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Insm1 (IA-1) is an essential component of the regulatory network that specifies monoaminergic neuronal phenotypes in the vertebrate hindbrain

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Monoaminergic neurons include the physiologically important central serotonergic and noradrenergic subtypes. Here, we identify the zinc-finger transcription factor, *Insm1*, as a crucial mediator of the differentiation of both subtypes, and in particular the acquisition of their neurotransmitter phenotype. Insm1 is expressed in hindbrain progenitors of monoaminergic neurons as they exit the cell cycle, in a pattern that partially overlaps with the expression of the proneural factor Ascl1. Consistent with this, a conserved cis-regulatory sequence associated with *Insm1* is bound by Ascl1 in the hindbrain, and Ascl1 is essential for the expression of Insm1 in the ventral hindbrain. In *Insm1*-null mutant mice, the expression of the serotonergic fate determinants *Pet1*, *Lmx1b* and *Gata2* is markedly downregulated. Nevertheless, serotonergic precursors begin to differentiate in *Insm1* mutants, but fail to produce serotonin because of a failure to activate expression of tryptophan hydroxylase 2 (Tph2), the key enzyme of serotonin biosynthesis. We find that both *Insm1* and *Ascl1* coordinately specify Tph2 expression. In brainstem noradrenergic centres of *Insm1* mutants, expression of tyrosine hydroxylase is delayed in the locus coeruleus and is markedly deficient in the medullary noradrenergic nuclei. However, *Insm1* is dispensable for the expression of a second key noradrenergic biosynthetic enzyme, dopamine β-hydroxylase, which is instead regulated by *Ascl1*. Thus, Insm1 regulates the synthesis of distinct monoaminergic neurotransmitters by acting combinatorially with, or independently of, Ascl1 in specific monoaminergic populations.

KEY WORDS: Hindbrain, Neuron, Serotonin, Noradrenaline, Mouse

INTRODUCTION

Differentiated neuronal subtypes are characterised by numerous molecular and morphological differences (Doyle et al., 2008; Heiman et al., 2008) that are specified, to a large extent, within the antecedent neural progenitors (Jessell, 2000). These cells undergo progressive commitment to specific neuronal fates through the cell-autonomous actions of transcription factor networks that regulate generic as well as subtype-specific properties of neurons. However, it has proven difficult to determine which mature phenotypic traits are specified by individual members of the gene regulatory network. Moreover, the mechanisms by which information regarding specific differentiation traits are transmitted from progenitors to their neuronal progeny are poorly understood. To address these issues, we have investigated the differentiation of monoaminergic neurons in the mammalian hindbrain (Goridis and Rohrer, 2002). These cell populations are defined and distinguished by their neurotransmitter phenotype: serotonin and noradrenaline. Both monoamine transmitters have a wide range of complementary actions and are implicated in the pathophysiology of many common neurological and psychiatric disorders (Jonnakuty and Gragnoli, 2008; Paterson et al., 2006; Prince, 2008; Weinshenker, 2008).

Several fate determinants have been identified that regulate the specification and differentiation of serotonergic (5HT) and noradrenergic (NA) neuronal subtypes. 5HT neural progenitors are specified around embryonic day 10.5 (E10.5), by the transcription factors Nkx2.2 (Briscoe et al., 1999), Foxa2 (Jacob et al., 2007) and Ascl1 (also called Mash1) (Pattyn et al., 2004). These progenitor-expressed factors in turn regulate the expression of the post-mitotically expressed transcription factors Pet1 (Hendricks et al., 1999; Hendricks et al., 2003), Lmx1b (Cheng et al., 2003; Ding et al., 2003), Gata2 (Craven et al., 2004) and Gata3 (van Doorninck et al., 1999), all of which are required for correct 5HT differentiation. There are several central NA nuclei, of which the largest by far is the locus coeruleus (LC). Its anlage forms around E9.0 (Pierce, 1973) when the constituent cells express Ascl1 (Hirsch et al., 1998), the orphan nuclear receptor Ear2 (also known as Nr2f6) (Warnecke et al., 2005), the transcription factors Tlx3 (also called Rnx) (Qian et al., 2001) and Phox2a (Morin et al., 1997) and the closely related protein, Phox2b (Pattyn et al., 2000). These divergent transcriptional pathways culminate in neuronal differentiation of the respective cell types, and the acquisiton of a specific neurotransmitter phenotype. Serotonin and noradrenaline are synthesised in multistep, enzymatically controlled pathways: serotonin is synthesised by the enzymes L-aromatic amino acid decarboxylase (Aadc), expressed by all monoaminergic neurons, and tryptophan hydroxylase 2 (Tph2), expressed exclusively in 5HT neurons (Walther et al., 2003; Zhang et al., 2004), whereas noradrenaline is produced by the activities of dopamine β-hydroxylase (Dbh) and tyrosine hydroxylase (Th).

A notable feature of the ontogeny of these distinct neuronal groups is the shared expression of *Ascl1* by their respective progenitors; this is a gene that regulates generic and subtype-specific neuronal properties, including the specification of neurotransmitter phenotype in the peripheral nervous system (PNS) (Guillemot et al., 1993; Hirsch et al., 1998; Lo et al., 1998; Mizuguchi et al., 2006; Nakada et al., 2004; Parras et al., 2002; Pattyn et al., 2004). However, it is not clear how the Ascl1 coding

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of neurotransmitter identity is relayed from progenitors to their neuronal progeny. The zinc-finger gene, *Insm1* (also known as *IA-1*) is widely expressed in the central nervous system (CNS), and is known to regulate the maturation of cortical progenitors (Farkas et al., 2008). It is also expressed in the PNS, where it functions downstream of Ascl1 in the transcriptional pathway of NA differentiation (Wildner et al., 2008). This prompted us to examine whether *Insm1* has an essential function in the ontogeny of central monoaminergic neurons. Here, we provide biochemical evidence that, in the hindbrain, *Insm1* is a direct target of Ascl1. Consistent with this, we find that Ascl1 is essential for the expression of Insm1 in the ventral hindbrain. Our genetic evidence shows that *Insm1* regulates multiple post-mitotic determinants of 5HT neurons, and is coordinately required with *Ascl1* for the expression of Tph2, and hence for serotonin expression itself. By contrast, in the LC, *Insm1* and *Ascl1* regulate sequential steps in NA synthesis. Together, these data suggest that *Ascl1* and *Insm1* constitute core regulatory components for monoaminergic neurotransmitter synthesis in the CNS.

