived from chick embryos at HH stage 17. Primer pairs used for the template isolation were 5'−ATGTTCCTCGCCGTCCTCTACTTGCCCTG−3' and 5'−GCTACAAGAGCAGCTAGTGATCCAG−3' for GFRα1, 5'−ATGTTCCTCGCCGTCCTCTACTTGCCCTG−3' and 5'−CTATAGAGGCTCTGCCAGGAGAA−3' for GFRα2, 5'−ATGGGGGATGCTTGGCAGG−3' and 5'−TACGACAGTGGCGTCTGACGAGCG−3' for Ascl1, and 5'−ATGTTCCTCGCCGTCCTCTACTTGCCCTG−3' and 5'−TACGACAGTGGCGTCTGACGAGCG−3' for engrailed1. Primer pairs used for the isolation of partial cDNA sequences of chick Ptf1a and Delta1 used for in situ hybridization were 5'−CAGGGGAGGGGTTGACGACGAG−3' and 5'−TCAGGAAGCGGGGTCAAGG−3' for Ptf1a, and 5'−CAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG−3' and 5'−TACGACAGTGGCGTCTGACGAGCG−3' for Delta1. The sequence information used for template isolation was derived from BBSRC chick EST database and NCBI nucleotide database.

We have not been able to isolate the full length chick Ptf1a cDNA and have used mouse Ptf1a sequence for the construction. Full length coding region of mouse Ptf1a was isolated by PCR from the E10 mouse first strand cDNA using the primer pair of 5'−ATGGGGGATGCTTGGCAGG−3' and 5'−TCAGGAAGCGGGGTCAAGG−3' and 5'−TACGACAGTGGCGTCTGACGAGCG−3'. Complementary DNA fragment of the herpesvirus VP16 activator domain was isolated from the genomic DNA of human.
herpesvirus, which was gift from Dr. Suzutani in the Department of Bacteriology in our institute, using primers, 5′-CTGTCGAG GCCCCCCAGCACCAGTCAACCCTG-3′ and 5′-CTACCCAGCTACTCGTAATTCACAGGACGTACCC-3′.

The PCR products were cloned into pCRII TOPO cloning vector (Invitrogen). Correct DNA sequences of obtained PCR products were confirmed using ABI 3100 sequencer.

Expression vector construction

We used the avian retrovirus-based vector RCAS(B) to express foreign genes in the spinal cord, because we favored the relatively weak promoter activity of this vector to avoid “super physiological levels” of expression.

Ncol site including initiation codon, Myc-tag sequence, and Csp45I site were added in frame with 5′-end of NeuroM-coding sequence with a primer 5′-CCATGCAGAGAACAACTCATCGAGGAATGAGATTCGAGAACG-3′, and so was SpeI site after the stop codon of NeuroM cDNA by PCR using Pfu DNA polymerase. The PCR product was cloned into pCRII TOPO Blunt (Invitrogen) and was sequenced. Following digestion with Ncol and SpeI, NeuroM-coding region with Myc-tag was transferred to the Sla13-Ncol adapter vector in which the original PsiI site was replaced with SpeI site in advance. Clal fragment containing the expression cassette was then transferred to RCAS(B) vector (Hollenbeck and Fekete, 2003).

For the construction of Nuc-GFP, NgN2, Ascl1, and Ptf1a expression vector, NeuroM-RCAS(B) vector was pre-digested with Csp45I and SpeI to remove NeuroM cDNA, but to leave Myc-tag sequence in the RCAS(B) vector. Csp45I site was added at the 5′-end of coding sequence of Nuc-GFP, NgN2, Ascl1 and Ptf1a by PCR in frame with Myc-tag sequence. Stop codon and SpeI site were also added at the 3′-end of the coding sequences. The PCR product with the Csp45I and SpeI sites was cloned into pCRII TOPO Blunt vector, and was sequenced. Following the digestion of the cloned vector by Csp45I and SpeI, the fragment was transferred to the pre-digested NeuroM RCAS(B) vector. The primer pairs used for the addition of Csp45I and SpeI sites were 5′-TCGAAATGTTGACACATGCAGAGAACAACTCATCGAGGAATGAGATTCGAGAACG-3′ and 5′-ACTAGTTTATCTAGATCCGGTGG-3′, and 5′-CTACCCAGCTACTCGTAATTCACAGGACGTACCC-3′ and 5′-ACTAGTGTGATCAGAATACATATTCCGGCTTGGCGGTAGCTACCC-3′.

For the construction of Nuc-GFP, NgN2, Ascl1, and Ptf1a expression vector, NeuroM-RCAS(B) vector was pre-digested with Csp45I and SpeI to remove NeuroM cDNA, but to leave Myc-tag sequence in the RCAS(B) vector. Csp45I site was added at the 5′-end of coding sequence of Nuc-GFP, NgN2, Ascl1 and Ptf1a by PCR in frame with Myc-tag sequence. Stop codon and SpeI site were also added at the 3′-end of the coding sequences. The PCR product with the Csp45I and SpeI sites was cloned into pCRII TOPO Blunt vector, and was sequenced. Following the digestion of the cloned vector by Csp45I and SpeI, the fragment was transferred to the pre-digested NeuroM RCAS(B) vector. The primer pairs used for the addition of Csp45I and SpeI sites were 5′-TCGAAATGTTGACACATGCAGAGAACAACTCATCGAGGAATGAGATTCGAGAACG-3′ and 5′-ACTAGTTTATCTAGATCCGGTGG-3′, and 5′-CTACCCAGCTACTCGTAATTCACAGGACGTACCC-3′ and 5′-ACTAGTGTGATCAGAATACATATTCCGGCTTGGCGGTAGCTACCC-3′.

Expression vector construction

We used the avian retrovirus-based vector RCAS(B) to express foreign genes in the spinal cord, because we favored the relatively weak promoter activity of this vector to avoid “super physiological levels” of expression.

