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Onecut factors control development of the Locus Coeruleus and of the mesencephalic trigeminal nucleus

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ABSTRACT

The Locus Coeruleus (LC), the main noradrenergic nucleus in the vertebrate CNS, contributes to the regulation of several processes including arousal, sleep, adaptative behaviors and stress. Regulators controlling the formation of the LC have been identified but factors involved in its maintenance remain unknown. Here, we show that members of the Onecut (OC) family of transcription factors, namely HNF-6, OC-2 and OC-3, are required for maintenance of the LC phenotype. Indeed, in embryos lacking any OC proteins, LC neurons properly differentiate but abnormally migrate and eventually lose their noradrenergic characteristics. Surprisingly, the expression of *Oc* genes in these neurons is restricted to the earliest differentiation stages, suggesting that OC factors may regulate maintenance of the LC in a non cell-autonomous manner. Accordingly, the OC factors are present throughout development in a population directly adjacent to the LC, the rhombencephalic portion of the mesencephalic trigeminal nucleus (MTN). In the absence of OC factors, rhombencephalic MTN neurons fail to be generated, suggesting that OC proteins cell-autonomously control their production. Hence, we propose that OC factors are required at early developmental stages for differentiation of the MTN neurons that are in turn necessary for maintenance of the LC.

Introduction

The Locus Coeruleus (LC) or A6 nucleus, located in the dorsal part of the pons, constitutes the main noradrenergic nucleus in the vertebrate CNS (Goridis and Rohrer, 2002). Via widespread projections throughout the encephalon, it contributes to the regulation of arousal, attention, sleep, awakeness and adaptative behaviors (Aston-Jones and Cohen, 2005; Bouret and Sara, 2005; Grzanna and Fritschy, 1991). It is also involved in controlling stress and anxiety, and has been implicated in anxiety disorders, depression and vulnerability to opiate abuse (Itoi and Sugimoto, 2010; Van Bockstaele et al., 2010).

LC neurons arise from the alar plate of the first rhombomere (r1), the rostral-most transient segmental division of the hindbrain visible around embryonic day (e)8.5 in rodent embryos. They are generated from e9.5 onward all along the rostrocaudal axis of r1. After initial radial migration toward the pial surface, they migrate ventrally and caudally to eventually settle in the lateral basal plate and form a compact population adjacent to the fourth ventricle around e14.5 (Aroca et al., 2006). The genetic cascade that regulates LC neuron differentiation has been extensively deciphered (Brunet and Pattyn, 2002). Like all noradrenergic neurons, the LC neurons express the transcriptional activators *Phox2a* and *Phox2b*, two paralogous homeodomain proteins of the Q50 paired-like class (Tiveron et al., 1996). Both factors are essential for proper differentiation of LC neurons. Phox2a is required for *Phox2b* activation that, in turn, is necessary to induce the expression of *Dopamine-β-Hydroxylase* (*Dbh*), the key enzyme of noradrenalin synthesis (Morin et al., 1997; Pattyn et al., 2000). Early transient expression of *Tlx3/Rnx* is required for *Dbh* expression in the caudal part of the LC (Qian et al., 2001), while *IA-1/Insm-1* is necessary for timely onset of the expression of *tyrosine hydroxylase* (*Th*), another enzyme involved in noradrenalin synthesis (Jacob et al., 2009). Additional factors control some aspects of the migration and survival of the LC neurons. Netrin1, produced in the floor plate, is required for survival and proper tangential migration of LC cells (Shi et al., 2008). Furthermore, *in vitro* studies suggest that TrkB ligands are also necessary for survival of LC cells and, together with Corticotropin-Releasing Factor, for acquisition of their noradrenergic phenotype (Holm et al., 2003; Traver et al., 2006).