MATERIALS AND METHODS

Mouse strains

The generation and genotyping of *Insm1*^{lacZ}, *Ascl1*, *Ascl1*^{Ngn2KI} and *Phox2b*^{lacZ} mutant lines has been described (Gierl et al., 2006; Parras et al., 2002; Pattyn et al., 1999).

Immunohistochemistry, in situ hybridisation and BrdU labelling

Immunohistochemistry was performed on horizontal cryosections (12-14 μm) of mouse embryos and on whole mounts as described (Stamataki et al., 2005). Primary antibodies raised against serotonin (Sigma), Islet 1 (Isl1) (Developmental Studies Hybridoma Bank), Phox2a and Phox2b (gifts from J-F. Brunet and Christo Goridis), Tbx20 (gift from J. Ericson), Foxa2 (Developmental Studies Hybridoma Bank), Nkx2.2 (Ericson et al., 1997), βIII-tubulin (Covance), BrdU (Developmental Studies Hybridoma Bank), Ascl1 (Lo et al., 1991), Tph2 (Sigma), TH (Chemicon), Dbh (Abcam), Tlx3 (Muller et al., 2005) and activated caspase 3 (Millipore) were used. A guinea-pig Insm1 antibody was raised against a bacterially expressed GST-fusion protein containing N-terminal amino acid sequences 2-167 of mouse Insm1, which was kindly provided by Gerard Gradwohl (INSERM, Strasbourg, France). In situ hybridisation was carried out using antisense, digoxigenin-labelled RNA probes that were obtained as described (Jacob et al., 2007). Additional probes include *Aadc* (*Ddc – Mouse Genome Informatics*) (Hermanson et al., 2003), *Tlx3* (gift from Q. Ma), *Hb9* (*Mnx1 – Mouse Genome Informatics*) (Tanabe et al., 1998), *Dbh*, *Phox2a* and *Chat* (gifts from C. Goridis and J-F Brunet). BrdU labelling was performed as described (Jacob et al., 2007). Sections, or whole mounts were imaged as described (Stamataki et al., 2005). Cell counts were performed unilaterally on sections obtained from between three and five embryos at each developmental stage, for each genotype. In E10.5 and E12.5 embryos, at least two sections from each relevant rhombomeric level were used for quantitation. In E16.5 embryos, serotonin-positive neurons at all anteroposterior (AP) levels, of every 12th hindbrain section were counted.

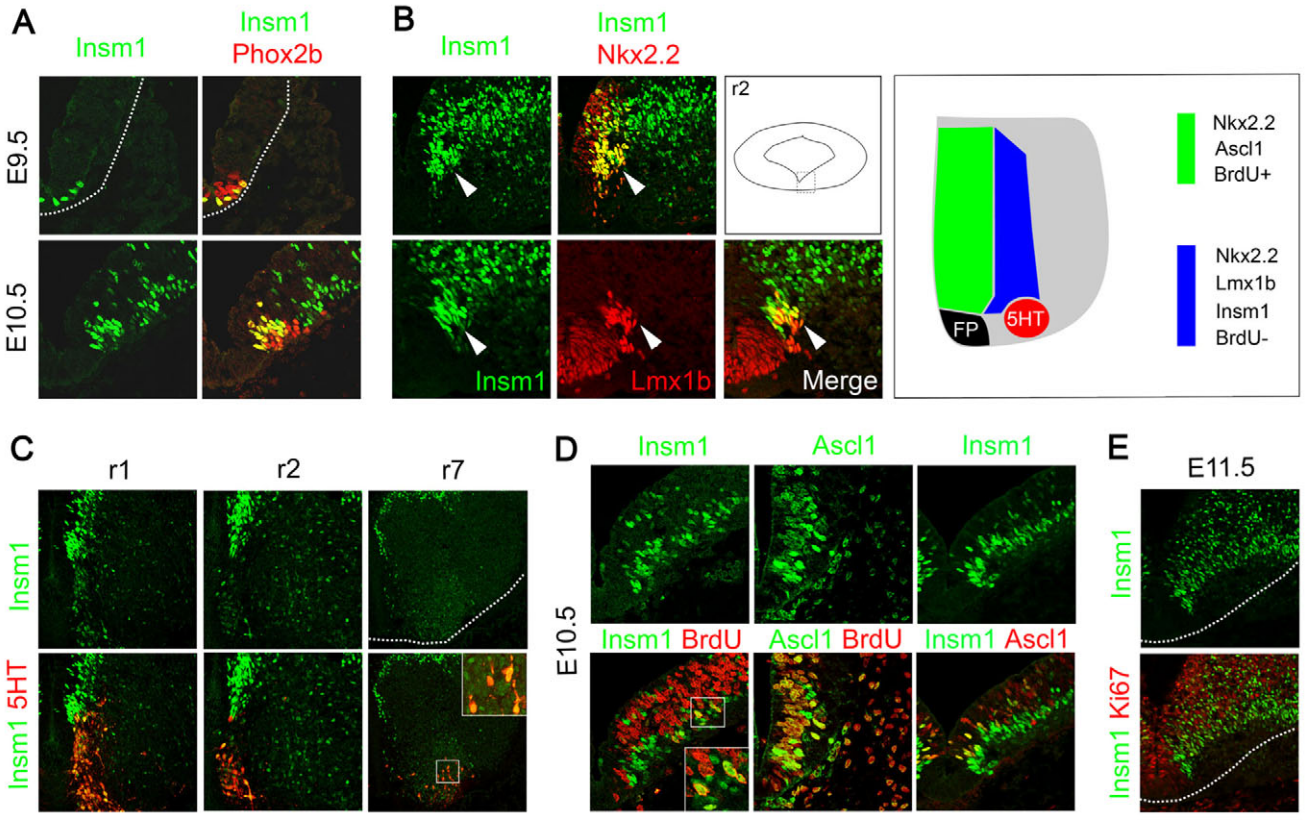

Fig. 1. Expression of Insm1 in the mouse hindbrain. (**A-C**) Insm1 (green) is detected in a subset of differentiating visceral motor (VM) neurons, marked by Phox2b (red) expression (A), in Nkx2.2$^+$ (red) cells and Lmx1b$^+$ (red) serotonergic (5HT) precursors at E11.5 (B, Insm1 co-expressing cells marked by arrowheads), and in 5HT (red) neurons at E12.5 (C). Inset in C shows a high-magnification view of the boxed area. (**D**) BrdU pulse-labelling of cycling hindbrain neural progenitors at E10.5, showing expression of Insm1 mainly in post-mitotic neurons, and occasionally in progenitors (boxed area), whereas Ascl1 is mainly confined to cycling progenitors. Inset shows high-magnification view of the boxed area. (**E**) Expression of Insm1 (green) and Ki67 (red), which is a marker of proliferating cells, at E11.5. The schematic alongside B summarises the expression of Insm1 in the ventral hindbrain in relation to markers of 5HT progenitors (Nkx2.2 and Ascl1) and differentiating 5HT neurons (Lmx1b). FP, floor plate. The dotted line indicates the pial surface of the hindbrain.