Ncol site including initiation codon, Myc-tag sequence, and Csp45I site were added in frame with 5′-end of NeuroM-coding sequence with a primer 5′-CCATGCAGAGAACAACTCATCGAGGAATGAGATTCGAGAACG-3′, and so was SpeI site after the stop codon of NeuroM cDNA by PCR using Pfu DNA polymerase. The PCR product was cloned into pCRII TOPO Blunt (Invitrogen) and was sequenced. Following digestion with Ncol and SpeI, NeuroM-coding region with Myc-tag was transferred to the Sla13-Ncol adapter vector in which the original PsiI site was replaced with SpeI site in advance. Clal fragment containing the expression cassette was then transferred to RCAS(B) vector (Hollenbeck and Fekete, 2003).

For the construction of Nuc-GFP, NgN2, Ascl1, and Ptf1a expression vector, NeuroM-RCAS(B) vector was pre-digested with Csp45I and SpeI to remove NeuroM cDNA, but to leave Myc-tag sequence in the RCAS(B) vector. Csp45I site was added at the 5′-end of coding sequence of Nuc-GFP, NgN2, Ascl1 and Ptf1a by PCR in frame with Myc-tag sequence. Stop codon and SpeI site were also added at the 3′-end of the coding sequences. The PCR product with the Csp45I and SpeI sites was cloned into pCRII TOPO Blunt vector, and was sequenced. Following the digestion of the cloned vector by Csp45I and SpeI, the fragment was transferred to the pre-digested NeuroM RCAS(B) vector. The primer pairs used for the addition of Csp45I and SpeI sites were 5′-TCGAAATGTTGACACATGCAGAGAACAACTCATCGAGGAATGAGATTCGAGAACG-3′ and 5′-ACTAGTTTATCTAGATCCGGTGG-3′, and 5′-CTACCCAGCTACTCGTAATTCACAGGACGTACCC-3′ and 5′-ACTAGTGTGATCAGAATACATATTCCGGCTTGGCGGTAGCTACCC-3′.

Expression vector construction

We used the avian retrovirus-based vector RCAS(B) to express foreign genes in the spinal cord, because we favored the relatively weak promoter activity of this vector to avoid “super physiological levels” of expression.

Ncol site including initiation codon, Myc-tag sequence, and Csp45I site were added in frame with 5′-end of NeuroM-coding sequence with a primer 5′-CCATGCAGAGAACAACTCATCGAGGAATGAGATTCGAGAACG-3′, and so was SpeI site after the stop codon of NeuroM cDNA by PCR using Pfu DNA polymerase. The PCR product was cloned into pCRII TOPO Blunt (Invitrogen) and was sequenced. Following digestion with Ncol and SpeI, NeuroM-coding region with Myc-tag was transferred to the Sla13-Ncol adapter vector in which the original PsiI site was replaced with SpeI site in advance. Clal fragment containing the expression cassette was then transferred to RCAS(B) vector (Hollenbeck and Fekete, 2003).

For the construction of Nuc-GFP, NgN2, Ascl1, and Ptf1a expression vector, NeuroM-RCAS(B) vector was pre-digested with Csp45I and SpeI to remove NeuroM cDNA, but to leave Myc-tag sequence in the RCAS(B) vector. Csp45I site was added at the 5′-end of coding sequence of Nuc-GFP, NgN2, Ascl1 and Ptf1a by PCR in frame with Myc-tag sequence. Stop codon and SpeI site were also added at the 3′-end of the coding sequences. The PCR product with the Csp45I and SpeI sites was cloned into pCRII TOPO Blunt vector, and was sequenced. Following the digestion of the cloned vector by Csp45I and SpeI, the fragment was transferred to the pre-digested NeuroM RCAS(B) vector. The primer pairs used for the addition of Csp45I and SpeI sites were 5′-TCGAAATGTTGACACATGCAGAGAACAACTCATCGAGGAATGAGATTCGAGAACG-3′ and 5′-ACTAGTTTATCTAGATCCGGTGG-3′, and 5′-CTACCCAGCTACTCGTAATTCACAGGACGTACCC-3′ and 5′-ACTAGTGTGATCAGAATACATATTCCGGCTTGGCGGTAGCTACCC-3′.
For double probe in situ hybridization, digoxigenin- and fluorescein-labeled RNA probes were simultaneously incubated with sections. After post-hybridization washing, anti-digoxigenin antibody immunohistochemistry was first carried out, and BM-purple substrate (Roche) was used as a substrate for conjugated alkaline phosphatase reaction (blue reaction product). After the alkaline phosphatase was inactivated by fixing sections in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) for 30 min, sections were again incubated in anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche) overnight at 4 °C. A second coloring reaction (red reaction product).

**Immunohistochemistry and TUNEL**

Embryos were fixed in 4% PFA in 0.1 M PB for 30 min at 4 °C, and immersed in 20% sucrose in 0.1 M phosphate buffered saline (PBS) overnight. The lumbar region of the embryo was frozen-embedded. Embryos were transversely sectioned (12 μm thickness).

Anti-neuron-specific nuclear protein antibody (NeuN) and anti-neuron-specific isform of β-tubulin antibody (Tuj1) were purchased from Chemicon and Promega, respectively. The mouse monoclonal anti-Myc and anti-p27 antibodies were from Santa Cruz and BD Bioscience, respectively. Anti-Lhx1/5 and anti-Islet-1 antibodies were obtained from the Developmental Studies Hybridoma Bank. The 5-BrDU antibody was from Seikagakukogyo (Japan). Myc-tag and 5-BrDU immunohistochemistry was carried out after in situ hybridization. Alkaline phosphatase reaction product of in situ hybridization was fixed in 4% PFA in 0.1 M PB for 30 min before immunohistochemistry. For 5-BrDU immunohistochemistry, nuclear DNA was denatured in 2N hydrochloric acid for 30 min at 37 °C. Sections were incubated in the primary antibodies overnight at 4 °C, and then in Histofine Simple Stain HRP-conjugated secondary antibody (Nissui, Japan) or fluorescent-conjugated secondary antibodies for 1 h at a room temperature. 3,3′-Diaminobenzidine (DAB) was used as a chromogen. For double detection of Myc-tag and p27, the biotinylated anti-Myc mouse monoclonal antibody (Santa-Cruz) was used after p27 immunohistochemistry.

TUNEL reaction was carried out as described in Gavrieli et al. (1992).

