Emerging roles for HOX proteins in synaptogenesis

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ABSTRACT Neural circuit formation requires the intricate orchestration of multiple developmental events including cell fate specification, cell migration, axon guidance, dendritic growth, synaptic target selection, and synaptogenesis. The HOX proteins are well-known transcriptional regulators that control embryonic development. Investigations into their action in the vertebrate central nervous system have demonstrated pivotal roles in specifying neural subpopulations, but also in several successive steps required for the assembly of neuronal circuitry, such as neuron migration, axon growth and pathfinding and synaptic target selection. Several lines of evidence suggest that the HOX transcription factors could also regulate synaptogenesis processes even after the process of axonal and dendritic guidance has concluded. Here we will review the current data on HOX proteins in neural circuit formation in order to evaluate their potential roles in establishing neuronal connectivity with specific emphasis on synapse formation and maturation.

KEY WORDS: transcription factor, neuronal connectivity, synapse assembly, synaptic refinement, synapse pathology

Introduction

The functions of the vertebrate nervous system are mediated by a complex and vast array of neural circuits that are formed during development and modulated by experiences. The assembly of these circuits during development relies on key sequential steps that include establishment of ordered patterns of neural specification; postmitotic neuron migration; axon growth, pathfinding and target selection; formation of synaptic connections; and synapse maturation (Colon-Ramos, 2009, Lu et al., 2009). Recent evidences suggest that the HOX transcription factors function at multiple steps during neural circuit formation in the brainstem and spinal cord, to shape and fine-tune circuits involved in vital behaviors, such as respiration, locomotion, motor coordination and several sensory modalities such as audition, nociception and proprioception (Di Bonito et al., 2013a, Narita and Rijli, 2009, Philippidou and Dasen, 2013).

The HOX family of transcription factors gathers key regulators of embryo patterning, organ development, and cell differentiation during animal development, but also throughout adult life (Mallo et al., 2010, Rezsohazy et al., 2015). In mammals, 39 Hox genes have been identified that are clustered in four genomic loci, the HoxA, HoxB, HoxC and HoxD complexes, and can be subdivided into 13 paralogy groups (PG) according to their sequence similarities and relative position along the clusters (Deschamps, 2007).

In their patterning function, Hox genes are expressed in a nested fashion following their order on the four chromosomal clusters. Their expression thus obeys a temporal and spatial colinearity rule according to which the genes located at the 3’ side of a complex are expressed earlier and more rostrally than those residing at the 5’ extremity of the complex (Kmita and Duboule, 2003). In early mammalian embryos the hindbrain (or rhombencephalon), which constitutes the main part of the brainstem, is organized along the 5'–3' axis (Deschamps et al., 2013a).

Abbreviations used in this paper: AVCN, anterior ventral cochlear nucleus; ASD, autism spectrum disorders; cKO, conditional knock-out; dPrV, dorsal principal trigeminal nucleus; E, embryonic day; MN, motoneuron; P, postnatal day; PG, paralogy groups; PMC, phrenic motor column; r, rhombomere; VPM, ventral posteromedial nucleus of the thalamus; vPrV, ventral principal trigeminal nucleus.

Glossary: Synaptogenesis: developmental process involving synapse formation, synapse stabilization and activity-dependent synapse refinement and elimination; Synapse formation: process that includes terminal branching of axons, development of dendrites, assembly of pre- and post-synaptic elements; Synapse maturation: activity-dependent synapse refinement through selective synapse strengthening or elimination (pruning); Synaptic plasticity: in the adult brain, refers to the property of synapses to strengthen or weaken in response to different patterns of neuronal activity; Topographic connectivity: specific feature of connection pattern in which at all levels of the pathway, the spatial arrangement of neurons and their afferent fibers provides a faithful representation of the physical distribution of source neurons.

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antero-posterior axis into 8 segments called rhombomeres, all of which having a unique profile of PG1-PG4 Hox genes expression, at the exception of rhombomere 1 (r1) where no Hox gene expression has been reported during embryogenesis (Tumpel et al., 2009). Later in central nervous system development, the caudalmost part of the rhombencephalon, that shows no visible segmentation, reveals a hidden metameric organization, comprising five pseudo-(or crypto-) rhombomeres (r7-r11) that also present a unique combination of Hox gene expression, including PG5-PG9 (Hutlet et al., 2016, Tomas-Roca et al., 2016). Caudally to the hindbrain, the expression of Hox genes within the spinal cord is also closely aligned with their position within the Hox cluster: PG4-8 Hox genes are expressed at brachial levels, PG8-9 Hox genes at thoracic and PG10-13 Hox genes at lumbar levels of the spinal cord (Dasen and Jessell, 2009, Dasen et al., 2005). The unique profile of Hox gene expression within each segment of the hindbrain and spinal cord appears central to several aspects of their functions in the nervous system. While their combinatorial expression is often required to specify neuronal progenitor domains, single Hox genes may be essential for acquisition of regional identity, as illustrated for Hoxa2, being the only Hox gene expressed in r2; or Hoxc9, which is sufficient to determine neuronal identity in the thoracic region (Jung et al., 2010).