Chromatin immunoprecipitation

Chromatin was obtained from the pooled hindbrains of 23 mouse embryos at E11.5. Hindbrain tissue was fixed in Hanks' balanced salt solution/1% formaldehyde for 15 minutes at room temperature and quenched with 125 mM glycine for 8 minutes at room temperature. Tissue was lysed in 1% SDS, 10 mM EDTA and 50 mM Tris pH 8.0 for 30 minutes at 4°C and sonicated for 30 minutes, using a Bioruptor Sonicator (Diagenode). Chromatin immunoprecipitation (ChIP) assays were performed as described (Castro et al., 2006) using mouse anti-Ascl1 (Lo et al., 2002) or no antibody (mock IP). Immunoprecipitated fragments were quantified by real-time PCR (Applied Biosystems 7500). Primer pairs used were: Insm1 5′-TTGGGTGAGCCTGTCTTAG-3′ and 5′-CCGGCCTTATCTTCAC-TTC-3′, Insm1 open reading frame (ORF) 5′-CAGGTGATCCTC-CTTCAGGT-3′ and 5′-CGCTCTCTCTTTGTGGGTCT-3′, β-actin ORF GCCATGTTCAATGGGGTACT-3′ and 5′-GGTGCTAAGAAGGCT-GTTCC-3′.

RESULTS

Insm1 is expressed in visceral motor and 5HT neuronal precursors

At E9.5 Insm1 protein could be detected only in a subset of Phox2b^{+} differentiating visceral motor (VM) neurons, the first cell type to be generated by common VM-5HT progenitors in the ventral hindbrain (Briscoe et al., 1999; Jacob et al., 2007; Pattyn et al., 2003) (Fig. 1A). By E10.5, Insm1 was additionally expressed more widely in a salt-and-pepper distribution, along the entire dorsoventral axis of the hindbrain, a finding consistent with earlier studies (Breslin et al., 2003; Mellitzer et al., 2006). One day later, at E11.5, Insm1 was strongly expressed by cells located laterally in the Nkx2.2^{+} region that give rise to 5HT neurons in rhombomere (r) 1-3 and r5-8 (Fig. 1B) (Briscoe et al., 1999). Direct confirmation that Insm1 is expressed by cells in the 5HT lineage was obtained by detecting co-localisation of Insm1 with Lmx1b (Ding et al., 2003) in cells adjacent to the floor plate (Fig. 1B). By E12.5, Insm1 was weakly expressed in serotonin-expressing neurons (Fig. 1C).

A 45-minute pulse of BrdU labelling at E10.5 revealed that Insm1 was first expressed by cells at, or after, the final S-phase, in contrast to Ascl1, which was expressed mainly in cycling progenitors (Fig. 1D). Consistent with these observations, only a small proportion of cells co-expressed Insm1 and Ascl1 (Fig. 1D). One day later, during the period of 5HT neurogenesis, Insm1 expression was confined to cells that do not express the proliferation marker Ki-67, indicating that they are post-mitotic (Fig. 1E).

Insm1 regulation in the hindbrain

Multiple basic helix-loop-helix (bHLH) family members can regulate the expression of *Insm1* (Breslin et al., 2003; Castro et al., 2006; Mellitzer et al., 2006). As Ascl1 is the only conventional proneural factor expressed by 5HT progenitors, we reasoned that it is likely to be the physiologically relevant member of this group (Pattyn et al., 2004). Consistent with this, the *Insm1* gene is associated with a highly conserved Ascl1/Brn motif, which mediates the direct regulation of this gene by Ascl1 in the ventral telencephalon (Castro et al., 2006). To test whether, in the hindbrain, the Insm1-associated Ascl1/Brn motif is also bound directly by Ascl1, we performed ChIP with chromatin obtained from the hindbrains of E11.5 mouse embryos. There was an approximately 20-fold enrichment of the Ascl1/Brn-containing sequence compared with the β-actin and Insm1 ORF controls, following immunoprecipitation with an anti-Ascl1 antibody (Fig. 2A). Thus, in the hindbrain, as in other CNS regions, *Insm1* is a direct target of Ascl1.

Next, we analysed the expression of Insm1 in *Ascl1*-null mutants to determine if Ascl1 is necessary for Insm1 expression in 5HT precursors (Guillemot et al., 1993). In *Ascl1*$^{-/-}$ embryos at E11.5 there was a striking downregulation of Insm1 expression only in laterally positioned Nkx2.2^{+} cells, which correspond to 5HT precursors (Fig. 2B; see Fig. S1 in the supplementary material). To address the specificity of this regulatory relationship, we took advantage of a mouse line in which the *Ascl1* coding sequence is replaced by the neurogenin 2 (*Ngn2*; *Neurog2* – Mouse Genome Informatics) sequence (*Ascl1*$^{Ngn2KI/KI}$) (Parras et al., 2002). In these mice, Ngn2 was expressed in a manner that recapitulated the spatiotemporal profile of Ascl1 expression. The co-expression of Insm1 in Nkx2.2^{+} cells of these mutant mice was rescued (Fig. 2C). This indicates that in the CNS, Insm1 can be regulated by alternative bHLH family members, providing an explanation for the expression of Insm1 in dorsoventral regions of the hindbrain that are devoid of Ascl1 expression, and the normal dorsal pattern of Insm1 expression in *Ascl1*-null mutants (see Fig. S1 in the