**Quantification and statistical analysis**

For quantification of Ngn2, NeuroM, and GFRα-positive cells, nuclei present within *in situ* hybridization-positive areas were counted in five to seven sections per embryo under camera lucida drawing tube. NeuN-, Islet-1-, Lhx1/5-, p27-, and Edu-positive nuclei were counted in captured digital images of five to seven sections per embryo. For all cases, statistics were derived from five embryos in total and were presented as the mean ± standard error of the mean (SEM). One-sided t-test was used for comparison.

**Results**

**Parallel expressions of GFRα1 and bHLH genes**

In the chick embryo spinal cord on embryonic day 3.5 (HH stage 20–21), GFRα1 mRNA expression was observed along the most medial margin of the mantle layer. GFRα1-positive cells were segregated into two groups, a small cluster in the dorsal region and a longitudinal stripe running dorsoventrally. A small number of GFRα1-positive cells were scattered between these two groups (Fig. 1A). Slightly later (embryonic day 4, E4, HH stage 24), apparent motoneurons expressed GFRα1 mRNA in the periphery of the ventral spinal cord, in addition to the above medial two expression domains (Fig. 1B). The localization of GFRα1 mRNA overlapped with the region containing a pan-neuronal marker (a neuron-specific isoform of β-tubulin, Tuj-1: Fig. 1B), but was largely excluded from cells that contained nuclei that had incorporated the thymidine analog (Edu), indicating that GFRα1-expressing cells are postmitotic neurons (Fig. 1C).

This characteristic expression pattern of GFRα1 was very similar to that of two basic helix-loop-helix (bHLH) transcriptional factors, NeuroM (Math3, NeuroD4: Fig. 1D) and Neurogenin2 (Ngn2: Fig. 1E). Double *in situ* hybridization of GFRα1 mRNA with NeuroM or Ngn2 mRNA revealed that the expression domains of GFRα1 corresponded closely to that of NeuroM and Ngn2 along the dorsoventral axis although the expression domain of NeuroM and Ngn2 were shifted towards the midline (Fig. 1F and G). We partitioned the transverse plane of the unilateral spinal cord into twenty segments along the dorsoventral axis and quantified the number of GFRα1-, NeuroM-, and Ngn2-positive cells within each segment, and found that they exhibited similar distribution patterns (Fig. 1H). During development, the positions of NeuroM and Ngn2 expression corresponded to that of GFRα1 (Suppl. Fig. 1).

Although GFRα4 expression was not a primary focus, it is of interest that along the dorsoventral axis of the spinal cord, GFRα4 mRNA expression in the mantle layer matched that of a transmembrane Notch ligand, Delta1 in the ventricular layer (Fig. 1I and J).

**NeuroM function regulates the induction of GFRα1 expression**

The above expression patterns suggest potential regulatory roles for NeuroM and Ngn2 in the expression of GFRα1. To test this possibility, we first electroporated a NeuroM expression vector into progenitor cells in the prospective lumbar spinal cord at HH stage 14 (two days incubation). Under the promotor activity of the retrorival long terminal repeat (LTR), the expression of GFP protein with Myc-tag and nuclear localization signal (Myc-Nuc-GFP) was observed mainly in the nuclei of proliferating ventricular cells and in some apparent differentiated neurons (Fig. 2A). Because NeuroM mRNA and protein are normally expressed in cells during the transitional period from proliferating cells to nascent neurons in the spinal cord (Roztocil et al., 1997; Bylund et al., 2003), NeuroM protein was precociously overexpressed in our experimental condition.

Compared to no alterations in the expression of GFRα1 mRNA after Nuc-GFP overexpression (Fig. 2A), excess expression of NeuroM protein in progenitor cells spatially and quantitatively increased GFRα1 mRNA expression along the entire dorsoventral axis of the lumbar spinal cord at HH stage 23–24 (approximately 2 day after electroporation: Fig. 2B and C). The distribution of increased GFRα1 expression was not laterally restricted, but also appeared even in the apparent ventricular layer (Fig. 2B). To analyze the identity of the increased GFRα1-positive cells, we pulse-labeled proliferating cell nuclei with Edu in the NeuroM-transfected spinal cord at HH stage 19–20 (about 24 h after electroporation) when progenitor cells are actively proliferating in the most regions of the spinal cord. Myc-NeuroM-positive nuclei appeared to be relatively concentrated, but were not confined to the lateral side of the spinal cord (Fig. 2D). We examined five to seven sections from each of five transfected spinal cords and never observed double-labeled nuclei with Edu and Myc-NeuroM immunohistochemistry (Fig. 2D). We also observed significantly fewer Edu-positive nuclei after NeuroM overexpression (Fig. 2D and E). However, the distribution and number of cells expressing a marker for cell-cycle exit, p27kip1 (p27), were not changed at the same developmental stage (HH stage...
Consistent with this observation, no alterations in the number and distribution of pan-neuronal makers (neuron-specific nuclear protein: NeuN, or Tuj1) were observed at a later developmental stage (e.g. HH stage 24) when the initial neuronal distribution pattern was established (Fig. 2Ha and I, and not shown). These results indicate that NeuroM biases progenitor cells toward differentiation, but does not promote their exit from the cell cycle. Taken together, it is likely that NeuroM overexpression induced precocious expression of GFRα1 mRNA in differentiating progenitor cells that are biased toward cell cycle exit. Moreover, after the overexpression of NeuroM, GFRα2 and GFRα4 expression was still observed, indicating that NeuroM does not override endogenous subtype specification programs for the expression of other GFRα subtypes (Suppl. Fig. 2).

It is of interest to determine whether the expression of Ret, a signaling component for GFRα1, is concomitantly upregulated after the overexpression of NeuroM. However, the expression of Ret in the spinal cord was reduced after NeuroM overexpression, indicating that NeuroM transcriprional factor is also involved in the regulation of Ret expression, albeit the mode of its action for Ret is different from that for GFRα1 in the spinal cord (Suppl. Fig. 3A).