During neurulation, combinatorial expression of Hox genes provides a segmental identity and antero-posterior patterning information to neural progenitors (Dasen and Jessell, 2009, Nolte and Krumlauf, 2007). A failure to establish the correct pattern of Hox gene expression at early stages of the nervous system development results in changes in neuronal identity that ultimately lead to defects in axon guidance and circuit formation. In addition to these well-described roles in neural mitotic progenitors, studies in the past decade have revealed Hox gene functions in postmitotic neurons, that include specification of neuronal subtype, stereotypic neuronal migration, axon pathfinding and neuronal circuit connectivity (Bechara et al., 2015, Catela et al., 2016, Di Bonito et al., 2013b, Geisen et al., 2008, Oury et al., 2006, Pasqualetti et al., 2007, Philippidou et al., 2012). The role of Hox genes in neuronal circuitries assembly during development has been investigated so far for neuronal migration and clustering, axon guidance, and axon branching (reviewed in Di Bonito et al., 2013a, Kratochwil et al., 2017, Narita and Rijli, 2009, Philippidou and Dasen, 2013). However, recent evidences, based on conditional mutant characterization and transcriptomic analysis suggest that HOX transcription factors are also required in postmitotic neurons for subsequent aspects of circuit formation, namely synapse formation and maturation which includes refinement and fine-tuned topographic connectivity of somatosensory circuits (Bechara et al., 2015, Karmakar et al., 2017, Lizen et al., 2017b). Within this review, after a brief overview of synaptogenesis and of Hox gene expression at postnatal stages, we will summarize and discuss these recent data, focusing on the mammalian central nervous system. To conclude, we will consider how new data on HOX regulatory networks could contribute to a better understanding of processes underlying synapse pathologies and neurological diseases.

**Synapse formation and maturation**

To function properly, the brain must be correctly wired during crucial periods in development, a process that relies on the establishment of precise connections between neurons and their pre- and postsynaptic targets.

As axons enter into their target field, they undergo extensive remodeling, ultimately leading to presynaptic assembly. Axonal remodeling involved terminal axonal branching, changes in growth cone morphology and its transformation into presynaptic boutons, a process supported by modification of axonal microtubule dynamics, leading to recruitment of molecules required for presynaptic differentiation. In parallel, formation and growth of dendritic spines, structures that primarily receive excitatory inputs, is observed on the postsynaptic dendrites, leading to recruitment of postsynaptic components, such as PSD95 clustering, and postsynaptic assembly (Garner et al., 2006, Lu et al., 2009, McAllister, 2007). For this early stage, postsynaptic and presynaptic neurons use a diverse set of cell surface and secreted proteins to guide and control the initiation of synapse formation (Fig. 1). Among the cell surface proteins involved in these processes, the best described are cell adhesion molecules such as the neurexin-neuroligin pairs, the synaptic cell adhesion molecules (SynCAM) and the cadherins (Basu et al., 2015, Bemben et al., 2015, Williams et al., 2010). Among the secreted molecules involved in synapse formation, the fibroblast growth factor (FGF)-22 is capable of inducing presynaptic differentiation in a variety of neuronal types (Umemori et al., 2004). A number of studies have shown that WNT signaling is also involved both in an anterograde and retrograde manner to support synapse assembly, notably through the action of WNT5A and WNT7A ligands (Dickins and Salinas, 2013, Inestrosa and Arenas, 2010).

The initial stages of synaptic differentiation occur within the first few hours after the establishment of the axo-dendritic contact. However, the development of a nascent synapse into a functional synapse involves the recruitment of hundreds of proteins, morphological changes and establishment of functional electrophysiological properties (Garner et al., 2006, Williams et al., 2010). This includes formation and cycling of synaptic vesicles, expression and exposition of postsynaptic receptors, Neurotransmitter release (Fig. 1) as well as generation of spontaneous and miniature excitatory postsynaptic currents (mEPSCs). As a paradox, the final stage of generation and maturation of neuronal synapses is also associated with a peak in synapse elimination, a process known as synaptic pruning, that has been proposed to be crucial for the maturation and strengthening of remaining synaptic connections (Ricciamagno and Kolodkin, 2015). Indeed, during development, synaptogenesis generates an excess of neuronal synapses, requiring their selective elimination and the maturation of surviving contacts to achieve adult synaptic architecture. This can be illustrated with the transition from multi-innervated to singly-innervated target cells, as observed for muscle fibers at the neuromuscular junction, or at the synapses between climbing fibers and Purkinje cells in the cerebellum (Nenisksyte and Gross, 2017). Studies on synaptic maturation indicate that this process is dependent on both genetics and synaptic activity, the weaker inputs being preferentially eliminated. Secreted and cell surface molecules figure among the emerging molecular players involved in synaptic maturation (Fig. 1). Synaptic maturation can also lead to modification in synaptic transmission through changes in neurotransmitter receptors and is associated to changes in cytoplasmic levels of Ca$^{2+}$, a potent activator of intracellular signaling cascades (Nenisksyte and Gross, 2017). Of note, circuit refinement appears crucial for one peculiar aspect of sensory circuits, their topographic organization, that ensure high-
fidelity relay of sensory inputs to nuclei in the brainstem, thalamus and finally cortical areas (Hirtz et al., 2012). Circuit refinement by the elimination of synapses was reported in two phases: a primary brain circuit refinement observed during the first three postnatal weeks in mice; and a secondary phase described around weeks 3-8 in mice, corresponding to adolescence in mammals (Neniskyte and Gross, 2017). The primary phase is required for the proper formation of sensory circuits that will be mainly discussed here.

