ただし、129SvEv バックグラウンドの場合には、ホモ型は生まれて来ないため、恐らくホモ型は胎生期か生直後に致死となると考えられた。それに対して、BALB/c バックグラウンドの場合には、ホモ型 *(del17/del17)* が生まれ育つことがわかった。従って、本研究では、BALB/c 系統のバックグラウンドの個体を用いて研究を遂行した。ちなみに、遺伝的バックグラウンドを変えるには7世代のバックスロスが必要であると一般的には言われているが、今回のBALB/c 系統との交配では、かけ合わせの2世代目（F2）で致死ではないホモ型 *(del17/del17)* マウスが得られている。

2. *Epi-IER2*$^{del17/del17}$マウスでの*Epi-IER2* mRNA及びタンパク質の発現。

我々は、抗EPI-IER2 抗体を用いた免疫染色法を行い、ホモ型 *(del17/del17)* マウス脳組織におけるEPI-IER2 のタンパク質の発現について調べた。抗EPI-IER2 抗体を用いてEPI-IER2 陽性細胞を緑色に、DAPI を用いて核を青色に染色した。野生型ではEPI-IER2 陽性神経細胞が脳組織の様々な領域で発現が見られたのに対し、ホモ型 *(del17/del17)* マウスでは、EPI-IER2 の発現は検出できなかった。以上の結果と過去の報告から、ホモ型 *(del17/del17)* マウスでは、正常なEPI-IER2 タンパク質はできていないと考え、*Epi-IER2*$^{del17/del17}$が*Epi-IER2* 遺伝子の機能に関してはヌル変異体であるとして、研究を行った。

3. *Epi-IER2*$^{del17/del17}$マウスの身体的特徴

生後10 日目のホモ型 *(del17/del17)* マウスは、野生型、ヘテロ型マウスに比べて、雄雌共に体重は半分程度で全体的に身体が小さい（図1）。 一方、ホモ型 *(del17/del17)* マウスの雄の体重は、野生型に追いつくことはなかった。

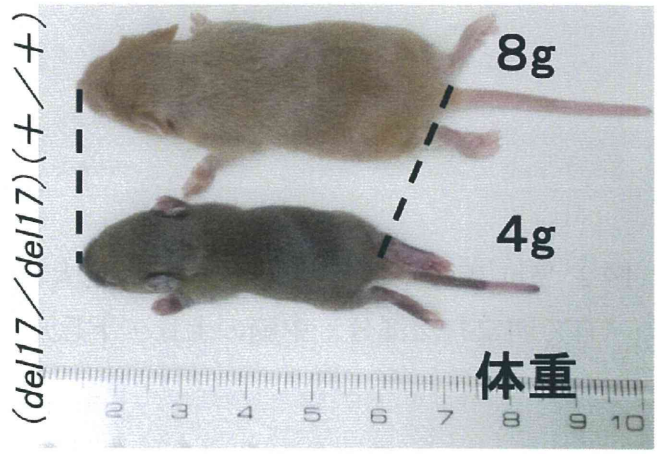


図1 *Epi-IER2*$^{del17/del17}$マウスの雄の外観 （生後10 日）（スケールバー：1000μm）

現段階で、雌雄差による成育の違いを説明することはできないが、性ホルモン等の影響が示唆される。また、雌は生殖能力を有するが、雄は生殖能力がほとんどないことが観察された。

4. *Epi-IER2*$^{del17/del17}$ マウスの脳の観察

成体のホモ型 *(del17/del17)* マウスでは、中脳の露出部が拡大していた。（図2）

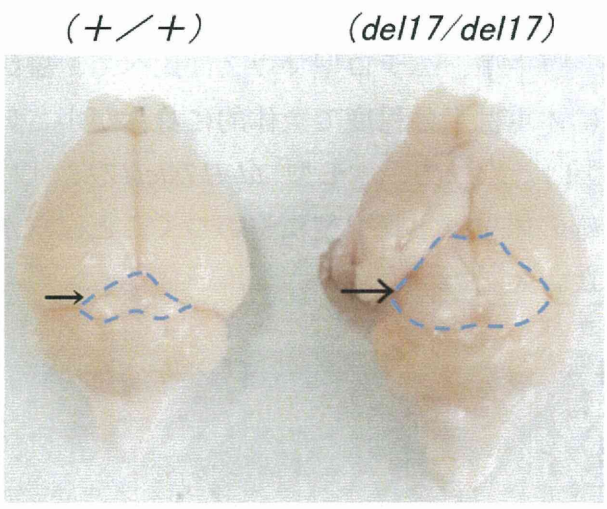


図 2　*Epi-IER2*$^{del17/del17}$ マウス中脳の肥大
（青で囲まれた部分：中脳の上丘・下丘）

どの部位が肥大しているのか調べるため、ルクソール・ファストブルー染色を行った。ルクソール・ファストブルー染色は、髄鞘を青く染色し、それによって、大まかな脳の構造を観察することができる　（図 3）

