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Plasticity of neural crest-placode interaction in the developing visceral nervous system

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Abstract

The reciprocal relationship between rhombomere (r)-derived cranial neural crest (NC) and epibranchial placodal cells derived from the adjacent branchial arch is critical for visceral motor and sensory gangliogenesis, respectively. However, it is unknown whether the positional match between these neurogenic precursors is hard-wired along the anterior-posterior (A/P) axis. Here we use the interaction between r4-derived NC and epibranchial placode-derived geniculate ganglion as a model to address this issue. In *Hoxa1*^{-/-}*bl*^{-/-} embryos, r2 NC compensates for the loss of r4 NC. Specifically, a population of r2 NC cells is redirected towards the geniculate ganglion, where they differentiate into postganglionic (motor) neurons. Reciprocally, the inward migration of the geniculate ganglion is associated with r2 NC. The ability of NC and placodal cells to respectively differentiate and migrate despite a positional mismatch along the A/P axis reflects the plasticity in the relationship between the two neurogenic precursors of the vertebrate head.

Keywords

Neural crest; epibranchial placode; migration; differentiation; Hox; Neurog

INTRODUCTION

The cellular diversification and identity of the animal body depends on the nested expression of the *Hox* genes and alignment of precursor cells along the anterior-posterior (A/P) axis (McGinnis and Krumlauf, 1992; Lumsden and Krumlauf, 1996; Trainor and Krumlauf, 2000b; Kiecker and Lumsden, 2005). In the developing vertebrate head, the interaction between pluripotent cranial neural crest (NC) cells and adjacent tissues of the developing head and neck areas is critical for the formation of musculoskeletal, connective, cardiovascular, respiratory lymphoid, endocrine, and neuronal tissues that constitute the head, neck, and thoracic-abdominal compartments of the body (Fontaine-Perus et al., 1988; Kirby, 1988; Manley and Capecchi, 1995; Gavalas et al., 1998; Gavalas et al., 2001; Trainor et al., 2002; Macatee et al., 2003; Arenkiel et al., 2004; Matsuoka et al., 2005; Oury et al., 2006; Park et al., 2006; Shiao et al., 2008; Coppola et al., 2010; Takano-Maruyama et al., 2010; Bertrand et al., 2011). The cranial NC is the central player that coordinates the cellular organization in these various tissue compartments. In the head, the cranial NC provides the source of many cell types, and through its interactions with cells in the adjacent branchial arches (ba) coordinates craniofacial morphogenesis. The nested expression of the *Hox* genes are superimposed on this NC-branchial arch interaction, which ensures that aligned cells are

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endowed with the positional information that are critical for their integration along the A/P axis (Lumsden et al., 1991; Krumlauf et al., 1993; Bell et al., 1999; Begbie and Graham, 2001; Oury et al., 2006).

In the developing cranial nervous system, the migration of NC cells originating from even-numbered rhombomeres (r) into the ba not only provides the source for the myriad of cell types that constitute the head, but they also act as a cellular conduit to integrate the periphery with the central nervous system (Lumsden and Krumlauf, 1996; Trainor and Krumlauf, 2000b; Trainor and Krumlauf, 2001; Coppola et al., 2010). For example, r4 NC cells migrate into the adjacent ba2 to form myelin forming Schwann cells that ensheath the axons of r4-derived facial motor neurons, which in turn innervate ba2-derived facial muscles (Arenkiel et al., 2003; Arenkiel et al., 2004). In the afferent pathway, r4 NC cells facilitate the inward migration and central connections of the ba2 placode-derived geniculate visceral ganglion (Begbie and Graham, 2001). The specification and formation of these motor and sensory pathways are dependent on the combinatorial activities of *Hoxa1* and *Hoxb1* (Goddard et al., 1996; Studer et al., 1996; Gavalas et al., 1998; Studer et al., 1998; Rossel and Capecchi, 1999; Gavalas et al., 2001; Gavalas et al., 2003; Arenkiel et al., 2004). The *Hox*-dependent positional integration of motor and sensory pathways appears to be a fundamental mechanism that is repeated along the A/P axis from the hindbrain to the spinal cord (Goddard et al., 1996; Studer et al., 1996; Carpenter et al., 1997; Gavalas et al., 1997; Tiret et al., 1998; Bell et al., 1999; Lance-Jones et al., 2001; McClintock et al., 2002; Dasen et al., 2003; Gaufo et al., 2003; Arenkiel et al., 2004; Gaufo et al., 2004; Dasen et al., 2005; Oury et al., 2006; Holstege et al., 2008; Chen et al., 2010).