In vivo imaging demonstrates that 70-90% of synapses in mature brain are then stable and persist for extended periods of time, perhaps for the lifespan of the animal. Synaptic activity is the key stimulus for maintaining the molecular composition of synapses,
required for axon and dendrite stability and the preservation of neuronal network (Leslie and Nedivi, 2011). Functional plasticity, through synaptic strengthening, weakening and/or pruning in mature synapses, allows the brain to adapt and change with experience. This plasticity mediates long-term structural and electrophysiological adaptations that take place in sensory, motor and somatosensory inputs, as well as in the process of learning and memory.

In summary, the delicate balance between synapse assembly, disassembly and maintenance is a highly regulated process controlled by a complex molecular dialogue between pre- and postsynaptic neurons. While synapse formation and arbor growth can proceed without active-regulated gene products, activity-regulated genes are essential mediators of the selective processes by which activity sculpts optimally functioning circuits. We will discuss herein evidences that HOX proteins are involved in these synaptogenesis processes.

**Hox gene expression at postnatal stages**

Although the expression of Hox genes has been well documented in the early embryonic stages, our knowledge of their expression at the fetal and postnatal stages is much more limited. After neurulation, a few individual reports revealed that early segmental Hox expression patterns were maintained up to late fetal stages and early postnatal stages in restricted neuronal subpopulations in the hindbrain (Di Bonito et al., 2013b, Geisen et al., 2008, Karmakar et al., 2017, Lizen et al., 2017a, Oury et al., 2006, Pasqualetti et al., 2007). In the fetal spinal cord, as in the hindbrain, Hox gene expression resolves into more restricted domains related to neurons groups, in particular subsets of motoneurons (MN) (Dasen et al., 2005). What happens at early postnatal stages and adulthood is even less described. However, studies have reported the presence of Hox gene transcripts in the postnatal and adult brain. Notably, RT-qPCR analysis of HOX expression in human samples revealed that 15 genes belonging to PG1-PG7 were expressed in adult brain extracts (Takahashi et al., 2004). More recently, two systematic analyses of the 39 Hox genes expression profiles have been performed in the hindbrain at perinatal stage and in the whole brain at adult stage (Hutlet et al., 2016, Tomas-Roca et al., 2016). They reveal that the majority of Hox genes remain expressed in the brain after birth and until adulthood. In the hindbrain, expression of PG1-PG8 Hox genes was detected in postmitotic neurons in a pattern correlated with segmental boundaries of rhombomeres and crypto-rhombomeres. Within the hindbrain, Hox gene transcripts were particularly enriched in precerebellar nuclei. These recent data suggest a functional relevance of these genes in the brain beyond their role of patterning genes, possibly related to synaptogenesis, as discussed below.

**Hox gene late functions in neural circuit formation**

Before 2000, it was well-known that Hox expression in the hindbrain regulated regional identity of neural progenitors along the anteroposterior axis, but it was not clear whether the early rhombomeric pattern of Hox expression has any influence on the establishment of the neuronal circuitry of the mature brainstem. The first observation that Hoxa1 loss-of-function led to long-term modification of hindbrain neural networks came in 2001, suggesting that Hox genes may provide a genetic basis for segment-specific modulation of neuronal development and connectivity (del Toro et al., 2001). This hypothesis prompted research on the late (fetal and/or adult) impact of Hox gene inactivation on neuronal circuitry, as illustrated by the study of Arenkiel et al., in 2004, in which they show that Hoxb1 directs the establishment of appropriate connectivity between hindbrain facial MNs and r4-derived neural crest cells (Arenkiel et al., 2004). However, as specific profiles of HOX transcription factors in neuronal progenitors and postmitotic cells define the unique molecular signatures that later orchestrate key aspects of circuit formation (migration, projection pattern, and synaptic specificity of neuronal subtypes), temporal conditional inactivation was required to fully discriminate between early and late functions of Hox genes (Philippidou and Dasen, 2013). To date, the question of multiple temporal roles have mainly been addressed for PG2 and PG5 Hox genes, for which transgenic mice with floxed alleles have been generated (Di Bonito et al., 2013b, Ren et al., 2002, Tabaries et al., 2007). These new genetic models of HOX function have provided valuable insights into their late specific roles in the central nervous system development, notably in the trigeminal and auditory sensory systems in the brainstem, in the pontine precerebellar system, and in the phrenic motor system in the spinal cord (Table 1).