図 3 に記した赤の点線で囲んでいる中脳の部位において、中脳全体が肥大していることが観察された。しかし、大脳や小脳の肥大は顕著ではなかった。

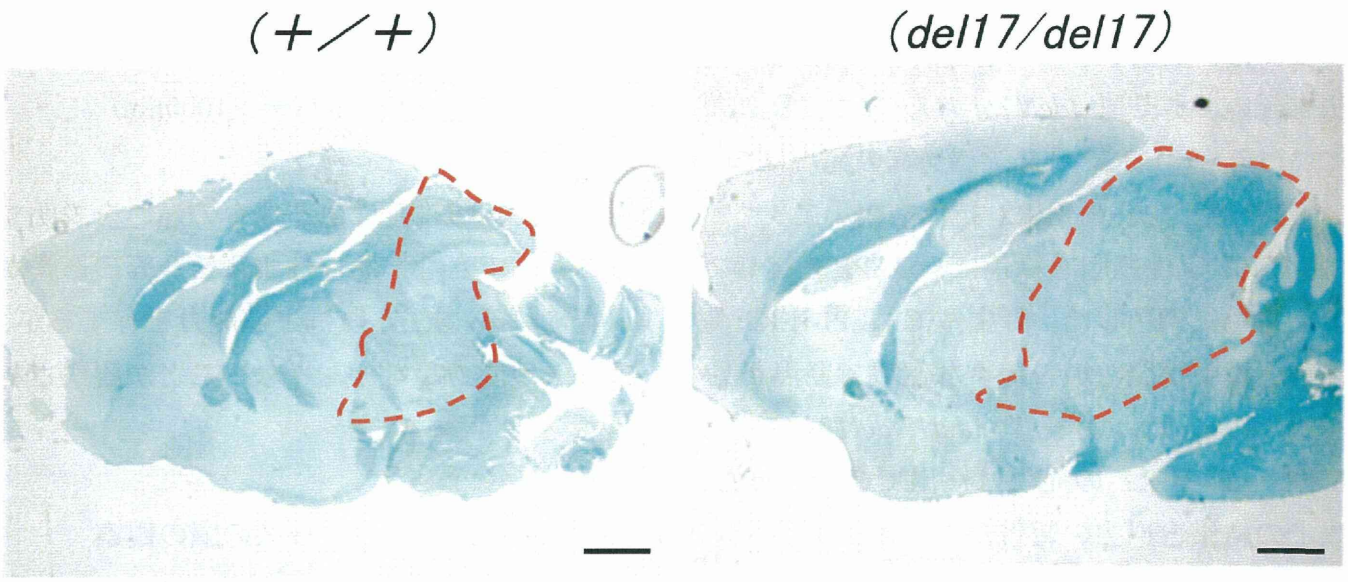


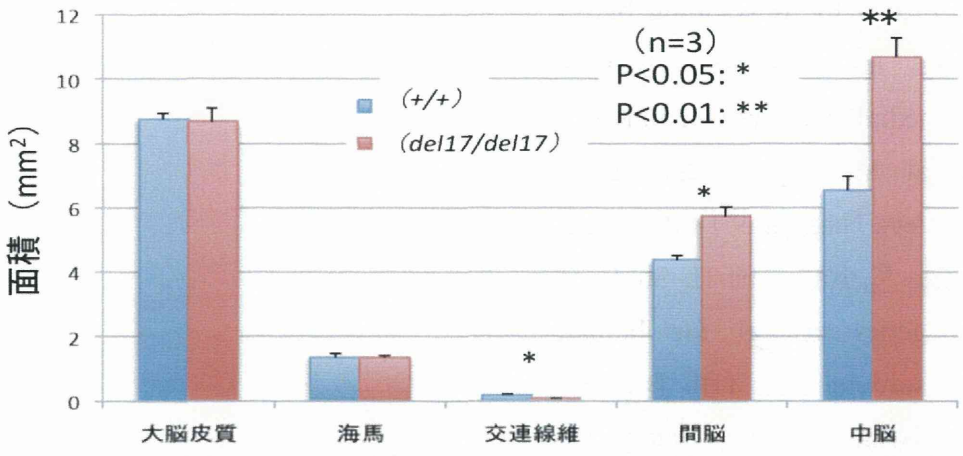
図 3　*Epi-IER2*$^{del17/del17}$ マウス中脳の肥大
（ルクソール・ファストブルー染色）（スケールバー：1000μm）

さらに、脳の各部位の大きさを定量するために、断面積を測定し比較した。前脳（大脳皮質・海馬・交連線維）、間脳、中脳に着目し lateral 0.8mm・lateral 1.0mm・lateral 1.2mm の 3 点におけるサジタル切片を作成し、各部位の断面積を解析ソフトの Metamorph を用いて計測した。（図 4）

生後１年のホモ *(del17/del17)* マウスにおいては、大脳皮質、海馬の断面積に変化は認められなかった。しかし、交連線維に関しては 0.44 倍と有意に小さくなっていた。また、間脳および中脳ではそれぞれ 1.31 倍、1.64 倍にと有意に増大していた。特に、中脳の肥大が著しい。

D. E.　考察と結論

本研究では、*Epi-IER* のファミリー分子である *Epi-IER2* の機能欠変異失体の表現型を解析した。C57B6 バックグラウンドでは、生後すぐに致死であったが、Balb/c 系統とのバッククロスによって生体まで生きることになり、生後の解析が可能となった。とはいえ、生後に発達不良が見られ、正常な発達ではないことが想像された。この分子は、神経系の神経細胞に広く発現している。機能欠変異失体の中枢神経系においては、中脳や間脳の肥大が認められたが、その原因については平成２５年度の研究で明らかにしていくつもりである。



	大脳皮質	海馬	交連繊維	間脳	中脳
(＋／＋)	8.76±0.51	1.36±0.28	0.18±0.07	4.38±0.32	6.54±1.20
(del17/del17)	8.68±1.20	1.36±0.18	0.08±0.01	5.74±0.84	10.69±1.70

図４　生後１年の *Epi-IER2*$^{del17/del17}$
マウス脳の面積比

G. 研究発表

1. 学会発表

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6) Koizumi K, Nakao K, Higashida H, Nakajima H. New Candidate Genes Critical for Human Developmental Disorders Such as Autism and Mental Retardation. 第 35 回日本分子生物学会年会, 福岡, 12.11-14, 2012.

