TITLE PAGE

Precerebellar cell groups in the hindbrain of the mouse defined by retrograde tracing and correlated with cumulative *Wnt1*-Cre genetic labeling

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Conflict of Interest Statement

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Abstract

The precerebellar nuclei are hindbrain and spinal cord centers that send fibers to the cerebellum. The neurons of the major hindbrain precerebellar nuclei are derived from the rhombic lip. *Wnt1*, a developmentally important gene involved in intercellular signalling, is expressed in the developing rhombic lip. We sought to investigate the relationship between the cell clusters expressing *Wnt1* and the precerebellar nuclei in the hindbrain. We therefore defined the hindbrain precerebellar nuclei by retrograde tracing, following cerebellar injections of HRP, and compared these results with the cell clusters expressing *Wnt1* in newborn mice. We found that 39 distinct hindbrain nuclei project to the cerebellum. Of these nuclei, all but three (namely the oral pontine reticular nucleus, the caudal pontine reticular nucleus, and the subcoeruleus nucleus) contain neurons expressing *Wnt1*. This shows a high degree of overlap between the precerebellar nuclei and the nuclei that express *Wnt1*. However, it should be noted that neurons expressing *Wnt1* are also found in the superior olivary complex, which is a basal plate derivative lacking cerebellar projections.

Keywords Hindbrain . Precerebellar nuclei . Wnt1 . Inferior olivary nucleus . Rhombic lip

Introduction

[1] *Abbreviations:* 4/5Cb, lobules 4 and 5 of the cerebellar vermis; 5N, motor trigeminal nucleus; 5PC, motor trigeminal nucleus, parvicellular part; 5Sol, trigeminal-solitary transition zone; 5Tr, trigeminal transition zone; 6Cb, lobule 6 of the cerebellar vermis; 7Cb, lobule 7 of the cerebellar vermis; 7n, facial nerve; 7N, facial nucleus; 8Cb, lobule 8 of the cerebellar vermis; Ar, arcuate nucleus; C, cochlear nuclei; Cb, cerebelum; chp, choroid plexus; Crus1, crus1 of the ansiform lobule; Crus2, crus2 of the ansiform lobule; Cu, cuneate nucleus; DC, dorsal cochlear nucleus; DMSp5, dorsomedial spinal trigeminal nucleus; DR, dorsal raphe nucleus; ECu, external cuneate nucleus; Gi, gigantocellular reticular nucleus; IC, inferior colliculus; In, intercalated nucleus; IO, inferior olivary nucleus; IOD, inferior olive dorsal nculeus; IODM, inferior olive dorsomedial cell group; IOM, inferior olive medial nucleus; IOPr, inferior olive principal nucleus; IRt, intermediate reticular zone; KF, Kölliker-Fuse nucleus; LC, locus coeruleus; lfp, longitudinal fasiculus of the pons; Li, linear nucleus; LPB, lateral parabrachial nucleus; LPBE, lateral parabrachial nucleus external part; LRt, lateral reticular nucleus; LVPO, lateroventral periolivary nucleus; mcp, middle cerebellar peduncle; MdD, medullary reticular nucleus, dorsal part; MdV, medullary reticular nucleus, ventral part; ml, medial lemniscus; MnR, median raphe nucleus; MPB, medial parabrachial nucleus; MVe, medial vestibular nucleus; MVeMC, medial vestibular nucleus, magnocellular part; MVePC, medial vestibular nucleus, parvicellular part; MVPO, medioventral periolivary nucleus; Mx, matrix region of the medulla; PCRtA, parvicelluar reticular nucleus, alpha part; PCRt, parvicelluar reticular nucleus; PM, paramedian lobule; PMn, paramedian reticular nucleus; PMnR, paramedian raphe nucleus; Pn, pontine nuclei; PnC, pontine reticular nucleus, caudal part; PnDL, pontine nuclei, dorsolateral part; PnL, pontine nculei, lateral part; PnM, pontine nuclei, medial part; PnO, pontine reticular nucleus, oral part; PnPed, pontine nuclei, peduncular part; PnR, pontine raphe nucleus; PnV, pontine nuclei, ventral part; Pr, prepositus nucleus; Pr5, principal sensory trigeminal nucleus; Pr5DM, principal sensory trigeminal nucleus dorsomedial part; Pr5VL principal sensory trigeminal nucleus ventrolateral part; py, pyramidal tract; r, rhombomere; RIP, raphe interpositus nucleus; RL, rhombic lip; RMg, raphe magnus nucleus; Ro, nucleus of Roller; ROb, raphe obscurus nucleus; RPa, raphe pallidus nucleus; RtTg, reticulotegmental nucleus of the pons; scp, superior cerebellar peduncle; Sim, simple lobule; Sol, solitary nucleus; Sp5C, spinal trigeminal nucleus, caudal part; Sp5I, spinal trigeminal nucleus, interpolar part; Sp5O, spinal trigeminal nucleus, oral part; SpVe, spinal vestibular nucleus; SubC, subcoeruleus nuclei; SuVe, superior vestibular nucleus; VCP, ventral cochlear nucleus posterior part; Ve, vestibular nuclei; X, nucleus X; xscp, decussation of the superior cerebellar peduncle; Y, nucleus Y.

The hindbrain and spinal cord cell groups that project to the cerebellar cortex are collectively referred to as the precerebellar nuclei. The axons of neurons of the precerebellar nuclei are of three types: climbing fibers which synapse with the dendrites of Purkinje cells; mossy fibers which synapse with the granule cells; and multilayer fibers which terminate diffusely in all layers of the cerebellar cortex. Almost all the precerebellar nuclei give rise to mossy fibers, the main exceptions being the inferior olivary nucleus which gives rise to climbing fibers, and the locus coeruleus which gives rise to multilayer fibers. Most recent studies on the hindbrain precerebellar nuclei focus only on five groups - the inferior olivary nucleus, pontine nuclei, reticulotegmental nucleus, lateral reticular nucleus, and the external cuneate nucleus [1-3]. These five groups have often been referred to as the major precerebellar nuclei because their efferents go almost entirely to the cerebellum. On this basis we find that the perihypoglossal nuclei and the parvicellular part of motor trigeminal nucleus should also be classified as major hindbrain precerebellar nuclei.

