Developmental Biology 369 (2012) 32–42

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

A novel role for FOXA2 and SHH in organizing midbrain signaling centers

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ARTICLE INFO

Article history:
Received 19 October 2011
Received in revised form
6 June 2012
Accepted 20 June 2012
Available online 27 June 2012

Keywords:
Floor plate
Midbrain–hindbrain boundary
Roof plate
LMX1B
BMP7
Signaling centers
Spinal cord

ABSTRACT

The floor plate (FP) is a midline signaling center, known to direct ventral cell fates and axon guidance in the neural tube. The recent identification of midbrain FP as a source of dopaminergic neurons has renewed interest in its specification and organization, which remain poorly understood. In this study, we have examined the chick midbrain and spinal FP and show that both can be partitioned into medial (MFP) and lateral (LFP) subdivisions. Although Hedgehog (HH) signaling is necessary and sufficient for LFP specification, it is not sufficient for MFP induction. By contrast, the transcription factor FOXA2 can execute the full midbrain and spinal cord FP program via HH-independent and dependent mechanisms. Interestingly, although HH-independent FOXA2 activity is necessary and sufficient for inducing MFP-specific gene expression (e.g., *LMX1B*, *BMP7*), it cannot confer ventral identity to midline cells without also turning on Sonic hedgehog (SHH).

We also note that the signaling centers of the midbrain, the FP, roof plate (RP) and the midbrain–hindbrain boundary (MHB) are physically contiguous, with each expressing *LMX1B* and *BMP7*. Possibly as a result, *SHH* or FOXA2 misexpression can transform the MHB into FP and also suppress RP induction. Conversely, HH or FOXA2 knockdown expands the endogenous RP and transforms the MFP into a RP and/or MHB fate. Finally, combined HH blockade and FOXA2 misexpression in ventral midbrain induces *LMX1B* expression, which triggers the specification of the RP, rather than the MFP. Thus we identify HH-independent and dependent roles for FOXA2 in specifying the FP. In addition, we elucidate for the first time, a novel role for SHH in determining whether a midbrain signaling center will become the FP, MHB or RP.

Introduction

During development, the ventral midline of the vertebrate neural tube is occupied by the FP, which secretes SHH and plays a critical role in cell fate specification and axon guidance (His, 1888; Kingsbury, 1920; Placzek and Briscoe, 2005; Strahle et al., 2004). Despite 100 years of study, questions remain unanswered with regard to the embryonic origin, specification and function of the FP across species and axial levels of the neural tube (Placzek and Briscoe, 2005).

The simplest functional organization of the FP to have emerged is its partition into medial (MFP) and lateral (LFP) subdivisions (Odenthal et al., 2000; Schauerte et al., 1998). These divisions are well established in the anamniote and avian spinal FP where the MFP and LFP differ from each other in their embryonic origin, and more controversially, in their dependence upon HH signaling (Charrier et al., 2002; Odenthal et al., 2000; Peyrot et al., 2011; Placzek and Briscoe, 2005; Strahle et al., 2004). HH signaling is required in the fish for specification of the neurectoderm-derived LFP, while the node-derived MFP depends upon Nodal signaling for its induction (Hatta et al., 1991; Odenthal et al., 2000; Schauerte et al., 1998; Strahle et al., 2004). However, these results are complicated by a requirement for HH signaling in the maintenance of MFP at later stages and by the presence of residual FP cells in the *nodal* (*cyclops*) mutant (Odenthal et al., 2000; Ribes et al., 2010).