Upregulation of GFRα1 mRNA expression following NeuroM overexpression might be indirectly mediated by homeodomain-containing factors that are expressed in the microdomains of the spinal cord. However, as previously shown, excess NeuroM expression does not alter the distribution or numbers of Islet-1-positive nuclei in the ventral spinal cord even though it induces ectopic GFRα1 mRNA expression in that region (Lee and Pfaff, 2003: Suppl. Fig. 3B and D), and Lhx1/5 expression was altered only in a specific microdomain in the dorsal spinal cord (Suppl. Fig. 3C and D). Thus, it is unlikely that increased GFRα1 mRNA expression in the entire spinal cord is an integration of local upregulation that is mediated by changes in the expression of homeodomain factors in the microdomain after NeuroM overexpression.

We next examined the effect of the suppression of NeuroM function on GFRα1 mRNA expression. To repress NeuroM activity,
we generated a repressor version of the NeuroM protein by fusing the bHLH domain of NeuroM with the repressor domain of chick engrailed1 protein (NeuroM-EnR). The NeuroM-EnR construct was then electroporated into ventricular cells in the lumbar spinal cord at HH stage 14. As revealed by the immunohistochemistry for Myc-tag, NeuroM-EnR protein was translocated into the nuclei, and the number of GFRα1 mRNA-expressing cells was significantly decreased two days after electroporation (Fig. 2 J and M). By contrast, the expression pattern and number of the other two GFRα mRNA-expressing cell types were not altered, indicating specific loss of GFRα1-positive cells after the suppression of NeuroM activity. (Fig. 2 K, L, and M). The number, but not distribution, of NeuN-positive nuclei was significantly reduced after the overexpression of NeuroM-EnR protein (Fig. 2N and O). TUNEL-positive nuclei (dying cells) did not appear to be increased after the expression of excess NeuroM-EnR protein (Fig. 2P). These results show that the loss of GFRα1 expression after NeuroM-EnR overexpression is due to the abrogation of neuronal differentiation specific to the GFRα1 mRNA-expressing neuronal lineage.

The overexpression of an obligate activator form of NeuroM (NeuroM bHLH domain fused with the viral VP16 activator domain) barely induced aberrant GFRα1 expression although the slight extension of GFRα1 expression domain into the normally GFRα1-sparse region in the dorsal spinal cord. This suggests, together with the effect of NeuroM-EnR, that NeuroM
may work as a transcriptional activator for an intermediate target gene involved in the regulation of GFRz1 expression (Suppl. Fig. 3E).

Changing the normal level of Ngn2 activity leads to aberrant induction of GFRz1 expression

We next analyzed the regulatory role of Ngn2 in GFRz1 expression. For this purpose we electroporated an expression vector coding the normal form of Ngn2 protein into the prospective lumbar spinal cord at HH stage 14. Two days after the electroporation, Ngn2 overexpression spatially and quantitatively increased GFRz1 mRNA expression, but not the number or distribution of post-mitotic pan-neuronal markers (NeuN and Tuj-1: Fig. 3A, B, C, D, and not shown). Although previous studies have demonstrated an increase of neuronal markers after Ngn2 overexpression in the spinal cord (Mizuguchi et al., 2001; Novitch et al. 2001; Bylund et al., 2003; Lee and Pfaff, 2003), we have not observed this using a RCASB vector. However, the expression of ectopic and increased neuronal markers were induced after the overexpression of Ngn2 using pCAGG vector, the promotor activity of which is much stronger than that of the viral LTR in RCAS vector (Niwa et al., 1991: Suppl. Fig. 4A). This indicates that a subtype specific marker (e.g. GFRz1) vs. a general neuronal marker requires different levels of Ngn2 expression. One day after the electroporation (HH stage 20), Ngn2-expressing (Myc-tag-positive nuclei) cells were concentrated in the lateral side of the spinal cord, and significantly fewer nuclei incorporated a thymidine analog, Edu (Fig. 3E and F). Nevertheless, consistent with there being no alteration of NeuN-positive cell numbers at HH stage 24, Ngn2 did not promote the cell cycle exit of progenitor cells, as shown by no changes in the number of p27-positive nuclei one day after the Ngn2 overexpression (HH stage 20: Fig. 3G and H). Thus, Ngn2 overexpression using the RCAS expression vector drove the cell cycle progression toward terminal differentiation, but never promoted exit from the cell cycle. Notably all these data indicate that increased GFRz1 expression by Ngn2 may be attributed to the precocious expression of GFRz1 in differentiating neuronal progenitor cells, but not to the over-production of GFRz1-positive neurons. In addition to the alterations of GFRz1 expression, GFRz2 expression was also changed after Ngn2 overexpression. This suggests that the expressions of other GFRz may be also regulated by bHLH factors including Ngn2 (Suppl. Fig. 2).

We next electroporated a construct expressing a repressor form of Ngn2 protein (Ngn2-EnR), in which the bHLH domain of Ngn2, indicating that our Ngn2-VP16 properly functions as a transcriptional repressor (Suppl. Fig. 4D). Thus, Ngn2 does not normally act as a simple transcriptional repressor for GFRz1 expression. Taken together with the above results, we hypothesized that a certain level of Ngn2 expression may be necessary for the proper expression of GFRz1 mRNA. Any deviation from an optimum level of Ngn2 expression may result in aberrant GFRz1 expression. On the contrary to no change in general neuronal production after Ngn2 overexpression, Ngn2-VP16 marginally but significantly increased neuronal production as indicated by the increased number of NeuN-positive nuclei (Fig. 3 M and N). This also supports the above result that different levels of Ngn2 expression are required to differentiate between general and subtype-specific neuronal specification.

Consistent with an inductive role of NeuroM in GFRz1 expression, NeuroM mRNA expression was upregulated after Ngn2, Ngn2-EnR and Ngn2-VP16 overexpression (Fig. 3O, P, and Q). Suppression of Ngn2 expression by Ascl1 leads to ectopic GFRz1 expression

Ascl1 is expressed in a complementary fashion to Ngn2 in many regions of the central nervous system. In the dorsal spinal cord of the chick embryo, Ngn2 and Ascl1 mRNA expression appear mutually exclusive, suggesting a possible inhibitory regulation between Ascl1 and Ngn2 (Suppl. Fig. 5A, B, and C). Although the suppression of Ascl1 expression by Ngn2 has been well documented, the overexpression of Ascl1 also diminished Ngn2 mRNA expression in retinal progenitor cells (Fode et al., 2000; Mao et al., 2009; Roybon et al., 2010). In our study, electroporation of an Ascl1 expression vector at HH stage 14 also resulted in the apparent reduction of Ngn2 mRNA expression (Fig. 4A). We have not been able to determine whether this reduction is owing to the direct suppression of Ngn2 expression by Ascl1, or to increased numbers of progenitor cells in which Ngn2 expression has been permissively reduced by Ascl1. However, the reduction ascribed to the narrowing expression domains, rather than to loss of Ngn2 mRNA expression, suggests an increase of Ngn2-negative progenitor cells (Fig. 4A). We next analyzed the effect of lowering Ngn2 expression levels on GFRz1 by overexpression of Ascl1.