Throughout development and during adulthood (Allen Brain Atlas http://mouse.brain-map.org), the rhombencephalic part of the mesencephalic trigeminal nucleus (MTN) lies in close vicinity to the LC. The MTN contains the cell bodies of trigeminal primary proprioceptors and is the sole sensory nucleus located inside the CNS (Lazarov, 2002, 2007). MTN neurons constitute a central component in the integration of the reflex control of oral and facial movements. The MTN cells are the earliest detectable postmitotic neurons in the CNS, born in the mesencephalon from e8.5 to e12.5 (Easter et al., 1993; Stainier and Gilbert, 1990) at a slow but steady rate. It is proposed that migration

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of a part of these neurons in the rhombencephalon leads to formation of the rhombencephalic portion of the MTN (Lazarov, 2002). MTN neurons express a specific combination of markers that includes the LIM homeodomain protein *Isl1* (Hunter et al., 2001), the intermediate filament *Peripherin* (Wang et al., 2007), the POU homeodomain protein *Brn3a* (Fedtsova and Turner, 1995) and the paired homeodomain protein *Drg11/Prrxl1* (Rebelo et al., 2007; Wang et al., 2007). Some of these factors have been shown to be necessary for MTN development. In *Brn3a* −/− mouse, no MTN neurons are detected at e18.5 (Ichikawa et al., 2005). In the absence of Drg11, MTN cells are normally generated but neuronal death during embryonic development leads to loss of the nucleus at birth (Wang et al., 2007).

In order to further unveil mechanisms that control the development of neuronal populations such as the LC and the MTN, description of additional markers and study of their roles during embryonic development are needed. To address this issue, we focused on a family of transcriptional activators called Onecut factors (OC) that, in mammals, contains three members, namely HNF-6 (also called OC-1), OC-2 and OC-3 (Jacquemin et al., 1999; Lannoy et al., 1998; Vanhorenbeeck et al., 2002). OC proteins are characterized by a bipartite DNA-binding domain constituted by a single Cut domain and a divergent homeodomain (Burglin and Cassata, 2002; Lannoy et al., 1998; Vanhorenbeeck et al., 2002). They have first been described during embryonic development in endoderm derivatives such as liver, pancreas, stomach and duodenum (Jacquemin et al., 2003; Landry et al., 1997; Vanhorenbeeck et al., 2002). Phenotypic analyzes of mouse embryos carrying single or compound inactivation of *Hnf6* and *Oc2* unveiled critical roles of these proteins in hepatic and pancreatic differentiation and morphogenesis (Clotman et al., 2002, 2005; Jacquemin et al., 2000; Margagliotti et al., 2007; Pierreux et al., 2006; Vanhorenbeeck et al., 2007). OC factors have also been found in the developing CNS of several species wherein they participate in neural development (Nguyen et al., 2000; Otim et al., 2004; Sasakura and Makabe, 2001). In mammals, they have been detected both in the PNS and in the CNS. In the PNS, HNF-6 and OC-2 are present in the trigeminal ganglion wherein OC-2 contributes to proper central projection of the sensory neurons (Hodge et al., 2007). In the CNS, the expression of the OC factors has been reported in the spinal cord where they are dynamically and differentially expressed in motor neurons and in subsets of ventral interneurons (Francius and Clotman, 2010; Stam et al., 2012), in different cell types in retina (Wu et al., 2012) and in the dopaminergic A13 nucleus. In the absence of OC factors, A13 neurons are normally generated but fail to form a cohesive nucleus and to eventually maintain their dopaminergic phenotype (Espana and Clotman, 2012). In these mutants, we also noticed a loss of MTN and of LC neurons in late-stage embryos. We therefore studied the distribution of the OC factors in both populations during embryonic development and we investigated their roles in the formation of these two nuclei.

Results

OC factors are transiently expressed in the LC but are continuously expressed in the MTN throughout embryonic development

To establish whether OC factors are expressed in the LC during embryonic development, sections of wildtype embryos were colabeled at different developmental stages for OC factors and for Phox2a, the generic marker of LC/noradrenergic neurons. At e9.5, LC cells have just started to be generated and only few differentiating neurons could be observed in r1, as evidenced by restricted expression of Phox2a in the rostral part of this rhombomere (Fig. 1A–C). At this very early stage, HNF-6 was present in all the LC neurons (magenta cells, Fig. 1A), whereas OC-2 or OC-3 were not detected (Fig. 1B, C). At e10.5, additional LC neurons had been generated. A majority of LC cells still expressed *Hnf6*, although at lower levels (Fig. 1.D, white arrowhead in inset), but Phox2a positive cells devoid of HNF-6 were observed (Fig. 1D, white arrow in inset). Only a small subset of LC cells expressed low levels of *Oc2* (Fig. 1E, arrowhead in inset), whereas OC-3 remained undetectable (Fig. 1F). At later stages, OC factors were not detected in LC neurons (Fig. 1G–L). Hence, *Hnf6* and, to a lesser extent, *Oc2* were transiently expressed during the early stages of LC neuron differentiation.