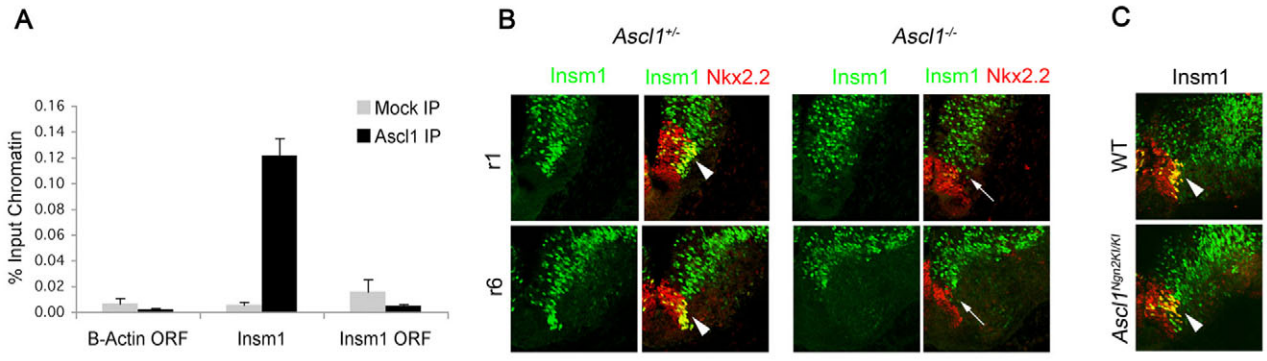

Fig. 2. Regulation of Insm1 expression in the mouse hindbrain. (**A**) ChIP demonstrates that Ascl1 binds to an *Insm1*-associated genomic sequence, which is located 4.5 kb 5′ of the transcription start site of the *Insm1* gene (Castro et al., 2006). Binding to the β-actin and Insm1 ORFs serve as controls. Chromatin was obtained from E11.5 mouse hindbrains. Data are presented as the mean±s.d. of six replicate amplifications from a single immunoprecipitation. (**B**) Downregulation of Insm1 (green) expression in the ventral hindbrain of *Ascl1*-null mutant mice at E11.5. The cells at the lateral boundary of the Nkx2.2-expressing (red) domain, which strongly express Insm1 in control embryos (arrowheads), lose or markedly downregulate Insm1 expression in *Ascl1* mutants (arrows) along the entire AP axis of the hindbrain. (**C**) Recovery of Insm1 expression in the ventral hindbrain of *Ascl1* mutants in which neurogenesis is rescued by the substitution of *Ngn2* (*Ascl1*$^{Ngn2KI/KI}$) (arrowhead).

supplementary material). Finally, we analysed Insm1 expression in the hindbrain of *Phox2b* mutant mice, as *Phox2b* has been reported to be an essential regulator of *Insm1* in the PNS (Wildner et al., 2008), and found that Insm1 expression persisted in these mice (data not shown). Together, these data show that Ascl1 is a direct and crucial regulator of *Insm1* expression in 5HT precursors, but that other proneural factors can regulate Insm1 expression.

The expression of 5HT fate determinants is reduced in *Insm1* mutants

To investigate the function of *Insm1* in the generation of ventral neuronal subtypes in the hindbrain we analysed embryos with a targeted mutation of *Insm1* (Gierl et al., 2006). VM and somatic motor (SM) neuronal development proceeded normally in *Insm1* mutant mice (see Fig. S1 in the supplementary material). Moreover, there was no marked difference in neurogenesis in the hindbrain, as judged by immunostaining for the pan-neuronal marker, βIII-tubulin (see Fig. S1 in the supplementary material).

We next examined the consequences of *Insm1* deletion on 5HT neuronal differentiation. Analysis at E12.5, when 5HT neurogenesis is virtually complete (Jacob et al., 2007; Pattyn et al., 2003), showed a severe deficit of serotonin expression at all axial levels, especially in r2-3 (Fig. 3A,D). In older embryos, 5HT neurons aggregate to form a mature multi-nuclear complex, designated B1-9 (Tork, 1990). Those neurons born in r1-3 populate the anterior, pontine nuclei B4-9, whereas posterior 5HT neurons are located in the B1-3 medullary nuclei (Jensen et al., 2008). At E16.5, there was a marked reduction in the size of most 5HT nuclei in mutants, particularly those nuclei that receive a contribution from r2 and r3, namely, B8, B9 and B5 (Fig. 3B,E). The reduction in 5HT expression cannot be accounted for by a change in progenitor specification, as Foxa2 and Nkx2.2 expression in progenitors is not affected in the mutants, nor by an increase in cell death in the mutant hindbrains, as shown by the absence of activated caspase 3 immunostaining at E12.5 (see Fig. S1 in the supplementary material) and E16.5 (data not shown).

To ascertain the differentiation status of cells in the 5HT lineage in *Insm1* mutants, we examined the expression of post-mitotic determinants of 5HT identity at E12.5 (Fig. 3C; see Fig. S2 in the supplementary material). *Lmx1b* expression was greatly reduced in the ventral hindbrain at anterior and posterior levels. However, ventral *Gata2* expression profiles varied depending on AP position. In the anterior hindbrain, *Gata2* expression was markedly reduced, but posteriorly its expression was not obviously affected. Expression of the 5HT neuronal-specific marker, *Pet1* (Hendricks et al., 1999) was also significantly reduced at all AP levels, although the magnitude of the deficit varied in accordance with AP position (Fig. 3F). In r2-3 the number of *Pet1*$^{+}$ cells was reduced by approximately 80%, but in r6-7 there was only a 25% reduction in *Pet1* expression. In comparison, there was a more pronounced reduction in serotonin-expressing neurons at anterior and posterior levels, ranging from an