In addition to these major precerebellar nuclei, there are a number of nuclei that have a less prominent projection to cerebellum [4]; these 'minor' precerebellar nuclei can be divided into three groups, the sensory precerebellar nuclei, the monamine precerebellar nuclei, and the reticular precerebellar nuclei. This paper provides a full account of the major and minor precerebellar nuclei in the mouse as defined by injections of retrograde tracer in the cerebellum.

The major precerebellar nuclei of the hindbrain develop from a zone of intense proliferation called the rhombic lip. The rhombic lip is formed from the thickened alar plate of the hindbrain, and lies along the border of the fourth ventricle [5]. The rhombic lip and adjacent ventricular zone have been shown to give rise to most of the neurons of the cerebellum, the major precerebellar nuclei of the hindbrain, and the roof structures of the fourth ventricle [1,6-9]. The cell groups that arise from the lower rhombic lip are arranged in three distinct dorsoventral zones, each characterized by a particular pattern of gene expression [1]. The dorsal zone, which gives rise to the roof structures, is characterized by strong *Wnt1, Lmx1a, and Gdf7* expression. The middle zone, which gives rise to most of the 'mossy' precerebellar nuclei and the granule cells of the cerebellum, is characterized by *Atoh1* (*Math1*) and *Wnt1* expression. The ventral zone, which gives rise to the inferior olivary cells, is characterized by *Ngn1* and *Wnt1* expression [1].

Like the tegmentum of the hindbrain, the rhombic lip is derived from a series of rostrocaudal rhombomeric domains [10]. The rhombomere 1 (r1) component of the rhombic lip has been named the

upper rhombic lip, and the remainder (r2-r8 components) has been named the lower rhombic lip [1]. It should be noted that many authors, including Landsberg et al (2005) [1] do not specifically recognize the isthmus as a distinct region between r1 and the midbrain, despite strong evidence that the rostral part of the mammalian 'r1' is homologous with the avian isthmus [11]. Authors who do not recognize isthmus generally refer to the combined isthmic/r1 region as 'greater r1.' We follow Aroca and Puelles [11], in that we distinguish the isthmus segment from the remainder of r1, so our use of the term 'r1' does not include the isthmus. Together, the isthmus and r1 give rise to cells of the external granule layer, neurons of the cerebellar nuclei, and unipolar brush cells [12, 13]. The rhombic lip region of r2-r5 gives rise to the neurons of cochlear nuclei [14], and the major precerebellar nuclei of Landsberg et al. (2005) are generated from cells of the lower (r6-r8) rhombic lip [1].

An earlier report by Rodriguez and Dymecki (2000) [15] found that only mossy fiber precerebellar nuclei (pontine, reticulotegmental, external cuneate, vestibular nuclei, and lateral reticular nuclei) belonged to *Wnt1* lineage, and that the main climbing fiber nucleus (the inferior olivary nucleus) was not labeled. However, in a later study, the same group reported *Wnt1* expression in the inferior olivary nucleus [1]. An important anatomical study by Nichols and Bruce (2006) [16] reported the pattern of *Wnt1* expression in a *Wnt1* transgenic strain expressing lacZ. This latter study provides a careful and detailed description of embryonic *Wnt1* expression in the mouse hindbrain.

Because *Wnt1* is expressed in all layers of the rhombic lip, we decided to examine whether or not the cell clusters expressing *Wnt1* in the hindbrain were exclusively associated with many or all nuclei that project to the cerebellum. *Wnt1* expressing cells could be the progeny of cells that expressed *Wnt1* at some stage during early development, or they could be cells which express *Wnt1* in late embryonic or postnatal stages. We were particularly interested in finding whether *Wnt1* was expressed in the minor precerebellar nuclei. To resolve this question, we have compared the results of a large series of tracing studies on the precerebellar nuclei with the cumulative expression seen in the *Wnt1*-Cre::ROSA26R mouse. We found that the correlation between the cell groups expressing *Wnt1* and the cerebellar projecting nuclei was strong, but not perfect.

Materials and Methods

Retrograde Tracing

C57BL/6J mice (8 weeks, 21–24 g, n=22) obtained from the Animal Resource Center (Perth, Western Australia) were used for cerebellar injections. Animal experimental procedures were in accordance with

The Australian Code for the Care and Use of Animals in Research and approved by the Animal Care and Ethics Committee of The University of New South Wales.

Mice were anaesthetized with ketamine (80mg/kg) and xylazine (5mg/kg) given by intraperitoneal injection. The occipital bone and atlas of the right side were exposed by retracting the suboccipital muscles. In the majority of cases, a retrograde tracer (HRP, fluorogold, or DiI (1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate)) was injected into all major regions of the right side of the cerebellum (Fig. 1-B-1); in some cases, the injection was restricted to smaller areas (Fig. 1-B-2). For the large injection series, small holes were drilled in occipital bone to allow the injections into the anterior vermis, posterior vermis, simple lobule, crus 1 and crus 2 of the ansiform lobule, paramedian lobule, paraflocculus, and flocculus (Fig. 1-B-1). Each injection contained one of the following – 0.2~0.3 µl 25% HRP (Sigma), 5% fluorogold (Fluorochrome LLC), or 0.1% DiI (Invitrogen D3899). Our results are based chiefly on a large series of HRP injections because it is readily taken up by fibers terminating in or passing through an injection site, thus ensuring the widest anatomical coverage within the hemi-cerebellum [17, 18].

In HRP injection cases (n=18), mice were deeply anaesthetized with pentobarbitone sodium after a survival period of 24 hours. They were perfused transcardially, firstly with heparin saline (40IU/50 ml saline, 37 °C), then 4% paraformaldehyde (4 °C, pH7.4), and finally 10% sucrose (4 °C). Perfused brains were kept in the same fixative for a further 2 hours, and then transferred to 30% sucrose-buffer solution at 4°C for 12 hours. Serial sections were cut at 40 µm on a freezing microtome, followed by mounting on gelatin-coated slides prior to the reaction with 3,3′,5,5′-tetramethylbenzidine (TMB) [19]. In fluorogold (n=2) or DiI injection cases (n=2), the mice were sacrificed after 5 days (fluorogold) or 3 weeks (DiI). The brains were cut with a cryostat (fluorogold) or a vibrotome (DiI). In all cases, every fifth section was Nissl stained to confirm the anatomical details.