**Lessons from conditional mutants: roles in synapse assembly and maturation**

The first investigation of late function of Hox genes in neural circuit formation by the temporal inducible inactivation using the LoxP/CreER2 system was in 2006 when Oury et al., characterize Hoxa2 multiple functions in the mouse trigeminal pathway (Oury et al., 2006). The trigeminal system is important in relaying somatosensory stimuli including touch, pain, and temperature from the face to the cortex. In rodents, a large portion of this system is devoted to conveying sensory inputs from the spatially-organized whiskers to the somatosensory cortex with high-fidelity. Sensory inputs from whiskers are collected through the maxillary branch of the trigeminal nerve whose cell bodies are localized in the trigeminal ganglion. These inputs topographically project onto the somatosensory cortex with relay in the ventral principal trigeminal nucleus (vPrV) in the hindbrain and then in the ventral postero-medial nucleus of the thalamus (VPM). At each relay, the spatial arrangements of neurons and their afferent fibers reproduce the physical distribution of the peripheral sensory receptors. This physical distribution of neurons and their afferent forms modules known as barrelettes, barreloids, and barrels in the brainstem, thalamus, and cortex, respectively (reviewed in Erzurumlu et al., 2010). During early hindbrain segmentation, Hoxa2 is the only Hox gene expressed in r2, although with a lower expression level than in r3 and more caudal rhombomeres. At fetal stages, Hoxa2 expression is differentially maintained in rhombomeres postmitotic progeny, being expressed in vPrV (derived from r3), although not in dorsal PrV (dPrV, derived from r2) (Bechara et al., 2015, Oury et al., 2006). Hoxa2 conditional inactivation in postmitotic neurons during axonal branching of primary sensory neurons (tamoxifen induction from embryonic day (E) 12.5 through E13.5) reduces arborization of afferents from whiskers to their target neurons in vPrV. Late removal of Hoxa2 also leads to topographic connection defects of vPrV neurons in the thalamus and loss of topographic connectivity in both the hindbrain and thalamus relays (Oury et al., 2006). These results show that in r3-derived vPrV neurons,
Hoxa2 promotes arborization of whisker-related afferents non-autonomously and topographic connectivity to the thalamus. Additional experiments revealed that ectopic expression of Hoxa2 in r2-derived postmitotic dPrV neurons is sufficient to change the program of dPrV neurons into vPrV barrelette neuron program (Bechara et al., 2015). Hoxa2 ectopic expression induces attraction of the whisker-related afferents, induces formation of asymmetrical dendrite arbors, and allows ectopic barrelette map formation. These defects can be related to synaptic processes occurring during late prenatal-early postnatal stages: terminal axonal branching and targeting, formation and growth of dendritic spines and synaptic specificity. In addition, ectopic Hoxa2 expression allows topographically directed targeting and perinatal refinement of dPrV axons with vPrV axons into a single whisker-related barreloid map in the thalamus (Bechara et al., 2015). Altogether, these data suggest that maintained expression of Hoxa2 in dPrV postmitotic neurons is sufficient to direct topographic axon targeting and refinement in the thalamus, a process that could result from regulation at the presynaptic level of molecules involved in activity-dependent synaptic pruning. Whether this regulation is directly HOXA2-dependent or a downstream consequence of the change in dPrV molecular program remains to be established.

Hox genes have also been involved in the establishment of topographic maps in brainstem auditory circuits. Indeed, tonotopy is a critical feature of the auditory system allowing to discriminate sound frequencies. In the auditory pathway, frequencies are first coded by the sensory receptors of hair cells located at different apical-to-basal levels in the cochlea. In turn hair cells activate specific spiral ganglion primary sensory neurons that topographically project onto the brainstem cochlear nuclear complex. Tonotopic organization is maintained at the different relays of the auditory pathway, in brainstem cochlear nuclei, midbrain, thalamus, and cortex (Kandler et al., 2009). Bushy cells, localized in the anterior ventral cochlear nucleus (AVCN), are glutamatergic neurons where the axon of one spiral ganglion neuron forms a unique and large synapse, the endbulb of Held. This single synapse input ensuring the fidelity of sound transmission is the result of fine-tuned tonotopic precision which required synaptic refinement of the spiral ganglion efferents. In the AVCN, which is mostly derived from r3 with a small contribution from r2, Hoxa2 and Hoxb2 (PG2 Hox genes) are expressed throughout pre- and post-natal development and at least up to 2 months of age (Narita and Rijli, 2009). As PG2 Hox genes are also expressed in mitotic progenitors, their conditional inactivation in Atoh1-derived postmitotic AVCN Bushy cells (Atoh1Hox2cKO) was used to investigate their potential involvement in topographic connectivity (Karmakar et al., 2017). Although the organization of primary sensory neuron inputs on AVCN neurons was preserved in Atoh1Hox2cKO mutants, the topographic precision of spiral ganglion axons prenatal targeting seemed compromised. This was functionally confirmed at adult stages and associated to the observation that individual AVCN Bushy cells in Atoh1Hox2cKO mutants receive multiple innervations instead of a single axonal input as observed in control animals. The endbulb of Held synapse maturation occurring during postnatal development was thus impaired in Atoh1Hox2cKO mutant AVCN Bushy cells. From a behavioral point of view, PG2 Hox mutants fail to discriminate two close pure-tone frequencies when they are tested by fear conditioning. These findings point to the involvement of PG2 Hox genes at postsynaptic level in AVCN.