At variance, OC proteins were continuously detected in cells directly adjacent to the LC neurons (Fig. 1A–L). We hypothesized that these cells corresponded to the sensory neurons of the rhombencephalic part of the MTN and we addressed this question by colabelings for OC factors and Isl1. At e9.5, all the early MTN neurons contained the three OC proteins (cyan cells in Fig. 1A–C). From e10.5 onward, *Oc2* and *Oc3* were expressed in a majority of the MTN neurons (Fig. 1, E–F, H–I, K–L) whereas HNF-6 was detected in a subset of these cells (Fig. 1. E, yellow arrowhead in inset; Fig. 1G, J). These observations were consistent with colabelings for the three OC factors (Fig. 1M–P). At e9.5, *Hnf6* expression was broader than that of *Oc2* and *Oc3*, OC-2 and OC-3 being exclusively detected in cells containing HNF-6 (Fig. 1M). At later stages, the OC factors were distributed in a partially overlapping pattern (Fig. 1N–P). From e10.5 onward, the expression pattern of the three *Oc* factors was partially overlapping, including cells expressing one (red, green or blue), two (cyan, magenta or yellow) or the three OC factors (white cells). Noticeably, at each stage of development, at least one OC factor was expressed in each MTN cell, although at variable levels (Fig. S1). In addition, the mesencephalic portion of the MTN also expressed OC factors (data not shown). Hence, the OC factors were transiently expressed in early LC neurons whereas they were continuously present in the MTN cells.

OC factors are dispensable for generation of the LC neurons but are required for maintenance of the LC

To assess potential role of the OC factor during LC development, we compared the distribution of LC markers including Phox2a, Phox2b, *Dbh* and TH at different developmental stages in control and in *Hnf6/Oc2* −/− embryos. Immunofluorescence analyzes on sections of control embryos at e10.5 showed that the LC cells characterized by the expression of *Phox2a* (Fig. 2A), *Phox2b* (Fig. 2J), *Th* (data not shown) and *Dbh* (Fig. 2P) have started to differentiate and were located beneath the pial surface in the rostral portion of r1. At later stages, LC neurons migrated caudally and ventrally toward their eventual location, adjacent to the fourth ventricle, where they formed a compact nucleus (Fig. 2B–D, Q–S; Fig. S2A). Noticeably, at e12.5, Phox2b was detected in LC cells and in the Phox2b positive/Phox2a negative lateral ventral band in r1 (lvb-r1) (Fig. 2L), as previously described (Aroca et al., 2006). In the absence of OC factors at e10.5, LC neurons expressing *Phox2a*, *Phox2b* and *Dbh* were detected in normal amount in the rostral portion of r1 (Fig. 2E, I, M, T). However, observations at later stages showed a sequential loss of the LC markers. First, Phox2a positive cells seemed less numerous at e11.5 (Fig. 2F) and very scarce or absent at later stages (Fig. 2G, H). Accordingly, quantifications of the number of Phox2a positive cells in r1 indicated a significant depletion of Phox2a positive neurons at e11.5 (84%) and e12.5 (97%) (Fig. 2I). Whereas the amount of LC neurons progressively increased in control embryos, the number of Phox2a positive cells significantly decreased at e11.5 and e12.5 in the absence of OC factors. At e11.5, Phox2b labeling was comparable in mutant and in control embryos (Fig. 2K, N) but one day later, the amount of Phox2b positive cells corresponding to LC neurons was reduced in *Hnf6/Oc2* −/− embryos (Fig. 2L, O). Similarly, progressive loss of *Dbh* positive cells was observed at e11.5 and e12.5 (Fig. 2U, V), leading to complete absence of *Dbh* expression at e14.5 (Fig. 2W). These observations suggested that LC neurons were progressively lost in the absence of OC factors.

To evaluate whether the loss of LC markers in r1 may be due to abnormal localization of the LC cells, TH distribution was also assessed by whole mount immunofluorescence labeling and 3D-reconstructions (Espana and Clotman, 2012). These analyses confirmed that LC cells