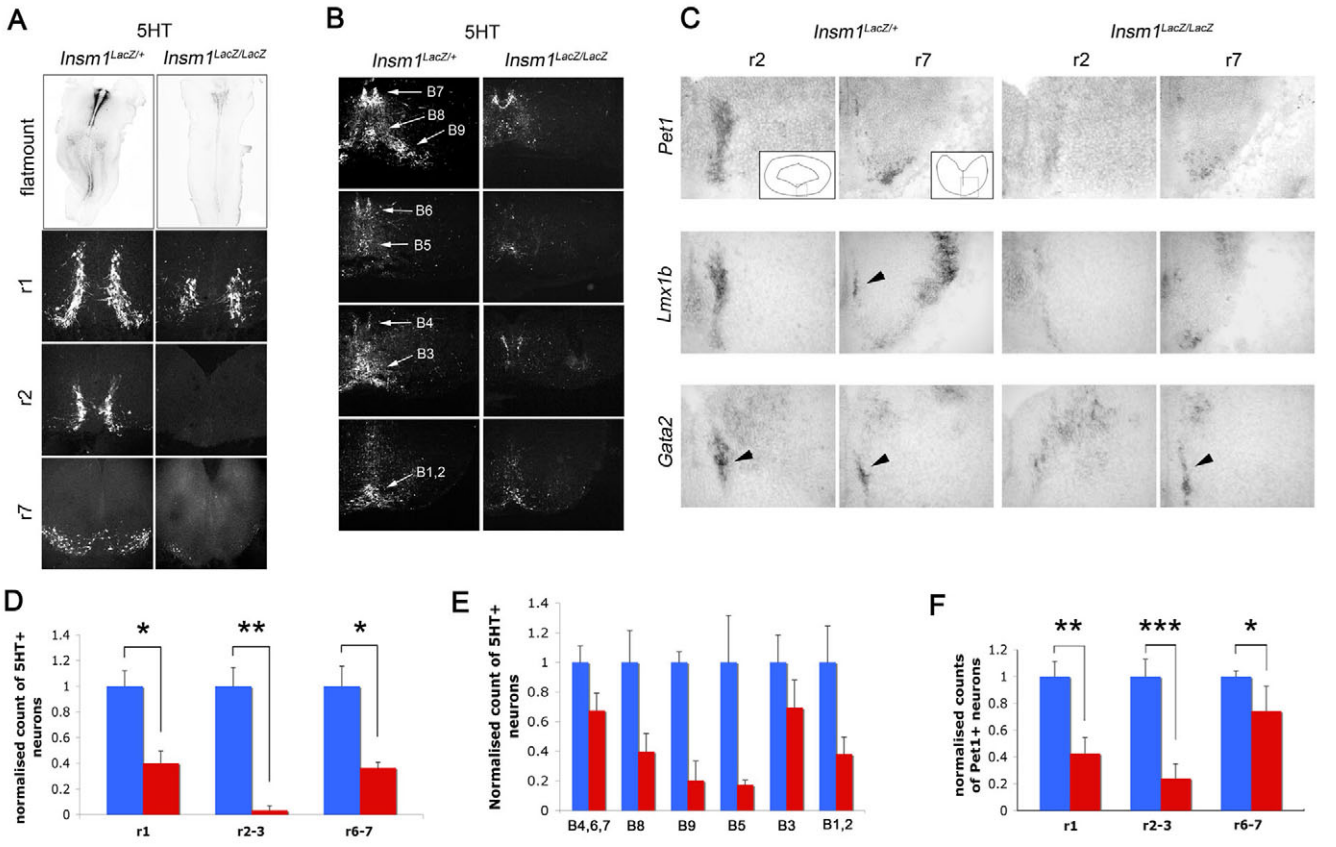

Fig. 3. Impaired differentiation of 5HT neurons in *Insm1* mutant mice. (**A**) Serotonin expression in flatmounts and sections of *Insm1* mutants and controls at E12.5. (**B**) Reduced size of 5HT nuclei, B1-9, in E16.5 mutant embryos (right-hand panels) compared with controls (left panels). (**C**) Expression of the indicated markers by RNA in situ hybridisation in control and mutant 5HT precursors (arrowheads indicate location of differentiating 5HT neurons) at E12.5. (**D**,**E**) Quantification of serotonin expression at E12.5 (D) (*, $P=3\times10^{-4}$; **, $P=3\times10^{-5}$; Student's *t*-test) and at E16.5 (E) in *Insm1* mutant and control embryos. (**F**) Quantification of *Pet1* expression at E12.5 in mutant and control embryos (*, $P=0.039$; **, $P=1.8\times10^{-3}$; ***, $P=7\times10^{-4}$). Cell counts are represented as normalised mean±s.d. Blue, controls; red, *Insm1* mutants.

approximately 60% loss in r1 to a greater than 90% reduction in r2-3 (Fig. 3D). Together, these findings indicate that the expression of serotonin and multiple 5HT fate determinants is impaired in the absence of *Insm1*.

Insm1 and *Ascl1* are coordinately required for serotonin biosynthesis

The discrepancy between the expression of the neurotransmitter serotonin and the 5HT fate determinant, *Pet1*, prompted an evaluation of the serotonin biosynthetic pathway. At E12.5, transcripts of the enzyme *Aadc* were normally expressed, or only slightly reduced, in the anterior hindbrain of *Insm1*-null mutants, and appeared unchanged posteriorly (Fig. 4A). However, in older *Insm1* mutants, at E16.5, there was a global reduction in *Aadc* expression within the serotonergic nuclear complex (Fig. 4B). This correlates with the decrease in *Pet1* expression observed at the same stage. By contrast, expression of Tph2, which is the rate-limiting enzyme of serotonin synthesis (Walther et al., 2003; Zhang et al., 2004), was markedly downregulated throughout the AP extent of the hindbrain of *Insm1* mutants from as early as E12.5, and matched the profile of serotonin expression (Fig. 4A). The normal early expression of *Aadc* in *Insm1* mutants demonstrates that presumptive 5HT neurons remain viable and execute part of a terminal differentiation programme. This suggests that the reason for the greater reduction in the expression of serotonin than that of *Pet1* is a block in differentiation at the level of Tph2 expression. The loss of serotonin production that resulted from the severe downregulation of Tph2 could be explained by a direct requirement for Insm1, or a secondary consequence of the loss of expression of the other post-mitotic 5HT fate determinants that are Insm1-dependent (Fig. 3C).

The requirement of Insm1 for Tph2 expression suggested that the type-specification role of *Ascl1* in conferring 5HT identity might, at least in part, be to regulate serotonin synthesis, perhaps via the induction of *Insm1* (Pattyn et al., 2004; Wildner et al., 2008). As a conventional loss-of-function approach to address this issue makes it impossible to distinguish between the proneural and subtype-specification activities of Ascl1, we took advantage of *Ascl1*$^{Ngn2KI/KI}$ mice (Parras et al., 2002). In this mutant line, neurogenesis in the ventral hindbrain is rescued, but 5HT neurons are absent and are replaced by neurons of unknown identity (Pattyn et al., 2004). As shown earlier, there was no change in Insm1 expression in the ventral hindbrain of *Ascl1*$^{Ngn2KI/KI}$ mutants at E11.5 (Fig. 2C), nor was *Aadc* expression affected. Notably, Tph2 expression was severely reduced in these embryos (Fig. 4C). We conclude that both *Insm1* and *Ascl1* coordinately regulate serotonin synthesis, via their direct or indirect co-regulation of Tph2 (Fig. 4D).