X-gal Staining

The *Wnt1*-Cre transgenic line, generated by pronuclear injection of the mouse *Wnt1* promoter and enhancer genomic DNA joined with the gene for Cre recombinase [20], was obtained from Andrew P. McMahon. The ROSA26R line was obtained from the Jackson laboratory (JAX mice Strain Name B6.129S4-Gt (ROSA) 26Sortm1Sor/J, Stock Number:003474), backcrossed to the outbred Swiss strain for several generations and maintained as a homozygous colony. *Wnt1*-Cre and ROSA26R genotyping was performed as previously described [21, 22]. These two lines of mice were crossed and maintained in

accordance with IACUC protocols approved by The University of Utah. The expression of lacZ in hindbrain neurons was studied in the newborn mice (n=3). We have determined that the topography of hindbrain nuclei in newborn mice is the same as in adult mice. The animals were anesthetized with isoflurane and transcardially perfused with PBS and then with 2% formaldehyde (EMS #15713) in 0.1 M Pipes, pH 6.9, 2 mM $MgCl_2$ and 1.25 mM EGTA. The brains were then dissected, postfixed for 2 hours at room temperature in the same solution, and then cryoprotected in PBS with 10% sucrose, 2mM $MgCl_2$, and subsequently in 30% sucrose, 2mM $MgCl_2$. The brains were then embedded in 2% gelatin (Sigma G2500), 0.9% NaCl and cryosectioned at 40 µm. The sections were mounted on Superfrost Plus Gold slides, permeabilized in PBS with 2 mM $MgCl_2$, 0.02% NP-40 and 0.01% sodium deoxycholate, and finally moved into X-gal solution (1 mg/ml X-gal, 25 mM $K_3Fe(CN)_6$, 25mM $K_4Fe\ (CN)_6 \cdot 3H_2O$, 2 mM $MgCl_2$, 0.02% NP-40, 0.01% sodium deoxycholate in PBS) for overnight staining at room temperature. The slides were then placed in 50% glycerol in PBS for clearing and mounted with Celvol 205.

Imaging and Mapping

Histological preparations (HRP, Nissl, and *Wn1*-Cre lacZ) were photographed using an Olympus AX70 microscope with a SPOT digital camera (14.2 Color Mosaic). The images were collated with Adobe Photoshop CS4 and mapped with the assistance of an atlas of the mouse brain [23].

Results

Retrograde Labeling of Hindbrain Nuclei after Cerebellar HRP Injections

After large HRP injections into one side of the cerebellar cortex, TMB labeled cells were easily identifiable in the hindbrain nuclei. Neurons with blue granular inclusions in the cytoplasm were identified as precerebellar nuclei. Different injection strategies were tried in an attempt to identify all of the precerebellar nuclei (Fig. 1-B). The best results were obtained when HRP was injected in a series of cerebellar penetrations over the whole hemi-cerebellum (n=10) (Fig. 1-B-1). However, in eight cases, small injections were made in different lobules (n=6) (Fig. 1-B-2) or in the vermis close to the midline (n=2) (Fig. 1-B-2) to supplement the data from cases with multiple injections. To confirm the HRP results, we performed additional injections with two other retrograde neuronal tracers, DiI and fluorogold. The results of DiI and fluorogold injections were the same as those derived from HRP injections. When we compared HRP with fluorogold and DiI, we found that HRP was more effective for the purposes of this study. The main disadvantage of using fluorogold and DiI is that, like all fluorescent

tracers, they fade with time, and so are less suitable for large scale mapping studies such as this. We appreciate that the use of fluorogold antibody could overcome the fading problem, but we found that this method was less sensitive than HRP. Although previous use of DiI has been confined to postmortem tracing experiments, we successfully followed recent reports on the use of DiI in *in vivo* studies in adult animals [24, 25].

The cumulative expression of *Wnt1* in the mouse brain was detected by X-gal staining in brains of newborn animals of a *Wnt1*-Cre lineage. A limitation of this technique is that it cannot reveal the separate lineages in relation to certain times during development. Overall, we have shown that the pattern of nuclei labeled after cerebellar injections is very similar to the pattern of *Wnt1* expressing cell clusters (Table 1).

<Put Fig. 1 here>

We will present the pattern of TMB labeled nuclei under two headings – the major precerebellar nuclei and the minor precerebellar nuclei.

Retrograde labeling in major precerebellar nuclei

As noted in the introduction, we use the term 'major precerebellar nuclei' to include the perihypoglossal and parvicellular part of the motor trigeminal nuclei in addition to the customarily recognized pontine, reticulotegmental, external cuneate, lateral reticular, and inferior olivary nuclei.

The *pontine nuclei* (Pn), located in the ventral hindbrain in the region of rhombomere 3 and 4, are the largest of the precerebellar nuclei in the mouse. The pontine nuclei can be divided on topographical grounds into rostromedial, peduncular, medial, ventral, ventromedial, dorsolateral, and lateral subdivisions. All but one of these subdivisions contained densely packed TMB labeled neurons. The exception was the peduncular subdivision, where labeled cells were sparse. Labeled cells were most numerous in the side contralateral to the injection, but were also present in all subdivisions of the ipsilateral side. The ipsilateral middle cerebellar peduncle was filled with TMB stained axons, which we presume to have arisen mainly from the contralateral and ipsilateral pontine nuclei (Fig. 2).

<Put Fig. 2 here>

The rostral part of the *reticulotegmental nucleus* (RtTg) is separated from the pontine nuclei by the medial lemniscus. RtTg is known to be closely associated with the pontine nuclei in functional terms [4]. The middle and caudal parts of the RtTg become progressively separated from the pontine nuclei, eventually forming a distinct group of cells just ventral to the central gray matter. Many TMB labeled

cells were found in all parts of the RtTg. As in the pontine nuclei, there was a contralateral preponderance, but in the case of RtTg this predominance was only slight.

