

Systematic expression analysis of *Hox* genes at adulthood reveals novel patterns in the central nervous system

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Abstract *Hox* proteins are key regulators of animal development, providing positional identity and patterning information to cells along the rostrocaudal axis of the embryo. Although their embryonic expression and function are well characterized, their presence and biological importance in adulthood remains poorly investigated. We provide here the first detailed quantitative and neuroanatomical characterization of the expression of the 39 *Hox* genes in the adult mouse brain. Using RT-qPCR we determined the expression of 24 *Hox* genes mainly in the brainstem of the adult brain, with low expression of a few genes in the cerebellum and the forebrain. Using in situ hybridization (ISH) we have demonstrated that expression of *Hox* genes is maintained in territories derived from the early segmental *Hox* expression domains in the hindbrain. Indeed, we show that expression of genes belonging to paralogy groups PG2-8 is maintained in the hindbrain derivatives at adulthood. The spatial colinearity, which characterizes the early embryonic expression of *Hox* genes, is still observed in sequential antero-posterior boundaries of expression. Moreover, the main mossy and climbing fibres

precerebellar nuclei express PG2-8 *Hox* genes according to their migration origins. Second, ISH confirms the presence of *Hox* gene transcripts in territories where they are not detected during development, suggesting neo-expression in these territories in adulthood. Within the forebrain, we have mapped *Hoxb1*, *Hoxb3*, *Hoxb4*, *Hoxd3* and *Hoxa5* expression in restricted areas of the sensory cerebral cortices as well as in specific thalamic relay nuclei. Our data thus suggest a requirement of *Hox* genes beyond their role of patterning genes, providing a new dimension to their functional relevance in the central nervous system.

Keywords *Hox* genes · Hindbrain · Forebrain · Precerebellar nuclei · Sensory circuits · RT-qPCR · In situ hybridization

Abbreviations

AMS	Anterior extramural migrating stream
AP	Area postrema
A/P	Antero-posterior
BS	Brainstem
Cb	Cerebellum
CNS	Central nervous system
Cx	Cerebral cortex
dmX	Dorsal nucleus of the vagus nerve
ECU	External cuneate
Hi	Hippocampus
Hy	Hypothalamus
IF5	Interfascicular trigeminal nucleus
IHC	Immunohistochemistry
IO	Inferior olive
ISH	In situ hybridization
PBS	Phosphate-buffered saline
IMS	Intramural migrating stream
LGd	Dorsal lateral geniculate nucleus

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LRN	Lateral reticular nucleus
MG	Medial geniculate nucleus
MNs	Motoneurons
MO	Medulla oblongata
NTS	Nucleus of the solitary tract
OA	Olfactory areas
PCs	Purkinje cells
PMS	Posterior extramural migrating stream
PG	Paralogy groups
PN	Pontine nucleus
Po	Pons
PrV	Principal sensory trigeminal nucleus
r	Rhombomere
RO	Raphe obscurus
RPA	Raphe pallidus
SC	Spinal cord
SpV	Spinal trigeminal nucleus
Th	Thalamus
TRN	Tegmental reticular nucleus
VPL	Ventral posterolateral nucleus of the thalamus
VPM	Ventral posteromedial nucleus of the thalamus

Introduction

Early in nervous system development, fate specification of neural progenitors occurs in the proliferative neuroepithelium, conditioning the appropriate differentiation of their post-mitotic descendants. Cell fate specification is imposed by patterning genes expressed in stripes or patches, very often homeogenes (Kiecker and Lumsden 2005). Post-mitotic neurons then migrate, either radially or tangentially, to reach their final location within the central nervous system (CNS) (Hatten 2002). During the phase of axonal guidance, developing axons find their way towards their appropriate target territories and, within these territories, recognize the proper target cells with which to form synapses. This phase is characterized in the sensory circuits by a spatial organization of neurons and their afferent fibres which is faithfully reproduced in every relay of the circuit, from the peripheral receptors up to the cerebral cortex, establishing the so-called topographic maps (Cang and Feldheim 2013). Both migrating cells and growing axons rely on environmental guidance cues belonging to the ephrin, netrin, slit and semaphorin families and their respective receptors (Dickson 2002; Evsyukova et al. 2013). After this activity-independent phase, during a postnatal “critical period”, the neuronal circuitry is intensively remodelled by individual experiences (Erzurumlu and Gaspar 2012; Hensch 2005).

It is becoming increasingly clear that Hox homeoproteins, encoded by *Hox* genes, are involved in the programs regulating most of these steps. *Hox* genes constitute an

evolutionary conserved gene family and encode transcription factors that play key roles in the establishment of the body plan during development. 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 positions along the clusters (Deschamps 2007). During embryonic development, their expression obeys a temporal and spatial colinearity rule according to which genes located at the 3' side of a complex are expressed earlier and more rostrally than those residing by the 5' extremity of the complexes (Kmita and Duboule 2003). From this expression pattern it appears that Hox proteins are active in a combinatorial fashion along the embryo main axes, as exemplified in the CNS (Alexander et al. 2009).

During neurulation, through their combinatorial expression, *Hox* genes provide segmental identity and antero-posterior (A/P) patterning information to proliferating neurons (Nolte and Krumlauf 2007). Between embryonic days E7.5–E9.5, ten *Hox* genes, members of PG1–4, have domains of expression in the CNS which map to the hindbrain and spinal cord, while genes from PG5–13 only display expression in the spinal cord. Within the hindbrain these ten *Hox* genes have a sharp anterior limit of expression corresponding to rhombomere (r) boundaries. More specifically, *Hoxa2* is expressed up to the r1/r2 boundary while *Hoxb2* is expressed up to the r2/r3 boundary; *Hoxa1* and *Hoxb1* are expressed up to the r3/r4 boundary, PG3 *Hox* genes up to the r4/r5, and PG4 *Hox* genes (at the exception of *Hoxc4*) up to the r6/r7. It has been demonstrated that these spatially restricted patterns of expression are associated with the establishment of rhombomeric territories and with cell fate determination (Nolte and Krumlauf 2007; Tumpel et al. 2009). After neurulation, early segmental *Hox* expression patterns are often maintained up to late foetal stages in restricted neuronal subpopulations in the hindbrain (Di Bonito et al. 2013b; Geisen et al. 2008; Oury et al. 2006; Pasqualetti et al. 2007). Recent work has started to uncover the importance of these late *Hox* expression programs in regulating several aspects of neural circuit formation, such as neuronal migration, axonal growth and pathfinding, target selection and topographic map formation (Erzurumlu et al. 2010). Such roles have been shown for some *Hox* genes in the hindbrain, notably for *Hoxa2* and *Hoxb2* which control tangential migration of precerebellar pontine neurons and for *Hoxa2* which is implicated in the development of a facial somatosensory map (Geisen et al. 2008; Oury et al. 2006). In the foetal spinal cord, as in the hindbrain, *Hox* gene expression resolves into more restricted domains related to neurons groups, in particular subsets of motoneurons (MNs). Through a systematic expression analysis of the 39 chicken *Hox* genes in post-mitotic MNs, it has been shown that Hox proteins not only specify distinct A/P motor pool identities but also contribute to the specificity of motor axon

projection, controlling precise patterns of target-muscle connectivity (Dasen et al. 2005).

What happens after birth is less well understood. Studies have reported the presence of *Hox* gene transcripts in the postnatal and adult brain, and in some cases, in areas where they were not expressed at earlier stages. Notably, RT-qPCR analysis of *HOX* expression in human samples revealed that 15 genes belonging to PG1-PG7 were expressed in the adult brain extracts (Takahashi et al. 2004). Using immunohistochemistry (IHC) or in situ hybridization (ISH) on mouse postnatal or adult brain, expressions of *Hoxd1*, *Hoxa2*, *Hoxa5*, *Hoxb7* and *Hoxb8* have been reported either in forebrain territories or in the cerebellum, derived from r1 (Greer and Capecchi 2002; Sanlioglu et al. 1998; Wolf et al. 2001). However, the question of their function at adulthood remains to be investigated.

Indeed, the elucidation of the embryonic functions of *Hox* genes, by analysing how genomic mutations in *Hox* genes in mice affect the shape of body structures, has been a prime objective of the “Hox community” during the last decades. Notably, several *Hox* gene mutants are characterized by homeotic transformation of rhombomeres and changes in neuronal identities (reviewed in Guthrie 2007). However, investigation of these mutant mice do not allow the study of *Hox* gene functions at later (foetal, postnatal and adult) stages. To bypass earlier developmental requirements for *Hox* genes the systematic analysis of spatio-temporally controlled knockout mouse models is required. Such an analysis would benefit from a map of *Hox* gene expression in the adult brain that would serve as a stepping stone for future functional genomic studies on the *Hox* family in the CNS. Moreover, describing when and where genes are transcribed is a powerful method to approach their function. In this paper, we therefore provide a neuroanatomical atlas of expression of the 39 *Hox* genes in the adult CNS, based on mRNA detection. Due to the neuroanatomical complexity of the adult brain, and since other groups are investigating Hox functions in the spinal cord (see Dasen and Jessell 2009), our study is limited to the brain. Our analysis reveals that the majority of *Hox* genes remain expressed in the brain at adulthood. For most of the *Hox* genes, the transcript profile fits with the maintenance of expression in their rhombomere-derived post-mitotic neurons. However, for a subset of genes belonging to PG1 to PG5, transcripts were also detected in unexpected territories, notably in the forebrain derivatives.

Materials and methods

Animals and tissue harvest

Adult male C57Bl/6J mice, purchased from Charles River, were maintained and fed in standard conditions on a 14 h

light/10 h dark cycle. Experimental procedures on animals were performed in accordance with the guidelines of the animal ethics committee of the Université catholique de Louvain and in agreement with the European directive 2010/63/UE. To minimize the impact of the environment on *Hox* gene expression, mice housing and brain collection were highly standardized. After a 4 h fasting period, 12-week-old males were killed by gas inhalation at 11–12 a.m. and whole brains were rapidly isolated. Together with the cervical spinal cord, brains for RT-qPCR were immediately dissected into seven subregions (Fig. 1a), frozen in liquid nitrogen and stored at -80°C . Brains for ISH were embedded in OCT mounting medium (Shandon Cryomatrix™, Thermo Electron, France), snap frozen on dry ice and stored at -80°C . For IHC procedures, animals were deeply anesthetized with an intra-peritoneal lethal injection of sodium pentobarbital (Nembutal, CEVA, France; 80–100 mg/kg) and then rapidly perfused transcardially with phosphate-buffered saline (PBS, pH 7.4), followed by ice-cold 4 % paraformaldehyde in PBS. After a last PBS wash, fixed brains were removed and cryo-preserved in graded solutions of 10–20–30 % sucrose in PBS at 4°C (about 24 h in each solution). Brains were embedded in OCT medium, frozen on dry ice and stored at -80°C .