Insm1 and Ascl1 regulate sequential steps in central noradrenaline biosynthesis

To compare the role of Insm1 in 5HT neurons with functionally related neuronal populations, we turned our attention to the NA subtype of monoaminergic neurons. *Insm1* mutant mice are thought to die, in part, because of a lack of peripheral noradrenaline synthesis (Wildner et al., 2008). However, the status of central NA centres is unclear. The major NA centre, the LC, originates from progenitors in the dorsal part of the metencephalon (r1). Double immunofluorescence labelling of the hindbrain in control embryos revealed strong overlapping expression of the noradrenaline synthetic enzyme Th and Insm1 (Fig. 5A). To confirm that Insm1 is expressed in the LC, we immunostained for Phox2a, which, in the

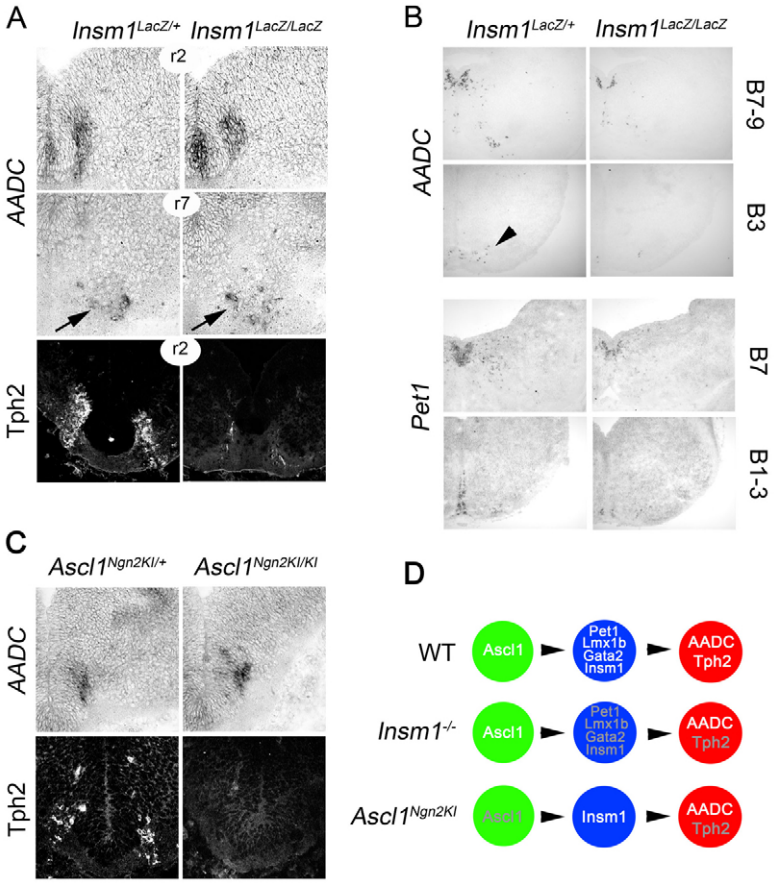

Fig. 4. Altered expression of serotonin biosynthetic enzymes in *Insm1* and *Ascl1* mutant mice. (**A**,**C**) At E12.5, *Aadc* expression, detected by RNA in situ hybridisation, is intact in *Insm1* (A) and *Ascl1*$^{Ngn2KI/KI}$ (C) mutant embryos, but Tph2 expression, detected by immunofluorescence, is drastically reduced. (**B**) By E16.5, *Aadc* expression is also reduced and correlates with the persistent downregulation of *Pet1* expression. (**D**) Summary of the effects of targeted mutation of *Insm1* and *Ascl1* on the specification and differentiation of 5HT neurons. White text denotes normal marker expression and grey text indicates loss of expression or reduced expression of markers. Green, blue and red circles represent neural progenitors, post-mitotic serotonergic precursors and 5HT neurons, respectively.

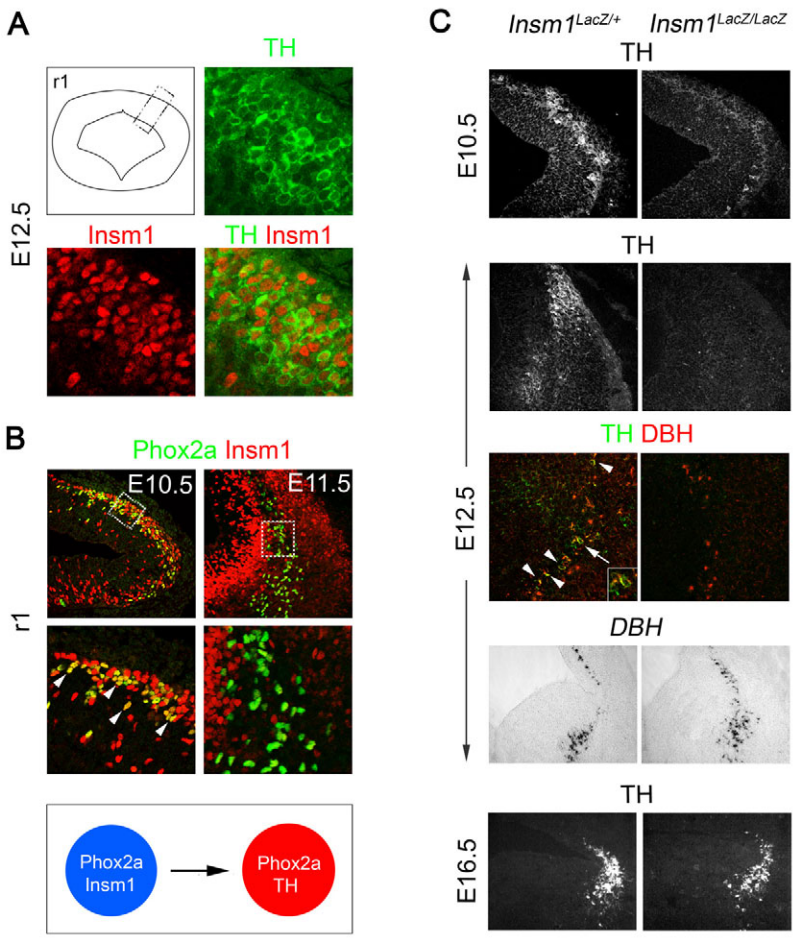

Fig. 5. Abnormal differentiation of the locus coeruleus in *Insm1* mutant mice. (**A**) Insm1 (red) is co-expressed with Th$^+$ (green) neurons in r1 of control embryos at E12.5. (**B**) Expression of Insm1 (red) in precursors of the locus coeruleus (LC), marked by Phox2a (green) in E10.5 and E11.5 wild-type embryos. The lower panels show high-magnification images of the boxed regions in the upper panels. At E10.5, all LC precursors, marked by Phox2a expression, co-express Insm1 (examples marked by arrowheads). One day later, Insm1 is downregulated in LC precursors. These changes are summarised in the schematic, in which the blue and red circles represent post-mitotic LC precursors and differentiated (Th$^+$) neurons, respectively. (**C**) In *Insm1* mutants, as compared with controls, Th expression in the dorsal region of r1 is greatly reduced at E10.5 (top panels) and E12.5 (middle), but shows a marked recovery by E16.5 (bottom). Dbh, assayed by immunofluorescence and by in situ hybridisation, is not altered in *Insm1* mutants at E12.5 (middle panels).

metencephalon, up to at least E13.5, is expressed exclusively in the LC (Tiveron et al., 1996; Valarche et al., 1993). At E10.5, all Phox2a$^+$ LC precursors co-expressed Insm1, but a day later, Insm1 was downregulated in LC neurons (Fig. 5B).