It is established that the cranial NC is critical for coordinating many aspects of craniofacial development, but equally important is the influence of the environment on determining many of its possible fates, thereby reflecting the plasticity of the cranial NC (Fontaine-Perus et al., 1988; Kirby, 1988; Saldivar et al., 1996; Trainor and Krumlauf, 2000a; Trainor et al., 2002; Le Douarin et al., 2004; Bogni et al., 2008; Coppola et al., 2010; Takano-Maruyama et al., 2010). Others and we have shown that the ba2 placode-derived geniculate visceral ganglion is essential for the survival and differentiation of r4 NC-derived visceral postganglionic (PG) precursors of the submandibular and sphenopalatine ganglia (Coppola et al., 2010; Takano-Maruyama et al., 2010). Together with the observation that the inward migration of the geniculate ganglion is dependent on r4-derived NC cells (Begbie and Graham, 2001; Barlow, 2002; Coppola et al., 2010; Takano-Maruyama et al., 2010), we suggested that the reciprocal interaction between positionally aligned cells is critical for the integration of precursors into a common visceral neuronal circuit. However, it is unknown whether this reciprocal interaction is restricted to this A/P position or whether cranial NC and placodal cells show plasticity in their ability to differentiate and migrate, respectively, under different positional conditions along the A/P axis.

Evidence from quail/chick studies have shown that non-neuronal cells, presumably of NC origin, contained within the placode-derived visceral sensory ganglion differentiate into PG neurons when ectopically grafted outside of its normal A/P position (Fontaine-Perus et al., 1988; Kirby, 1988; Le Douarin et al., 2004). These findings provide evidence for the plasticity of the cranial NC, showing that it can differentiate into PG neurons under different positional conditions. In contrast, the geniculate ganglion fails to migrate inwards to the hindbrain when r4 is specifically ablated in the chick embryo (Begbie and Graham, 2001). This suggests that the dependence of the inward migration of the geniculate ganglion is specific to r4 NC cells because neighboring NC cells, which are presumably left intact with this specific surgical ablation, are unable to compensate for the loss of r4 NC cells.

Here we use the mouse embryo harboring mutations for *Hoxa1* and *Hoxb1* – genes that are required for the formation of r4 NC cells, but not the ba2-derived geniculate ganglion (Gavalas et al., 1998; Studer et al., 1998; Rossel and Capecchi, 1999; Gavalas et al., 2001; Gavalas et al., 2003) – as a model to test whether PG neurons differentiate and geniculate ganglion migrate in the absence of the r4 NC population that normally facilitates these processes. In *Hoxa1*^{-/-}*b1*^{-/-} embryos, we found that r2-derived NC cells compensate for the loss of r4 NC cells. In this context, r2 NC cells are able to migrate into the geniculate ganglion and differentiate into PG neurons, and associate with the inward migration of the geniculate ganglion. Our study provides evidence for the plasticity of the cranial NC fated for the PG neuronal lineage, and reveals an unanticipated plasticity of the geniculate visceral sensory ganglion in its migratory behavior in the absence of a normal, positionally matched NC population.

RESULTS

Differentiation of postganglionic neurons in the absence of their normal rhombomeric source and migratory environment

Fate mapping of cranial NC in mouse and quail/chick embryos have shown that parasympathetic PG neurons of the submandibular and sphenopalatine ganglia originate from r4, part of the preotic region of the hindbrain (D'Amico-Martel and Noden, 1983; Arenkiel et al., 2003; Yang et al., 2008; Takano-Maruyama et al., 2010). Based on these findings, we predicted that the submandibular and sphenopalatine ganglia would fail to form in mouse embryos harboring compound mutations of *Hoxa1* and *Hoxb1* due to the absence of r4 NC cells in these mutant embryos (Gavalas et al., 1998; Studer et al., 1998; Rossel and Capecchi, 1999; Gavalas et al., 2001; Gavalas et al., 2003). We unexpectedly found the presence of Phox2b⁺ PG neurons of the submandibular and sphenopalatine ganglia at e12.5, albeit at significantly reduced numbers and expression levels (Fig. 1A, D; n=4).

Postganglionic neurons of the otic ganglion were also observed in reduced numbers in *Hoxa1*^{-/-}*b1*^{-/-} embryos, and were situated in their normal position just ventral to the geniculate ganglion (Fig. 1G) (D'Amico-Martel and Noden, 1983; Enomoto et al., 2000). These findings suggest that NC precursors of the submandibular, sphenopalatine, and otic ganglia are able to migrate rostrally through an abnormal branchial arch environment to reach their target areas. Furthermore, the presence of the placode-derived geniculate ganglion in its normal position close to the hindbrain indicates that the inward migration of this ganglion occurs in the absence of r4-derived NC cells (Fig. 1H, I). This is in contrast to a study in the chick embryo in which r4 was specifically ablated, and the placode-derived geniculate ganglion failed to migrate inwards to the hindbrain (Begbie and Graham, 2001).