H. 知的財産権の出願・登録状況

なし

III. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ
Hoshino M, Seto Y, Yamada M	Specification of Cerebellar and Precerebellar Neurons	Manto M, Gruol D, Schmahmann J, Koibuchi N, Rossi F	Handbook of the Cerebellum and Cerebellar Disorders	Springer	Netherlands	2013	131-136

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hori K, Hoshino M	GABAergic neuron specification in the spinal cord,the cerebellum and the cochlear nucleus	*Neural Plast*	2012 号	Article ID 921732 11pages	2012
Arai A, Saito T, Hanai S, Sukigara S, Nabatame S, Otsuki T, Nakagawa E, Takahashi A, Kaneko Y, Kaido T, Saito Y, Sugai K, Sasaki M, Goto Y, Itoh M	Abnormal maturation and differentiation of neocortical neurons in epileptogenic cortical malformation: unique distribution of layer-specific marker cells of focal cortical dysplasia and hemimegalencephaly	*Brain Res*	1407 巻	89-97	2012
Sakakibara T, Sukigara S, Otsuki T, Takahashi A, Kaneko Y, Kaido T, Saito Y, Sato N, Nakagawa E, Sugai K, Sasaki M, Goto Y, Itoh M	Imbalance of interneuron distribution between neocortex and basal ganglia: Consideration of epileptogenesis of focal cortical dysplasia	*J Neurol Sci*	323 巻	128-133	2012

IV. 研究成果の刊行物・別刷

Specification of Cerebellar and Precerebellar Neurons

8

Mikio Hoshino, Yusuke Seto, and Mayumi Yamada

Abstract

The cerebellum is thought to participate in the regulation of movement and is comprised of various types of neurons in the cerebellar cortex and nuclei. Each type of neurons has morphologically, immunohistochemically, and electrophysiologically distinct characteristics. In addition, there are two precerebellar afferent systems, the mossy fiber (MF) system and the climbing fiber (CF) system. MF neurons are located in various nuclei throughout the brainstem and send their axons to cerebellar granule cells, whereas CF neurons reside exclusively in the inferior olivary nucleus (ION) and project to Purkinje cells. Recently developed genetic lineage-tracing methods as well as gene-transfer technologies have accelerated the studies on the molecular machinery to specify neuronal subtypes in the cerebellum and the precerebellar systems.

Specification of Cerebellar Neurons

The cerebellum consists of three parts: cortex, white matter, and nucleus. The cerebellar cortex contains Purkinje, Golgi, Lugaro, stellate, basket, granule, and unipolar brush cells. The latter two cell types are glutamatergic excitatory neurons, while the others are all GABAergic inhibitory neurons. The cerebellar nucleus (CN) includes three types of neurons: large glutamatergic projection neurons (CN-Glu neurons), mid-sized GABAergic inhibitory projection neurons (CN-GABA-ION

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M. Manto, D.L. Gruol, J.D. Schmahmann, N. Koibuchi, F. Rossi (eds.),
Handbook of the Cerebellum and Cerebellar Disorders,
DOI 10.1007/978-94-007-1333-8_8,

neurons), and small GABAergic interneurons (CN-GABA interneurons). CN-GABA-ION neurons extend their axons to the inferior olivary nucleus (ION) (Carletti and Rossi 2008), while CN-Glu neurons send their axons to nuclei outside the cerebellum, including the red nucleus and the thalamus.

It is believed that all types of cerebellar neurons are generated from the neuroepithelium of the alar plate of rhombomere 1 (r1) during development (Millet et al. 1996; Wingate and Hatten 1999; Chizhikov and Millen 2003; Zervas et al. 2004). The dorsal-most part of the r1 neuroepithelium, that is, the roof plate, does not produce neurons but cells of the choroid plexus (Chizhikov et al. 2006). Neuroepithelium that produces cerebellar neurons can be divided into two regions: the rhombic lip (RL) and the ventricular zone (VZ). These two regions can be morphologically discriminated by a notch located on the border.

Although the history of studies on the cerebellum is very long (Ramón y Cajal 1911), the molecular machinery underlying cerebellar neuron development is still unclear. In 1997, Ben-Arie et al. reported that a basic-helix-loop-helix type (bHLH) transcription factor, Atoh1 (also called Math1), is expressed in the RL and involved in producing cerebellar granule cells (Ben-Arie et al. 1997). However, the development of the other types of neurons in the cerebellum remained elusive until three breakthrough papers were published in 2005.

While generating certain transgenic lines, Hoshino et al. found a novel mutant mouse line, *cerebelless*, which lacked the entire cerebellar cortex. In this mutant, all types of GABAergic neurons are not produced in the cerebellum, which leads to the secondary loss of glutamatergic granule cells and eventually, the entire cerebellar cortex (Hoshino et al. 2005). The responsible gene was identified as *pancreatic transcription factor 1a* (*Ptf1a*), which was known to participate in pancreatic development and to encode a bHLH transcription factor. This gene is expressed in the neuroepithelium of the VZ but not of the RL and its expression is lost in the *cerebelless* mutants. Cre-loxP recombination-based lineage tracing analysis revealed that all types of cerebellar GABAergic neurons are derived from Ptf1a-expressing neuroepithelial cells in the VZ, but glutamatergic neurons, such as granule cells and CN-Glu neurons, are not. Loss of *Ptf1a* expression in *cerebelless* as well as *Ptf1a*-knock out mice resulted in inhibition of the production of GABAergic neurons in the cerebellar primordium. Furthermore, ectopic introduction of *Ptf1a* by means of in utero electroporation resulted in the abnormal production of neurons with GABAergic characteristics from the dorsal telencephalon that should only produce glutamatergic neurons under normal conditions. In addition, Pascual et al. reported that, in the *Ptf1a*-null mutants, the fate of neurons produced from the VZ is changed to that of granule cells (Pascual et al. 2007). These observations suggested that Ptf1a, expressed in the cerebellar VZ, determines GABAergic neuronal fate in the cerebellum. *PTF1A* was also identified as a causative gene for a human disease that exhibits permanent neonatal diabetes mellitus and cerebellar agenesis (Sellick et al. 2004).