Gene expression studies: RT-qPCR

To allow reliable and unequivocal interpretation of our data, all steps have been realized in conformity with the MIQE guidelines (Bustin et al. 2009). Quality, quantity and integrity of extracted RNAs (Tri Reagent, Sigma T9424) were evaluated by gel electrophoresis, spectrophotometry (Nanodrop) and microfluidic analysis (Agilent Bioanalyzer). Reverse transcription steps were carried out using Quantitect Reverse Transcription Kit (Qiagen 205311). To allow for specific, efficient and comparable amplification of transcripts of *Hox* genes, we have designed and tested several primer pairs per gene, from which 39 were selected (Table S1). The selection was based both on the efficiency of the amplification reaction and on the specificity of the amplification, as controlled by sequencing of the amplicon. To provide the most complete representation of *Hox* mRNAs, we used an external cDNA standard, transcribed from a pool of RNAs extracted from C57Bl/6J embryos at different embryonic stages (whole embryos at E8.5, 9.5, 10.5 plus limbs dissected from E12.5 fetuses) covering the principal stages/structures of all *Hox* genes expression. A large amount of this standard was prepared and was used repeatedly as a basis to compare the results, thus increasing the reproducibility of the measurements. This standard was used for optimization and validation of primers and as an internal calibrator to create a dilution series (standard curve) with arbitrary units for relative quantification

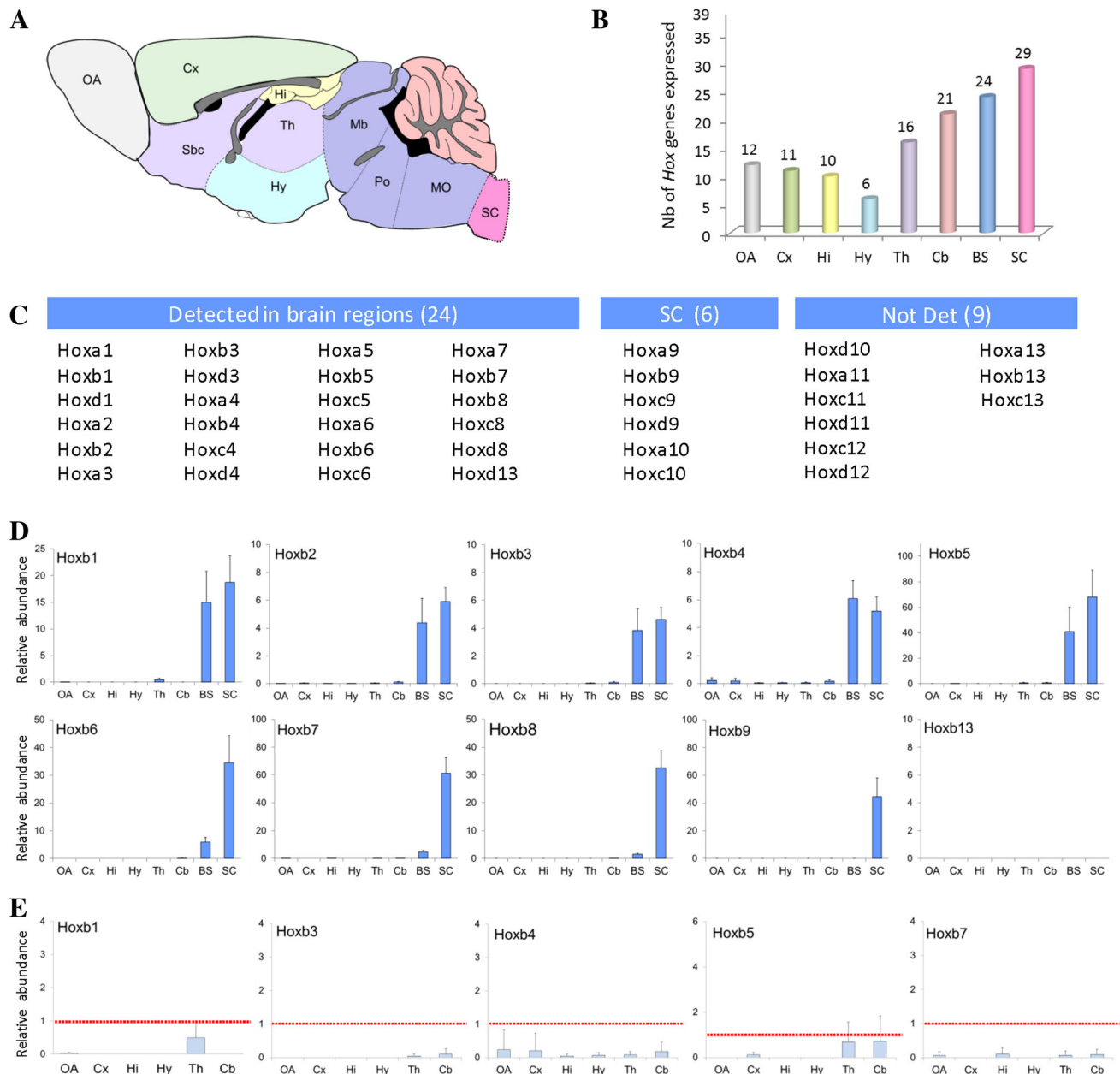


Fig. 1 Quantitative analysis of expression of *Hox* genes in the central nervous system (CNS) of adult C57Bl/6J mice. **a** Schematic representation of the eight regions dissected for the RT-qPCR analysis. **b** Summary of the number of *Hox* genes expressed in each of the 8 regions analysed, with colour corresponding to the scheme provided in **a**. **c** List of the 24 *Hox* genes expressed in at least one of the 8 CNS regions analysed (left columns), of the 6 genes expressed in the cervical spinal cord only (SC—middle column) and of the 9 genes for which expression was not detected (Not Det.) in our analysis (right columns). **d** The RT-qPCR data are illustrated for the *HoxB* cluster and are expressed according to the relative quantification of the $\Delta\Delta C_T$

method where a mix of embryo cDNA, collected at several developmental stages (whole embryos at E8.5, 9.5, 10.5 plus limbs dissected from E12.5 foetuses), was used as the standard calibrator (relative expression = 1). Data are reported as means \pm SEM, $n = 6$ –10. **e** Enlargement of the RT-qPCR data in anterior areas of the brain for a subset of *Hoxb* genes. The red dotted line marks the abundance of transcripts measured in the embryonic samples. OA olfactory areas, Cx cerebral cortex, Hi hippocampus, Hy hypothalamus, Th thalamus and subcortical areas, BS brainstem, including the midbrain (Mb), pons (Po) and medulla oblongata (MO), Cb cerebellum, SC cervical spinal cord

(Bustin 2000; Bustin et al. 2009). Normalization is an essential component for a reliable qPCR assay and guidelines recommend normalizing mRNA concentration against

the geometric average of a minimum of 2–3 reference genes (Derveaux et al. 2009; Vandesompele et al. 2002). After control of abundances and stable expression in 42

CNS samples, three genes were selected for normalization: *36B4* (GeneID: 11837), *H2A* (GeneID: 100039319) and *I8S* (GeneID: 19791) (Fig. S1).

Hox expression was then assessed on a StepOne + (ABI) using SYBR Green (Qiagen 204143) as the detection method, and the geometric mean of the three reference genes as internal normalization reference. Normalized mRNA expression levels (mean \pm SEM from 6 to 10 mice) were compared to the standard calibrator which has a relative expression of 1 (see above). The resulting relative expression levels are obtained by the $\Delta\Delta C_q$ method.

Processing of data

Raw C_q values were individually examined and curated to ensure data quality and accuracy. All samples were amplified in duplicates to minimize experimental variation. C_q higher than 35 cycles were not considered accurate enough for further analysis and values were set to zero. In the relative quantification ($2^{-\Delta\Delta C_q}$), only values higher than 0.005 were further considered, others were set to zero. Finally, only amplification detected in 4 or more samples of a particular region (out of 6–10) were considered reproducible enough to be used for further analysis.

Gene expression studies: non radioactive ISH

For ISH, 12 sets of 18 μm thick serial coronal or sagittal cryosections per brain were cut on a Leica CM 3050S cryostat. Gene expression was detected using digoxigenin-labelled RNA probes, as previously described (Chotteau-Lelievre et al. 2006). This procedure has been previously validated in the context of the European consortium Eumorphia and is available on the EMPRESS website (<http://empress.har.mrc.ac.uk/about.html>). In addition, this procedure has been optimized to increase its sensitivity in the context of our study: brain sections, analysis of highly homologous genes, and detection of low-abundant transcripts. This includes the following pre-hybridization treatments: acetylation of positively charged amino groups to reduce the electrostatic binding of probe to sections; and de-lipidization of the sections with chloroform. Hybridization was carried out at moderate stringency (65 °C, that is ≥ 25 °C below the T_m calculated for all probes), thus minimizing non-specific hybridization of probes to unrelated sequences (Angerer and Angerer 1992). Commercial Hybmix (Ambion, B8807G) was used as it appeared to increase sensitivity and it allowed standardization of the procedure. Washes were performed at high stringency to restrict hybridization to the sequence homologous to the probe. To increase the sensitivity of the reaction for probes

that give a weak signal, 5 % (w/v) of high molecular weight polyvinyl alcohol (PVA- Sigma, P1763) was added in the staining solution (De Block and Debrouwer 1993). The addition of this polymer enhances the alkaline phosphatase reaction and prevents diffusion of reaction intermediates that can occur during long incubation, resulting in a significant increase in sensitivity without increasing the background. Therefore, incubations up to 45 h were possible with moderate background generation (see Fig. S4). For synthesis of probes, cDNA fragments of the gene of interest cloned into a plasmid vector that allows in vitro transcription with SP6, T3 or T7 polymerases were obtained from our collaborators (P. Dollé, F. Rijli, R. Rezsöházy, M. Kmita, M. Torres, J. Deschamps, D. Duboule, M. Capecchi, S. Stadler, Z. Lele, C. Gu). New plasmids were prepared in house for *Hoxc8*, *Hoxc13* and *Hoxc5*. For several genes, templates synthesized by RT-PCR were used, prepared according to the GenePaint procedures as described online at <http://www.genepaint.org/>, to produce a second independent probe (kindly provided by G. Eichele). A detailed list of data regarding plasmids and template is available upon request. All probes were internally sequenced for specificity and were first validated by ISH on embryos/foetuses for sensitivity (Fig. S2–S3). After ISH on brain sections, extensive annotation was carried out for brain anatomical regions spanning forebrain, midbrain and hindbrain (for nomenclature see the Allen Brain Atlas, Mouse brain reference atlas—<http://mouse.brain-map.org/static/atlas> and Hof et al. 2000), as previously described (Gofflot et al. 2007). Double ISH was performed on cryostat sections using Digoxigenin- and Fluorescein-labelled probes as described (Mayer et al. 2010). Hybridized sections were observed on a Leica DM2500 microscope, and pictures were captured with a Leica DFC420C camera.

Annotation of expression

Expression thresholds versus background were established for each ISH taking into account the appropriate control and the duration of the staining reaction. A frequently used control for specific hybridization to mRNA is a sense-strand probe which should not specifically anneal to the target RNA. However, many *Hox* loci are transcribed from the antisense strand. An alternative is to use an antisense probe from a gene not expressed in the tissue. In this study we used either a *Hox* sense probe (e.g., *Hoxb6S* and *Hoxb8S*) or a probe for a *Hox* gene not expressed in the brain (e.g., *Hoxa10* or *Hoxd10*) (Fig. S4–S5). For low expression signals, thresholds were confirmed by two researchers after examination of a minimum of 3 independent hybridizations.

Protein localization studies: immunohistochemistry IHC

For IHC, 18 μm thick serial cryosections were cut as above. After washes in TBS-Tx (Tris-buffered saline 0.05 M, Triton X-100 1 %, pH 7.4), sections were blocked in a solution of 5 % milk in TBS-Tx (1 h at room temperature, RT). Sections were incubated overnight at 4 °C with primary antibody solution (antibody diluted in TBS-Tx 1 % milk) in a humid chamber. The following primary antibodies were used: anti-Hoxb3 (1:50, Santa Cruz sc-28606, rabbit polyclonal IgG), anti-Hoxb4 (1:50, Developmental Studies Hybridoma Bank I12, rat fusion mouse monoclonal IgG2a). After incubation with primary antibody, sections were rinsed with TBS-Tx and incubated for 1 h at RT with the appropriate secondary antibody (1:500 in TBS-Tx 1 % milk). Secondary antibodies were used as follows: Alexa Fluor 546 goat anti-rat IgG (H+L) (1:500, Invitrogen A-11081), Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (1:500, Invitrogen A-21206). After TBS-Tx washes, sections were stained with TB-DAPI (1:20000 for 10 min) then mounted with Fluorescence Mounting Medium (Dako). The immunostained samples were examined with an Axioskop2 fluorescence microscope (Zeiss Instruments).

Results

Hox expression profiling by RT-qPCR

To provide a global and quantitative profile of the expression of *Hox* genes in the adult mouse CNS, RT-qPCR was performed on 8 isolated subregions that include the olfactory areas (OA), cerebral cortex (Cx), hippocampus (Hi), hypothalamus (Hy), thalamus and basal ganglia (Th), cerebellum (Cb), brainstem (midbrain, pons and medulla oblongata) (BS) and cervical spinal cord (SC) (Fig. 1a). In this analysis transcripts of 30 *Hox* genes were detected in at least one region of the CNS analysed (Fig. 1c, raw data of relative abundance provided in Table S2). Indeed, we showed the presence of transcripts for 24 *Hox* genes in the brain. Six genes were expressed only in the cervical SC samples, while expression of 9 genes was not detected in any of the analysed samples. As the thoracic and lumbar SC regions were not represented in the samples analysed, these results do not exclude the potential expression of these nine genes at a more caudal level of the adult CNS.