Analysis of *Hoxa1*^{-/-}*b1*^{-/-} embryos at a later stage (e13.5) showed that the Phox2b⁺ PG neurons in the submandibular, sphenopalatine, and otic ganglia remained reduced both in number and expression levels compared to control littermates (Fig. 1B, C, E, F, H, I). The persistence of Phox2b at low expression levels suggests these PG precursors may not fully mature into PG neurons, which normally expresses high levels of Phox2b at this stage. Nevertheless, despite the absence of r4 NC and abnormal branchial arch tissues, NC and placodal cells have the capacity to differentiate and migrate to their normal target areas. This suggests a compensatory mechanism to support the early differentiation of PG neurons and facilitate the inward migration of the geniculate ganglion.

Migration of the geniculate ganglion from an A/P mismatch of neural crest and placodal cells

To explore a possible compensatory mechanism, we reexamined hindbrain and NC patterning in *Hoxa1*^{-/-}*b1*^{-/-} embryos. Previous reports have shown that the combined loss

of *Hoxa1* and *Hoxb1* resulted in a residual r4 territory, complete loss of NC cells from r4 to the caudal hindbrain and associated NC-derived ganglia, loss of ba2, and fusion of placode-derived geniculate and trigeminal sensory ganglia (Gavalas et al., 1998; Studer et al., 1998; Rossel and Capecchi, 1999; Gavalas et al., 2001). The latter phenotype suggests that the ba2 cleft, the source of the geniculate ganglion, is intact in *Hoxa1*^{-/-}*b1*^{-/-} embryos. Indeed, in e10.5 *Hoxa1*^{-/-}*b1*^{-/-} embryos, we observed the ba2 cleft, from which *Isl1*⁺*Phox2b*⁺ neurons of the geniculate ganglion emerged (Fig. 2A, B; asterisk). In whole-mount preparations, we observed fusion of the *Isl1*⁺*Phox2b*⁺ geniculate and *Isl1*⁺ trigeminal ganglia, confirming a previous study (Gavalas et al., 2001). At this level of analysis it is difficult to assess whether the geniculate ganglion has migrated inwards to the hindbrain or remained in a sub-ectodermal position. The latter scenario is possible since the inward migration of the geniculate ganglion is dependent on r4 NC cells (Begbie et al., 1999; Begbie and Graham, 2001). Alternatively, since r3 and r4 are eliminated in *Hoxa1*^{-/-}*b1*^{-/-} embryos, the geniculate ganglion may adapt to a new environment that facilitates its inward migration to a more rostral rhombomere. This would suggest that a more rostral NC population associates with the inward migration of the geniculate ganglion.

To test this possibility, we analyzed *Hoxa1*^{-/-}*b1*^{-/-} embryos at e9.5, the time when precursors of ba2 placode-derived geniculate ganglion are initially engaged with r4 NC cells (Begbie and Graham, 2001; Takano-Maruyama et al., 2010). In sagittal sections of control embryos, *Phox2b*⁺*Isl1*⁺ precursors of the geniculate ganglion have delaminated from the ba2 cleft and aggregated as a sphere directed towards r4 (Fig. 2C, asterisk). A clear boundary is seen between the geniculate ganglion and the more rostral trigeminal ganglion (Fig. 2C, double arrow). In *Hoxa1*^{-/-}*b1*^{-/-} embryos, the *Phox2b*⁺*Isl1*⁺ geniculate ganglion also delaminates from the ba2 cleft and aggregates as a sphere directed towards the hindbrain (Fig. 2D, asterisk). However, the separation between the geniculate and trigeminal ganglia is missing. Instead, these ganglia are fused, with the geniculate ganglion directed towards r2, the entry point of the central processes of the trigeminal ganglion. Furthermore, we verified that the migratory path of the *Phox2b*⁺ geniculate precursors, from the ba2 cleft to r4, is completely circumscribed by *Sox10*⁺ NC cells in control embryos (Gavalas et al., 1998; Begbie and Graham, 2001; Takano-Maruyama et al., 2010). In contrast, the inward migratory path of the *Phox2b*⁺ geniculate precursors in *Hoxa1*^{-/-}*b1*^{-/-} embryos were associated with *Sox10*⁺ NC cells that stream from r2 to the ba2 cleft (Fig. 2F, asterisk). These findings suggest that in the absence of r3 and r4, the inward migration of the geniculate ganglion appears to be redirected towards r2 by a more rostral r2 NC population normally associated with the trigeminal ganglion.