Immunostaining against Th in *Insm1* mutants at E10.5 revealed a marked reduction in Th expression in the dorsal part of r1, and 2 days later, the expression of TH was reduced further in this region (Fig. 5C). As, at these mid-embryonic stages, Th is also expressed by non-monoaminergic neuronal populations, we used Dbh expression in r1 as a precise marker of the LC. In r1 of control embryos at E12.5, a subset of Th-expressing neurons also expressed Dbh, confirming that neurons of the LC have reached an advanced stage of differentiation by this time point. By contrast, age-matched *Insm1* mutant embryos specifically lacked Th expression in the LC. As in the sympathetic chain (Wildner et al., 2008), the deficit in Th expression was transient, and by E16.5 there was a substantial recovery in Th expression in the LC of *Insm1* mutants (Fig. 5C).

To determine the extent to which differentiation of the LC is perturbed in *Insm1* mutants, we analysed the expression of additional markers. By E10.5, multiple markers of the forming LC were expressed in control embryos (see Fig. S3 in the supplementary material). No alteration in *Phox2a*, Phox2b, *Tlx3* and *Dbh* expression were observed in *Insm1* mutant embryos at the same stage. We also counted the number of Ascl1$^+$ cells in dorsal r1 and found there was no significant difference between control and *Insm1* mutant mice (see Fig. S3 in the supplementary material). Similarly, at E12.5 the expression of *Dbh*, *Phox2a*, Phox2b and *Tlx3* expression was indistinguishable in *Insm1* mutant and control mice (Fig. 5C; Fig. 6A; see Fig. S3 in the supplementary material). Therefore, the absence of Insm1 does not result in a global delay in differentiation of the LC.

Is the differentiation of the other brainstem NA centres (Dahlström and Fuxe, 1964) affected by the loss of *Insm1*? Smaller, scattered groups of NA cells located at more posterior levels of the brainstem include the pontine, A5 nucleus, which expresses Th normally at the same stage (data not shown) and the medullary NA groups, A1 and A2, which have reduced expression of Th (Fig. 6B), without any alteration in *Dbh* and *Phox2a* expression in E16.5 *Insm1* mutant mice (see Fig. S3 in the supplementary material). Therefore, *Insm1* is essential for normal spatiotemporal expression of the central NA neurotransmitter phenotype, via its regulation of Th (Fig. 6D). In the LC, *Insm1* controls the timely onset of expression of Th, and in the medullary nuclei, *Insm1* is indispensable for normal Th expression.

Next, we asked whether Insm1 also acts combinatorially with Ascl1 to regulate noradrenaline biosynthesis in the LC. We addressed this question by re-examining NA differentiation in the LC of *Ascl1*$^{Ngn2KI/KI}$ mice, in which *Dbh* expression was previously reported to be lost (Parras et al., 2002). We confirmed the loss of Phox2b and *Dbh* expression reported previously in the dorsal part of r1 in *Ascl1*$^{Ngn2KI/KI}$ mice at E11.5 (Parras et al., 2002) (data not shown), but strikingly, expression of Tlx3 was intact in the same region. Consistent with this, at E13.5 these mutant mice expressed Th in the normal spatial pattern characteristic of the LC (Fig. 6C). These findings imply that cells of the presumptive LC can partially differentiate when Ascl1 function is substituted by a different bHLH