On the other hand, two other groups revealed a molecular fate map of the derivatives of Atoh1-expressing neuroepithelial cells in the cerebellar RL (Machold and Fishell 2005; Wang et al. 2005). They showed that not only granule cells but

also, at least in part, some neurons in the CN are derived from the RL, although they did not discriminate between neuron types in the CN. In their studies, the development of RL-derived CN neurons was shown to be disrupted in the *Atoh1*-null mice. Because Hoshino et al. reported that GABAergic but not glutamatergic CN neurons are derived from Ptf1a-expressing neuroepithelial cells in the VZ (Hoshino et al. 2005), their findings suggest that cerebellar glutamatergic neurons such as granule cells and CN-Glu neurons are derived from the RL. Accordingly, unipolar brush cells, which are glutamatergic, were also shown to emerge from the RL (Englund et al. 2006).

Together, these studies indicate the presence of two molecularly defined neuroepithelial areas in the cerebellum, the Atoh1-expressing RL and the Ptf1a-expressing VZ, which generate glutamatergic and GABAergic neurons, respectively. Each bHLH transcription factor is involved in producing the corresponding neuronal subtype in the cerebellum. This suggests a model in which the cerebellar neuroepithelium is regionalized into two distinct regions, the VZ and the RL, by the two bHLH transcription factors (Hoshino 2006). During embryonic development, the ventral part of the cerebellar neuroepithelium expresses Ptf1a, leading to the acquirement of cerebellar VZ characteristics to generate GABAergic neurons, while the dorsal part of cerebellar neuroepithelium expresses Atoh1 and becomes the cerebellar RL, producing glutamatergic neurons. In the telencephalon, similar regionalization by bHLH transcription factors takes place. Glutamatergic neurons emerge from dorsal neuroepithelium expressing Neurogenin 1/2 (Ngn 1/2), and GABAergic neurons are produced from ventral neuroepithelium expressing Mash1 (Wilson and Rubenstein 2000).

How are these neuroepithelial areas formed? In general, the roof plate can affect the dorsal structure of the neural tube (Lee et al. 2000; Millonig et al. 2000). Chizhikov et al. revealed that the roof plate plays an important role in the formation of the cerebellar dorsoventral domain formation by analyzing cerebellar mutants that lack the roof plate (Chizhikov et al. 2006). Moreover, it has been suggested that bone morphogenetic proteins (BMPs) secreted from the roof plate as well as Notch signaling are involved in the formation of the RL and the VZ (Machold et al. 2007). A recent study that induced Purkinje cells from ES cells suggested that loss of sonic hedgehog signaling may give the dorsoventral spatial information of the cerebellar VZ to the cerebellar neuroepithelium which eventually leads to the expression of Ptf1a (Muguruma et al. 2010).

Although the machinery governing GABAergic and glutamatergic neuronal subtype specification by transcription factors has been clarified to some extent, molecular mechanisms to specify each member of GABAergic (e.g., Purkinje, Golgi, basket, stellate cells and CN-ION, CN-interneurons) or glutamatergic (e.g., granule, unipolar brush cells, and CN-Glu neurons) subtype remain unclear. However, birthdating studies using ^{3}H-thymidine and BrdU (Chan-Palay et al. 1977; Batini et al. 1992; De Zeeuw and Berrebi 1995; Sultan et al. 2003; Leto et al. 2006) as well as adenovirus (Hashimoto and Mikoshiba 2003) revealed that each type of neuron is generated at distinct developmental stages. As to GABAergic neurons, Purkinje cells are produced at an early stage (embryonic day (E) 10.5–13.5 in

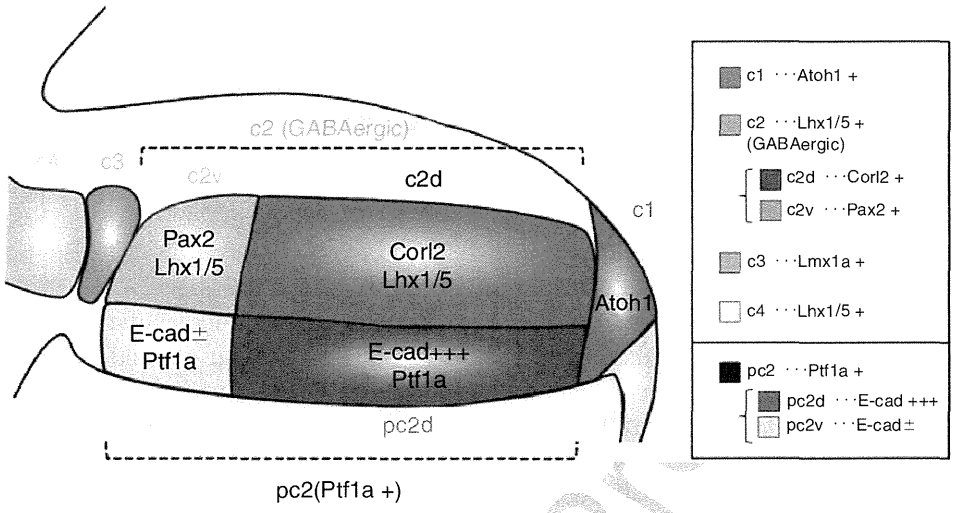

Fig. 8.1 Domain structure of the cerebellar primordium. The c1 domain, expressing Atoh1, corresponds to the rhombic lip that produces all types of glutamatergic neurons in the cerebellum. The pc2 is the Ptf1a-expressing neuroepithelial domain that generates all types of GABAergic cerebellar neurons. At early neurogenesis stages, such as E12.5, the pc2 domain can be subdivided into pc2d and pc2v subdomains, which expresses E-cadherin strongly and weakly, respectively. The c2 domain, expressing Lhx1/5, consists of immature GABAergic neurons putatively generated from pc2 neuroepithelial domain. This domain can also be subdivided into two subdomains, c2d and c2v. The c2d subdomain consists of corl2-expressing neurons or Purkinje cells, whereas the c2v subdomain includes Pax2-positive cerebellar GABAergic interneurons. Although c3 and c4 domains are Lmx1a- and Lhx1/5-positive, respectively, cell types which consist these domains are unknown. The roof plate (RP) is located most dorsally, and plays prominent roles in organizing this cerebellar domain structure