The *lateral reticular nucleus* (LRt) is a prominent cell group located in the caudal hindbrain lateral to the inferior olivary nucleus. TMB labeled neurons were found bilaterally in the subtrigeminal, magnocellular, and parvicellular parts of LRt, but in the caudal part there was an obvious ipsilateral predominance (Fig. 3). The linear nucleus of the hindbrain, which is almost certainly a rostral extension of LRt [26], is prominently labeled on both sides after unilateral cerebellar injections.

The *inferior olivary nucleus* (IO) is the major source of climbing fibers projecting to the Purkinje cells of the cerebellum [27, 28]. The climbing fibers of the individual subdivisions of the IO are topographically organized into a number of sagittally oriented zones [29]. As expected, we found strongly labeled cells in all the subnuclei of the contralateral IO after large cerebellar injections. The axons of these labeled cells could be easily traced across the midline into the contralateral inferior cerebellar peduncle. A small cluster of labeled neurons was often found on the ventral surface of the pyramid of the hindbrain. In many cases this cell group was continuous with groups of labeled neurons in the inferior olivary nucleus. We consider the neurons on the external surface of the pyramid to be the rodent homolog of the human arcuate nucleus (Fig. 3C).

<Put Fig. 3 here>

HRP labeled cells were present bilaterally in the *external cuneate nucleus* (ECu) with an obvious ipsilateral preponderance. Large numbers of neurons were found in ECu in both multiple and single injection cases, which suggests that each neuron projects to a wide area of cerebellar cortex.

The *parvicellular part of motor trigeminal nucleus* (5PC) is a diffuse cell group located between the motor trigeminal nucleus and the principal sensory trigeminal nucleus, with its neurons mingled with the fiber bundles of the emerging trigeminal motor nerve. Labeled neurons were found bilaterally in all areas of the 5PC.

The *perihypoglossal nuclei* (the *prepositus nucleus*, the *intercalatus nucleus*, and the *nucleus of Roller*) enclose the rostral part of the hypoglossal nucleus. These three nuclei are involved in eye movement coordination, and have been shown to project to a widespread area of the cerebellum in cat [4, 30]. We found labeled neurons bilaterally in all three of the perihypoglossal nuclei, but with a slight ipsilateral preponderance, which is consistent with the finding of Røste (1989) [30]. Most of the labeled

perihypoglossal neurons were located in the nucleus of Roller and the caudal (magnocellular) portion of the prepositus nucleus.

Retrograde labeling in sensory precerebellar nuclei

A number of *sensory nuclei of the hindbrain* contain labeled cells after cerebellar injections. In this group, we place nuclei whose major role is the processing of sensory information but which also project to the cerebellum. This group includes the trigeminal, solitary, vestibular, cochlear, cuneate, hindbrain matrix, and parabrachial nuclei.

Labeled cells were found in all parts of the *trigeminal sensory nuclei*. Among the major sensory trigeminal nuclei, the density of labeled cells was greatest in the ipsilateral principal sensory trigeminal nucleus and the interpolar part of the ipsilateral spinal trigeminal nucleus (Pr5 and Sp5I), and least in the caudal part of the ipsilateral spinal trigeminal nucleus (Sp5C) (Fig. 1). In addition to the major trigeminal subdivisions, a few labeled cells were present in the dorsomedial spinal trigeminal nucleus (DMSp5), the trigeminal transition zone (5Tr), and the trigeminal-solitary transition zone (5Sol). No labeled neurons were found in the mesencephalic trigeminal nucleus.

The *solitary nucleus* is the visceral sensory nucleus of the hindbrain. It contains numerous labeled neurons, which were concentrated in a long wedge-shaped mediolateral band.

A few labeled cells were found in the *superior, medial, lateral, and spinal vestibular nuclei*. We found labeled neurons in the *nucleus Y* (Y) in only two cases, which might indicate that Y projects to a restricted area of the cerebellum and therefore it can be easily missed in a tracer study. Nucleus Y has been shown to project to the flocculus in cat [31], rhesus macaque [32], and rat [33], but the large cerebellar injections used in this study did not allow us to determine the fine topographical relationships of this or other individual precerebellar nuclei. Labeled cells were also found in *nucleus X* (X) on both sides. The labeled cells were concentrated in the caudal half of the nucleus.

In two of our cases there were small numbers of labeled neurons in the dorsal and ventral *cochlear nuclei* of both sides. HRP labeled cells were also present bilaterally in the *cuneate nucleus* (Cu) but with an obvious ipsilateral preponderance. The *matrix region of the medulla* (Mx) is an area between the cuneate nucleus and the spinal trigeminal nucleus [23]. A few labeled neurons were found bilaterally in this area, especially in its rostral portion.

A few labeled neurons were found bilaterally in the *medial parabrachial nucleus*, including its *external part*. There were fewer labeled neurons in the *lateral parabrachial nucleus*, mainly concentrated in its

external part. A few labeled neurons were found bilaterally in the *Kölliker-Fuse nucleus*, which lies adjacent to the parabrachial complex.

Two monoamine groups (the raphe nuclei, the locus coeruleus and neighbouring nuclei) and the nuclei of the reticular formation have also been shown to project to the cerebellum in our experiments.

The *raphe nuclei* are a series of median or paramedian cell groups extending from the rostral hindbrain to the junction of the hindbrain with the spinal cord. All of the raphe nuclei are serotonergic except the raphe interpositus nucleus [34, 35]. An earlier study in the rat suggested that the raphe nuclei might be a source of extraolivary climbing fibers projecting to the cerebellar cortex in the rat [36, 37]. However, we have not found any recent studies that have reported a climbing fiber projection from the raphe nuclei, and we have assumed that their axons end as mossy fibers. Following cerebellar HRP injections in this study, the majority of the raphe nuclei were found to contain cerebellar projecting cells. Nuclei containing labeled cells were the dorsal raphe (DR), the median raphe (MnR), the paramedian raphe (PMnR), the raphe magnus (RMg), the pontine raphe (PnR), the raphe interpositus (RIP), the raphe pallidus (RPa), and the raphe obscurus (ROb). In coronal sections, most labeled neurons in the raphe had long olive-shaped somata oriented parallel to the midline. The intensity of TMB in labeled cells of the raphe nuclei was much less than that in the neurons of the major precerebellar nuclei. No labeled cells were found in the caudal linear nucleus of the raphe.