Differentiation of postganglionic neurons from an A/P mismatch of neural crest and placodal cells

The differentiation of PG neurons and inward migration of the geniculate ganglion have been shown to depend on the reciprocal interaction between r4-derived NC and ba2 placode-derived geniculate ganglion, respectively (Begbie and Graham, 2001; Barlow, 2002; Coppola et al., 2010; Takano-Maruyama et al., 2010). Since the inward migration of the geniculate ganglion occurs in the presence of the more rostral r2 NC population in *Hoxa1*^{-/-}*b1*^{-/-} embryos, we reasoned that by reciprocity the r2 NC engaged with the geniculate ganglion could differentiate into PG neurons. To explore this possibility, we performed immunolabeling for *Sox10* and *Phox2b* to identify whether r2 NC cells differentiate into PG precursors within the geniculate ganglion (Takano-Maruyama et al., 2010). In control embryos, the *Phox2b*⁺ geniculate ganglion is clearly demarcated from the more ventral *Sox10*⁺*Phox2b*⁺ postotic NC-derived otic ganglion (Fig. 3A, C, E; the orientation of the panels show the otic ganglion to the right of the geniculate ganglion). In *Hoxa1*^{-/-}*b1*^{-/-} embryos, we observed a population of *Sox10*^{High}*Phox2b*^{Low} precursors that

appeared to stream from the geniculate ganglion into the area normally occupied by the otic ganglion (Fig. 3B, D, F). This expression profile of Sox10 and Phox2b is indicative of early differentiating r4 NC-derived PG precursors of the sphenopalatine and submandibular ganglia as they migrate and emerge from the geniculate ganglion (Takano-Maruyama et al., 2010). However, the position of the PG precursors and the abnormal morphology of branchial arches in *Hoxa1*^{-/-}*b1*^{-/-} embryos make it unclear whether these precursors contribute to the rostral sphenopalatine ganglion or the more ventral otic ganglion.

To clarify this issue, we analyzed transverse sections of e11.5 embryos to better visualize the relationship between precursors of the sphenopalatine ganglion that emerge from the geniculate ganglion, and the more ventrally situated otic ganglion. In control embryos, the precursors of the sphenopalatine ganglion emerge from the ventromedial side of the geniculate ganglion and migrate rostrally alongside the sphenopalatine artery (Fig. 3G, I, K; see arrow in I). The more differentiated otic ganglion, as defined by the combination of Sox10^{High}Phox2b^{High} expression, is located directly ventral to the geniculate ganglion. This relationship of geniculate, sphenopalatine, and otic precursors in the transverse plane is also observed in *Hoxa1*^{-/-}*b1*^{-/-} embryos (Fig. 3H, J, L). A sagittal section through the geniculate ganglion would therefore capture the otic ganglion in the same plane, but miss the more medially located precursors of the sphenopalatine ganglion. Based on this anatomical criterion, the aforementioned stream of Sox10^{High}Phox2b^{Low} precursors in *Hoxa1*^{-/-}*b1*^{-/-} embryos likely contribute to the otic ganglion (Fig. 3B, D, F). Altogether, these findings indicate that the more rostral r2 NC cells engaged with the geniculate ganglion have the capacity to differentiate into PG precursors of the sphenopalatine and otic ganglia, and likely the submandibular ganglion.

Multi-rhombomeric origin of preganglionic precursors of the otic ganglion

Our finding that r2 NC cells in *Hoxa1*^{-/-}*b1*^{-/-} embryos migrate through the geniculate ganglion to give rise to the sphenopalatine, submandibular, and otic ganglia reflects the plasticity of the cranial NC. Previous genetic lineage labeling of *Hoxb1* demonstrated that precursors of the sphenopalatine and submandibular ganglia originated from r4 (Yang et al., 2008; Takano-Maruyama et al., 2010), whereas the otic ganglion, as revealed by a chick/quail transplantation study, showed that its precursors originated from postotic levels of the hindbrain (posterior to r4) (D'Amico-Martel and Noden, 1983). The emergence of PG precursors of the otic ganglion from the geniculate ganglion suggests that this may be a consequence of the reorganization of the hindbrain and craniofacial area in *Hoxa1*^{-/-}*b1*^{-/-} embryos (Rossel and Capecchi, 1999), or a possible normal contribution from an unappreciated preotic source (i.e., r4).