mice), Golgi cells at middle stages (E14.5~), and stellate/basket cells at a late stage (Perinatal~). Regarding glutamatergic neurons, in addition to the experiment above, molecule-based lineage tracing analyses (Machold and Fishell 2005; Wang et al. 2005; Englund et al. 2006) have clarified that CN-Glu neurons leave the cerebellar RL at early stages (E10.5–12.5) and granule cells and unipolar brush cells at middle to late stages (granule cell:E12.5~, ubc: E12.5–E18.5). In addition, somatic recombination-based clonal analyses suggested that Purkinje, Golgi, and basket/stellate cells as well as some CN neurons (probably GABAergic) belong to the same lineage (Mathis et al. 1997; Mathis and Nicolas 2003). These data indicate that some temporal information in the neuroepithelium may be involved in specification of neuronal types in the RL and VZ, respectively. However, the underlying molecular mechanisms have not yet been clarified.

Some scientists tried to divide the structure of the cerebellar primordium into several domains (Fig. 8.1). Chizhikov et al. defined four cellular populations (denoted c1–c4 domains) in the cerebellar primordium by the expression of a few transcription factors (Chizhikov et al. 2006). c1 corresponds to the Atoh1-expressing RL and c2 is located just above the Ptf1a-expressing VZ (denoted

pc2), indicating that c2 cells mainly consist of GABAergic inhibitory neurons. Although c3 and c4 express Lmx1a and Lhx1/5 respectively, their neuronal subtypes remain to be determined. This subdomain structure is disrupted when the roof plate was removed (Chizhikov et al. 2006). Furthermore, at the early neurogenesis stage (e.g., E12.5 in mice), Minaki et al. subdivided the c2 domain into dorsally (c2d) and ventrally (c2v) located subdomains that express corl2 and Pax2, respectively (Minaki et al. 2008). While corl2 is exclusively expressed in immature and mature Purkinje cells (Minaki et al. 2008), Pax2 is expressed in GABAergic interneurons (e.g., Golgi, stellate, basket, CN-GABA neurons) in the cerebellum (Maricich and Herrup 1999; Weisheit et al. 2006). They also subdivided the Ptf1a-expressing neuroepithelial domain (pc2) into pc2d and pc2v, which strongly and weakly express E-cadherin, respectively. From the positions of the neuroepithelial and neuronal subdomains, they suggested that the pc2d neuroepithelial subdomain produces cells in the c2d domain which give rise to Purkinje cells and pc2v subdomain generates cells in the c2v that become GABAergic interneurons (Mizuhara et al. 2010). As development proceeds, pc2d and pc2v subdomains become smaller and larger, respectively, and by E14.5 in mice, the Ptf1a-expressing pc2 domain is comprised only by the pc2v subdomain which expresses E-cadherin weakly. This correlates with the fact that, at E14.5 in mice, Ptf1a-expressing neuroepithelium does not produce Purkinje cells but Pax2-positive interneurons (Maricich and Herrup 1999; Hashimoto and Mikoshiba 2003). Expression patterns of several other transcription factors in the cerebellar VZ during development were also reported. For example, Zordan et al. reported the expression patterns of proneural bHLH transcription factors, such as Ngn1, Ngn2, and Mash1 in the cerebellar VZ although their function in cerebellar development is still unclear (Zordan et al. 2008; Lundell et al. 2009). However, it has been recently reported that Pax2-positive neurons, but not Purkinje cells, are reduced in the *Mash1*-null cerebellum (Grimaldi et al. 2009), suggesting that these bHLH transcription factors may play distinct roles in cerebellar development.

In addition, several transcription factors have been reported to participate in the development of a certain type of cerebellar neurons. Double knockout of *Lhx1* and *Lhx5* as well as the targeted disruption of their cofactor *Ldb1* resulted in lack of Purkinje cell production in the cerebellum although Pax2-positive interneurons did not seem to be affected. Because Lhx1 and Lhx5 are expressed in post-mitotic cells, this suggests that Lhx1, Lhx5, and Ldb1 are post-mitotically involved in Purkinje cell specification (Zhao et al. 2007). In addition, targeted disruption of *cyclin D2* caused loss of stellate cells in the cerebellar molecular layer, suggesting its involvement in the development of stellate cells (Huard et al. 1999).

From the RL, several types of glutamatergic neurons, such as CN-Glu neurons, granule cells, and unipolar brush cells, are generated. CN-Glu neurons leave the RL at early neurogenesis stages. Some transcription factors, such as Tbr1, Irx3, Meis2, Lhx2, and Lhx9 have been found to be expressed in post-mitotic progenitors of CN-Glu neurons, but their roles have not been clarified (Morales and Hatten 2006). Other molecules, such as Zic1 (Aruga et al. 1998), have been reported to play important roles in the migration, maturation, and survival of granule cells, but the

molecular machinery underlying the specification of granule cell identity is unknown. Although unipolar brush cells strongly express Tbr2, its function is also elusive.