The organization of *Hox* genes in PG along the chromosomes seems to be associated with their expression profiles in the CNS at adult stages, as illustrated in Fig. 1d for the *HoxB* cluster and for all clusters in Fig. S6. Indeed,

transcripts for all genes belonging to PG1-8 were amplified in the brain, while with the exception of *Hoxd13*, no transcripts of genes belonging to PG9-13 were detected in the brain samples. With the exception of *Hoxd10*, transcripts of PG9-10 genes were amplified in the cervical SC sample, while with the exception of *Hoxd13*, expression of all PG11-13 genes was absent in the brain and the cervical SC (Table S2). These data are in agreement with previous study showing detection of *Hox* transcripts in antero-posterior subregions of the adult SC (Sabourin et al. 2009). Our results also revealed the presence of transcripts of *Hox* genes belonging to PG5-8 in the brainstem at adulthood, whereas no expression of these genes is observed in hindbrain territories during embryogenesis (Fig. S6; Table S2). However, these data fit with the anterior expansion of expression from the SC into the caudal hindbrain observed between E10.5 and E11.5 for *Hoxb5-b8*, as well as for *Hoxa5-a6* (Ahn et al. 2014; Oosterveen et al. 2003, 2004). Our data suggest that this dynamic process probably also occurs for the other genes/clusters at some point during foetal development.

Unexpectedly, transcripts of 21 *Hox* genes, all belonging to PG1-8, were amplified from the isolated Cb (Fig. 1b; Table S2), a derivative of r1 where no *Hox* gene expression has been reported during embryogenesis. These findings are consistent with previous RT-qPCR data showing expression of 6 *HOX* genes belonging to PG1-4 in human Cb samples (Takahashi et al. 2004). Finally, a significant number of gene transcripts were detected at a relatively low level in regions derived from the forebrain, where no *Hox* gene expression is observed at any developmental stage (Fig. 1b; Table S2). The domains of expression thus seem to extend more anteriorly at adulthood than during embryogenesis.

The relative quantification method employed allowed the calculation of the abundance of individual *Hox* gene expression for each region relative to its own expression within whole embryos, and thus to compare the expression in adulthood with its global embryonic level of expression. However, despite particular care taken in the design of the experiment, expression data cannot be compared between genes as amplification parameters may vary. Among the eight regions analysed, the relative abundance of *Hox* transcripts decreased from posterior to more anterior regions in the CNS (Figs. 1d, S6). In the brainstem samples, the relative level of expression of *Hox* genes was thus higher (up to 40 times for *Hoxb5*) or in the same magnitude than their level measured in whole embryos (Fig. 1d; Table S2). In contrast, the relative level of expression in the cerebellum varied from 1.5 (*Hoxa5*) to 0.01 as compared to whole embryo expression levels. In the anterior territories, for all detected transcripts, the relative expression was always lower than the global embryonic expression levels,

varying from 0.2 to 0.01 (5–100 times lower) (examples in Fig. 1e).

In conclusion, this standardized quantitative analysis of all 39 murine *Hox* genes revealed for the first time the expression of 24 *Hox* genes in the adult mouse brain. In addition, our data revealed the presence of transcripts of *Hox* genes in brain regions derived from territories where expression has not been reported during embryonic and foetal development, namely the cerebellum and the forebrain. These data prompted us to characterize their neuroanatomical localization within the complex brain architecture.

Anatomical localization of *Hox* expression by in situ hybridization (ISH)

ISH is an invaluable molecular tool to visualize nucleic acids in their cellular environment, especially in a structurally complex tissue such as the brain. However, it is not quantitative, and as compared to RT-qPCR its applicability can be limited by its restricted detection sensitivity. Indeed, the low amount of *Hox* transcripts amplified by RT-qPCR in anterior territories of the adult mouse brain made their detection by ISH challenging. Despite high stringency conditions, long exposures were necessary to reveal non-abundant transcripts, increasing the risk of non-specific background generation. To increase the robustness of the results, ISH was reproduced on 3–10 different brains for each gene, and, whenever possible, with two independent probes. No signal was detected for *Hox* genes in the subcortical areas (caudate putamen, accumbens nucleus, globus pallidus) and in the hypothalamus. In addition, ISH is not appropriate for detecting low expression in the hippocampus and olfactory bulbs, which are prone to background in the granular layer (Fig. S4). For these reasons these four regions are not detailed below and only areas presenting reliable and reproducible labelling are described.

Hindbrain

In the adult, the hindbrain is an important centre for coordinating motor activity, posture, equilibrium, sleep patterns, and essential unconscious functions, such as breathing rhythms and blood circulation. It is composed of the Cb, the pons (Po) and the medulla oblongata (MO). In agreement with RT-qPCR data, we did not detect ISH signal for *Hox* genes belonging to PG9–13 in the hindbrain derivatives, nor in any region of the brain. For the majority of the other genes, we detected labelling in hindbrain nuclei.

First, we observed sequential A/P boundaries of expression suggesting that the spatial colinearity is

maintained at adulthood. Indeed, in sagittal sections, a clear boundary was observed between a caudal territory of widespread labelling and an anterior territory with signal restricted to specific nuclei. According to the position of the gene within the cluster the boundary was located at different A/P levels: e.g., the boundary for *Hoxb5* was more anterior than the boundary of *Hoxb6* expression, which was anterior to that of *Hoxb8* (Fig. 2a, f, k). This was confirmed in coronal sections, where PG2 to PG8 *Hox* genes showed nested overlapping domains within the rostrocaudal subnuclei constituting the spinal trigeminal nucleus, SpVO, SpVI and SpVC (Fig. 2b–d, g–i, l–n). In the dorsal hindbrain, sequential anterior boundaries of expression were also observed for different paralog *Hox* genes, as illustrated at the level of the area postrema (AP) and the caudal nucleus of the solitary tract (NTS) (Fig. 2e, j, o). At this level, we also detected expression of all *Hox* genes belonging to PG3–5, plus *Hoxb6* and *Hoxb7*, in the dorsal nucleus of the vagus nerve (dmX) which serves parasympathetic vagal functions and has its origin in r6–r7 (Kiecker and Lumsden 2005). In most of the hindbrain nuclei, *Hox* expression appeared scattered with only a fraction of neurons being labelled. This fraction varied from about 80 % (e.g., AP) to 50 % (e.g., NTS) or less in nuclei located at the anterior boundaries (e.g., SpVO). Finally, in the core region of the medulla, neuron assemblies which generate specific behaviours such as feeding and breathing are located in the central reticular formation. For the majority of *Hox* genes, labelled cells were found in a number of nuclei belonging to this hindbrain reticular formation, such as the gigantoreticular and magnocellular reticular nuclei (not illustrated). However, as these large cells also appear labelled with control probes after long incubation (see Fig. S4), we cannot completely exclude at that stage unspecific labelling of these neurons.

Rostral to their main boundary, *Hox* genes expression was also observed in highly restricted areas, as illustrated for *Hoxb5* and *Hoxb6* in the pontine nuclei (blue arrows in Fig. 2a, f). Strikingly, labelled cells were almost exclusively restricted to precerebellar nuclei. The precerebellar system provides the principal input to the Cb and is essential for coordinated motor activity. Neurons of the major precerebellar nuclei give rise to mossy fibres that synapse onto the cerebellar granule cells (Fu et al. 2013; Rodriguez and Dymecki 2000). Among those nuclei, we observed labelling for almost all genes belonging to PG2–PG5, plus *Hoxb6*, in the pontine nucleus (PN), the tegmental reticular nucleus (TRN) and the interfascicular trigeminal nucleus (IF5) located among the emerging fibres of the motor root of the trigeminal nucleus, in the anterior hindbrain (ventral r1–r2) (Fig. 3; Table 1). Associated with IF5, large scattered cells were also labelled in the principal sensory trigeminal nucleus (PrV, blue arrows

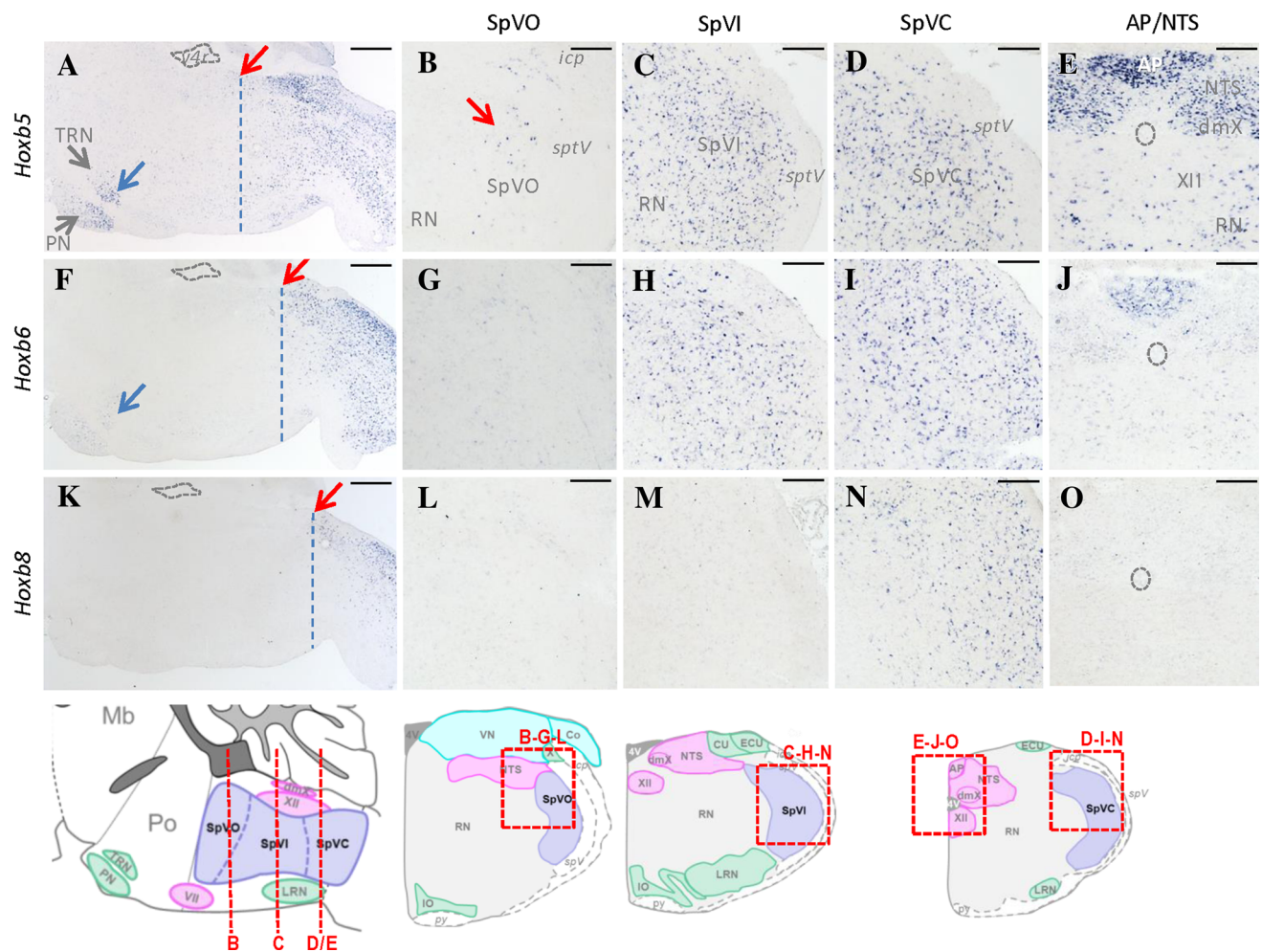


Fig. 2 *Hox* gene expression in the hindbrain of adult C57Bl/6J mice. In situ hybridization of *Hoxb5*, *Hoxb6* and *Hoxb8* on sagittal and coronal cryosections of adult mouse hindbrain. Schematic views of sagittal and coronal hindbrain sections in the *bottom* row indicate the localization of pictures. **a, f, k** On sagittal sections, a border of expression is observed at distinct antero-posterior levels for the three genes (*dotted blue line*). The anterior limit is clearly visible on the dorsal region (*red arrows*). Anterior to this border, expression is also detected in discrete areas, such as the pontine nuclei (PN) and the tegmental reticular nuclei (TRN) (*blue arrows*). **b–d, g–i, l–n** On coronal sections, expression within the antero-posterior subnuclei constituting the spinal trigeminal nucleus (SpV) showed nested overlapping domains with sequential anterior boundaries. *Hoxb5* is expressed in the oral part (SpVO *red arrow*), the intermediate part