As expected from elevated GFRz1 expression after the suppression of Ngn2 activity, the downregulation of Ngn2 expression by Ascl1 overexpression also resulted in spatial and quantitative up-regulation of GFRz1 mRNA expression in the spinal cord at stage HH 23-24, regardless of the location of endogenous Ascl1 expression (Fig. 4B and C, and Suppl. Fig. 5). GFRz1 mRNA expression appeared to be segregated from the localization of 5BrdU-positive nuclei and to be confined within the mantle layer, suggesting increased production of GFRz1 mRNA-positive neurons (Fig. 4B and D). This is quite different from the localization of GFRz1 expression following the overexpression of NeuroM, which is coincident with 5-BrdU-positive nuclei (not shown, but compare Fig. 4B to Fig. 2B). Nevertheless, Ascl1 overexpression significantly reduced the total number of NeuN-positive nuclei at HH stage 23-24 (Fig. 4E and F). In addition, the absence of commissural fibers made the electroporated side of the spinal cord appear smaller than normal (Fig. 4E and not shown). It is somewhat puzzling that the effect of Ascl1 overexpression decreased neuronal production whereas GFRz1-positive neurons...
Fig. 3. Ngn2 overexpression promotes GFRα1 expression. (A and B) Increased GFRα1 in situ hybridization at HH stage 24 after Ngn2 overexpression (brown precipitate) in A. GFRα1–positive cells are significantly increased in B. **p < 0.001. (C and D) No change of NeuN-positive nuclei (red) in distribution in C (HH stage 24: blue fluorescence, Myc-tag immunohistochemistry) and in numbers in D. (E and F) Lateral concentration and no EdU incorporation (red) of Ngn2-positive nuclei (green) at HH stage 20 in E. Significant reduction in the number of EdU-incorporated nuclei (**p < 0.001), compared to the contralateral side (Contra.) in F. (G and H) p27-positive nuclei are unchanged in the distribution (red) in G and in number in H at HH stage 20. The overexpression of both obligate suppressor and activator forms of Ngn2 promote GFRα1 expression. (I and J) Upregulation of GFRα1 in situ hybridization after Ngn2-EnR overexpression at HH stage 24 in I. No change in the distribution of NeuN-positive nuclei (red) in J. Brown or blue nuclei, Myc-tag immunohistochemistry for Ngn2-EnR. (K) Quantification of GFRα1-positive cells and NeuN-positive nuclei. ***p < 0.001. (L to N) GFRα1 in situ hybridization is upregulated even after Ngn2-VP16 overexpression (brown nuclei) at HH stage 24 in L. The number of NeuN-positive nuclei (green) is increased after Ngn2-VP16 overexpression (red) in M. In N, ***p < 0.001 and *p < 0.05. (O to Q) NeuroM in situ hybridization at HH stage 24 after Ngn2, Ngn2-EnR, and Ngn2-VP16 overexpression in O, P, and Q, respectively.
were increased. When analyzed less than one day after the electroporation of Ascl1 (HH stage 18–19), we observed: (1) more and more ectopic p27-positive nuclei [Fig. 4G and H]; (2) lateral localization of Myc-tag (Ascl1-expressing nuclei: Fig. 4I); and (3) lower incorporation of EdU (Fig. 4I and J). These data demonstrate that Ascl1 promotes neurogenesis. Because Ascl1 overexpression did not increase apoptotic nuclei (not shown), we reasoned that Ascl1 overexpression may accelerate
neuronal production. Ascl1 overexpression also reduced GFRα, but not GFRβ (Suppl. Fig. 2).

As previously shown, NeuroM expression is closely associated with that of GFRα. However, NeuroM mRNA expression was downregulated following Ascl1 overexpression although Ascl1 increased GFRα expression. Like Ngn2 following Ascl1 overexpression, this downregulation of NeuroM also appears to be due to narrowing the expression domain, and not to the loss of expression (Fig. 4K). Taken together with the previous results, this downregulation of NeuroM indicates that NeuroM is sufficient, but not necessary for GFRα expression. Thus, NeuroM is not a direct inducer for GFRα expression.

Although the above results clearly demonstrate that Ascl1 alone is sufficient for GFRα expression, the onset and expression pattern of Ascl1 is not related to that of GFRα (Suppl. Fig. 5B, C, D, E, and F). This difference suggests that Ascl1 may compete and intervene in the regulatory sequence of a distinct determinant involved in GFRα expression, rather than Ascl1 itself working as a direct specification factor by binding its own specific target sequence for transcription. This possibility was examined in the next experiment, in which Ascl1 function was blocked by the introduction of an expression vector carrying the suppressor form of Ascl1 (Ascl1-EnR). Ascl1-EnR electroporation at HH stage 14 abolished the markers of neuronal differentiation including GFRα mRNA (Fig. 4L and M), NeuN (Fig. 4N and O), and Tuj1 (not shown) expression. This suppression occurred even in the region, in which endogenous Ascl1 expression was not observed, thus demonstrating the presence of a different determinant for GFRα expression, which Ascl1 can competitively modify. Furthermore, after the overexpression of an obligate activator form of Ascl1 (Ascl1-VP16), GFRα expression was also diminished regardless of endogenous Ascl1-expression domain (Suppl. Fig. 6A). Ngn2 expression appeared not to be largely affected (Suppl. Fig. 6B). Since the dominant activator form of Ascl1 reversed the effect of Ascl1, Ascl1 may act as a competitive transcriptional repressor for the target gene of a putative determinant for GFRα expression in the normal context of GFRα differentiation.