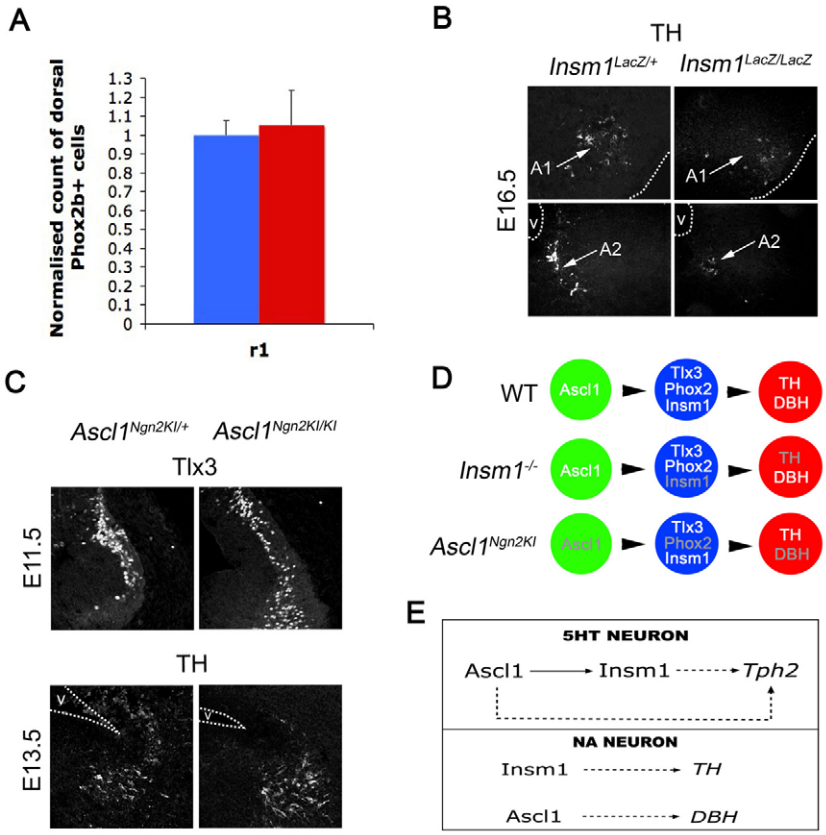

Fig. 6. Insm1 and Ascl1 regulate distinct enzymatic components of central noradrenaline biosynthesis. (**A**) Quantification of Phox2b expression at E12.5 shows that similar numbers of LC neurons are present in *Insm1* mutant (red bar) and control (blue bar) mice (P=0.8). (**B**) Persistent reduction of Th expression as shown by immunofluorescence in medullary noradrenergic (NA) nuclei A1-2 of *Insm1* mutants at E16.5. v, fourth ventricle. (**C**) Expression of Tlx3 and Th is retained in the LC of *Ascl1*$^{Ngn2KI/KI}$ mutants. (**D**) Summary of the effect of *Insm1*- and *Ascl1*-targeted mutations on the specification and differentiation of the LC. As in Fig. 4D, green, blue and red circles represent progenitor, early post-mitotic and differentiated neurons, respectively. Phox2 refers to Phox2a and Phox2b. (**E**) Specification of monoaminergic neurotransmitter identity by Ascl1 and Insm1. In 5HT neurons, these two transcription factors specify *Tph2* in a combinatorial manner, whereas in NA neurons they independently specify distinct neurotransmitter biosynthetic enzymes. The solid arrow indicates direct positive regulation, and dashed arrows indicate that it is not known whether the genetic interaction is direct or indirect.

factor. In particular, Th induction can occur independently of Ascl1 when neurogenesis is rescued in the presumptive LC (Fig. 6D). We conclude that *Insm1* and *Ascl1* independently regulate sequential steps in the enzymatic synthesis of noradrenaline in the CNS (Fig. 6E).

DISCUSSION

These data provide new insight into the transcriptional control of the differentiation of 5HT and NA neuronal subtypes. Our finding of a common requirement for *Insm1* enhances the ontogenetic relatedness of these two neuronal subtypes, and identifies a regulatory link between 5HT and NA progenitor specification by Ascl1, and the subsequent acquistion of a monoaminergic phenotype. An initial step in this process is the direct regulation of *Insm1* by Ascl1, which results in the early post-mitotic expression of Insm1. We find that Insm1 is a crucial mediator of some of the type-specification functions of Ascl1 – in particular the selection of neurotransmitter identity within the CNS.

Specification of monoaminergic neurotransmitter identity by Ascl1 and Insm1

Superficially, the common requirement for *Insm1* in 5HT and NA neuronal subtypes might suggest mechanistic similarities in its regulation of the differentiation of both neuronal subtypes, but there are two important differences. First, *Insm1* is essential for the expression of post-mitotic fate determinants during 5HT, but not NA, differentiation, which suggests that it forms an essential part of the regulatory network in the former context, but might have a more circumscribed function during NA differentiation. Specifically, our data support the notion that Insm1 and the set of genes that it regulates during NA differentiation constitute a genetic subprogramme for the acquisition of NA neurotransmitter identity, separate from the determination of other cellular traits. Second, *Insm1* acts combinatorially with *Ascl1* to specify the 5HT transmitter phenotype, but independently of *Ascl1* in the selection of NA transmitter identity (Fig. 6E). During 5HT differentiation, Ascl1 and Insm1 regulate *Tph2* expression via a genetic pathway that resembles a feedforward loop. However, forced expression of *Ascl1*, *Insm1* or *Ascl1* and *Insm1* in ovo are not sufficient to ectopically activate Tph2 expression (data not shown), which indicates that these transcription factors are unable to instruct 5HT neurotransmitter identity. This is not surprising given studies that implicate Pet1 and Lmx1b in the regulation of serotonin expression (Cheng et al., 2003; Hendricks et al., 2003). An important regulatory difference is that Pet1 and Lmx1b regulate the expression of both enzymes in the serotonin synthesis pathway, in contrast to Ascl1 and Insm1, which exclusively regulate Tph2 expression (Fig. 4). Moreover, Pet1-binding sites have been identified in human and mouse *Tph* and in human *Aadc* (Hendricks et al., 1999). Therefore, our observation that Tph2, but not *Aadc* expression is affected in *Insm1* mutants gives credence to the notion that Insm1 might regulate *Tph2* directly, rather than indirectly via the induction of *Pet1* and *Lmx1b* (Fig. 3C). To confirm this, characterisation of the regulatory elements of the Tph2 gene will be required. By contrast, as Ascl1 is predominantly expressed in dividing progenitors, it seems likely that it regulates *Tph2* indirectly in post-mitotic neurons. The downstream mediator(s) of this aspect of Ascl1 function awaits identification.

The evidence for independent roles for *Ascl1* and *Insm1* in encoding transmitter phenotype in the central NA system reveals how versatile these factors are. This versatility is also evident in the specification of NA identity during the development of the

sympathetic nervous system. In contrast to the role of Insm1 in the regulation of central noradrenaline synthesis, in the periphery, *Insm1* mediates the correct activation of *Th* and *Dbh* by Ascl1 (Wildner et al., 2008). Similar combinatorial and independent coding of peptidergic neuronal identity by unrelated transcription factors also occurs in the CNS of *Drosophila* (Allan et al., 2005; Baumgardt et al., 2007), which adds further support to the idea that the transcriptional logic of at least some important aspects of neuronal differentiation is conserved in bilaterians.

The retention of a small, but significant proportion of 5HT neurons in a position-dependent manner in *Insm1* mutants (Fig. 3D) suggests there are Insm1-independent pathways of 5HT differentiation. One possible mechanism might be functional compensation by related family members. The transcription factor Insm2 is expressed in the embryonic mouse brain (Gong et al., 2003) in a pattern that is reported to be largely non-overlapping with Insm1 (Duggan et al., 2008). Further detailed analysis of Insm2 expression in relation to Insm1 and other 5HT markers will begin to address the mechanism underlying the partial compensation.

Multiple functions of Insm1 during neuronal development

A recent study has shown that in the developing neocortex, Insm1 expression is most prominent in the proliferative zone and is specifically required for the formation of basal progenitors (Farkas et al., 2008). The ability of Insm1 to promote proliferation might be explained by its regulation of Stat3, which is known to be strongly expressed in neocortical ventricular zone and subventricular zone progenitors (Farkas et al., 2008). By contrast, in the hindbrain (this study) and spinal cord (Gierl et al., 2006), Insm1 is expressed mainly in differentiating neurons. Only in the sympathetic nervous system, in which Insm1 is also expressed in differentiating neurons, is it known to regulate both proliferation and differentiation. In this lineage, neurons undergo proliferation at early developmental stages (Rohrer and Thoenen, 1987). In the absence of *Insm1*, sympathetic neuronal proliferation is reduced and their differentiation is delayed (Wildner et al., 2008). Further investigation of the region-specific regulation and molecular targets of Insm1 should provide insight into the mechanisms underlying its pleiotropic effects during neuronal development.

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Supplementary material

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