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Differences in the spatiotemporal expression and epistatic gene regulation of the mesodiencephalic dopaminergic precursor marker *PITX3* during chicken and mouse development

Ruth Klafke[a], A. Alwin Prem Anand[b], Wolfgang Wurst[a,c,d,e§], Nilima Prakash[a,c,1§] and Andrea Wizenmann[b§]

[a] Institute of Developmental Genetics, Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH), Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

[b] Institute of Clinical Anatomy and Cell Analysis, University of Tübingen, Oesterbergstrasse 3, 72074 Tuebingen, Germany

[c] Technische Universität München-Weihenstephan, Lehrstuhl für Entwicklungsgenetik c/o Helmholtz Zentrum München, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

[d] Deutsches Zentrum für Neurodegenerative Erkrankungen (DZNE) Standort München, Schillerstr. 44, 80336 München, Germany

[e] Munich Cluster for Systems Neurology (SyNergy), Adolf-Butenandt-Institut, Ludwig-Maximilians-Universität München, Schillerstrasse 44, 80336 München, Germany

[§] *Corresponding Authors:*

*Andrea Wizenmann, Institute of Anatomy, University of Tübingen, Oesterbergstrasse 3, D-72074 Tuebingen, Germany. Tel.: +49-7071-2972177, Fax: +49-*7071-295124*, E-mail: andrea.wizenmann@ uni-tuebingen.de*

Wolfgang Wurst, Institute of Developmental Genetics, Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt GmbH, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany. Tel.: +49-89-3187-4110, Fax: +49-89-3187-3099, E-mail: wurst@helmholtz-muenchen.de;

Nilima Prakash, Hochschule Hamm-Lippstadt, University of Applied Sciences, Marker Allee

76-78, 59063, Hamm, Germany. Tel.: +49-2381-8789-512, E-mail: nilima.prakash@hshl.de

Present address:

[1] Hochschule Hamm-Lippstadt, University of Applied Sciences, Marker Allee 76-78, D-59063, Hamm, Germany

Email addresses:

Ruth Klafke: ruth.klafke@med.uni-muenchen.de
Alwin Prem Anand Asir Paulraj: alwinprem@gmail.com
Wolfgang Wurst: wurst@helmholtz-muenchen.de
Andrea Wizenmann: awizenmann@anatom.uni-tuebingen.de
Nilima Prakash: nilima.prakash@hshl.de

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In chick, dopaminergic progenitor marker *PITX3* is one of the earliest mdDA marker genes and is pivotally controlled by the SHH signaling pathway downstream of WNT9A.

Abstract

Mesodiencephalic dopaminergic (mdDA) neurons are located in the ventral mesencephalon and caudal diencephalon of all tetrapod species studied so far. They are the most prominent DA neuronal population and are implicated in control and modulation of motor, cognitive and rewarding/affective behaviors. Their degeneration or dysfunction is intimately linked to several neurological and neuropsychiatric human diseases. To gain further insights into their generation, we studied spatiotemporal expression patterns and epistatic interactions in chick embryos of selected marker genes and signaling pathways associated with mdDA neuron development in mouse. We detected striking differences in the expression patterns of the chick orthologs of the mouse mdDA marker genes *Pitx3* and *Aldh1a1*, which suggests important differences between the species in the generation/generating of these cells. We also discovered that the Sonic hedgehog signaling pathway is both, necessary and sufficient for the induction of ectopic *PITX3* expression in chick mesencephalon downstream of *WNT9A* induced *LMX1a* transcription. These aspects of early chicken development resemble the ontogeny of zebrafish diencephalic DA neuronal populations, and suggest a divergence between birds and mammals during evolution. .

Keywords (6): Dopamine precursors; chicken; mouse; *PITX3*; *WNT9a*; *SHH*

Introduction

Neurons producing the neurotransmitter/-modulator dopamine (DA) are present in the central nervous system of all chordates and in most other invertebrate phyla (Barron et al., 2010; Yamamoto and Vernier, 2011). The most prominent DA-synthesizing neuronal population, however, has evolved in tetrapods (amphibians, reptiles, birds and mammals) (Marin et al., 1998; Yamamoto and Vernier, 2011). These neurons emerge from ventral mesencephalon and diencephalon (prosomeres 1-3 (p1-p3)), and have been termed mesodiencephalic dopaminergic (mdDA) neurons (Puelles et al., 2013; Smidt and Burbach, 2007). The implication of mdDA neuron degeneration and/or dysfunction in several human neurological and psychiatric diseases, like Parkinson's disease, addictive disorders, schizophrenia, attention deficit/hyperactivity disorders and depression (Baik, 2013; Dauer and Przedborski, 2003; Howes and Kapur, 2009; Hyman et al., 2006; Nieoullon, 2002), has led to a strong clinical interest not only in deciphering the normal physiology and pathophysiology of these neurons, but also the cues responsible for their generation and survival during development and in adulthood.