One day after electroporation of Ascl1-EnR, more and more ectopic p27-positive nuclei were observed (HH stage 19: Fig. 4P and Q). The number of progenitor cells passing through the S-phase was also significantly increased, as shown by increased ratio of double-labeled nuclei with Ascl1-EnR (Myc-tag) and EdU/Ascl1-EnR-positive nuclei, compared to that of Nuc-GFP- and EdU-double positive nuclei/Nuc-GFP-positive nuclei (Fig. 4R and S). Thus, the suppression of Ascl1 function accelerates cell cycle progress and exit. Apoptosis was not increased after the suppression of Ascl1 activity (not shown). These data indicate that abrogated neuronal differentiation, including GFRα expression following the overexpression of Ascl1-EnR, is due to the direct suppression of neuronal phenotype expression, rather than to arresting the progression of the cell cycle or to inducing cell death of progenitor cells. Moreover, these data further indicate that two aspects of the neurogenic function of Ascl1 are separable: Ascl1 is necessary for the expression of GFRα neuronal subtype, but is unnecessary for cell cycle exit.

The expression of NeuroM and Ngn2 mRNA were also abrogated by the overexpression of Ascl1-EnR (Fig. 4T and U). Thus, the suppression study using Ascl1-EnR further supports an epistatic role of Ascl1 over Ngn2 downregulation: Ascl1 may act at an earlier time window to permissively downregulate Ngn2 expression during the differentiation of progenitor cells. Promoting Ngn2 expression by Ptf1a induces ectopic GFRα1 expression

We next asked whether increasing Ngn2 expression by other transcriptional factors might also induce aberrant GFRα1 expression. A recent study has demonstrated that a bHLH transcriptional factor, pancreatic transcriptional factor 1a (Ptf1a), is a direct transcriptional activator of Ngn2 genes in the dorsal spinal cord (Henke et al., 2009). When we overexpressed Ptf1a in the chick embryo spinal cord by electroporation at HH stage 14, an upregulation of Ngn2 mRNA was observed at HH stage 24 in both dorsal and ventral spinal cord (Fig. 5A). Concomitantly, GFRα1 expression was also spatially and quantitatively upregulated in the apparent mantle layer of the entire spinal cord (Fig. 5B and C). Consistent with this, the localization of increased GFRα1 expression did not overlap with 5-BrdU-labeled nuclei, as observed after Ascl1 overexpression (not shown). However, one difference is that Ptf1a alone cannot promote the cell cycle exit of progenitor cells. Rather, our results indicate that Ptf1a appears to retard cell cycle progression: (1) Ptf1a overexpression significantly decreased p27-positive nuclei at HH stage 18 (Fig. 5D and E) and NeuN-positive nuclei two days after electroporation (Fig. 5F and G); nevertheless, (2) Ptf1a drove progenitor cells toward cell cycle exit as indicated by lateral settlement of Myc-tag-positive nuclei, the lack of incorporation of EdU into Myc-tag-positive nuclei, and significantly fewer EdU-positive nuclei (Fig. 5H and I). From these data, we conclude that elevated levels of Ngn2 by Ptf1a led to ectopic GFRα1 expression within a fixed number of differentiated neurons. Ptf1a also reduced the expression of GFRα2, as NeuroM, Ngn2 and Ascl1 did (Suppl. Fig. 2).

The downregulation of Ptf1a activity using a suppressor form of Ptf1a (Ptf1a-EnR) resulted in an effect similar to that after the overexpression of Ascl1-EnR. The overexpression of Ptf1a-EnR significantly reduced the expression of neuronal markers including GFRα1, NeuN, and Tuj1 expression without any apparent increase in apoptosis (Fig. 5J, K, L, and not shown). NeuroM and Ngn2 expression were also reduced (Fig. 5M and not shown). One day after electroporation of the Ptf1a-EnR expression vector (HH stage 20), the ratio of Ptf1a-EnR- and EdU-double positive nuclei/Ptf1a-EnR-positive nuclei was significantly increased, compared to that of Nuc-GFP- and EdU-double positive nuclei/Nuc-GFP-positive nuclei (Fig. 5N and O). Additionally, ectopic and significantly more p27-positive nuclei were observed (Fig. 5P and Q). Thus, like Ascl1-EnR does, Ptf1a-EnR also inhibits neuronal differentiation, but not cell cycle progression or exit.

As with Ascl1, the onset and expression pattern of Ptf1a does not match that of GFRα1, even though it has an ability to induce GFRα1 expression (Suppl. Fig. 7). The suppression of Ptf1a activity also resulted in the abrogation of neuronal differentiation in the entire spinal cord, as observed after Ascl1 overexpression. These data indicate that Ptf1a may also competitively interact with a preexisting determinant for GFRα1 expression similar to Ascl1. Being consistent with this, Ptf1a-VP16 overexpression also resulted in the upregulation of Ngn2 and GFRα1, regardless of endogenous expression pattern of Ptf1a (Suppl. Fig. 6D and E). Thus, Ptf1a may act as a competitive transcriptional activator for a GFRα1-determinant.

Discussion

Developmental roles of GFRα1 in differentiating neurons

GFRα1 is expressed soon after the differentiation of neurons in the spinal cord. Because the axons of the nascent neurons have not reached their targets, GFRα1 plays a different role from canonical trophic function involving target-derived neurotrophic factors. In the ventral forebrain, GFRα1 participates in the
differentiation and migration of a subpopulation of the cortical interneurons via Ret- and NCAM-independent manner after the production of cortical inhibitory interneurons is determined by Ascl1 (Pozas and Ibáñez, 2005; Canty et al., 2009). In the early spinal cord, GDNF mRNA is detected in the floor plate (Homma et al., 2000). Likewise in the spinal cord, GFRα1 may play a role in the differentiation of a neuronal subpopulation, or migration of a neuronal subpopulation, possibly by means of ligand-induced cell adhesion (Ledda et al., 2007). From the view of an early developmental role for GFRα1, we can not know which possibility early GFRα1 expression reflects, that the GFRα1 expression in an immature neuron is maintained even after the settlement in a final destination, or that GFRα1 expression is transient in nascent neurons and after the migration to the final locations, maturated neurons re-express GFRα1.