(SpVI) and the caudal part (SpVC). *Hoxb6* is expressed in the SpVI and SpVC, and *Hoxb8* is expressed only in SpVC. **e, j, o** On coronal sections, expression within the dorsal region of the caudal adult hindbrain showed partially overlapping patterns. *Hoxb5* is expressed in the area postrema (AP), the nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus nerve (dmX). *Hoxb6* is expressed only in the AP and scattered cells in the NTS, while no labelling is detected for *Hoxb8*. *Dotted circles* mark the ventricle. On sagittal sections, anterior is on the *left*; on coronal sections medial is on the *left*. Fibre tracts are in *italic*. *v4r* fourth ventricle, lateral recess; *sptV* spinal tract of the trigeminal nerve; *icp* inferior cerebellar peduncle; *RN* reticular nucleus; *XII* hypoglossal nucleus. *Scale bar* 500 μ m in **a, f** and **k**; 200 μ m in others

in Fig. 3b, f, j), which likely correspond to precerebellar neurons previously identified by retrograde labelling (Fu et al. 2013). Expression of the same genes, together with a few genes belonging to PG6–PG8, was also detected in the nucleus x, the lateral reticular nucleus (LRN) and the external cuneate (ECU), that are located more caudally (r7–r8) (Fig. 3; Table 1). Within the pontine nuclei, regionalized patterns were observed according to PG groups (data not shown), as previously reported (Di

Meglio et al. 2013; Geisen et al. 2008). Neurons of the precerebellar mossy fibres nuclei arise from tangential migration of the progenitors issued from the caudal part of the lower rhombic lip (r6–r8) (Farago et al. 2006; Rodriguez and Dymecki 2000), more precisely from the dorsal aspect of the rhombic lip (Ray and Dymecki 2009). It was of note that signal was also observed in the climbing fibres precerebellar nuclei, forming the inferior olive (IO), whose neurons synapse directly on Purkinje cells. Although

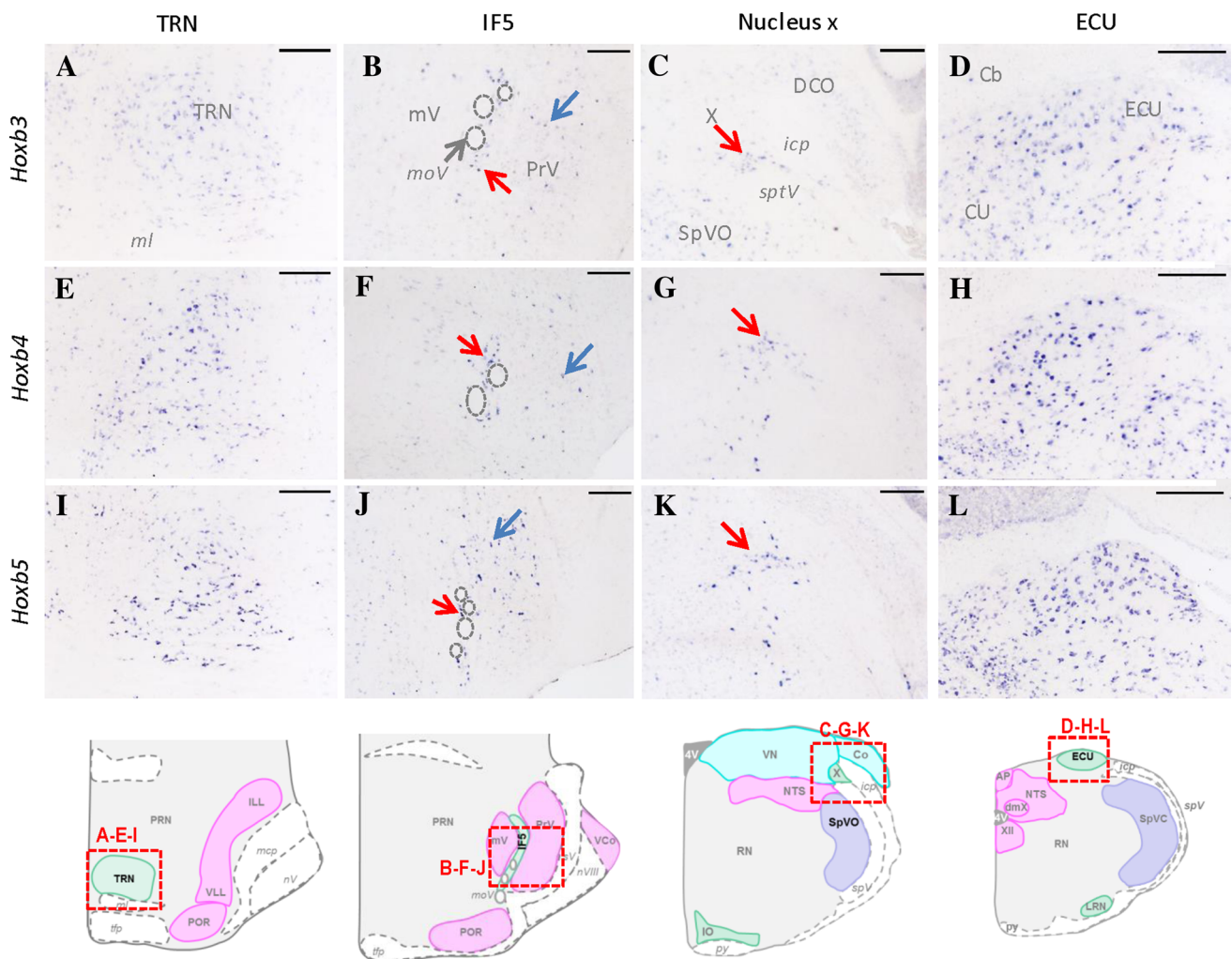


Fig. 3 *Hox* gene expression in mossy fibres precerebellar nuclei. In situ hybridization of *Hoxb3*, *Hoxb4* and *Hoxb5* on coronal cryosections of adult mouse hindbrain. Schematic views of coronal hindbrain sections in the *bottom row* indicate the localization of pictures. **a, e, i** Expression in the tegmental reticular nucleus (TRN) located in the pons. **b, f, j** Expression in the interfascicular trigeminal nucleus (IF5 *red arrows*) located among the emerging fibres of the motor root of the trigeminal nucleus (*moV dotted circles*). Note that scattered cells are labelled in the principal sensory nucleus of the

trigeminal (PrV *blue arrows*). **c, g, k** Expression in the nucleus x (*red arrows*), located dorsally in the medulla oblongata close to the oral part of the spinal nucleus of the trigeminal (SpVO). **d, h, l** Expression in the external cuneate nucleus (ECU), located dorsally in the caudal medulla oblongata. Medial is on the *left*, lateral is on the *right*. Fibre tracts are in *italic*. *ml* medial lemniscus, *mV* motor nucleus of the trigeminal *sptV* spinal tract of the trigeminal nerve, *icp* inferior cerebellar peduncle, *DCO* dorsal cochlear nucleus, *Cb* cerebellum, *CU* cuneate nucleus. Scale bar 200 μ m

precerebellar climbing fibre neurons also arise from migration of neurons from the lower rhombic lip (r7-r8), they are issued from progenitors located in the ventral aspect of the rhombic lip (Ray and Dymecki 2009). In this nucleus, only a subset of *Hox* genes were expressed, among which were all PG1-8 members of the *HoxD* cluster, namely *Hoxd1*, *Hoxd3*, *Hoxd4*, *Hoxd8* and some members of the *HoxB* cluster, namely *Hoxb2*, *Hoxb3* and *Hoxb5*, plus *Hoxa3* (Fig. 4; Table 1). Strikingly, no members of the *HoxC* cluster were expressed in this nucleus. It is worth mentioning that the only ISH labelling of *Hoxd1* was detected in the IO and the neighbouring

raphe obscurus (RO), and that expression of *Hoxd1* has not been reported in the mouse hindbrain at embryonic stages (Frohman and Martin 1992). Close to the IO, we also detected regionalized labelling for the same *Hox* genes in serotonergic raphe nuclei: the posterior part of the RO, the raphe pallidus (RPA) and raphe magnus (Fig. 4 and data not shown). It has been previously reported that expression of *Hox* genes belonging to PG2-5 is maintained at foetal stages in migrating and settling pontine neurons (Geisen et al. 2008). We show here that expression is maintained till adulthood, and is also observed in other precerebellar nuclei, including both mossy and climbing

Table 1 Expression of *Hox* genes in major precerebellar nuclei, classified according to their migration stream

	Mossy fibers nuclei						Climbing fibres nuclei
	AMS			PMS			IMS
	PN	TRN	IF5	Nu x	LRN	ECU	IO
PG1							
Hoxa1	O	O	O	O	O	O	O
Hoxb1	O	O	O	O	O	O	O
Hoxd1	O	O	O	O	O	O	X
PG2							
Hoxa2	X	X	X	X	X	X	O
Hoxb2	X	X	X	X	X	X	X
PG3							
Hoxa3	X	X	X	X	X	X	X
Hoxb3	X	X	X	X	X	X	X
Hoxd3	O	O	X	X	O	X	X
PG4							
Hoxa4	X	X	X	X	X	X	O
Hoxb4	X	X	X	X	X	X	O
Hoxc4	X	X	X	X	X	X	O
Hoxd4	X	X	X	X	X	X	X
PG5							
Hoxa5	X	X	X	X	X	X	O
Hoxb5	X	X	X	X	X	X	X
Hoxc5	X	X	X	X	X	X	O
PG6							
Hoxa6	O	O	O	O	O	O	O
Hoxb6	X	X	O	O	X	O	O
Hoxc6	O	O	O	X	X	X	O
PG7							
Hoxa7	O	O	O	O	X	X	O
Hoxb7	O	O	O	O	O	O	O
PG8							
Hoxb8	O	O	O	O	O	O	O
Hoxc8	O	O	O	O	O	O	O
Hoxd8	O	O	O	X	X	X	X
PG9							
Hoxa9	O	O	O	O	O	O	O
Hoxb9	O	O	O	O	O	O	O
Hoxc9	O	O	O	O	O	O	O
Hoxd9	O	O	O	O	O	O	O
PG10							
Hoxa10	O	O	O	O	O	O	O
Hoxc10	O	O	O	O	O	O	O
Hoxd10	O	O	O	O	O	O	O
PG11							
Hoxa11	O	O	O	O	O	O	O
Hoxc11	O	O	O	O	O	O	O
Hoxd11	O	O	O	O	O	O	O

Table 1 continued

	Mossy fibers nuclei						Climbing fibres nuclei
	AMS			PMS			IMS
	PN	TRN	IF5	Nu x	LRN	ECU	IO
PG12							
Hoxc12	O	O	O	O	O	O	O
Hoxd12	O	O	O	O	O	O	O
PG13							
Hoxa13	O	O	O	O	O	O	O
Hoxb13	O	O	O	O	O	O	O
Hoxc13	O	O	O	O	O	O	O
Hoxd13	O	O	O	O	O	O	O

Cross indicates the presence of ISH labelled cells within the nucleus (either scattered, regionalized or ubiquitous) while “O” indicates the absence of reliable signal within the nucleus. On the basis of our observation of *Hox* expression profiles, we have here associated IF5 to the AMS

AMS anterior extramural migrating stream, PMS posterior extramural migrating stream, IMS intramural migrating stream, PN pontine nuclei, TRN tegmental reticular nucleus, IF interfascicular trigeminal nucleus, Nu x nucleus x, LRN lateral reticular nucleus, ECU external cuneate nucleus, IO inferior olive, PG paralogy group

fibres nuclei. Our data also suggest that all area of expression of *Hox* genes in the adult MO and Po derived from their embryonic/foetal expression domain, as rostral nuclei expressing *Hox* genes originate from tangential migration of progenitors from the caudal hindbrain.