To address this, we performed genetic lineage analyses with the *Hoxa3-Cre* and *Hoxb1-Cre* drivers coupled with the *ROSA-YFP* reporter to label NC cells from r5 (postotic) and r4 (preotic and postotic) to the caudal hindbrain, respectively (Srinivas et al., 2001; Arenkiel et al., 2003; Macatee et al., 2003). We confirmed that PG neurons of the sphenopalatine and submandibular ganglia originate from r4-derived NC cells in e12.5 *Hoxa3*^{Cre/+};*ROSA*^{YFP/+} and *Hoxb1*^{Cre/+};*ROSA*^{YFP/+} embryos (Fig. 4A–D) (D'Amico-Martel and Noden, 1983; Arenkiel et al., 2003; Yang et al., 2008; Takano-Maruyama et al., 2010). In *Hoxa3* lineage-labeled embryos, which label postotic NC cells from r5 to the caudal hindbrain, no GFP-labeling was observed among Sox10⁺Phox2b⁺ PG neurons of the sphenopalatine and submandibular ganglia (Fig. 4A, C). In *Hoxb1* lineage-labeled embryos, which label r4 and postotic NC cells, GFP labeled all Sox10⁺Phox2b⁺ PG neurons of the sphenopalatine and submandibular ganglia. These findings suggest that all PG neurons of the sphenopalatine and submandibular ganglia originate from r4 NC. The NC origin of PG neurons of the otic ganglion revealed a more complex picture. In *Hoxa3* lineage-labeled embryos, the majority of PG neurons in the otic ganglion were triple-labeled with GFP, Sox10 and Phox2b (Fig.

4E). However, we identified a small population of Sox10⁺Phox2b⁺ devoid of GFP expression. The absence of GFP expression in these cells may be due to the efficiency of the Cre recombinase, or the possibility that they arise from a preotic source. To address this, we analyzed the otic ganglion in *Hoxb1* lineage-labeled embryos. In these embryos, we observed a greater number of triple-labeled cells in the otic ganglion compared to *Hoxa3* lineage-labeled embryos (Fig. 4F). This suggests that the small population of PG neurons that were unlabeled with GFP in *Hoxa3* lineage-labeled embryos may originate from r4.

Since the differentiation of r4 NC-derived PG precursors of the submandibular and sphenopalatine ganglia has been shown to depend on their interaction with the geniculate ganglion (Coppola et al., 2010; Takano-Maruyama et al., 2010), we reasoned that the population of r4 NC cells that contribute to the otic ganglion could also depend on the interaction with the geniculate ganglion. To address this, we generated *Neurog2*^{-/-};*Hoxa3*^{Cre/+};*ROSA*^{YFP/+} embryos to monitor the fate of lineage-labeled postotic NC cells in the absence of the geniculate ganglion. In e12.5 control sagittal sections labeled for Phox2b, Sox10, and GFP, we observed both triple-labeled Phox2b⁺Sox10⁺GFP⁺ and double-labeled Phox2b⁺Sox10⁺ precursors in the otic ganglion (Fig. 4G). In contrast, only triple-labeled Phox2b⁺Sox10⁺GFP⁺ precursors were observed in the otic ganglion of *Neurog2*^{-/-};*Hoxa3*^{Cre/+};*ROSA*^{YFP/+} embryos (Fig. 4H). The absence of double-labeled Phox2b⁺Sox10⁺ precursors suggests that the r4 NC-derived population that contributes to the otic ganglion may be dependent on the geniculate ganglion for differentiation. However, the 5-fold reduction of PG neurons in the otic ganglion of *Neurog2* mutant embryos cannot be simply due to the loss of the r4 NC population (Fig. 4I). This dramatic reduction might be due to a dependence of postotic NC-derived precursors on the placode-derived petrosal ganglion, which is also missing in *Neurog2* mutant embryos (Fode et al., 1998). These findings suggest that a small r4 NC population contributes to the otic ganglion, and imply that the ectopic differentiation of PG neurons in the otic ganglion of *Hoxa1*^{-/-}*b1*^{-/-} embryos is mediated through the interaction of r2-derived NC cells with the geniculate ganglion. Furthermore, since *Hoxa1*^{-/-}*b1*^{-/-} embryos were reported to be deficient in postotic NC cells (Gavalas et al., 1998), it is unlikely that the postotic hindbrain provides the source of PG neurons in the otic ganglion.

DISCUSSION

In this study we provide genetic evidence that the alignment and interaction of r4-derived NC and ba2-derived placodal cells along the A/P axis is not absolutely required for the differentiation of PG neurons of the submandibular and sphenopalatine ganglia and inward migration of the geniculate ganglion, respectively. In the absence of r4 NC cells in *Hoxa1*^{-/-}*b1*^{-/-} embryos, the r2 NC compensates, showing that it has the capacity to migrate towards and differentiate within the geniculate ganglion. The ability of the r2 NC to differentiate into PG neurons is consistent with the idea that the cranial NC is not prepatterned, but is dependent on environmental signals (Fontaine-Perus et al., 1988; Kirby, 1988; Saldivar et al., 1996; Trainor et al., 2002; Le Douarin et al., 2004; Bogni et al., 2008). However, it remains to be determined whether this phenomenon is unique to NC cells derived from even-numbered rhombomeres, or whether NC cells from other A/P axial levels have the potential to be induced into PG neurons by the placode-derived visceral sensory ganglion. This idea also puts into question whether other placode- or NC-derived ganglion related to other sensory modalities can induce NC cells to differentiate into PG neurons. Our data suggest that the epibranchial placode-derived visceral sensory ganglion may be unique in this process. For example, although the placode-derived vestibulocochlear (balance and hearing) and placode/NC-derived trigeminal (somatosensory) ganglia are intact in *Neurog2*^{-/-} embryos, these ganglia fail to provide the compensatory environment to attract or induce the differentiation of PG neuronal precursors (Coppola et al., 2010; Takano-