In addition to genetic analyses, heterotopic and heterochronic transplantation studies have also provided important clues to understanding cerebellar development (Carletti and Rossi 2008). When tissues from embryonic and postnatal cerebella were mixed and transplanted to the fourth ventricle of an adult mouse, the postnatal-derived cells differentiated only into interneurons such as granule, basket, and stellate cells, but not projection neurons, such as Purkinje cells, whereas the embryonic-derived cells were capable of becoming all types of cerebellar neurons (Jankovski et al. 1996). In addition, it was shown that dissociated cells taken from cerebellar primordium at early neurogenesis stages could differentiate into all major types of cerebellar neurons, but those from postnatal cerebellum differentiated only to Pax2-positive interneurons (Carletti et al. 2002). These findings suggest that the differentiation competence of cerebellar progenitors becomes restricted as development proceeds. However, the molecular mechanisms underlying this fate restriction process have not yet been clarified. Interestingly, Leto et al. suggested that pax2-positive interneurons, such as Golgi, stellate, basket cells, and CN-GABA interneurons are derived from same progenitor pool (Leto et al. 2006).

Specification of Precerebellar Neurons

There are two types of precerebellar afferent systems: mossy fiber (MF) and climbing fiber (CF) systems. MF neurons are located in several nuclei throughout the brain stem and extend their glutamatergic projections to granule cells conveying peripheral and cortical information to the cerebellum. Four major nuclei containing MF neurons are the pontine gray nucleus (PGN), the reticulotegmental nucleus (RTN), the lateral reticular nucleus (LRN), and the external cuneate nucleus (ECN) in the hindbrain (Altman and Bayer 1987). In addition, some MF neurons are also located in the spinal trigeminal nucleus (Sp5) in the hindbrain and Clarke's column in the spinal cord. In contrast, CF neurons reside exclusively in the inferior olive nucleus (ION), which receive input from the cerebral cortex, the red nucleus, spinal cord, and other brain stem nuclei and send glutamatergic projections to Purkinje cells (Ruigrok et al. 1995). Both types of precerebellar neurons also send branch axons to the neurons in the CN. These precerebellar systems are thought to transmit the external and internal information to the cerebellar cortex to modulate cerebellar function, including regulation of animal movement.

Previous birthdating studies in mice revealed that CF neurons are generated at relatively early neurogenesis stages (E9.5–11.5) and MF neurons are produced at slightly later stages (E10.5–16.5) (Pierce 1973). Along the rostrocaudal axis, both MF and CF neurons in the hindbrain are generated from the caudal hindbrain, around rhombomeres 6–8 (r6–8), as suggested by avian grafting studies as well as mammalian fate map analyses (Ambrosiani et al. 1996; Cambronero and Puelles

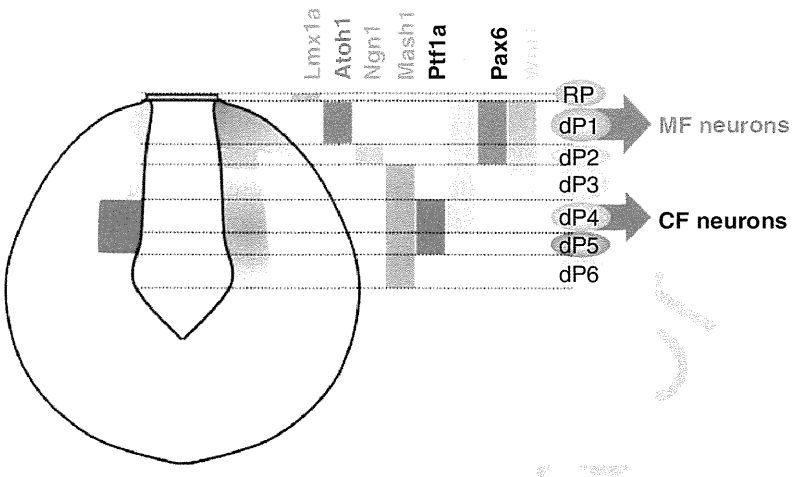

Fig. 8.2 Neuroepithelial domain structure in the caudal hindbrain. In the caudal hindbrain (r6–8), several transcription factors are expressed within the dorsal neuroepithelium during embryonic development. The dorsal-most part, the roof plate (RP), expresses Lmx1a. Other than the roof plate, the dorsal neuroepithelium can be divided into six domains (dP1–dP6) according to the expression pattern of transcription factors such as Atoh1, Ngn1, Pax6, Mash1, Ptf1a, and Olig3. While mossy fiber (MF) neurons are derived from the dP1 domain expressing Atoh1, climbing fiber (CF) neurons are generated from the dP4 domain expressing Ptf1a and Olig3

2000; Farago et al. 2006; Kawauchi et al. 2006). By contrast, MF neurons in the Clarke's nucleus are generated in the spinal cord (Bermingham et al. 2001). Classic anatomical and immunohistochemical studies have suggested that these precerebellar nuclei neurons in the hindbrain emerge from the dorsal part of the hindbrain and migrate tangentially or circumferentially to their final loci (Bloch-Gallego et al. 1999; Yee et al. 1999; Kyriakopoulou et al. 2002). However, they take slightly different paths from each other; MF and CF neurons move extramurally and intramurally, respectively. Introduction of a GFP-expressing vector into the embryonic dorsal hindbrain allowed the dramatic visualization of migrating precerebellar nuclei neurons during development (Kawauchi et al. 2006; Okada et al. 2007).

Many groups have reported transcription factors that are expressed within the dorsal neuroepithelium of the caudal (r6–8) hindbrain during embryonic development, trying to define domains along the dorsoventral axis. The dorsal-most part expressing Lmx1a corresponds to the roof plate which gives rise to the choroid plexus (Chizhikov et al. 2006). Other than the roof plate, the dorsal neuroepithelium can be divided into six domains (dP1–dP6) according to the expression pattern of the transcription factors, such as Atoh1, Ngn1, Mash1, Ptf1a, and Olig3 (Fig. 8.2). As to the precerebellar nuclei neurons, a series of studies have tried to clarify the precise origins of MF and CF neurons by genetic lineage-tracing methods.