However, RT-qPCR data also revealed the presence of transcripts in the Cb, located in the most anterior part of the dorsal hindbrain and implicated in movement coordination. The mature Cb is made up of several distinct cell types: the giant Purkinje cells (PCs), the principal output neurons; and the very small granule cells. As discussed above, ISH does not allow the reliable detection of expression in granule cell layers and we cannot conclude at this stage as to the presence (or absence) of low expression of *Hox* genes in cerebellar granule cells. In PCs we could detect ISH signal for *Hoxa5* (data not shown), which had the highest relative expression in RT-qPCR (Table S2). For all other genes for which we detected expression by RT-qPCR at levels lower than the embryonic expression level, we did not observe reliable ISH labelling. Indeed, although we could in some experiments observe labelling after long incubations, we could not make conclusions as to specific expression, as PCs were also labelled with control probes after long incubations (see Fig S4).

RT-qPCR analysis did not allow the evaluation of expression in the midbrain, included in brainstem samples, as no precise anatomical landmark allows its reliable

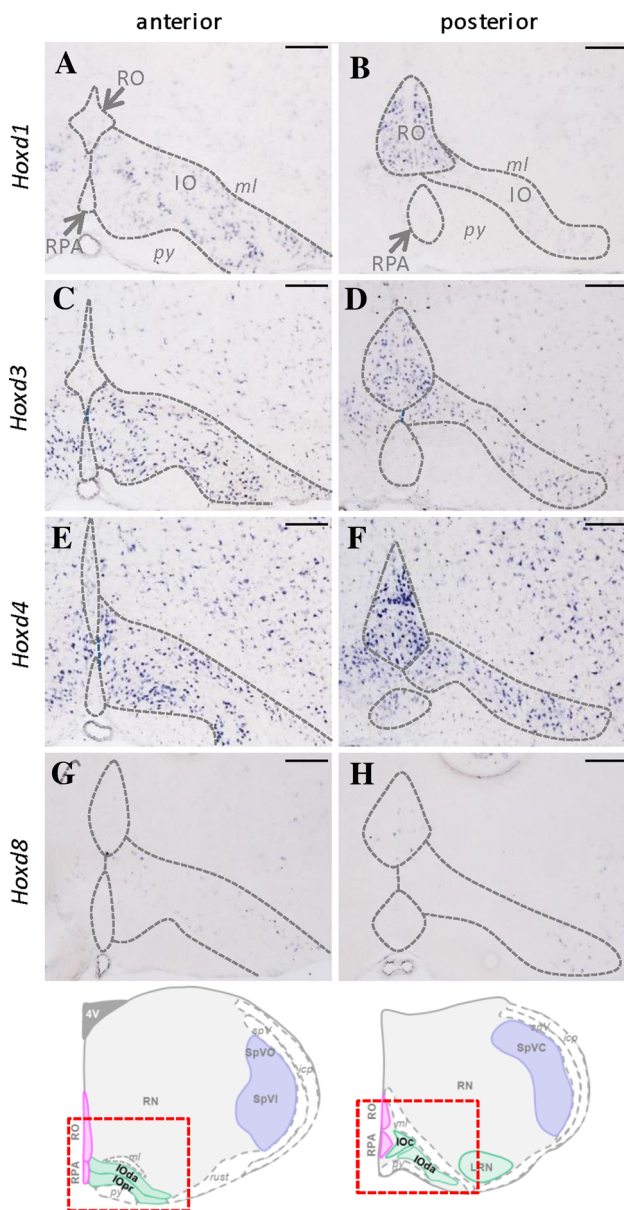


Fig. 4 *Hox* gene expression in climbing fibres precerebellar nuclei, the inferior olive (IO). In situ hybridization of *Hoxd1*, *Hoxd3*, *Hoxd4* and *Hoxd8* on coronal cryosections of adult mouse hindbrain. Schematic views of coronal hindbrain sections in the bottom row indicate the localization of pictures. **a, c, e, g** In the anterior region of the IO labelling is observed for the four *Hoxd* genes, although with variable expression level for *Hoxd1* and with scattered pattern for *Hoxd8*. At that level, no labelling is observed in the neighbouring raphe nuclei, the raphe obscurus (RO) and raphe pallidus (RPA). **b, d, f** In the caudal region of the IO, labelling is not detected for *Hoxd1* but is still present for *Hoxd3*, *Hoxd4* and *Hoxd8*. In addition, at that level labelling is detected in the RO, but not in the RPA. Medial is on the left, lateral is on the right. Fibre tracts are in italic. *ml* medial lemniscus; *py* pyramid. Scale bar 200 μ m

dissection without contamination from neighbouring areas. However, no ISH signal over background level was detected in the midbrain for any *Hox* gene.

Forebrain

The mouse neocortex is a highly organized, six-layered structure that contains glia and radially extending neurons. The neurons of each layer are born concurrently and as a result their gene expression and subsequent growth share key similarities (Greig et al. 2013; Molyneaux et al. 2007). In sensory regions, layer 4 (granular layer) is prominent, and is the principal destination of thalamo-cortical fibres. Layer 5 contains the larger pyramidal neurons of the cortex, which project axons to cortical or sub-cortical targets. In addition, functional areas have been identified in the cortex, mainly on the basis of their relationship with specific thalamic nuclei.

ISH confirmed the presence of transcripts of a subset of *Hox* genes within the neocortex that includes *Hoxb1*, *Hoxb3*, *Hoxb4*, *Hoxd3*, and *Hoxa5*. For *Hoxb1*, *Hoxb3* and *Hoxa5*, labelling was observed in the majority of brains analysed ($n = 7-10$), while for *Hoxb4* and *Hoxd3*, labelling was more variable from one brain to another, a potential consequence of the low level of expression. All genes showed a similar expression pattern, with mRNA restricted to a narrow row of cells closely associated to layer 5 as illustrated in the somatosensory and visual cortices (Fig. 5a–h). Although *Hox* gene expression seems to be associated to cortical layer and not to cortical area identities, the expression appeared restricted to functional sensory areas characterized by a prominent granular layer 4 (somatosensory, visual, auditory), and was not observed in the agranular cortex (e.g., see Fig. S7), suggesting a link with layer 4 neurons. In addition, in motor areas, no signal above background levels was observed.

Over the past decade high-throughput efforts to define laminar and subtype-specific gene expression in the neocortex have led to the identification of an increasing number of molecular markers of cortical layers. To gain information about the layer(s), and cortical subpopulations, expressing *Hox* genes in the adult neocortex, we applied double ISH with *Cux2*, labelling layers 2–4 (Zimmer et al. 2004); *Ctip2*, mainly labelling layer 5/6 and the subplate (Hevner et al. 2003); and *Plxnd1*, labelling layers 2–5a (Watakabe et al. 2006). Double ISH are illustrated with *Hoxb3* as the representative gene (Fig. 6). In the primary somatosensory cortex, comparison of *Hoxb3* with *Cux2* showed that *Hoxb3*+ cells were distinct from *Cux2*+ cells, although they were located very close to the border of *Cux2* expression. However, in double ISH with *Ctip2*, labelling deep layers 5/6, *Hoxb3*+ cells were also negative for *Ctip2* (data not shown). According to Molyneaux et al. (2007), the gap between *Ctip2* and *Cux2* expression domains in the adult cortical layers would correspond to sublayer 5a. Double ISH with *Plxnd1*, which expression pattern in the adult mouse neocortex includes layer 5a, revealed that

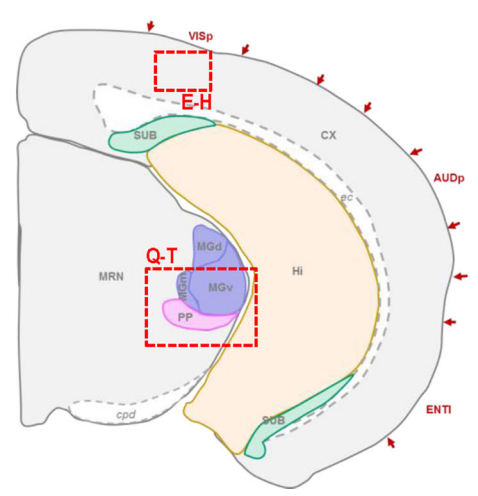
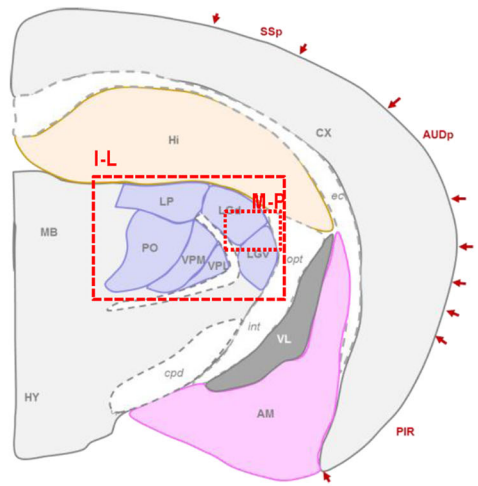
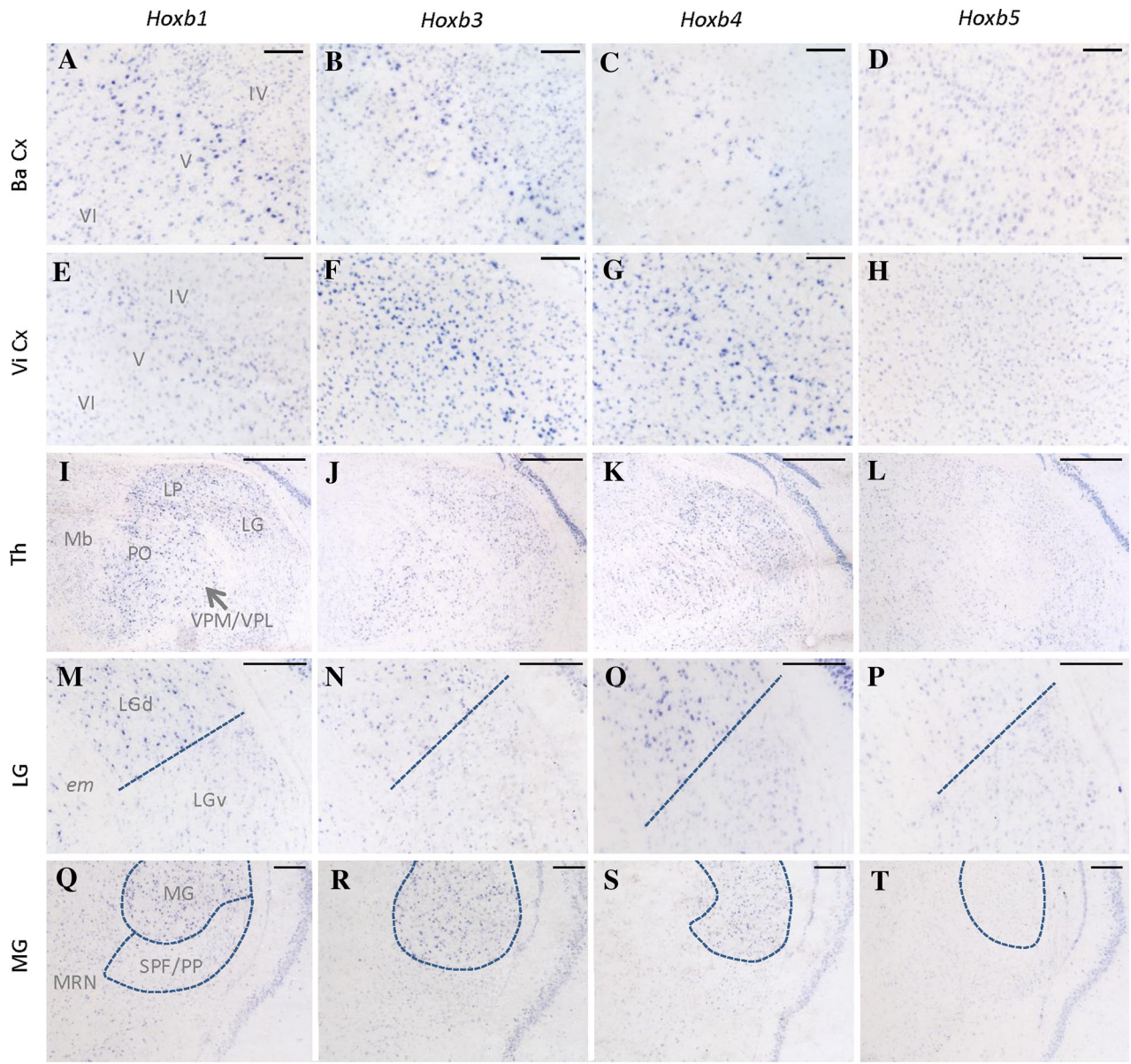