Maruyama et al., 2010). This suggests that the geniculate ganglion has molecular features – perhaps shared by other epibranchial placode-derived visceral sensory ganglia – that are critical for the attraction, differentiation, and/or survival of the NC fated for the PG neuronal lineage (Pattyn et al., 1999; Enomoto et al., 2000; Dauger et al., 2003; Bogni et al., 2008; Shiau et al., 2008; Coppola et al., 2010). This interaction may be specific for the parasympathetic PG neuronal lineage, as NC cells derived from the trunk spinal cord differentiate into sympathetic PG neurons in the absence of the dorsal root ganglia in *Neurog1*^{-/-} embryos (Ma et al., 1999). How about PG neurons of the sacral division of the parasympathetic nervous system? Are NC cells from this caudal level of the neural tube dependent on a placode-like cellular environment to differentiate into PG neurons? A detailed analysis of the cellular environment encountered by the migratory NC population at this level of the A/P level axis could provide insight into this question, and possibly to a broader mechanism that regulates the specification of both cranial and sacral parasympathetic PG neurons. At this point, our findings indicate that the interaction between the NC and the epibranchial placode-derived sensory visceral ganglion may be unique to the differentiation of cranial parasympathetic PG neurons.

Our study also reveals an unanticipated plasticity of the placode-derived geniculate ganglion – the ability to associate with an A/P mismatched NC population and migrate inwards to the hindbrain. Previous studies have shown that surgical removal of r4 in the chick embryo resulted in failure of the geniculate ganglion to migrate inwards to the hindbrain, indicating an essential role for r4-derived NC cells in this migratory process (Begbie et al., 1999; Begbie and Graham, 2001). These findings imply that neighboring NC cells that are intact in the chick embryos, presumably those derived from r2, are unable to compensate for the loss of r4. In contrast, we observed that in r4 NC-deficient *Hoxa1*^{-/-}*Hoxb1*^{-/-} embryos (Gavalas et al., 1998; Studer et al., 1998; Rossel and Capecchi, 1999; Gavalas et al., 2001), r2 NC cells compensate by associating with the inward migration of the geniculate ganglion, but whether this association is functional remains to be tested. Nevertheless, the discrepancy between the reported studies (Begbie et al., 1999; Begbie and Graham, 2001) and our findings may reflect how the hindbrain and surrounding tissues respond to surgical versus genetic manipulation. The inability of r2-derived NC cells to compensate for the surgical loss of r4 may depend on a time window in which r2 NC cells and the geniculate ganglion are able to respond to one another, or simply to the severity of the surgical ablation along the A/P axis (Begbie et al., 1999; Begbie and Graham, 2001; Trainor et al., 2002). With the loss of *Hoxa1* and *Hoxb1* in the germline, it is possible that the plasticity that we observed for the geniculate ganglion is a consequence of an early coordinated reorganization of the hindbrain and surrounding branchial arch tissues (Rossel and Capecchi, 1999). Alternatively, the loss of r3 and the boundary between the trigeminal and geniculate ganglia in *Hoxa1*^{-/-}*Hoxb1*^{-/-} embryos (Fig. 2C–F), which may be a manifestation of this reorganization, eliminates a non-permissive migratory boundary (Lumsden and Guthrie, 1991). This in turn may allow r2-derived NC cells to engage the geniculate ganglion, thereby facilitating its inward migration. Short of resolving this issue, future work will focus on identifying the time window and the molecular requirements that control the full repertoire of cell types that can differentiate from the even-numbered rhombomere NC and epibranchial placode-derived cell interaction (Fontaine-Perus et al., 1988; Trainor et al., 2002; Le Douarin et al., 2004).

EXPERIMENTAL PROCEDURES

Mouse Lines

The *Hoxa1*, *Hoxb1*, and *Neurog2* mutant mice, and *Hoxb1-Ires-Cre*, *Hoxa3-Ires-Cre*, and *ROSA-YFP* reporter mice have been reported (Ma et al., 1999; Rossel and Capecchi, 1999; Srinivas et al., 2001; Arenkiel et al., 2003; Macatee et al., 2003).