We demonstrated that the overexpression of bHLH factors affected mainly GFRα2 expression and that GFRα4 expression is related to a Notch ligand, Delta. These indicate that bHLH factors and related molecules are also involved in the regulation of these two receptor expressions. In this study, however, we focus on the regulatory roles of bHLH factors for GFRα1.

**NeuroM may act as a differentiation factor for the GFRα1 phenotype**

Combinatorial actions of bHLH and homeodomain transcriptional factors have been shown to control the local production of specific classes of neurons in various regions of the nervous system (Bertland et al., 2002). In the ventral spinal cord, NeuroM has no effect on the differentiation of the neuronal subtype, but induces motorneuron differentiation together with LIM-homeodomain
expression, or it may suppress the action of an inhibitory factor (Roztocil et al., 1997; Pleasure et al., 2000; Tomita et al., 2000). In the present study, we have demonstrated in the chick embryo spinal cord that: (1) the expression patterns of GFRα1 and NeuroM are very similar; (2) the overexpression of NeuroM alone is sufficient to induce precocious expression of GFRα1 mRNA in differentiating progenitor cells; and (3) NeuroM protein activity is required specifically for the differentiation of the GFRα1 neuronal lineage. All these results demonstrate that NeuroM may act as a differentiation factor for the GFRα1 phenotype in the spinal cord. However, it is unlikely that GFRα1 gene is a direct transcriptional target of NeuroM protein. Our overexpression studies demonstrate that NeuroM is required for the neuronal production of GFRα1 subtype, but that NeuroM alone is not sufficient for this purpose, indicating that NeuroM is involved, together with other differentiation factors, in promoting the maturation of nascent GFRα1-positive neurons. Moreover, Ascl1 overexpression upregulates GFRα1 expression even though it decreases NeuroM mRNA expression, indicating that NeuroM is sufficient but not essential for the induction of GFRα1 expression. Ascl1 may bypass the action of NeuroM in the induction of GFRα1 expression, or it may suppress the action of an inhibitory factor in the regulatory machinery of GFRα1 expression. Thus, NeuroM may be a constituent of the regulatory machinery for GFRα1 expression.

An appropriate level of Ngn2 expression is important for GFRα1 expression

Previous studies have extensively demonstrated that Ngn2 alone is sufficient for general neuronal production, whereas, for subtype specification function, Ngn2 requires co-factors, which are expressed in regionally restricted microdomains, ensuring that fate specification role of Ngn2 is separable from the general proneural function (Bertrand et al., 2002; Guillemot, 2007). We have asked how the GFRα1 phenotype, which is shared by neuronal subpopulations localized in multiple microdomains in the spinal cord, is specified. The results demonstrate that Ngn2 alone is sufficient for this purpose, but that it requires optimal levels of expression for proper GFRα1 specification, and further that variable levels of Ngn2 expression may separate fate specification from the proneural role of Ngn2. The exact nature and levels of Ngn2 required for the correct differentiation of GFRα1 is not clear from our study. Previous studies have shown that ectopic expression of Ngn2 in progenitor cells of the ventral forebrain differed, depending on the means of Ngn2 misexpression. In the ventral forebrain of the transgenic mouse in which Ascl1 gene is replaced by Ngn2, no dorsal forebrain markers are induced (Fode et al., 2000). However, in utero electroparation of a Ngn2 expression vector induces dorsal forebrain makers in the ventral forebrain (Mattar et al., 2008). In vitro transfer of Ngn2 gene in progenitor cells from the ventral forebrain also results in the expression of dorsal neuronal markers (Roybon et al., 2010). These differences may be ascribed to various expression levels of Ngn2 driven by different promoter activities (Roybon et al., 2010). Thus, in accord with our results, these data indicate that the level of Ngn2 expression is important for subtype specification.

A recent study has reported that Ngn2 expression in neuronal progenitor cells is dynamic in that Ngn2 expression is oscillating in progenitor cells in the developing mouse cortex and that a sustained upregulation of Ngn2 may be necessary for neuronal differentiation (Kageyama et al., 2008; Shimojo et al., 2008). The oscillation of another bHLH factor, Hes7 is also observed in developing somites and serves as a segmentation clock (Dubrulle and Pourquié, 2004; Kageyama et al., 2007). Interestingly, perturbations of this oscillation by loss of Hes7 activity or by sustained expression of Hes7 result in the same phenotype of fused somites (Bessho et al., 2001; Hirata et al., 2004). In our study, both the promotion and suppression of Ngn2 activity also led to aberrant GFRα1 expression. Although we have no idea whether Ngn2 expression is oscillating in the chick embryo spinal cord, we can only speculate that increased GFRα1 expression after changing Ngn2 activity could reflect such a dynamic temporal regulation of Ngn2.

A variety of extracellular and intracellular molecules regulate the expression levels of Ngn2. During neurogenesis, Notch signaling pathways negatively regulate Ngn2 expression levels, whereas Wnt signaling pathways positively regulate Ngn2 expression (Backman et al., 2005; Hirabayashi et al., 2004; Yoon and Gaiano, 2005; Machon et al., 2007). As shown in our study, other bHLH factors (Ascl1 and Ptf1a) also regulate Ngn2 expression levels, directly or indirectly. Our study further demonstrates that perturbation of Ngn2 expression levels affects the specification of GFRα1. These indicate that Ngn2 is not a direct inducer of GFRα1 expression, but rather may serve as readout to control the expression of other effector molecules that work as differentiation factors for GFRα1. For the expression of GFRα1, integrated Ngn2 expression levels appear to be converted into a differentiation program in which NeuroM is involved. Several previous studies pointed out that NeuroM is downstream of Ngn2 in mouse cranial sensory neurons, the retina, and the cortex (Fode et al., 1998; Akagi et al., 2004; Schuurmans et al., 2004; Mattar et al., 2008). Our study has also shown that the upregulation of NeuroM occurs together with the upregulation of GFRα1 expression following the perturbation of Ngn2. Because perturbation of Ngn2 expression always led to the upregulation of NeuroM protein activity of Ascl1 is always associated with one particular neuronal subtype, but that NeuroM alone is not sufficient for this purpose, indicating that NeuroM is involved, together with other differentiation factors, in promoting the maturation of nascent GFRα1-positive neurons. Moreover, Ascl1 overexpression upregulates GFRα1 expression even though it decreases NeuroM mRNA expression, indicating that NeuroM is sufficient but not essential for the induction of GFRα1 expression. Ascl1 may bypass the action of NeuroM in the induction of GFRα1 expression, or it may suppress the action of an inhibitory factor in the regulatory machinery of GFRα1 expression. Thus, NeuroM may be a constituent of the regulatory machinery for GFRα1 expression.

