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

John Jacob1,2,†, Robert Storm3,*, Diogo S. Castro1,*, Christopher Milton1, Patrick Pla4, François Guillemot1, Carmen Birchmeier5 and James Briscoe1,†

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 enzymatically controlled pathways: serotonin is synthesised by the key enzyme of serotonin biosynthesis. 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 acquisition 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

†Authors for correspondence (e-mails: jjacob@nimr.mrc.ac.uk; jbrisco@nimr.mrc.ac.uk)
*These authors contributed equally to this work
*These authors for correspondence (e-mails: jjacob@nimr.mrc.ac.uk; jbrisco@nimr.mrc.ac.uk)

Accepted 6 May 2009
of neurotransmitter identity is relayed from progenitors to their neuronal progeny. The zinc-finger gene, *Insm1* (also known as *IA-I*) 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 SHT 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 *Insm1lacZ*, *Ascl1*, *Ascl1Ngn2KI* and *Phox2blacZ* 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) serotoninergic (SHT) precursors at E11.5 (B, Insm1 co-expressing cells marked by arrowheads), and in SHT (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 SHT progenitors (Nkx2.2 and Ascl1) and differentiating SHT neurons (Lmx1b). FP, floor plate. The dotted line indicates the pial surface of the hindbrain.
Insm1 regulates monoaminergic neurotransmitter identity

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 (Ascl1Ngn2KI/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 (arrows) in which neurogenesis is rescued by the substitution of Ngn2 (Ascl1Ngn2KI/KI) (arrowhead).
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). Interestingly, 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...
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<sup>Ngn2<sub>KI</sub></sup> 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<sup>Ngn2<sub>KI</sub></sup> 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
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 Ascl1Ngn2KI/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 Ascl1Ngn2Ki/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
factor. In particular, Th induction can occur independently of Ascl1 when neurogenesis is rescued in the presumptive LC (Fig. 6D). We conclude that *Ins1* 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 *Ins1* 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 acquisition of a monoaminergic phenotype. An initial step in this process is the direct regulation of *Ins1* by Ascl1, which results in the early post-mitotic expression of *Ins1*. We find that *Ins1* 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 Ins1**
Superficially, the common requirement for *Ins1* 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, *Ins1* is essential for the expression of post-mitotic fate determinants during 5HT, but not NA, differentiation, which 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 *Ins1* 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, *Ins1* 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 Ins1 regulate Tph2 expression via a genetic pathway that resembles a feedforward loop. However, forced expression of *Ascl1*, *Ins1* or *Ascl1* and *Ins1* 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 Ins1, 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 *Ins1* mutants gives credence to the notion that *Ins1* 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 *Ins1* 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 Ins1 in the regulation of central noradrenaline synthesis, in the periphery, Ins1 mediates the correct activation of Th and Dbh by Asc1 (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 Ins1 mutants (Fig. 3D) suggests there are Ins1-independent pathways of 5HT differentiation. One possible mechanism might be functional compensation by related family members. The transcription factor Ins2 is expressed in the embryonic mouse brain (Gong et al., 2003) in a pattern that is reported to be largely non-overlapping with Ins1 (Duggan et al., 2008). Further detailed analysis of Ins2 expression in relation to Ins1 and other 5HT markers will begin to address the mechanism underlying the partial compensation.

Multiple functions of Ins1 during neuronal development

A recent study has shown that in the developing neocortex, Ins1 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 Ins1 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 (Gierli et al., 2006), Ins1 is expressed mainly in differentiating neurons. Only in the sympathetic nervous system, in which Ins1 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 Ins1, 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 Ins1 should provide insight into the mechanisms underlying its pleiotropic effects during neuronal development.

Acknowledgements

We gratefully acknowledge Jean-François Brunet and Christo Goridis for the Phox2b mutant mice and for comments on the manuscript. This work was funded by the UK Medical Research Council. Deposited in PMC for release after 6 months.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/14/42477/DC1

References


Lo, L. C., Tiveron, M. C. and Anderson, D. J. (1998). MASH1 activates expression of the paired homodomain transcription factor Phox2a, and couples pan-
neuronal and subtype-specific components of autonomic neuronal identity. Development 125, 609-620.