Labeled cells were found in the *locus coeruleus and subcoeruleus nucleus* on both sides after unilateral cerebellar injections. A few neurons were also found in the subcoeruleus nuclei of both sides. Previous studies have shown that locus coeruleus axons that project to the cerebellum are neither mossy nor climbing fibers; instead they spread diffusely through all layers of the cerebellar cortex, and have been called multilayered fibers [38-40].

Labeled cells were found in a number of nuclei belonging to *the hindbrain reticular formation*. We consider the hindbrain reticular formation proper to consist of three zones: a medial gigantocellular zone (oral part of the pontine reticular nucleus (PnO), caudal part of the pontine reticular nucleus (PnC), gigantocellular reticular nucleus (Gi), paramedian reticular nucleus (PMn), and ventral medullary reticular nucleus (MdV)), an intermediate zone (intermediate reticular zone (IRt)), and a lateral small cell zone (parvicelluar reticular nucleus (PCRt) and dorsal medullary reticular nucleus (MdD)). It must be noted that there have been attempts to add many other hindbrain nuclei to the list of those that make up the reticular formation. The nuclei that are often lumped with the reticular formation include the

reticulotegmental nucleus, the lateral reticular nucleus, the raphe nuclei, and the locus coeruleus. Like Brodal (1981) [4], we do not see the point in grouping together nuclei with quite disparate anatomy and function simply because there is no other convenient place to put them. For this reason we treat the reticulotegmental nucleus and the lateral reticular nucleus as major precerebellar nuclei, since they have little in common with the main parts of the reticular formation. Likewise, we do not include the raphe nuclei or the locus coeruleus in the reticular formation, because they are monoanminergic.

The *oral part of the pontine reticular nucleus* and the *caudal part of the pontine reticular nucleus* contained few labeled cells after tracer injection into the cerebellum. The labeled cells were found both ipsilaterally and contralaterally. A similar pattern of sparsely labeled cells was found in the *gigantocellular reticular nucleus* and the *ventral medullary reticular nucleus.* In the lateral reticular formation, which is dominated by small cells, a few labeled cells were found on both sides in the *parvicellular reticular nucleus* and its *alpha part* (PCRtA), but were not seen in the dorsal part of medullary reticular nucleus. A few labeled cells were found in the *intermediate reticular zone* and the *paramedian reticular nucleus*.

<Put Table 1 here>

Hindbrain Cell Clusters Expressing *Wnt1*

We have mapped the distribution of *Wnt1* expression by recording the position of X-gal labeled cells in the hindbrain in sections of animals of the *Wnt1*-Cre lineage. The distribution of X-gal staining is shown in Table 1.

Wnt1 expression in the major precerebellar nuclei

The *pontine nuclei, reticulotegmental nucleus, lateral reticular nucleus,* the *external cuneate nucleus,* and the parvicellular part of the motor trigeminal nucleus showed intense X-gal staining. The X-gal staining in the *parvicellular part of the motor trigeminal nucleus* (5PC) was very intense, similar to the intensity of staining in the pontine and reticulotegmental nuclei. The area of intense X-gal staining was much larger than the region occupied by the HRP labeled neurons after cerebellar injection (Fig. 4 row 4). X-gal labeled area is also considerably larger than the area identified as 5PC in the atlas of Franklin and Paxinos (2008). All three of the *perihypoglossal nuclei* showed X-gal staining. The staining was strongest in the intercalatus nucleus and lightest in the prepositus nucleus.

X-gal staining in the *inferior olivary nucleus* was sparser and lighter compared with the other major precerebellar nuclei, but the caudal pole of the inferior olivary nucleus showed relatively strong X-gal

staining (Fig. 4 row 1). The arcuate nucleus, which we believe to be an outlying part of the inferior olivary nucleus, showed light X-gal staining.

<Put Fig. 4 here>

Wnt1 expression in the minor precerebellar nuclei

Wnt1 expression, as evidenced by X-gal staining, is present in almost all of the minor hindbrain precerebellar nuclei (Table 1).

All the *sensory nuclei of the hindbrain* that project to the cerebellum, except the gracile nucleus, show X-gal staining. The *trigeminal sensory nuclei* show X-gal staining, with the most intense staining in the cells of the caudal part of the spinal trigeminal nucleus (Fig. 4). The neurons of the *solitary nucleus*, the *cochlear nuclei*, and the major *vestibular nuclei* were stained with X-gal. Among the vestibular nuclei, the X-gal staining was more intense in the *spinal vestibular nucleus* and the *parvicellular part of the medial vestibular nucleus* (Fig. 5). The *matrix region of the medulla* and *cuneate nucleus* showed intense X-gal staining. The *medial and lateral parabrachial nucleus* showed moderate or intensive X-gal staining. Light staining was present in the cells of the *Kölliker-Fuse nucleus.*

All the *monoamine nuclei of the hindbrain* that project to the cerebellum, except the *subcoeruleus nucleus*, show X-gal stain. The X-gal staining in the *raphe nuclei* was mostly light or moderate, but was more intense in the *dorsal raphe nucleus*. The *locus coeruleus* also contained X-gal stained cells.

Although all major parts of the *hindbrain reticular formation* were shown to project to the cerebellum, some of these nuclei (the *oral and caudal parts of the pontine reticular nucleus*) did not show X-gal staining. X-gal staining was light or moderate in the *gigantocellular reticular nucleus*, *ventral part of the medullary reticular nucleus, intermediate reticular zone*, *alpha part of the parvicellular reticular nucleus, parvicelluar reticular nucleus,* and *paramedian reticular nucleus*.