Possible regulatory roles of Ascl1 and Ptf1a in GFRα1 expression

In the central and peripheral nervous system, Ascl1 has been shown to act as a neuronal-subtype specification factor (Bertrand et al., 2002). The misexpression of Ascl1 induces ventral neuronal makers in the dorsal forebrain (Fode et al. 2000) and Ascl1 also induces noradrenergic differentiation in the sympathetic neuronal lineage (Lo et al., 1998). Because the subtype-specification activity of Ascl1 is always associated with one particular neuronal phenotype depending on the region of the nervous system, the action of Ascl1 is context-specific. Our overexpression study demonstrates that Ascl1 induces GFRα1 expression in the spinal cord, regardless of the localization of endogenous Ascl1 expression. Therefore, the specification function of Ascl1 for GFRα1 is not coupled with co-factors regionally expressed at the site of endogenous Ascl1 expression, but is highly likely to associate with a more generalized context in the spinal cord.

Misexpression of Ptf1a in the dorsal telencephalon induces a ventral phenotype, which also occurs after dorsal expansion of Ascl1 in the telencephalon of Ngn2-mutant mouse (Fode et al., 2000; Parras et al., 2002; Hoshino et al., 2005). In our study, the overexpression of Ptf1a also mimics the effect of Ascl1 overexpression with regard to the upregulation of GFRα1. Our results demonstrate that Ascl1 is necessary but not sufficient for Ptf1a expression (Wildner et al., 2006: Suppl. Fig. 7C and D). These data indicate that Ptf1a may act together with Ascl1 as a specification
or determination factor for GFRα1-positive neuronal subtype. Expression patterns of Ascl1 and Ptf1a indicate that this may occur during a limited spatial and temporal window. However, similar to Ascl1, Ptf1a also has the ability to induce GFRα1 in spinal cord regions lacking endogenous Ptf1a. This indicates that Ptf1a is more likely to interact with a common regulatory program for GFRα1 expression in the spinal cord, rather than directly acting with Ascl1 in a specific location.

When two different transcriptional factors competitively bind to a common regulatory target such as an enhancer region of DNA, the deletion of one factor results in no changes in phenotype because of compensatory binding by the other factor. By contrast, the overexpression of a repressor form, which is composed of the engrafted repressor domain and bHLH DNA-binding domain, results in the total loss of downstream function because of full occupation of the binding site by the repressor acting as a dominant negative. This is what we observe in our overexpression experiments using the suppressor forms of Ascl1 and Ptf1a (Ascl1/C18Ptf1a). Interestingly, even in the regions of the spinal cord where no Ascl1/Ptf1a is endogenously expressed, the overexpression of the suppressor forms of Ascl1/Ptf1a abrogates neuronal differentiation including GFRα1 expression. This clearly points to the presence of a distinct determinant for GFRα1 expression, the target sequence of which the binding domain of Ascl1/Ptf1a can competitively perturb, although the identity of this competitive determinant is not currently clear. Further evidence for a distinctive determinant comes from the fact that the overexpression of both normal and suppressor forms of Ascl1 resulted in the downregulation of Ngn2 expression. Although this appears contradictory, it could also occur if the suppressor works as a dominant negative form to antagonize the function of a competitive determinant. Thus, Ngn2 is apparently positioned downstream of this competitor in the regulatory transcriptional cascade for GFRα1 expression. Therefore, we do not favor the idea that two different regulatory pathways (Ascl1/Ptf1a-dependent and independent) for GFRα1 expression co-exist and work at different developmental periods and cell lineages even though the mismatch between Ascl1/Ptf1a and GFRα1 might so indicate. Rather, we favor a model in which Ascl1/Ptf1a serve as competitive modulators to stochastically alter default Ngn2 expression levels in the regulation of GFRα1 expression.

We have demonstrated that Ascl1 suppresses Ngn2 expression, although the mechanism for this is not clear, and that Ascl1/C18Ptf1a overexpression reversed this effect of Ascl1. Henke et al. (2009) demonstrated that Ptf1a is a direct transcriptional activator of Ngn2 expression and we have also shown that both Ptf1a and Ptf1a/C18Ptf1a overexpression upregulate Ngn2 expression in vivo. These data indicate that Ascl1/Ptf1a may be counteracting molecules for regulating the level of Ngn2 expression and therefore may modulate pre-existing levels of Ngn2, depending on how much Ascl1/Ptf1a is available at a particular time and places. Our results also show the appropriate levels of Ngn2 are critical for GFRα1 expression. Changing the default levels of Ngn2 may lead to variable levels of GFRα1 expression. Even very subtle changes of Ngn2 expression could result in significant changes of GFRα1 expression in the spinal cord. It is important to identify determinants for default Ngn2 expression, with which Ascl1/Ptf1a may cooperate in the spinal cord.

Finally, the role of Ascl1 in GFRα1 expression in the embryonic spinal cord is reminiscent of the close link of the polymorphism in Ascl1 protein to the susceptibility to Parkinson’s disease, although it is not clear whether these bHLH factors are also involved in the regulation of GFRα1 expression in adult DA neurons, the survival of which in the adult CNS is supported by GDNF (Ide et al., 2005; Pascual et al., 2008; Deng et al., 2010).

Acknowledgment

This work is supported by Grant-in-Aid for Scientific Research (C: 15500243) from Japan Society for the Promotion of Science. We thank Ronald W. Oppenheim for comments on an earlier draft of this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.08.002.

References


Wildner, H., Müller, T., Cho, S.H., Bröhl, D., Cepko, C.L., Guillemot, F., Birchmeier, C., 2006. dILA neurons in the dorsal spinal cord are the product of terminal and non-terminal asymmetric progenitor cell divisions, and require Mash1 for their development. Development 133, 2105–2113.