Fig. 5 *Hox* gene expression in the forebrain of adult C57Bl/6J mice. In situ hybridization of *Hoxb1*, *Hoxb3*, *Hoxb4* and *Hoxb5* on coronal cryosections of adult mouse forebrain. Schematic views of coronal forebrain sections in the *bottom row* indicate the localization of the pictures. **a–h** In the barrel field cortex (Ba Cx) and visual cortex (Vi Cx) blue labelled cells are observed in a stripe located medially between layer IV and layer V for *Hoxb1*, *Hoxb3* and *Hoxb4*. No signal is observed for *Hoxb5*. **i–l** In the posterior thalamus (Th), the signal is observed dorsally in the lateral geniculate complex (LG), as well as in the somatosensory nuclei, the ventral postero-medial (VPM) and the ventral posterolateral (VPL) nuclei for *Hoxb1*, *Hoxb3* and *Hoxb4*. No signal is observed in the midbrain (Mb). **m–p** In the LG, regionalized labelling was observed for *Hoxb1*, *Hoxb3* and *Hoxb4*, as only the dorsal nucleus (LGd) but not the ventral nucleus (LGv) is labelled. **q–t** In the most posterior region of the thalamus, the medial geniculate complex (MG) is clearly labelled for *Hoxb1*, *Hoxb3* and *Hoxb4*, while the suprafascicular/peripeduncular (SPF/PP) nucleus is not labelled. Note that no labelling is detected for *Hoxb5* which is presented to illustrate the background level in these regions. Medial is on the *left*, lateral is on the *right*. Fibre tracts are in *italic. em* external medullary lamina, *LP* lateral posterior nucleus, *MRN* midbrain reticular nucleus. Scale bar 100 μ m in **a–h**; 500 μ m in **i–l**; 200 μ m in **m–t**

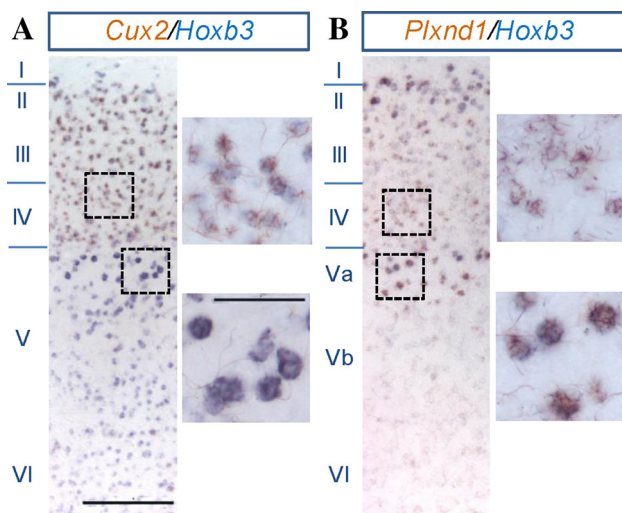


Fig. 6 *Hoxb3* expression in the cortex of adult C57Bl/6J mice. Double in situ hybridization of *Hoxb3* with *Cux2* and *Plxnd1* on coronal cryosections of the adult mouse somatosensory cortex. **a** *Hoxb3* (blue labelling) and *Cux2* (brown labelling) expression in the 6 layers of the somatosensory cortex (*left panel*) show a distinct pattern for both genes. *Square boxes* refer to magnification images on the *right panel* confirming the absence of co-localization. **b** *Hoxb3* (blue labelling) and *Plxnd1* (brown labelling) expression in the 6 layers of the somatosensory cortex (*left panel*) show a partly overlapping pattern in layer 5a, which is visible in the magnified picture on the right, showing superposition of blue and brown precipitates. Scale bar: 200 μ m in *left panels*; 50 μ m in magnifications in the *right panels*

most of *Hoxb3*+ cells were also *Plxnd1*+. Altogether these data suggest that *Hox* gene expression is restricted to neurons located in layer 5a in sensory cortices.

It should be mentioned that transcripts of the same *Hox* genes were detected in the piriform cortex, the primary

olfactory cortex, and in the entorhinal cortex, the interface between the hippocampus and neocortex (Fig. S7). Being more primitive regions of the cerebral cortex, these areas display only three distinct layers. *Hox* expression was again restricted to a stripe of cells located at the external side of layer 2 (layer 2a).

Finally, low but reproducible signal above the background level was observed in the thalamus. The thalamus receives input from touch, auditory and visual pathways, among others, and sends fibres to the cerebral cortex (Jones 1998). The thalamus is thus the most significant subcortical input to the neocortex, and in turn receives an even larger cortico-thalamic projection. Specific afferents terminate in all layers, but concentrate in layer 4. The axons specifically target principal neurons and interneurons, and maintain a strong topographic organization in sensory projections. It was noticeable that all *Hox* genes for which we detected transcripts in the neocortex also showed staining within the thalamus, i.e., *Hoxb1*, *Hoxb3*, *Hoxb4*, *Hoxd3*, *Hoxa5*. Particular attention was paid to the sensory relay projecting to the area of the neocortex described above: the part of the thalamus that receives sensory information from the eye is called the dorsal lateral geniculate nucleus (LGd); the somatosensory (touch sensation) nuclei of the thalamus are the VPM and VPL; and the medial geniculate nuclei (MG) form the auditory thalamus. All these nuclei contained labelled neurons (Fig. 5i–t; Table S3). In contrast, we never observed labelled cells within some thalamic nuclei, such as the reticular and reunions nuclei (Table S3). As observed in the hindbrain, *Hox* expression in thalamic nuclei appeared scattered with only a fraction of neurons being labelled.

In conclusion, within the range of sensitivity of ISH, we have confirmed the presence of *Hox* gene transcripts at adulthood in anterior territories of the brain, derived from areas in which expression of *Hox* genes has not been reported at embryonic stages. As cortical and thalamic neurons are born within the forebrain, these domains of expression cannot be explained by migration of progenitors expressing *Hox* at embryonic stages, and would suggest de novo transcription of *Hox* loci after birth.

To gain insight into the dynamic of expression of these *Hox* genes, we carried out ISH on foetal and postnatal brains. Expression in the caudal hindbrain/MO was observed at all stages analysed (from E16.5 till postnatal day P21), confirming the maintenance of *Hox* expression from embryonic to adult stage in rhombencephalon derivatives. Expression in the pons was already present at E16.5, in agreement with the timing of precerebellar neurons migration (Fig. 7a, b). However, no signal was observed in forebrain areas (Cx and Th) at foetal and early postnatal stages, as illustrated for *Hoxb3* (Fig. 7c, e, g). Between P12 and P16, faint signal could be observed on some sections

for a few genes (e.g. *Hoxa5*); however, it was often variable and not reproducible. The first reliable and reproducible signal was detected at P18, both in the Th and Cx, for all genes analysed (Fig. 7d, f, h). These data support the hypothesis that expression in the anterior territories is due to the neo-activation of a few *Hox* genes transcription during the third postnatal week.

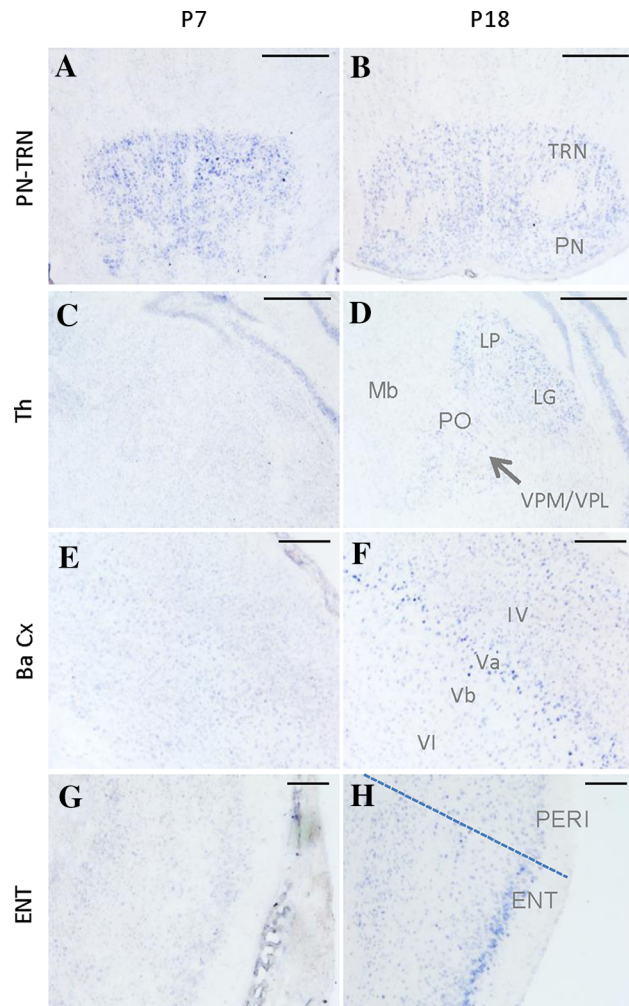
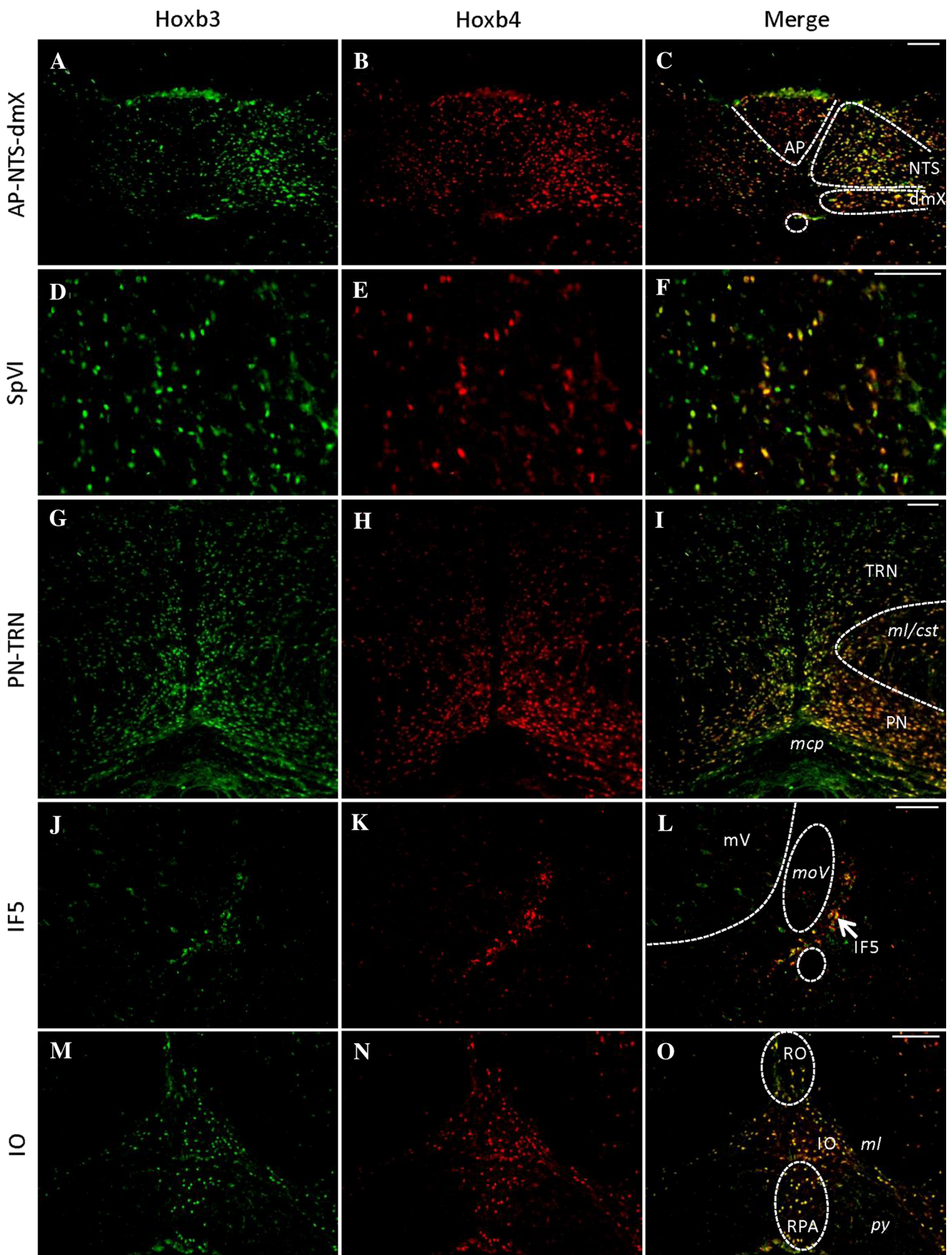