Wnt1 expression in hindbrain nuclei that do not project to the cerebellum

X-gal staining was not found in hindbrain nuclei that do not project to the cerebellum, with a small number of exceptions. These are the lateroventral periolivary nucleus (LVPO), the medioventral periolivary nucleus (MVPO), and the lateral superior olive (LSO), which showed moderate X-gal staining) (Fig. 5), and the gracile nucleus, which showed intense X-gal staining. We did not find HRP labeled cells in any of these nuclei after cerebellar injections. In the case of the gracile nucleus, we could not determine whether the stain was located in neurons of the gracile nucleus, incoming fibers of the gracile fasciculus, or both.

<Put Fig. 5 here>

Wnt1 Expression in Areas other than the Hindbrain

This paper is focused on the precerebellar nuclei of the hindbrain. For this reason, we will not report on the evidence of extensive *Wnt1* expression in the mesencephalon, diencephalon, hypothalamus, roof structures of the fourth ventricle, and the dorsal regions of the spinal cord. The cerebellum is a part of the hindbrain and shows intense X-gal staining (Fig. 4), but for the sake of consistency we report only on *Wnt1* expression in the isthmic and rhombomeric parts of the hindbrain.

Discussion

Identifying and Classifying the Precerebellar Nuclei in the Hindbrain of the Mouse

We believe that this paper provides the first detailed account of the pattern of hindbrain nuclei that project to the cerebellum in the mouse. In the case of the major precerebellar nuclei, our findings are consistent with previous reports, but we have expanded the list of nuclei in this category to include 5PC, the Li part of the lateral reticular nucleus, and the perihypoglossal nuclei. In addition, we have mapped the projection from a large number of minor precerebellar nuclei. We have identified a nucleus that appears to be the rodent homolog of the human arcuate nucleus, which we believe is a member of the inferior olive complex. In most cases, our findings on the minor precerebellar nuclei are consistent with previous studies, but we believe that we are the first to describe cerebellar projections in the mouse from 5PC, nucleus Y, PCRtA, and the matrix region of the medulla. Although the spinal cord was not a subject of detailed analysis in this study, we noted in passing that labeled neurons were found in the central cervical nucleus, the dorsal nucleus of the spinal cord (Clarke's column), and specific parts of the lamina 7 in lower lumbar segments. Such projections have been found in many other mammalian studies [41-44].

Previous studies have not identified 5PC as a precerebellar nucleus. The projection to the cerebellum from 5PC is something of a surprise, since neurons in this area have been shown to supply the tensor tympani muscle in the cat [45], guinea pig [46], and mouse [47]. The labeled neurons of the nucleus we identify as 5PC are widely dispersed, from the region between motor and sensory trigeminal nuclei to the region of emergence of the trigeminal nerve. We have not found a description of this projection in any previous study. 5PC was heavily stained with X-gal, indicating that it contains a considerable number of neurons expressing *Wnt1*.

In a recent study, we have documented the projection of the linear nucleus to the cerebellum [26]. The linear nucleus is a very distinctive cell group in rodents, but is not consistently found in other mammals [26]. The linear nucleus appears to be a specialized extension of the lateral reticular nucleus.

The arcuate nucleus is a large cell group in human brains that has long been recognized as a precerebellar nucleus, but its existence has not been documented in rodent brains. We found that the arcuate nucleus is an inconsistent cell group on the surface of the hindbrain pyramid in the C57BL/6J mouse. The arcuate cells are labeled following cerebellar injections. We believe that the arcuate cells are a displaced component of the inferior olivary nucleus. This is the subject of further investigation in our laboratory.

The Possible Functions of Precerebellar Nuclei

There are obvious anatomical and functional differences between many of the nuclear groups that project to the cerebellum. For example, many of the precerebellar nuclei serve to provide direct sensory information to the cerebellum. These include the dorsal nucleus of the spinal cord and the external cuneate, vestibular, cochlear, and trigeminal nuclei. The lateral reticular nucleus may also serve to integrate spinal cord and primary sensorimotor cortex inputs to the cerebellum [4]. While the inferior olivary nucleus does relay some sensory information to the cerebellum, it has been identified as a source of timing signals for operation of the Purkinje cell system [48]. The pontine and reticulotegmental nuclei serve to link the cerebral neocortex to the cerebellum - a role that has become increasingly important in mammalian evolution. The locus coeruleus and raphe nuclei send diffuse projections to the cerebellum that may link the cerebellum to general behavioural states dominated by noradrenaline and serotonin.

The Functions of Wnt1 in Early Development of The Central Nervous System

Wnt1 appears to play a number of distinct roles during early development of the central nervous system. Thomas and Capecchi (1990) showed that Wnt1 was essential for development of the midbrain and cerebellum in mice [49]. The importance of Wnt1 in cerebellar development is summarized in a valuable review paper by Wingate [50]. Wnt1 plays a role in dorsalization of the spinal cord [51] and is also a powerful mitogen [52]. Our paper has focused on the expression of *Wnt1* in the rhombic lip, but only in respect to the hindbrain nuclei that derive from the rhombic lip.

The rhombic lip is an important neuroepithelial proliferative zone in the embryonic brain. It is the most dorsal part of the alar plate of the hindbrain, forming the larger margin of the fourth ventricle [5]. The rhombic lip can be divided into an upper part, which gives rise to the cerebellum, and a lower part,

which extends from rhombomere 2 to rhombomere 8 [1]. The rhombic lip is a site of early *Pax6* expression, but its development is later dominated by *Wnt1* expression [1].

Prenatal Expression of *Wnt1* in the Central Nervous System

The major precerebellar nuclei arise from the lower rhombic lip [1], and this led Landsberg et al. (2005) to assert that the upper rhombic lip does not give rise to precerebellar nuclei. However, we have shown that a number of minor precerebellar nuclei are located in the region formed by the developing isthmus and r1, which are in turn partly formed by the upper rhombic lip. We found, for example, that the dorsal raphe nucleus (an isthmic derivative) [53] and the locus coeruleus (an r1 derivative) [54] both project to the cerebellum; the upper rhombic lip is therefore also a source of precerebellar nuclei in the broadest sense.