### Table 1

SYNTHESIS OF IN VIVO DATA SUPPORTING ROLES OF HOX GENES IN SYNAPTOGENESIS

<table>
<thead>
<tr>
<th>Process</th>
<th>Neural system</th>
<th>Hox gene(s)</th>
<th>Model</th>
<th>Phenotype</th>
<th>ref</th>
</tr>
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<tbody>
<tr>
<td>Synapse formation</td>
<td>Trigeminal circuit</td>
<td>Hoxa2</td>
<td>cKO in vPrV neurons</td>
<td>Postsynaptic: late removal in the hindbrain reduces the arborization of axons from the maxillary branch of the trigeminal in r3-derived vPrV (collaterals formation) at E14.5</td>
<td>Oury et al., 2006</td>
</tr>
<tr>
<td></td>
<td>Trigeminal circuit</td>
<td>Hoxa2</td>
<td>ectopic expression in dPrV</td>
<td>Postsynaptic: Ectopic postmitotic expression in r2-derived dPrV attracts axons from the maxillary branch of the trigeminal nerve</td>
<td>Bechara et al., 2015</td>
</tr>
<tr>
<td></td>
<td>Phrenic system</td>
<td>Hoxa2/c5</td>
<td>cKO in PMC MNs</td>
<td>Presynaptic: Removal at E13.5 in PMC neurons reduces the number of terminal branches/arborization of PMC axons to the diaphragm</td>
<td>Philippidou et al., 2012; Truchon et al., 2017</td>
</tr>
<tr>
<td>Development of dendrites</td>
<td>Trigeminal circuit</td>
<td>Hoxa2</td>
<td>ectopic expression in dPrV</td>
<td>Postsynaptic: Ectopic postmitotic expression in r2-derived PrV influences dendritic organization of target neurons.</td>
<td>Bechara et al., 2015</td>
</tr>
<tr>
<td>Synapse maturation</td>
<td>Trigeminal circuit</td>
<td>Hoxa2</td>
<td>ectopic expression in dPrV</td>
<td>Presynaptic: Ectopic postmitotic expression in r2-derived dPrV results in an ectopic topographic map of whiskers in VPM nucleus at P7. Impact on synaptic refinement occurring between P0 and P7.</td>
<td>Bechara et al., 2015</td>
</tr>
<tr>
<td></td>
<td>Auditory circuit</td>
<td>Hoxa2/b2</td>
<td>cKO in AVCN Bushy cells</td>
<td>Presynaptic: correct organization of spiral ganglion fibers projections in AVCN neurons, but with reduced precision. Defects in refinement of afferents from multiple innervations to a single axonal input on Bushy cells.</td>
<td>Karamkar et al., 2017</td>
</tr>
<tr>
<td></td>
<td>Trigeminal circuit</td>
<td>Hoxa2</td>
<td>cKO in vPrV neurons</td>
<td>Post-synaptic: late removal in the hindbrain affects the topographic representation of afferent inputs in r3-derived vPrV at P4</td>
<td>Oury et al., 2006</td>
</tr>
</tbody>
</table>

AVCN: anterior ventral cochlear nucleus; cKO: conditional inactivation; dPrV: dorsal principal trigeminal nucleus; E: embryonic day; MN: motoneuron; P: postnatal day; PMC: phrenic motor column; r: rhombomere; VPM: ventral posterolateral nucleus of the thalamus; vPrV: ventral principal trigeminal nucleus.
Bushy cells to influence the refinement of afferents from the spiral ganglion neurons (Karmakar et al., 2017).

The third neural system in which specific function of Hox genes in postmitotic neurons has been demonstrated is the phrenic motor system. In mammals, respiration relies on the rhythmic contraction of the diaphragm which is controlled by a single input supplied by MNs in the phrenic motor column (PMC) in the spinal cord. MNs in the PMC are generated in the rostral cervical spinal cord, where they form a single clustered population spanning approximately three segments. Most PMC axons exit the spinal cord at the C4 level, converge with other cervical axons at the brachial plexus then extend ventrally through the thoracic cavity toward the primordial diaphragm. On reaching their target at E14.5, phrenic axons defasciculate from the main nerve and split into multiple finer branches before forming synapses across the muscle length (Allan and Greer, 1997). As in the hindbrain, following the early stages at which expression is in continuous longitudinal domains, Hox gene expression in the spinal cord resolves into more restricted domains related to neuron groups, in particular subsets of MNs (Dansen et al., 2005). Mutations in PG5–PG10 Hox genes result in transformation or reduction of distinct motor columns, and revealed a selective role for PG5 Hox genes during the specification of phrenic MNs (reviewed in Philippidou and Dasen, 2013). While Hoxb5 is normally excluded from PMC motor neurons, Hoxa5 and Hoxc5 gene expression is required in PMC neurons for multiple aspects of their development. Conditional deletion of Hoxa5 from MNs in a Hoxc5 knockout background showed a loss of PMC molecular determinants reflecting the involvement of Hoxa5/c5 to define positional identity of PMC neurons (Philippidou et al., 2012). These PG5 Hox gene compound mutants, Hoxa5 removal after the peak of motor neuron generation and after axons initial trajectories selection results in a reduction of PMC size and defects in the terminal arborization of PMC axons in the diaphragm. These data suggest requirement of PG5 Hox genes in PMC postmitotic neurons at fetal stages to regulate molecular programs involved in terminal branching of axons in the diaphragm, an early process in synapse assembly (Landry-Truchon et al., 2017, Philippidou et al., 2012).

Lessons from transcriptomic analyses: downstream pathways

These data from conditional inactivation in the central nervous system suggest that the HOX transcription factors could be recruited at different stages of the neuron life to sustain its diverse requirements, through the regulation of appropriate molecular programs. Very few studies have investigated the HOX proteins downstream pathways at a global level, and this hold true for their late functions in the central nervous system. Recently, a RNA-Seq analysis aimed at identifying the transcriptional programs downstream of HOX5 in the postnatal brainstem was performed (Lizen et al., 2017b, and data herein). Using an inducible conditional loss-of-function mouse model (Hoxa5 cKO), Hoxa5 was inactivated just after birth (postnatal days P1-P4) and the transcriptome of the brainstem was established at P21. Although we cannot totally exclude changes in RNA accumulated in axonal projections of HOXA5-expressing neurons located in other parts of the central nervous system (e.g. spinal cord), this paradigm allowed to detect changes in RNA expression levels mainly in brainstem HOX5-expressing neurons, and most likely in the precerebellar system where Hoxa5 expression was enriched (Lizen et al., 2015, Lizen et al., 2017a). As detailed below, the transcriptomic analysis revealed downregulation of several genes associated with synaptic function in Hoxa5 mutant samples, including key actors involved in the glutamatergic and GABAergic synapses and in calcium signaling. In parallel, to gain insights into the molecular programs regulated by PG2 HOX transcription factors in AVCN Bushy cells that might influence in a non-cell autonomous fashion early steps of presynaptic maturation of end bulb of Held (see above), Karmakar et al. performed a comparative transcriptomic analysis on Bushy cells isolated from mutant and control newborn animals by fluorescence-activated cell sorting (FACS) (Karmakar et al., 2017). Building upon these data and others available in literature we will discuss here the potential regulatory roles of HOX proteins in successive steps of synaptogenesis.