Gene expression patterns also support a partition of the mouse FP into medial and lateral subdivisions (Odenthal et al., 2000). However, the entire FP disappears following the loss of HH signaling,

Abbreviations: bi, Bilaterally electroporated; CYC, Cyclopamine; DI, Diencephalon; E, Embryonic day; EP, Electroporated; FP, Floor plate; HH, Hedgehog; H&H, Hamburger & Hamilton; HB, Hindbrain; hn, Hensen's Node; LFP, Lateral floor plate; MB, Midbrain; me, Mesendoderm; MFP, Medial floor plate; MHB, Midbrain–hindbrain boundary; ne, Neurectoderm; RP, Roof plate; TEC, Tectum

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http://dx.doi.org/10.1016/j.ydbio.2012.06.018

leaving unanswered the question of whether these subregions differentially require HH signaling for maintenance and/or induction as they do in the fish (Chiang et al., 1996; Fogel et al., 2008; Odenthal et al., 2000).

The chick spinal FP also displays node and neurectoderm-derived MFP and LFP subdivisions (Catala et al., 2000; Charrier et al., 2002). Here, SHH misexpression cannot induce an ectopic MFP, while notochord transplants readily do so via unidentified mechanisms (Catala et al., 2000; Charrier et al., 2002; Gray and Dale, 2010). More recent studies have suggested that early and transient exposure to SHH is capable of inducing a FP in all species, including the bird, fish and mouse (Ribes et al., 2010). But whether SHH is necessary and sufficient for the full FP program has not been addressed.

Even less is known about the specification of anterior (midbrain, rostral hindbrain) FP, which is distinct from the spinal FP in some respects (Patten et al., 2003; Placzek and Briscoe, 2005). For example, in addition to contributions from the node and neurectoderm, the chick anterior FP is also colonized by a unique population of cells emerging from ''area a'', a region anterior to Hensen's node (Patten et al., 2003; Schoenwolf and Sheard, 1990). Interestingly, ''area a'' explants can be induced to a FP fate in the absence of the notochord by a brief exposure to prechordal plate-derived SHH and Nodal activity (Patten et al., 2003). But whether the anterior FP cell-types are organized into medial and lateral subdivisions and whether they differentially utilize HH and Nodal signaling is not known.

This question has recently acquired significance with the identification of an MFP-like ventral midline region as the predominant source of midbrain dopaminergic (mDA) neurons, which help regulate voluntary movements and the reward-reinforcement circuitry of the brain (Bayly et al., 2007; Blaess et al., 2011; Joksimovic et al., 2009; Ono et al., 2007). Recent work, including ours, suggests that HH signaling may not be necessary for patterning this MFP-like region (Hynes et al., 2000; Kittappa et al., 2007; Lin et al., 2009; Ye et al., 1998). Instead, some studies have reported that the winged helix transcription factor FOXA2 may be a more potent inducer of mDA progenitors than SHH (Kittappa et al., 2007; Lin et al., 2009; Norton et al., 2005; Ribes et al., 2010; Sasaki and Hogan, 1993).

Since SHH and FOXA2 transcriptionally upregulate each other, the differences between their inductive abilities are not well understood (Kittappa et al., 2007; Sasaki et al., 1997). FP studies in the fish suggest that FOXA2 activity can be regulated by, and interacts with, both the SHH and TGFß/Nodal signaling cascades (Strahle et al., 2004). However, the role of FOXA2 in fish FP specification is complicated by several observations. Although FOXA2 can rescue the MFP in the fish *nodal* (*cyclops*) mutant, *nodal*-dependent induction of FP occurs in the absence of FOXA2 (Norton et al., 2005; Rastegar et al., 2002). Despite its ability to induce the fish MFP, FOXA2 mutants display relatively mild phenotypes consisting mainly of a failure of the FP to fully differentiate (Norton et al., 2005). Although these observations suggest a role for FOXA2 in elaborating, rather than inducing the fish FP, they may also reflect functional redundancy among Foxa2 homologs. This idea is supported by the concurrent downregulation of *Foxa2* and *Foxa3* in the fish, which results in a complete loss of node-derived structures, including the notochord and MFP (Dal-Pra et al., 2011). An identical phenotype is seen in *Foxa2*$^{-}$/$^{-}$ mutant mice where the absence of the node and notochord also precludes FP specification (Ang and Rossant, 1994; Norton et al., 2005).