The cells of the cerebellar cortex in amniote vertebrates arise from the isthmus and the first rhombomere [55, 56]. The chick/quail grafting study of Marin and Puelles [56] suggested that the auricle of the chick cerebellum (equivalent to the flocculonodular node of mammals) arises from cells in r2 boundary, but in the absence of confirmatory data in mammals this suggestion must be considered speculative. The intense X-gal staining in the cerebellum is consistent with studies showing that *Wnt1* is critical for cerebellar development [57].

The *Wnt1* expression in this Cre lineage does not assist with the analysis of the possible sequence of different *Wnt1* functions during brain development. The separate developmental roles of *Wnt1* are obscured by the expression picture revealed in this Cre lineage, since this method gives nothing more than a cumulative picture summarizing a sequence of separate events. Dissection of the temporal sequence of *Wnt1* expression in early development requires different methodologies, such as tamoxifen control of an ER lineage [2]. We therefore cannot link the relationship between the intensity of *Wnt1* expression with its variable roles in the developmental stages.

Is *Wnt1* A Genetic Marker for Precerebellar Nuclei?

We found that almost all of the nuclei projecting to the cerebellum express *Wnt1*, the only exceptions being the oral and caudal pontine reticular nuclei and the subcoeruleus nuclei. However, we should point out that there was not a good correlation between the intensity of *Wnt1* expression in a particular nucleus and the number of neurons in the same nucleus labeled after cerebellar injection (Table 1). For example, the caudal spinal trigeminal nucleus (Sp5C) contains only a few labeled precerebellar cells and the gracile nucleus has no labeled cells, but both show intensive X-gal staining. The strong X-gal signal in

Sp5C and the gracile nucleus seems to be related to the intense *Wnt1* activity in the spinal and caudal hindbrain alar plate derivatives – the dorsal column nuclei, the dorsal horn and the dorsal funiculus. This pattern of expression may be distinct from the expression of *Wnt1* in hindbrain precerebellar nuclei.

This is the first comprehensive report of mapping of the cumulative expression of *Wnt1* as seen in cell groups in the post-natal mouse hindbrain. Two previous studies of *Wnt1* lineage derivatives from the rhombic lip found *Wnt1* expression in the major mossy precerebellar nuclei, but not in the major climbing fiber nucleus, the inferior olivary nucleus [1, 15]. The failure to identify *Wnt1* expression in the inferior olivary nucleus is almost certainly attributable to technical issues. Nichols and Bruce [16] used a "straight" *Wnt1*-lacZ transgene and the failure to detect X-gal signal in the inferior olivary nucleus suggests that *Wnt1*-lacZ expression is transient in this nucleus. Rodriguez and Dymecki [15] used FLP-mediated recombination to generate their *Wnt1*-FLP lineage. The efficiency of FLP recombinase in mouse tissues is fairly low relative to Cre (Wu and Capecchi, personal communication) and the lack of staining was likely due to the fact that it was below the detection limit of that system. This appears to be confirmed by the fact that a later study by the same group using a new FLPe variant with higher activity [1] did identify *Wnt1* expression in the inferior olivary nucleus. None of these studies reported on *Wnt1* expression in the minor precerebellar nuclei. Because the *Wnt1*-cre methodology reveals the cumulative expression of *Wnt1* before P0, our study has demonstrated a wider range of cell groups that express *Wnt1*.

In many cases, the majority of cells in a particular nucleus (e.g. pontine nuclei) project to the cerebellum, and the majority of cells in that nucleus also express *Wnt1*. In such cases, we think it is likely that the retrograde tracer and *Wnt1* marker are marking the same cell population. However, it must be pointed out that we do not have direct evidence of colocalization in such cases. It is possible that some cells that project to the cerebellum may lack *Wnt1* expression, and that some *Wnt1* positive cells do not project to the cerebellum. In the latter case, it is possible that local interneurons might express *Wnt1*, yet fail to project the cerebellum. This question could be resolved in the future with a study of potential colocalization of *Wnt1* and cerebellum projection markers.

Wnt1 expression in the hindbrain is largely, but not entirely, restricted to the precerebellar nuclei. As noted above, *Wnt1* is also expressed in three nuclei of the superior olive complex (lateroventral periolivary nucleus, medioventral periolivary nucleus, and lateral superior olive) and the gracile nucleus, which did not contain labeled neurons after cerebellar injections. We assume that the expression in the

gracile nucleus is related to the role of *Wnt1* expression in the alar derivatives of the spinal cord and caudal hindbrain.

In classifying precerebellar nuclei into major and minor groups, we emphasize the difference between nuclei whose existence is almost entirely tied to the cerebellum, and those with other primary functions that happen to send secondary projections to the cerebellum. In this regard, the best examples of major precerebellar nuclei are perhaps the pontine and inferior olivary nucleus, because their size in different mammals seems to be correlated with the size of the cerebellum. While *Wnt1* is expressed in almost all of the minor precerebellar nuclei we have identified, the strong expression in the major precerebellar nuclei is the main feature that links the hindbrain expression of this gene to the development of the precerebellar system.

Conclusion

We have mapped the hindbrain precerebellar nuclei in the mouse using injections of retrograde tracer into the cerebellum. We were able to define 39 distinct nuclei that project to the cerebellum, a number of which have not previously been identified as precerebellar nuclei. *Wnt1* is most strongly expressed in the major precerebellar nuclei, but all the hindbrain precerebellar nuclei, except the pontine reticular nuclei and the subcoeruleus nuclei were shown to contain cells expressing *Wnt1*.

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Table 1 Cell groups labeled after HRP cerebellar injections compared with cumulative *Wnt1*-Cre genetic labeling in mouse hindbrain

Figure legends

Fig. 1 - A1-A12. A rostrocaudal series of coronal sections of mouse hindbrain summarizing the location of labeled neurons after large HRP injections in the right side of the cerebellum. Large numbers of labeled cells are seen in the pontine nuclei (Pn - A1), reticulotegmental nucleus (RtTg - A1-A3), and inferior olivary nucleus (IO - A7-A12) of the contralateral side, and in the trigeminal sensory nuclei (Pr5, DMSp5, Sp5O, Sp5I, Sp5C, 5Tr - A2-A12), external cuneate nucleus (ECu - A8-A11), and lateral reticular nucleus (LRt - A7-A12) of the ipsilateral side. A full description of the location of labeled cells is to be found in the text. B1. Cerebellar injection sites (black circles on a photograph of the dorsal surface of the cerebellum) in a case with multiple injections. B2. A summary of the sites of cerebellar injections in cases when only small localized injections (white circles) were made. This illustration summarizes the injections made in six different cases. The scale bar represents 1mm.