By analyzing genetically engineered mice that express *lacZ* or *Cre recombinase* under the control of the endogenous or exogenous *Atoh1* promoter, MF neurons of PGN, RTN, LRN, and ECN were shown to emerge from the Atoh1-expressing neuroepithelial domain (dP1, Ben-Arie et al. 2000; Rodriguez and Dymecki 2000;

Landsberg et al. 2005; Wang et al. 2005). Targeted disruption of the *Atoh1* gene resulted in loss of these MF neurons, suggesting an involvement of *Atoh1* in the MF neuron development.

Atoh1 regulates the expression of the transcription factor Barhl1 (Mbh2) that is expressed in MF neurons. Loss of Barhl1 expression resulted in a decrease of MF neurons, leading to a decrease in the size of MF precerebellar nuclei (Li et al. 2004). In addition, Flora et al. reported that one of the E-proteins, Tcf4, interacts with Atoh1 and regulates differentiation of a specific subset (PGN, RTN) of MF neurons (Flora et al. 2007).

Landsberg et al. also performed lineage trace analysis by using two variants of FLP (Flipperase recombinase) with different recombinase activities that were expressed under the control of the *Wnt-1* promoter whose strength is the highest at the dorsal-most part and gradually decreases ventrally. They demonstrated that CF neurons are derived from the neuroepithelial region where *Wnt-1* is very weakly expressed, whereas MF neurons emerge from the strongly Wnt-1-expressing region (Landsberg et al. 2005). In addition, Nichols and Bruce generated transgenic mice carrying a *Wnt-1*-enhancer/*lacZ* transgene and observed that MF neurons but not CF neurons were labeled by β-gal in those mice (Nichols and Bruce 2006). These findings suggested that CF neurons are generated from the neuroepithelial region ventral to the Atoh1-expressing domain.

By Cre-loxP-based lineage trace analysis, Yamada et al. showed that all CF neurons in the ION are derived from the Ptf1a-expressing neuroepithelial region (Yamada et al. 2007). Loss of the *Ptf1a* gene resulted in the fate change of some CF neurons to MF neurons, suggesting that Ptf1a plays a critical role in fate determination of CF neurons. They also showed an involvement of Ptf1a in migration, differentiation, and survival of CF neurons. Storm et al. reported that not only MF neurons but also CF neurons are derived from the Olig3-expressing neuroepithelial region that broadly expands within the dorsal hindbrain (Storm et al. 2009) by Cre-loxP-based linage tracing. Targeted disruption of the *Olig3* gene caused the disorganized development of MF neurons and complete loss of CF neurons (Liu et al. 2008; Storm et al. 2009). Moreover, the ectopic co-expression of Olig3 and Ptf1a induced cells expressing a CF neuron marker in chick embryos (Storm et al. 2009). These findings suggest that CF neurons emerge from the Ptf1a/Olig3-expressing neuroepithelial domain (dP4) and that Ptf1a and Olig3 are cooperatively involved in the development of CF neurons. Domain structure of the dorsal neuroepithelium in the caudal hindbrain region is shown in Fig. 8.2.

Conclusions and Future Directions

Various types of neurons are generated from the dorsal hindbrain. As described above, the dorsal neuroepithelium of the rostral hindbrain (r1) produces all types of cerebellar neurons, while the dorsal regions of the caudal hindbrain (r6–8) generate neurons that include the precerebellar system neurons, such as MF and CF neurons. In addition, histological observations suggested that the dorsal part of the middle

hindbrain produces neurons of the cochlear nucleus, where auditory information is processed and relayed to the brain (Pierce 1967; Ivanova and Yuasa 1998). More directly, genetic-fate-mapping studies using transgenic mice confirmed that neurons of the cochlear nucleus are derived from the dorsal part of r2–5 in mice (Farago et al. 2006), although in avians, they were shown to emerge from a broader part (r3–8) by grafting studies (Tan and Le Douarin 1991; Cambronero and Puelles 2000; Cramer et al. 2000). As to neuronal subtypes, Fujiyama et al. identified origins of inhibitory and excitatory neurons of the cochlear nucleus; inhibitory (glycinergic and GABAergic) and excitatory (glutamatergic) neurons are derived from Ptf1a- and Atoh1-expressing neuroepithelial regions, respectively (Fujiyama et al. 2009), and their development is dependent on the corresponding bHLH proteins.

In the hindbrain from r1 to r8, there are dorsoventral domain structures defined by several transcription factors, which are longitudinally expressed throughout the hindbrain. Especially, two bHLH transcription factors, Atoh1 and Ptf1a seem to play important roles in specifying distinct neuronal subtypes. These two proteins are expressed in different neuroepithelial regions throughout the hindbrain