Immunohistochemistry and Antibody Generation

Embryos were dissected in cold phosphate-buffered saline (PBS) and fixed in cold 4% paraformaldehyde (Sigma, St. Louis, MO) for 30 min at 4°C and cryoprotected in 30% sucrose at 4°C, followed by embedding in OCT compound (Tissue-Tek). The cryostat sections were blocked in PBS-T (phosphate-buffered saline with 0.02% TritonX-100) with 5% skimmed milk and 5% normal goat sera for 5 min at room temperature, followed by incubation with the appropriate dilution of primary antibody in PBS-T containing 2% skimmed milk and 5% goat sera overnight at 4°C. The following primary antibodies used for this study were mouse anti-Isl1/2 (Developmental Studies Hybridoma Bank, Iowa, IA), mouse anti-Sox10 (a gift from D. Anderson), and sheep anti-GFP (Invitrogen). The rabbit polyclonal antibody was generated against a 15-amino acid mouse Phox2b peptide sequence conjugated to cysteine, CPNGAKAALVKSSMF (Pattyn et al., 1997). The synthesis and purification of the Phox2b peptide, and subsequent immunization and bleeding of rabbits were conducted at the Covance facilities. For visualization of antibody binding, the sections were incubated with Alexa 594 conjugated goat anti-rabbit (Fab)₂ fragment (Invitrogen), Alexa 647 conjugated goat anti-mouse (Fab)₂ fragment (Invitrogen) and Alexa 488 conjugated goat anti-mouse (Fab)₂ (Invitrogen) for 45min at 4°C. Detection of apoptotic cells was performed with In situ cell death detection kit (Roche Applied Science, Indianapolis, IN). The sections were incubated with TdT and secondary antibodies for 1hr at 37°C. The slides were examined with a Zeiss LSM 5 PASCAL confocal microscope. Cell counting was performed using Imaris software (Bitplane AG, Zurich, Switzerland) in combination with manual verification of the automated results.

Statistical Analysis

Results are expressed as means $\pm$ SD. Statistical analysis of differences between two groups was performed using the unpaired Student's t-test (two-tailed analysis). Differences with P values of <0.05 were considered statistically significant.

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Fig. 1. Differentiation of postganglionic neurons in *Hoxa1*^{-/-}*b1*^{-/-} mutant embryos in the absence of a normal rhombomere source
 (A, D, G) Statistical analysis of Phox2b-expressing postganglionic (PG) neurons in the submandibular, sphenopalatine, and otic ganglia in e12.5 control (green bar) and *Hoxa1*^{-/-}*b1*^{-/-} mutant (red bar, n=4) embryos. The values for *Hoxa1*^{-/-}*b1*^{-/-} mutant embryos are expressed as a percentage relative to control embryos, which was set at 100%. The submandibular (23.6% ± 13.4 S.D, p<0.001), sphenopalatine (30.0% ± 14.1 S.D, p<0.002), and otic (3.8% ± 1.0 S.D, p<5×10⁻⁷), ganglia of *Hoxa1*^{-/-}*b1*^{-/-} mutant embryos (n=4) were significantly reduced compared to control embryos (n=4). The majority of PG neurons in the submandibular, sphenopalatine, and otic ganglia of e12.5 *Hoxa1*^{-/-}*b1*^{-/-}

mutant embryos expressed Phox2b at low levels, indicative of early differentiating PG precursors (Takano-Maruyama et al., 2010).

(B, C, E, F, H, I) Sagittal sections of e13.5 control (n=5) and *Hoxa1*^{-/-}*b1*^{-/-} mutant (n=5) embryos immunolabeled for Phox2b. The control embryo shows that the majority of PG neurons in the submandibular, sphenopalatine, and otic ganglia maintain express high levels of Phox2b. In the *Hoxa1*^{-/-}*b1*^{-/-} mutant embryo, the majority of PG neurons express low levels of Phox2b, suggesting that PG neurons are at an early stage of differentiation.

Abbreviations: Gg, geniculate ganglion; Og, otic ganglion.

Scale Bar: Panels B, C, E, F, H, I – 50 μ m

Fig. 2. Positional mismatch of cranial neural crest and epibranchial placode is conducive to the inward migration of the geniculate ganglion

(A, B) Whole-mount e10.5 control (n=3) and *Hoxa1*^{-/-}*b1*^{-/-} mutant (n=3) embryos immunolabeled for Isl (green) and Phox2b (red). The juxtaposed Isl1⁺Phox2b⁺ geniculate and Isl1⁺ vestibulocochlear ganglia are separated from the more rostral Isl1⁺ trigeminal ganglion in the control embryo, whereas these ganglia are fused in the *Hoxa1*^{-/-}*b1*^{-/-} mutant embryo.