Fig. 2 HRP-TMB labeled neurons in the pontine nuclei after a large series of HRP injections in the right side of the cerebellum in one multiple injection case. 2A shows the overall pattern of labeled cells in the pontine nuclei. The fibers of the middle cerebellar peduncle (mcp) of the right side are strongly labeled. The scale bar represents 200 µm. 2B Magnified images of labeled cells in the contralateral dorsolateral pontine nucleus (PnDL - 2Bi, 2Bii), peduncular pontine nucleus (PnPed - 2Biii), lateral pontine nucleus (PnL - 2Biv), medial pontine nucleus (PnM - 2Bv), and the ventral pontine nucleus (PnV - 2Bvi). The scale bar represents 20µm.

Fig. 3 HRP-TMB labeled neurons in the inferior olivary nucleus (IO) and the lateral reticular nucleus (LRt) after a large series of HRP injections in the right side of the cerebellum. A and B represent low power images of the hindbrain at the level of the rostral IO and the caudal IO, respectively. Labeling in the IO is almost entirely contralateral. Labeled cells are present in the LRt and Li of both sides (3B, 3A), with an ipsilateral predominance. The scale bars in A and B represent 200µm. C is a magnified image of the boxed area in A. Labeled cells are seen in four subnuclei of the IO (IODM, IOM, IOPr, IOD). The arcuate nucleus of the hindbrain (Ar), located ventral to the pyramid, contains labeled cells. The scale bar represents 100 µm.

Fig. 4 A series of sagittal sections of adult mouse hindbrain showing HRP-TMB labeled precerebellar nuclei (left column) compared with a series of similar sagittal sections of a newborn mouse showing the cell groups expressing *Wnt1* stained with X-gal. Note that all nuclei containing HRP labeled cells (left) are also labeled with X-gal in the series on the right. However, the staining intensity in the nuclei of the Wnt1 series is often very different from that in the HRP series; some nuclei are very strongly lableled with X-gal (Cu, Sp5C) but have very few HRP labeled cells. The position of each section pair relative to the midline is indicated in the upper left hand corner of each HRP-TMB section. The scale bar represents 0.5mm in each case.

Fig. 5 The intensity of X-gal staining in the vestibular nuclei (5A, 5B) varies from heavy (SpVe and MVePC), to medium (SuVe), to light (MVeMC). 5C shows prominent X-gal staining of cells in the LVPO, MVPO, and LSO, none of which projects to the cerebellum. The scale bar represents 0.2 mm.

Table 1 Cell groups labeled after HRP cerebellar injections compared with cumulative *Wnt1*-Cre genetic labeling in mouse hindbrain

		Precerebellar Nuclei	Ipsilateral Labeled Neurons*	Contralateral Labeled Neurons*	*Wnt1* Expression **
Major precerebellar nuclei		Pontine nuclei (Pn)	⊙	●	+++
		Reticulotegmental nucleus of the pons (RtTg)	●	●	+++
		Lateral reticular nucleus (LRt)	●	⊙	+++
		Inferior olivary nucleus (IO)	○	●	+
		External cuneate nucleus (ECu)	●	◉	+++
		Motor trigeminal nucleus, parvicellular part (5PC)	●	●	+++
		Prepositus nucleus (Pr)	●	●	+
		Nucleus of Roller (Ro)	●	●	++
		Intercalated nucleus (In)	◉	◉	+++
Minor precere-bellar nuclei	Hindbrain sensory nuclei	Cuneate nucleus (Cu)	●	◉	+++
		Cochlear nuclei (C)	◉	◉	+++
		Vestibular nuclei (Ve)	◉	◉	+ or ++ or +++
		Nucleus Y (Y)	○	◉	++
		Nucleus X (X)	⊙	⊙	++
		Solitary nucleus (Sol)	●	●	++
		Principal sensory trigeminal nucleus (Pr5)	●	⊙	+
		Spinal trigeminal nucleus (Sp5)	●	⊙	+ or ++ or +++
		Matrix region of the medulla (Mx)	◉	◉	+++
		Medial parabrachial nucleus (MPB)	⊙	◉	++
		Lateral parabrachial nucleus (LPB)	◉	◉	+++
		Kölliker-Fuse nucleus (KF)	◉	◉	+
	Hindbrain monoamine nuclei	Median raphe nucleus (MnR)	◉	◉	++
		Paramedian raphe nucleus (PMnR)	⊙	⊙	++
		Pontine raphe nucleus (PnR)	◉	◉	+
		Raphe interpositus nucleus (RIP)	◉	◉	+
		Raphe obscurus nucleus (ROb)	⊙	⊙	++
		Raphe magnus nucleus (RMg)	◉	◉	+
		Raphe pallidus nucleus (RPa)	◉	◉	+
		Dorsal raphe nucleus (DR)	◉	◉	+++
		Locus coeruleus (LC)	⊙	⊙	+++
		Subcoeruleus nucleus (SubC)	◉	◉	–
	Hindbrain reticular nuclei	Pontine reticular nucleus, caudal part (PnC)	◉	◉	–
		Pontine reticular nucleus, oral part (PnO)	◉	◉	–
		Gigantocellular reticular nucleus (Gi)	⊙	⊙	+
		Medullary reticular nucleus, ventral part (MdV)	⊙	⊙	+
		Paramedian reticular nucleus (PMn)	⊙	⊙	++
		Intermediate reticular zone (IRt)	⊙	⊙	+
		Parvicelluar reticular nucleus, alpha part (PCRtA)	◉	◉	+
		Parvicelluar reticular nucleus (PCRt)	◉	◉	+

*○no labeled cells; ◉ few labeled neurons; ⊙ moderate number of labeled neurons; ●numerous labeled neurons.

** *Wnt1* expression is represented by grades of X-gal staining, from light (+) to solid dark blue (+++).