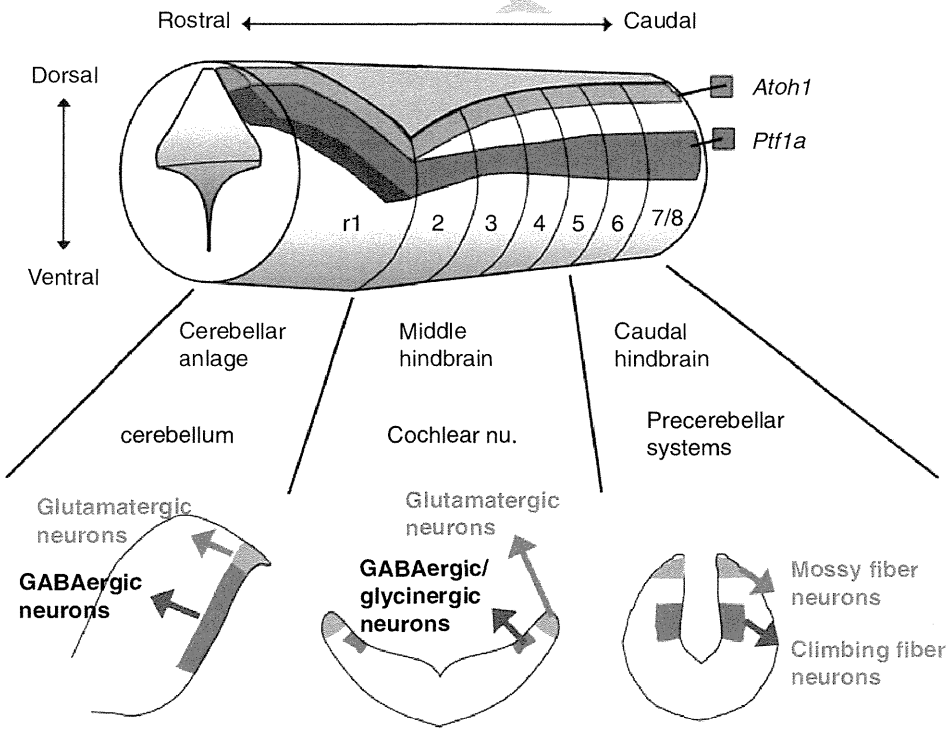

Fig. 8.3 Basic HLH proteins and neurons produced from the dorsal hindbrain. Atoh1 and Ptf1a are expressed in distinct neuroepithelial regions throughout the rhombomeres 1–8 (r1–8). Each number represents the rhombomeric number. *Upper* side is dorsal, *lower* is ventral. *Left* side is rostral, *right* side is caudal. Neuronal subtypes generated from the dorsal neuroepithelium of the rostral, middle, and caudal hindbrain regions are shown

(Fig. 8.3). In both the rostral (r1) and middle hindbrain (r2–5 in mice), Atoh1 and Ptf1a participate in generating excitatory and inhibitory neurons, respectively. However, this rule is not applicable to the caudal hindbrain. The Ptf1a neuroepithelial domain in the caudal hindbrain (r6–8 in mice) produces not only inhibitory neurons (local circuit neurons) but also glutamatergic neurons (CF neurons, Yamada et al. 2007), while the Atoh1 domain generates glutamatergic MF neurons. This raises the possibility that the rostral/middle (r1–5) and caudal (r6–8) hindbrain subregions have distinct characteristics. Overall, throughout the hindbrain regions, transcription factors, such as Atoh1 and Ptf1a, seem to define neuroepithelial domains along the dorsoventral axis and participate in specifying distinct neuronal subtypes according to the rostrocaudal spatial information (Fig. 8.3).

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Review Article

GABAergic Neuron Specification in the Spinal Cord, the Cerebellum, and the Cochlear Nucleus

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In the nervous system, there are a wide variety of neuronal cell types that have morphologically, physiologically, and histochemically different characteristics. These various types of neurons can be classified into two groups: excitatory and inhibitory neurons. The elaborate balance of the activities of the two types is very important to elicit higher brain function, because its imbalance may cause neurological disorders, such as epilepsy and hyperalgesia. In the central nervous system, inhibitory neurons are mainly represented by GABAergic ones with some exceptions such as glycinergic. Although the machinery to specify GABAergic neurons was first studied in the telencephalon, identification of key molecules, such as pancreatic transcription factor 1a (Ptf1a), as well as recently developed genetic lineage-tracing methods led to the better understanding of GABAergic specification in other brain regions, such as the spinal cord, the cerebellum, and the cochlear nucleus.

1. Introduction

The mammalian brain is a complex, highly organized structure that has a wide variety of morphologically and physiologically different neuronal cell types and diverse types of glia. Higher brain function is primarily accomplished by assembly of neural circuits with specific patterns of synaptic connectivity between diverse neuronal cell types. This fundamental process begins with cell fate determination, whereby progenitor cells in the ventricular zone exit the cell cycle and differentiate into distinct cell types with specific neuronal identities, followed by migration of the neuronal cells to proper regions in the brain, and axon guidance that extends to and recognizes their targets. There are two broad types of neurons, excitatory neurons and inhibitory neurons. In the central nervous system, excitatory neurons are mainly glutamatergic neurons that transmit information between different regions in the brain whereas inhibitory neurons are mainly composed of GABAergic and glycinergic neurons, make local connections, and are thought to act as a cellular elements coordinating and balancing excitatory activity. Indeed, previous studies have revealed that severe impairment of the GABAergic inhibitory system caused by deficits of genes regulating the development of GABAergic interneurons (e.g., transcription factor ARX [1] and Nkx2-1 [2]) or function of GABAergic neurotransmission (e.g., channels and transporters [3]) leads to pathological hyperexcitability and can result in severe epilepsy [4]. In other CNS regions, other types of neurotransmitters, such as histamine and taurine released from the hypothalamic inhibitory interneurons, also exert inhibitory actions [5–7]. Furthermore, shift of the neurotransmitter phenotype from GABAergic predominance to mainly glycinergic (or co-released from single synaptic terminals) neurotransmission occurs in some neurons, such as the interneurons projecting onto spinal motoneurons and lateral superior olive auditory relay neurons in the brainstem during postnatal maturation of inhibitory system [8–10]. The impairment of the maturation of GABAergic neurotransmission to motoneurons in the spinal cord and brainstem is thought to induce neurological dysfunctions such as hyperekplexia and amyotrophic lateral sclerosis [8]. In addition to the principal inhibitory role of