(C, D) Sagittal section through e9.5 control (n=9) and *Hoxa1*^{-/-}*b1*^{-/-} mutant (n=9) embryos immunolabeled for Isl1 (green) and Phox2b (red). The Isl1⁺Phox2b⁺ geniculate ganglion is separated from the more rostral Isl1⁺ trigeminal ganglion in the control embryo (double

arrow). The area between the geniculate and trigeminal ganglia is missing in the *Hoxa1*^{-/-}*b1*^{-/-} mutant embryo, resulting in the juxtaposition of the geniculate and trigeminal ganglia.

(E, F) Sagittal section through e9.5 control (n=9) and *Hoxa1*^{-/-}*b1*^{-/-} mutant (n=9) embryos immunolabeled for Sox10 (green) and Phox2b (red). In the control embryo, the Phox2b⁺ geniculate ganglion is associated with the stream of Sox10⁺ neural crest cells from r4 to the second branchial cleft (asterisk), the origin of the geniculate ganglion. In the *Hoxa1*^{-/-}*b1*^{-/-} mutant embryo, the Phox2b⁺ geniculate ganglion is directed rostrally, and is associated with the stream of Sox10⁺ neural crest cells from r2 to the second branchial cleft.

Abbreviations: ov, otic vesicle; 5g, trigeminal ganglion; 7g, geniculate ganglion; r, rhombomere.

Scale Bar: Panels A, B – 200 µm; Panels C–F – 50 µm.

Fig. 3. Positional mismatch of cranial neural crest and the geniculate ganglion is conducive to the differentiation of postganglionic neurons

(A–F) Sagittal sections of e12.5 control and *Hoxa1*^{-/-}*b1*^{-/-} embryos immunolabeled for Sox10 and Phox2b.

(G–L) Transverse sections of e11.5 control and *Hoxa1*^{-/-}*b1*^{-/-} embryos immunolabeled for Sox10 and Phox2b.

Boxed areas in panels A, B, G, and H (10x) are magnified in panels C, D, I, J, E, F, K, and L (40x).

Abbreviations: 5g, trigeminal ganglion; 8g, vestibulocochlear ganglion; 7g, geniculate ganglion; Og, otic ganglion; R, rostral; C, caudal; D, dorsal; V, ventral.

Scale Bar: panels A, B, G, H – 100 μ m; panels C, D, E, F, I, J, K, L – 50 μ m.

Fig. 4. Contribution of r4 neural crest to postganglionic neurons of the otic ganglion is dependent on the geniculate ganglion

(A, C, E) Sagittal sections of e12.5 *Hoxa3*^{Cre/+}; *ROSA*^{YFP/+} immunolabeled for Phox2b, Sox10, and GFP. The Phox2b⁺Sox10⁺ postganglionic (PG) neurons in the sphenopalatine and submandibular ganglia are devoid of GFP expression, whereas the otic ganglion is mostly triple-labeled with a small population of Phox2b⁺Sox10⁺ double-positive PG neurons.

(B, D, F) Sagittal sections of e12.5 *Hoxb1*^{Cre/+}; *ROSA*^{YFP/+} immunolabeled for Phox2b, Sox10, and GFP. The Phox2b⁺Sox10⁺ PG neurons in the sphenopalatine and submandibular ganglia express GFP, indicating that they are all derived from r4 NC cells. The otic ganglion has a greater number of triple-labeled PG neurons compared to *Hoxa3*^{Cre/+}; *ROSA*^{YFP/+} embryos, indicating that the double-positive PG neurons in the *Hoxa3*^{Cre/+}; *ROSA*^{YFP/+} embryo arise r4.

(G, H, I) Sagittal sections of e12.5 control (*Neurog2*^{+/+}; *Hoxa3*^{Cre/+}; *ROSA*^{YFP/+}) and *Neurog2*^{-/-} *Hoxa3*^{Cre/+}; *ROSA*^{YFP/+} embryos immunolabeled for Phox2b, Sox10, and GFP. The otic ganglion in the control embryo contains both triple-labeled Phox2b⁺Sox10⁺GFP⁺ and double-labeled Phox2b⁺Sox10⁺ PG neurons. The *Neurog2*^{-/-} embryo contains only triple-labeled Phox2b⁺Sox10⁺GFP⁺ PG neurons. Statistical analysis show an 80.7% reduction in the number of triple-labeled Phox2b⁺Sox10⁺GFP⁺ PG neurons in the otic ganglion of *Neurog2*^{-/-} embryos compared to control embryos (1146 ± 98 S.D. vs. 221 ± 166 S.D. neurons/ganglion, *p* < 0.001; *n* = 3).