Although conditional *Foxa2* or *Foxa1/2* knockouts targeting the ventral midbrain have been created in mice, they are not informative because they disrupt FOXA1/2 activity around E9, when ventral midbrain specification is well underway (Bayly et al., 2007; Blaess et al., 2006; Ferri et al., 2007; Fogel et al., 2008; Lin et al., 2009; Perez-Balaguer et al., 2009). Thus, the precise requirement for SHH and FOXA2 in FP specification remains poorly understood.

In this study, we have approached this problem by comparing the role of SHH and FOXA2 in midbrain and spinal FP specification in the chick, a species in which *FOXA1* or *FOXA3* genes have not been identified. We show that the midbrain FP can be clearly distinguished into MFP and LFP according to multiple criteria. Early manipulations of SHH and FOXA2 exclusively targeted to the neural plate suggest that SHH is not sufficient to induce the MFP in either the midbrain or spinal cord. By contrast, FOXA2 is necessary and sufficient for full MFP and LFP specification and does so via HH-dependent and independent mechanisms.

Notably, we show for the first time that the midbrain signaling centers express a common set of genes, and can take on each others' identities in a SHH-dependent manner. Thus, by regulating the identity of orthogonal signaling centers, MFP signals direct 3-dimensional patterning in the midbrain and hindbrain.

Materials and methods

Chick embryos

Fertilized Leghorn eggs (Ideal Poultry, Texas) were incubated at 38 °C. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Expression vectors

Embryos were electroporated with EGFP (EFX-EGFP), *Ptc1*$^{\Delta loop2}$ (pCIG-*Ptc1*$^{\Delta loop2}$), *Foxa2* (pMes-Foxa2-IRES-EGFP/EFX-Foxa2), *SHH* (XEX-SHH; pMes-SHH-ires-RFP), dominant negative *Foxa2* (pCAGGS-Fkh^{a2}-IRES-EGFP, pCAGGS-Fkh^{a2}-EnR–IRES-EGFP) or *FOXA2* RNAi (pSilencer-*FOXA2* shRNA) expression vectors (Agarwala and Ragsdale, 2002; Agarwala et al., 2001; Bayly et al., 2007; Briscoe et al., 2001; Jacob et al., 2007). EFX-*Foxa2* was constructed by ligating mouse *Foxa2* cDNA (pBSSK-*mHnf3b*) into EFX3C (Agarwala et al., 2001; Sasaki and Hogan, 1993). pMes-*Foxa2*-IRES-EGFP and pMes-*SHH*-ires-RFP were created by subcloning *Foxa2* or *SHH* cDNAs into the pMes-IRES-EGFP and pMes-IRES-RFP vectors.

FOXA2 shRNA constructs

Four short-hairpin RNA constructs were created by cloning into the pSilencer vector (Ambion). Of these, only one construct (FOXA2-1663) produced effective knockdown of FOXA2 mRNA (Supplementary Fig. S1A, B) and was generated using the following forward:

5′-CTCCTCCTAAAGGCAAAGGTTCAAGAGACCTTTGCCTTTAGGAGGAGTTTTTT-3′ and reverse primers: 5′-AATTAAAAAACTCCTCCTAAAGGCAAAGGTCTCTTGAACCTTTGCCTTTAGGAGGAGGGCC-3′.

RNAi electroporations (4 μg/μl) typically produced small effects, but replicated the larger effects of the dnFOXA2 constructs described above (Jacob et al., 2007). These data were therefore pooled.

In ovo electroporation

1–5 μg/μl DNA was electroporated into H&H stages 4–11 midbrains and H&H 8–11 spinal cords according to previous protocols (Agarwala and Ragsdale, 2002; Agarwala et al., 2001; Briscoe et al., 2001; Eom et al., 2011; Momose et al., 1999).

Midbrain explants

Whole embryo (H&H 3–6) or midbrain–hindbrain (H&H 7–9) explants were cultured for 24 h in 100 μM cyclopamine (Sigma)