At the postsynaptic levels, HOX proteins could influence (1) the maturation of afferents through the regulation of secreted molecules; (2) the recognition and contact of pre- and post-synaptic elements through the regulation of cell surface and/or secreted molecules; and (3) synapse strengthening/elimination through regulation of neurotransmission and/or calcium-signaling pathways. Candidate target genes identified in the two RNA-Seq analyses support such roles for both HOXA5 in the precerebellar system and HOXA2/HOXB2 proteins in the auditory system. Among target genes coding for secreted factors several members of the WNT family of signaling molecules were identified as neuronal targets of HOX proteins (Table 2). Wnt7a was found downregulated in Hoxa5 cKO brains, while Wnt3a is a target of HOXA2/HOXB2 proteins in AVCN Bushy cells (Bami et al., 2011, Karmakar et al., 2017, Lizen et al., 2017b). This is of particular interest, as WNT signaling has been highlighted as important player in synaptic assembly, function and maintenance (Dickins and Salinas, 2013, Inestrosa and Arenas, 2010). In the context of the cortico- ponto-cerebellar system, Wnt7a is expressed by granule cells and acts at the postsynaptic level by inducing the morphological maturation of mossy fibers afferents (Hall et al., 2000). As in granule cells, its induction by HOX5 in precerebellar mossy fiber neurons could influence the maturation of afferents such as cortical inputs in the pons (Fig. 2A). The extracellular matrix glycoprotein REELIN (RELN), that has previously been involved in the fine tuning of topographically organized neural circuits during postnatal development (Antonioli-Santos et al., 2017, D’Arcangelo, 2014) was also downregulated in Hoxa5 cKO samples. Among other secreted factors downregulated in Hoxa5 cKO samples, GDF10, belonging to the TGFβ superfamily, is of particular interest as it was shown that TGFβ ligands are able to induce retino-geniculate refinement (Bialas and Stevens, 2013). Finally, let’s mention the synapse organizer CBLN1 which interact with the glutamate receptor subunit GRID2 and with Neurexin to form a trimolecular trans-synaptic organizer of the glutamatergic synapse (Hirai et al., 2005, Matsuda et al., 2010, Ryu et al., 2012, Uemura et al., 2010) and was also downregulated in Hoxa5 cKO samples.

Among cell adhesion molecules, cadherins (Cdh) are well-known as important mediators in the formation of specific neural circuits (Basu et al., 2015), and several have also been identified in HOX downstream pathways (Table 2), notably Cdh15 and the Fat atypical cadherin 2 (Fat2), were identified downstream of HOX5 in the postnatal brainstem, while Cdh4-Cdh11-Cdh13-Cdh7 were candidate target genes of HOX2/HOXB2 in the AVCN Bushy cells. Hoxa5 cKO RNA-seq analysis also highlighted a downregulation of Svep1, coding for a ligand mediating cell adhesion in an integrin-dependent...
manner (Sato-Nishiuchi et al., 2012) (Fig. 2B). Interestingly, all these genes were reported as upregulated in Atoh1Δex2Δ3 KO Bushy cells during their maturation (Karmakar et al., 2017). The opposite activity of HOX proteins on similar downstream effectors suggests a complex, albeit extremely efficient, strategy to support formation of neuronal circuits, such as the cortico-pontine connectivity along the anteroposterior axis of the pons (Kratochwil et al., 2017). Such differential regulation of expression by HOX proteins has already been described for Unc5b to specialize pontine neurons during their migration: while dorsally-migrating Hox2-expressing neurons show high level of Unc5b expression, Hox5 genes negatively regulate Unc5b in ventrally-migrating neurons (Di Meglio et al., 2013). Other genes coding for cell surface molecules involved in cell adhesion of neuronal circuits, such as the cortico-pontine connectivity along the anteroposterior axis of the pons (Kratochwil et al., 2017). Such differential regulation of expression by HOX proteins has already been described for Unc5b to specialize pontine neurons during their migration: while dorsally-migrating Hox2-expressing neurons show high level of Unc5b expression, Hox5 genes negatively regulate Unc5b in ventrally-migrating neurons (Di Meglio et al., 2013). Other genes coding for cell surface molecules involved in cell adhesion

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AVCN: anterior ventral cochlear nucleus; E: embryonic day; P: postnatal day; Tam: tamoxifen; ES: embryonic stem cells.
were upregulated in the Hoxa5 cKO RNA-Seq analysis such as the kirre like nephrin family adhesion molecule 2 (Kirrel2) and Claudin 1 (Cldn1). Kirrel2 belongs to the immunoglobulin domain-containing family of adhesion molecules (Sellin et al., 2003, Yogev and Shen, 2014), that contains two other members, Kirrel1 and Kirrel3, the latter being involved in axonal sorting as well as in synaptogenesis (Gerke et al., 2006, Nishida et al., 2011, Serizawa et al., 2006).