Fig. 7 Dynamics of *Hoxb3* expression at postnatal stages. In situ hybridization of *Hoxb3* on coronal cryosections of mouse brains at postnatal stages P7 and P18. **a, b** *Hoxb3* is expressed at both stages in the pontine nuclei (PN) and tegmental reticular nuclei (TRN). **c, e, g** At stage P7 no signal is detected in the thalamus and cortex. **d, f, h** At stage P18 signal is detected in the thalamus and cortex in accordance with the expression pattern observed in the adult brain. Medial is on the *left*, lateral is on the *right* for **c–h**. *Th* thalamus, *Mb* midbrain, *PO* posterior complex of the thalamus, *LP* lateral posterior nucleus, *LG* lateral geniculate complex, *VPM/VPL* ventral posteromedial/ventral posterolateral nuclei; *Ba Cx* barrel field cortex, *ENT* entorhinal cortex, *PERI* perirhinal cortex. Scale bar 500 μ m in **a–d**; 200 μ m in **e–h**

Fig. 8 Hox protein detection in the hindbrain of adult C57Bl/6J mice. Coronal cryosections of adult mouse hindbrain immunolabeled for Hoxb3 and Hoxb4. **a–f** In the caudal hindbrain, Hoxb3 and Hoxb4 proteins are present in scattered cells of the area postrema (AP), in the nucleus of the solitary tract (NTS), in the dorsal motor nucleus of the vagus nerve (dmX) and in the intermediate part of the spinal trigeminal nucleus (SpVI). The proteins largely co-localize in these nuclei. **g–l** In mossy fibres precerebellar nuclei, Hoxb3 and Hoxb4 co-localize in the pontine nuclei (PN), the tegmental reticular nucleus (TRN) and the interfascicular trigeminal nucleus (IF5). **m–o** In climbing fibres precerebellar nuclei, the proteins co-localize in the inferior olive (IO). Labelling is also present in the raphe obscurus (RO) and raphe pallidus (RPA). Medial is on the *left*, lateral is on the *right* for **d–f** and **j–l**. Fibre tracts are in *italic*. *ml* medial lemniscus, *cst* corticospinal tract, *mcp* middle cerebellar peduncle, *mV* motor nucleus of the trigeminal, *moV* motor root of the trigeminal nerve, *py* pyramid. Scale bar 100 μ m

Hox proteins in the brain

Due to the systematic nature of our project, and to the difficulties in obtaining specific and efficient antibodies for each Hox protein, we analysed in this study the presence of *Hox* gene transcripts. In the final part of the study we nevertheless aimed to verify the presence of Hox proteins in the adult brain. Based on published data, we selected antibodies directed at Hoxb3 and Hoxb4 (Calonge et al. 2007; Gould et al. 1998). In the caudal hindbrain, we observed the presence of both proteins in the majority of nuclei labelled in ISH and described above, including the SpV, AP, NTS, dmX (Fig. 8a–f). We also confirmed the presence of Hoxb3 and Hoxb4 proteins within precerebellar nuclei, both in mossy fibres and climbing fibres neurons, as illustrated for PN, TRN, IF5 and IO (Fig. 8g–o). In all these nuclei, we could observe single- and/or co-labelled cells, suggesting that Hoxb3 and Hoxb4 proteins partly co-localize. In contrast, we could not detect IHC signal in the forebrain areas (Cx and Th). As antibodies have been validated and used mainly on embryonic tissues, this discrepancy between mRNA and protein detection could be explained by a lower sensitivity of the procedure. Alternatively, *Hox* genes are known to have complex transcription patterns relying on the use of multiple promoters, differential splicing of RNAs from a common promoter, and antisense transcription (Brock et al. 2009; Mainguy et al. 2007). Notably, the presence of multiple transcripts generated by alternative promoter usage and alternative splicing has been reported for *Hoxb3* and *Hoxa5*, and in the case of *Hoxa5*, it has been demonstrated that the alternative transcripts are non-coding (Coulombe et al. 2010; Sham et al. 1992). Long non-coding RNAs (lncRNAs) have been reported to have, on average, a lower expression level than protein coding genes, and to have cell type-specific expression (Batista and Chang 2013). The transcripts detected in anterior territories of the brain at adulthood could thus be lncRNAs.



Discussion

This work addressed the poorly investigated question of the presence of *Hox* transcription in the adult CNS. Our study provides several lines of evidence supporting the idea that *Hox* loci are transcribed in the brain at adulthood, thus strongly suggesting that *Hox* genes are recruited to serve new functions in mature neurons. Indeed, we demonstrate that expression of all *Hox* genes is maintained at adulthood in territories derived from the early segmental *Hox* expression domains in the hindbrain. For two *Hox* genes, we confirm the production of the protein in these territories. In addition, our data show the presence of transcripts of *Hox* genes in territories where they have not been described during embryonic and foetal development, suggesting neo-expression in these territories at adulthood. Before discussion of the results, we will first address issues regarding data interpretation.

Expression data analysis

Part of this paper deals with low expression signals, supposedly reflecting the low abundance of target transcripts, as measured by RT-qPCR or visualized by ISH. Whatever the procedure involved, low signal always needs to be carefully evaluated, raising the unavoidable questions of specificity and sensitivity. The procedures used in this paper are highly standardized and have been optimized for the brain and for the analysis of rare transcripts, as detailed in the “Materials and Methods”. Nevertheless, there are potential caveats that could interfere with the data interpretation. Regarding RT-qPCR analysis, one could question cross-contamination of samples during small mouse brain dissection. For the anterior part of the brain the risk is low as no territories of high *Hox* expression are in proximity to the Cx. However, during Cb isolation, cells from the underlying BS could contaminate the sample. Amplification of genes which expression is present only in the BS and not in the Cb was used as a control of potential contamination, and three potentially contaminated samples were discarded. Similarly, Hi and Hy samples could be contaminated by the neighbouring thalamus. As ISH did not confirm expression, we did not discuss these two regions in this paper. Regarding ISH, the most common problem is non-specific hybridization of probe to tissues that can lead to artefactual signal. As detailed in the “Materials and Methods”, negative control probes were always used allowing us to establish a “brain map” of common background and potential artefacts (see Fig. S4–S5), resulting in the exclusion of a few brain regions from the analysis (OB, Hi, Cb granular cell layer). Nevertheless, the final interpretation of specific signal versus unspecific background relies on the impartial expertise of researchers.

This remains true even for automated signal detection such as those used in the Allen Brain Atlas, where expression thresholds are calibrated through expert confirmation (Lein et al. 2007). Keeping this in mind, we have curated our data to ensure their quality and accuracy, and we are discussing here only the results for which expression data were reliable and consistent between both procedures.

Maintenance of *Hox* expression in the adult hindbrain

Within the embryonic hindbrain, PG1–PG4 *Hox* genes display nested overlapping domains of expression with rhombomere-specific boundaries (Nolte and Krumlauf 2007). During foetal development, changes in anterior expression boundaries reported for the *HoxB* cluster result in the presence of increasing numbers of *Hox* transcripts in caudal rhombomeres (r7/r8) (Oosterveen et al. 2003). In parallel tangential migration processes reported for *Hox* PG2–PG5 expressing cells result in *Hox* expression within pontine nuclei located in the rostral hindbrain (Geisen et al. 2008). The data presented in this paper go one step further, unravelling expression of *Hox* genes in the hindbrain well after the foetal and postnatal stages. Indeed, our systematic analysis performed on 12-week-old mice revealed the presence of 24 *Hox* genes transcripts, which are most likely translated into proteins, as demonstrated for *Hoxb3* and *Hoxb4*. The neuroanatomical patterns, together with the spatio-temporal analysis, strongly suggest maintenance of transcription at *Hox* loci from embryonic to adult stages.

In the BS the RT-qPCR allowed amplification of transcripts of 24 *Hox* genes, belonging to PG1 to PG8, at a relative abundance that is close or higher than their expression level in whole embryos. The neuroanatomical localization of transcripts by ISH confirmed the expression of the majority of genes belonging to PG1 to PG8 in the adult hindbrain with specific patterns. As a general rule, the expression of *Hox* genes was observed in their rhombomere-derived post-mitotic neurons, with the anterior boundary still visible at adulthood (see Fig. 2). Posterior to this boundary, the labelling was often widespread within the MO. These sequential A/P expression patterns in the adult hindbrain suggest the maintenance of the spatial colinearity rule at adulthood. They also imply that post-mitotic cells within the adult MO maintain expression of specific *Hox* gene combinations suggesting that the “Hox code” may be required for particular neuronal functions.

Interestingly, our analysis also revealed that, except for *Hoxc8*, all *Hox* genes belonging to PG5–PG8 are expressed in the caudal hindbrain at adulthood, that are not only *HoxB* genes as previously reported (Oosterveen et al. 2003, 2004), but also genes belonging to *HoxA*, *HoxC* and *HoxD* clusters. Although similar observations have recently been made for *Hoxa5* and *Hoxa6*, no anterior shift was observed

for *Hoxa7*, nor reported for *HoxC* and *HoxD* genes (Ahn et al. 2014). This anterior expansion is dependent upon retinoic acid signalling, and as recently demonstrated, relies on long-range regulation by combined RARE elements (Ahn et al. 2014; Oosterveen et al. 2003). Based on our data, we can propose that expansion occurs later than E11.5 for the other genes, but most likely before E14.5. Indeed, expression of *Hoxa7* and *Hoxd8* in precerebellar nuclei (Table 1) suggests that they were expressed in the caudal hindbrain at the time of tangential migration. This later shift could potentially rely on the RARE conserved region described 5' to all *Hox4* paralogs (a4u-, b4u-, c4u and d4u-RARE) (Ahn et al. 2014). Finally, based on their expression patterns, notably in the precerebellar nuclei, we can estimate that all these genes are expressed in r8, and for a few of them (e.g., *Hoxb6*), also in r7.

Another key feature of our analysis in the adult hindbrain is the importance of *Hox* gene transcripts within the main precerebellar nuclei. Previous studies reported that migrating pontine neurons at foetal stages express a *Hox* program characteristic of the r6–r8 axial origin, namely *Hox* PG2–5 genes (Di Meglio et al. 2013; Geisen et al. 2008). The PN migrating neurons form the anterior extramural migrating stream (AMS) (Ray and Dymecki 2009). Here, we show that expression of these genes is maintained in the PN until adulthood. Moreover, we report expression of *Hox* PG2–PG8 genes in the caudal precerebellar nuclei (ECU, LRN), that arise from the posterior extramural migrating stream (PMS). The observation that PG6–PG8 *Hox* genes are for the majority not expressed in the AMS and transcribed in the PMS derived nuclei suggest that the AMS is issued from r6 to r7, while PMS is issued from r7 to r8, in agreement with former hypotheses (Farago et al. 2006). We also reported for the first time the presence of transcripts of *Hox* genes in the inferior olive nuclei. IO migrating cells form the intramural migrating stream (IMS), issued from the r7 to r8 lower rhombic lip, and more specifically from the ventral aspect of the rhombic lip. As we mainly observed the expression of *HoxD* and *HoxB* genes belonging to PG1–PG8 in these cells, and not of *HoxC* genes, it suggests that during foetal development, the dorso-ventral expression of the genes varied between clusters. It would be interesting to evaluate the processes regulated by the IO in *HoxD* mutants, e.g., motor learning and control. Indeed, although *HoxD* mutants are mostly viable, no CNS or behavioural phenotype has been reported so far (Condie and Capecchi 1993; Guo et al. 2011; Horan et al. 1995).

Hox neo-expression in anterior brain territories

Thanks to the sensitivity and robustness of the RT-qPCR analysis, our study also revealed the expression of *Hox*

genes in adult brain samples derived from territories where no *Hox* gene expression has been reported during embryogenesis. Indeed, transcripts of 21 genes belonging to PG1–8 were amplified from the Cb, transcripts of 11 *Hox* genes were amplified from the isolated Cx, and 16 *Hox* genes transcripts were amplified from samples containing the Th. The relative abundance of these transcripts was always lower than the expression level measured in whole embryo samples. However, the high-resolution ISH revealed region and cellular-specific expression patterns for a few *Hox* genes within these territories. As compared to qPCR, ISH has a lower sensitivity threshold, which could explain discrepancies between both procedures in the number of genes detected in these territories. The temporal analysis of expression patterns revealed that transcription at *Hox* loci in these territories started at postnatal stages, most likely during the third postnatal week. Currently, we have no indication that *Hox* proteins are translated from these transcripts. Nevertheless, our analyses underline key features of *Hox* genes expression in the Cb and in forebrain-derived structures that are discussed here.

The Cb is derived from r1 (Zervas et al. 2005). During embryonic development *Hoxa2*, the most anterior *Hox*, is expressed up to the r1/r2 boundary, while *Hoxa1* and *Hoxb1* are expressed up to the r3/r4 boundary (Nolte and Krumlauf 2007). No *Hox* gene expression has thus been described in r1 at any developmental stage. In this territory expression of *Hox* genes detected by RT-qPCR can hardly be explained by migration of cells from caudal rhombomeres, thus suggesting neo-expression of the 21 *Hox* genes within cerebellar cells. For *Hoxa5* which expression is detected at a relative level close to embryonic expression level, ISH localized expression principally within the PCs, the main output neurons of the Cb. Our results are thus in agreement with previous data reporting *Hoxa5* expression in the Cb, at both RNA and protein level (Sanlioglu et al. 1998). These authors also identified a direct target of *Hoxa5*, the *Pcp2* Purkinje-specific gene, which has been involved in dendrites growth and synaptogenesis (Redd et al. 2002; Zhang et al. 2002). Our analysis suggests that other *Hox* genes may have important roles to play in cerebellum activity at adulthood.

An unexpected feature of our analysis is the presence of *Hox* transcripts within the adult forebrain, with a unique pattern that is summarized in Fig. 9. In this structure, ISH allowed us to characterize the neuroanatomical expression patterns of five *Hox* genes, namely *Hoxb1*, *Hoxb3*, *Hoxb4*, *Hoxd3*, and *Hoxa5*. In the neocortex, *Hox* genes are expressed in a layered pattern, with mRNA restricted to a narrow row of cells localized within layer 5a by double labelling. In addition, restricted expression was present as a stripe of cells at the external side of layer 2 in more primitive regions of the cortex. Finally, specific relay

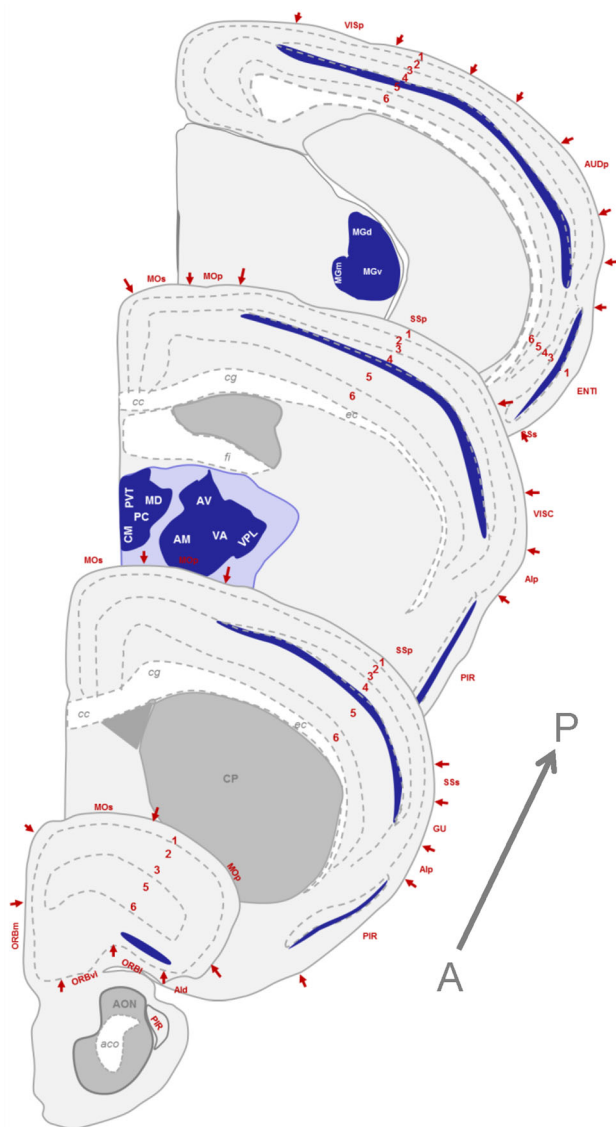


Fig. 9 Summary of the expression of *Hox* genes in the forebrain territories of adult mice. Schematic views of coronal brain sections along the antero-posterior (A/P) axis of the forebrain synthesizing the expression pattern observed for *Hox* genes in the cortex and in the thalamus (dark blue labelling). Similar pattern was observed for *Hoxb1*, *Hoxb3*, *Hoxb4*, *Hoxa5* and *Hoxd3*

nuclei in the thalamus were labelled. The cortical areas where signal was detected (visual, auditory, somatosensory), the close association of labelled cells with layer 4, and the associated expression in thalamic sensory relay nuclei could suggest a functional association with thalamo-cortical or cortico-thalamic neuronal circuits. However, the absence of co-localization with *Cux2*, expressed in granular cells (layer 4), shows that *Hox* genes are not expressed in neurons that receive the main thalamic input. Moreover, the absence of co-localization with *Ctip2* excludes expression of *Hox* genes from subcerebral projection

neurons-SCPN (layer 5b), whose subcortical targets include pons, superior colliculus, and spinal cord (Wise and Jones 1977; Killackey et al. 1989; Legg et al. 1989). Co-localization experiments with *Plxnd1* supported the localization of *Hox* transcripts in layer 5a, formed of sparse medium-sized pyramidal cells. In the rat somatosensory cortex (barrel field), it was suggested that layer 5a neurons predominantly integrate information from layer 4 and layer 5a, playing a role in intracortical processing of sensory information (Schubert et al. 2006). Gene expression patterns from molecular markers in layer 5 also support the presence of deep layer callosal projection neurons within layer 5, that would be *Ctip2*- and *Otx1*-, and could correspond to layer 5a (Greig et al. 2013; Molyneaux et al. 2007; Weimann et al. 1999; Woodworth et al. 2012). Further studies are now required to precisely establish the identity and connectivity of *Hox* expressing neurons. In addition, it is important to establish whether proteins are produced at low levels, or if transcripts detected here are lncRNAs. If the presence of these transcripts is confirmed, it would open the path for new investigations, as it is now acknowledged that lncRNAs play key regulatory functions (e.g., Batista and Chang 2013; Ponting et al. 2009).

Hox biological functions in the adult CNS

In the CNS, early embryonic expression of *Hox* genes in progenitors determines cell identities and cell fates of neurons that will participate in all hindbrain circuits (e.g., auditory, trigeminal, vestibular, precerebellar) as well as of spinal neuron populations. Cell identity then defines intrinsic features that direct selective patterns of axonal projection and target innervation. In addition to this early function, the maintenance of *Hox* gene expression in specific neuronal subpopulations during foetal stages is also required for circuit formation and neuronal connectivity (Di Bonito et al. 2013a). This has been well studied for *Hox* genes in the hindbrain, and particularly well for *Hoxa2*. In the precerebellar system, *Hoxa2* and *Hoxb2* are required for the normal rostral migration of precerebellar pontine neurons, a role that was associated with the direct regulation of *Robo2* expression in migrating pontine neurons by *Hoxa2* (Geisen et al. 2008). Indeed, the chemotrophic molecules of the Slit family and their Roundabout (*Robo*) receptors are important factors for pontine neuron migration. Moreover, it was recently shown that *Hox* PG5 repress *Unc5b*, a Netrin-binding receptor required in mediating repulsive response to Netrin in neurons that migrate in the ventral AMS, while *Unc5b* is positively regulated by *Hox* PG2 (Di Meglio et al. 2013). In the auditory system, *Hoxa2* acts on the connectivity of the anteroventral cochlear nucleus. Indeed, proper axonal pathfinding is disregulated in conditional *Hoxa2* mutants,

due to down-regulation of the Slit receptor Rig1/Robo3, known to control midline crossing by commissural axons in the hindbrain (Di Bonito et al. 2013b). *Hoxa2* also plays multiple roles in the assembly of the trigeminal circuit. It is required for proper targeting and arborisation of axonal projections, as well as for the establishment of the normal topographic pattern of PrV neurons (Oury et al. 2006). As *Hoxa2* is known to positively regulate *EphA4* and *EphA7* expression in PrV neurons, it has been suggested that it controls topographic connectivity through ephrin/Eph signalling in the PrV (Di Bonito et al. 2013a; Oury et al. 2006). In conclusion, it appears that Hox late foetal functions in neural circuit formation partly rely on their ability to directly regulate the expression of receptors for specific guidance molecules. In the spinal cord, *Hox* expression in MNs activates downstream transcription factor expression, some of them being necessary for regulation of axon guidance cues, such as Eph receptors, Cadherins and Semaphorins (Dasen et al. 2008; Dasen and Jessell 2009). However, no direct roles in the control of specific guidance receptors have been demonstrated so far.

In adulthood, guidance systems involving ephrin/Eph, Slit/Robo, Semaphorin/Plxn are still present, suggesting functions beyond their canonical role of axon guidance. In agreement, recent evidence indicates an important role for many of these guidance-related proteins in synapse formation and plasticity (Blockus and Chedotal 2014; Hruska and Dalva 2012; Pasterkamp and Giger 2009; Shen and Cowan 2010). Indeed, functionally mature synapses are not static, and can alter their strength and number in response to experience to facilitate complex behavioural plasticity. Although these processes are more active in young neurons, they are also observed in mature neurons. An attractive hypothesis is that *Hox* genes could thus be maintained to function during the refinement of topographic connectivity, acting during processes that are thought to be regulated by experience-dependent neural activity during early postnatal life, such as synaptic pruning and maturation, or even in refinement/reinforcement of synapses observed during the whole life. In support of this hypothesis, in addition to the above-mentioned target genes, other targets involved in neuritogenesis and synaptogenesis/synaptic plasticity, such as *Pcp2* (see above), *Pleiotrophin* (*Ptn*) (Chen et al. 2005; Tanaka et al. 2003) or *calbindin-28 K* (*Calb1*) (Bami et al. 2011) have been identified. Although no such functions have been reported for Hox proteins so far, our data can be related to discoveries made on other homeoproteins involved in the regulation of postnatal maturation of circuits, such as *Otx1*, in the refinement of axonal projection in layer 5 neurons during early postnatal life (Weimann et al. 1999), and *Otx2* in maturation of the visual circuit (Sugiyama et al. 2009).

Concluding remarks

This paper provides the first detailed account of expression of all *Hox* genes in the brain at adulthood. Our data suggest a functional relevance of these genes in the brain beyond their role of patterning genes, possibly with respect to cellular and synaptic plasticity. All potential functions of *Hox* genes in the postnatal and adult brain discussed here are speculative. Development of new mouse models allowing temporally-induced inactivation of *Hox* genes with in depth nervous and behavioural phenotyping is now required to fully elucidate the late biological functions of *Hox* genes in the CNS.

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