Finally, several postsynaptic players of glutamatergic synapses such as the glutamate receptors subunits GRM4, GRIN2C, and GRID2, were downregulated in Hoxa5 cKO neurons (Lizen et al., 2017b). GRM5, GRIN1 and GRIN2a were also identified down-stream of HOXA2/HOXB2 and HOXB1 proteins (Table 2). In parallel, differential expression of GABA receptor subunits was detected in these three studies, pointing to HOX proteins as common regula-
tors of postsynaptic neurotransmission at the glutamatergic and GABAergic synapses. Downstream of the signal reception, several actors of calcium signaling were identified as candidate targets of HOX proteins. The calcium release mediator ITPR1 was identified in the HOXA5 downstream pathway, together with three of its interacting proteins, namely Homer3, Car8 and Calb1 (Fig. 2C) (Hirota et al., 2003, Tu et al., 1998). Several other proteins whose activity is influenced by calcium, such as CAMK4, ADCY1, PRKCG, and ITPKA were additionally identified. In Atoh1\textsuperscript{Hoxc5KO} Bushy cells, Camk4 was similarly downregulated while Camk2b expression was upregulated. The regulation of these postsynaptic players could modulate strengthening of appropriate synapses through calcium signaling in the Hox-expressing neurons, such as the HOXA2/ HOXB2-positive AVCN Bushy cells and the HOXA5-positive precerebellar neurons. Such role of postsynaptic players in synaptic maturation can be illustrated with ADCY1 which plays a role in the regulation of synapse stabilization/elimination during the late stage of fine topography of sensory circuits (Nicol and Gaspar, 2014).

Alternatively, HOX target genes coding for cell surface and secreted molecules could also act at the presynaptic level to mediate axonal guidance and terminal arborization, as well as synapse formation and stabilization. In the context of precerebellar circuit formation during postnatal development, secreted molecules from mossy fibers could influence both the migration of granule cells in the internal granular layer and/or the subsequent connections between mossy fibers and granular cells. Secretion of RELN from HOXA5-positive precerebellar neurons could participate to the development and maturation of the dendrites and the spines of granule cells as RELN is implicated these processes (Lee and D’Arcangelo, 2016, Niu et al., 2008)(Fig. 3A). Downregulation of the WNT receptor Fzd7 was also detected in Hoxa5 cKO samples. This receptor could mediate Wnt7a signaling in mossy fibers to induce the morphological maturation of the glomerular rosettes, multisynaptic structures formed between mossy fiber axons and dendrites from cerebellar granule cells (Hall et al., 2000). In the context of ponto-cerebellar subcircuit maturation, the regulation of specific cell surface molecules by HOXA5 in pontine neurons could thus

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Fig. 2. Model of synaptic assembly and maturation through HOXA5 regulation of target genes. Illustrated for cortico-pontine projections. (A) The upregulation of specific postsynaptic secreted molecules could participate to synapse assembly and strengthening. (B) The regulation of specific cell surface molecules by HOXA5 could also mediate synaptic recognition with cortical afferents and influence synapse strengthening and elimination during cortico-pontine subcircuit maturation. (C) The upregulation of selected postsynaptic players of glutamatergic synapses illustrated here could facilitate strengthening of appropriate synaptic connections through calcium signaling in the HOXA5 positive pontine neurons. Glu: glutamate.
as well as other HOX proteins (Table 2) support the hypothesis that HOX proteins triggers specific developmental programs at later stages of neural circuit formation, aimed at promoting synaptogenesis.

**Synapse pathologies and neurodevelopmental disorders**

Given the critical role synapses play in normal neurophysiology, it is not surprising that loss of synaptic integrity may underlie many of the most common neurodegenerative diseases, such as Alzheimer disease or Parkinson’s disease (Henstridge et al., 2016). As this review is focused on the first steps of synapse assembly and maturation, it has also been suggested that insufficient or excessive synaptic pruning may underlie several neurodevelopmental disorders, including autism, schizophrenia, epilepsy and mental retardation (Neniskyte and Gross, 2017, van Spronsen and Hoogenraad, 2010).

Among those, autism spectrum disorders (ASD) are usually diagnosed in the first three years of life, a period that overlaps with the initial phase of cortical and cerebellar synaptogenesis in humans. Recent findings suggest a link between a failure to eliminate synapses and to appropriately strengthen other synaptic connections as a basis for functional deficits in mouse models of ASD (Neniskyte and Gross, 2017). In this context, it is of interest that several genes downregulated in the brainstem of Hoxa5 cKO mouse at P21, such as Cadps2, Itp1, Cbln1, Gmr4, Camk4, Reln, En2, Grid2 et Chd7; have been associated to autism traits either in humans or in mouse models (Becker et al., 2014, Genestine et al., 2015, Jongmans et al., 2006, Krishnan et al., 2017, Lammert and Howell, 2016, Sadakata and Furuichi, 2009, Schaaf et al., 2011, Schmunk et al., 2017, Waltes et al., 2014). As previously mentioned, some of these genes are downstream targets of other HOX transcription factors (Table 2). These data point to HOX5, and potentially other HOX proteins, as regulators in pathways that are affected in neurological diseases such as ASD. Indeed, our data suggest that HOX5 is regulating synaptogenesis in mossy fibers neurons, which provide the main source of input to the cerebellum. In parallel it is now well acknowledged that ASD is both associated to synapse deficits at early life stage and to alteration of cerebellar development and function (Ebert and Greenberg, 2013, Hampson and Blatt, 2015, Wang et al., 2014). Of interest, mutations and allelic variants within the human HOX1 gene have been linked to ASD risk (Ingram et al., 2000, Raznahan et al., 2012, Tischfield et al., 2005). However, due to the early restricted expression of this gene in the hindbrain, the etiology is most likely linked to altered prenatal neurodevelopment, such as early deficits in hindbrain patterning and cerebellar development, rather than to synaptogenesis defects.

If HOX proteins have such multitask molecular functions in neuronal circuit connectivity, it may be intriguing that only a relatively limited number of human HOX disorders have been reported so far, and very few being associated to neurological diseases (Quinonez and Innis, 2014). This limited number could be due to both

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**Fig. 3. Model of synapse maturation through the HOXA5 regulation of target genes, illustrated for the ponto-cerebellar projections. (A) The regulation of specific cell surface and secreted molecules by HOXA5 could mediate synaptic recognition with granule cells and influence synaptic strengthening and elimination during ponto-cerebellar subcircuit maturation. (B) The upregulation of some of the presynaptic actors of glutamatergic synapses could be involved in the maturation of ponto-cerebellar synapses, as illustrated for a few candidate target genes. Glu: glutamate.**

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mediate synaptic recognition and influence synaptic strengthening and elimination (Fig. 3A). In the RNA-Seq analysis of Hoxa5 cKO samples, several proteins encoded by HOXA5 candidate target genes within the glutamatergic synapse term were presynaptic (Lizen et al., 2017b). Among these presynaptic players, we detected SLC17A7 (VGLUT1), which has an essential role during postnatal development by loading presynaptic vesicle with glutamate allowing a regulation of neurotransmission (Fig. 3B)(Wojcik et al., 2004). We also identified CADPS2 involved in the release of the neurotrophic factor BDNF, which is essential for normal postnatal cerebellar development and is involved in the functional maturation of the glutamatergic synapses (Gottmann et al., 2009, Sadakata and Furuichi, 2009). Notably, CADPS2 was identified downstream of HOX2 proteins in two other transcriptomic analyses (Table 2). By regulating these different players in precerebellar nuclei, and notably in pontine nuclei, HOXA5 could be involved in the postnatal development of the cerebellum through the maturation of mossy fiber connections with granular cells (Fig. 3B).

In conclusion, available data reported in the literature for HOXA2/ HOXB2 and HOXA5 (Karmakar et al., 2017, Lizen et al., 2017b)
the overlapping expression domains of paralogy groups, which would complement single loss of function alleles, and the current limits of non-systematic molecular testing in patients with malformation syndromes. Moreover, most of the disorders due to loss-of-function of HOX genes are associated to complex syndromes, likely due to functions of HOX proteins in multiple tissues and at multiple developmental stages (Grier et al., 2005, Quinonez and Innis, 2014). Loss-of-function HOX genes mutations would thus likely be incompatible with life in humans.

As epigenetic and transcriptional deregulation of gene expression are increasingly being identified in the etiology of neurodevelopmental disorders, one could expect that spatial and/or temporal deregulation of HOX genes expression in the central nervous system could participate to the development of neuropathologies. A few recent reports support this hypothesis. First, a study suggests that HOX A5 is a key element in the CHARGE and Kabuki syndromes, two complex human syndromes that include intellectual disabilities and autistic-like behaviors, and which result, respectively, from a loss of function of the CHD7 and KMT2D proteins (Butcher et al., 2017, Jongmans et al., 2006). A DNA gain of methylation at the level of the human HOX A5 promoter has been demonstrated in these two syndromes, which would result in a decrease in its expression. Second, recent data suggest that DNA hypermethylation across an extended HOX A gene region is associated with Alzheimer disease in humans, with the strongest effect in the vicinity of HOX A3 (Smith et al., 2018). Finally, upregulation of microRNAs located in the HOX gene clusters was identified in brain samples from Huntington disease patients. Correlated to these changes, expression of 14 HOX genes was found significantly upregulated in these samples, among which HOX A5 (Hoss et al., 2014). It would thus be interesting to further investigate whether genetic variants and epigenetic dysregulation of HOX genes could be involved in synapse pathologies.

Concluding remarks

Emerging evidences suggest a role for HOX transcription factors in synaptogenesis during late fetal and early postnatal life. As discussed in this review, alteration of HOX activity at those stages in mouse models has been associated to defects in synapse assembly, synapse refinement and topographic connectivity. Recent transcriptomic analyses revealed that HOX proteins regulate expression of genes involved in both postsynaptic and presynaptic assembly and maturation. To confirm this hypothesis, and extend the data collected here for a few Hox genes, more Hox conditional mutants should be generated and characterized. Moreover, deepened phenotypic analyses to evaluate the behavioral consequences of conditional inactivation are required.

Current data thus support a model in which HOX transcription factors are molecular multitask proteins that orchestrate the interdependent development and innervation of circuits, directing successive developmental steps that range from cell identity and neuronal migration to synapse assembly and maturation. As suggested by others, it would be interesting to investigate whether late-stage Hox gene expression could also be involved in activity-dependent transcriptional regulation (Karmakar et al., 2017). Moreover, Hox genes continue to be expressed in the adult brain, suggesting a role in the mature nervous system. Whilst their function is virtually unknown, data gathered here could suggest a role in synapse maintenance and plasticity during all life. Indeed, even after circuits have matured, synaptic pruning continues to maintain brain plasticity to support cognitive functions such as learning and memory. Increased understanding of how HOX transcription factors are integrated to regulate synaptogenesis will also provide us with an increasingly clear picture of synapse assembly and maturation that is pre-requisite for apprehending the pathophysiological mechanism underlying synapse pathologies.

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