In mouse, the intersection of three secreted morphogens: *Fgf8, Wnt1 and Shh*, controls the induction of proliferating mdDA progenitors in the ventral mesodiencephalic area (Hegarty et al., 2013; Smidt and Burbach, 2007). The mid-hindbrain boundary (MHB) releases Fgf8 and Wnt1, although Wnt1, however, additionally shows a localized expression in ventral mes- and diencephalon (Hegarty et al., 2013; Smidt and Burbach, 2007; Wurst and Prakash, 2014). *Shh* secreted from the FP (floor plate) is required for the establishment of progenitor domains in ventral mesencephalon(Blaess et al., 2006; Perez-Balaguer et al., 2009). However, to generate mdDA neurons WNT/β-catenin signaling has to suppress expression of *Shh* in this region (Joksimovic et al., 2009);(Joksimovic and Awatramani, 2014; Wurst and Prakash, 2014). Wnt1/β-catenin signaling also sequentially induces the expression of several transcription factors (TFs) in postmitotic mdDA precursors such as *Lmx1a* and *Pitx3*, of which both are necessary for proper mdDA neuron development (Blaess

and Ang, 2015; Hegarty et al., 2013; Veenvliet and Smidt, 2014; Wurst and Prakash, 2014). *Pitx3* regulates the transcription of several genes necessary to ensure the correct differentiation, function and survival of the substantia nigra pars compacta (SNc) mdDA neurons (Jacobs et al., 2009; Jacobs et al., 2007; Luk et al., 2013; Maxwell et al., 2005; Peng et al., 2011; Veenvliet et al., 2013). *Pitx3* in this area has been found to cooperate with the nuclear receptor *Nr4a2* (*Nurr1*) and with *Aldh1a1* (*Raldh1*, *Ahd2*) (Jacobs et al., 2009; Jacobs et al., 2007). Further genes implicated in mouse mdDA neuron development include the TFs *Lmx1b*, Engrailed (*En1/2*, and *Neurog2* (*Ngn2*) (Blaess and Ang, 2015; Hegarty et al., 2013; Veenvliet and Smidt, 2014).

In chick, the first neurons expressing the rate-limiting enzyme for DA synthesis, tyrosine hydroxylase (TH), appear in ventrocaudal diencephalon on embryonic day (E) 5.5-6, and only at E8 in the midbrain tegmentum (Puelles and Medina, 1994); (Smeets and Gonzalez, 2000). Previous studies in chicken embryos suggested a time-course similar to that in the mouse embryo as well as a SHH-mediated regulation of mdDA neuron generation (Agarwala et al., 2005; Andersson et al., 2006b; Bayly et al., 2007; Watanabe and Nakamura, 2000). However, little if anything is known about the early steps of mdDA neuron development in chick embryos. We, therefore, embarked on a detailed description of the early spatiotemporal expression pattern of the chicken ortholog of mouse *Pitx3* and an analysis of the epistatic relationships between this TF and WNT and SHH signaling pathways in the mesodiencephalic region. We show significant differences between the spatiotemporal expression patterns of selected mouse and chicken mdDA marker genes, and the epistatic regulation of *Pitx3* transcription. These results might reflect the evolutionary divergence of the mdDA neuronal population in birds and mammals.

Results

PITX3 is one of the first mdDA orthologs expressed in E2.5 chicken ventral diencephalon

We first determined the expression patterns of the chick orthologs to the mouse mdDA marker genes *PITX3*, *NR4A2* and *ALDH1A1* in relation to *LMX1A and LMX1B, both* markers for mdDA precursors in chick (Andersson et al., 2006b), *and NGN2,* a generic marker for mdDA neurogenesis (Andersson et al., 2006a; Kele et al., 2006) . At HH 16/17 (E2.5) *PITX3* was expressed in a small domain within the ventral diencephalon (Fig. 1B,B'). whereas neither *NR4A2* nor *ALDH1A1* were detected in the mesodiencephalic region (Fig. 1C-D'). *LMX1B*, was present along the entire mesodiencephalic area while *LMX1A* and *NGN2 were* only found rostrally in this region (*Lmx1a*, *Ngn2*; Fig. 1E-G'). Coronal sections showed that *PITX3*, *LMX1A/B* and *NGN2* dermarcate an overlapping area along the dorso-ventral axis of the diencephalic domain (Fig. 1B'-G'). At HH 21 (E3.5) the postmitotic mdDA marker *NR4A2* initiated expression in the caudally expanded *PITX3*$^{+}$ diencephalic area (Fig. 1H-J). At this time, *PITX3* was mostly confined to ventral diencephalic p1/2 domain, which is delimited by the *SHH*$^{+}$ zona limitans intrathalamica (ZLI) and the *PAX6*$^{+}$ expression at the di-/mesencephalic boundary (Kiecker and Lumsden, 2005; Puelles and Rubenstein, 2003)(Fig. 1K,L). As development progressed, *TH* mRNA appeared at HH 29 (E6.5) within the *EN1*$^{+}$ ventral midbrain (Fig. 1M-N', (Puelles and Medina, 1994)), and the expression of *PITX3* abutted the *FGF8*$^{+}$ domain at the MHB (Fig. 1N''). Coronal sections revealed that the *TH*$^{+}$ territory overlapped only laterally with *PITX3* and *NR4A2* (Fig. 1N'-O',O'',P''). At E9 (HH 35), *PITX3* expression overlapped with *TH* and *NR4A2* in the mesodiencephalic A9 (SNc) and A10 (ventral tegmental) areas, and a subset of the pretectal and hypothalamic A12/A14 DA cell groups in the chick (Fig. S1 A-K). Notably, *PITX3* and *NR4A2* were not expressed in the dorsal diencephalic A11 DA cells (Fig.S1 C,D,H,I) and *PITX3* is not exclusively expressed in NR4A2 or TH positive cells (Fig.S1 H,J). Strikingly, no expression of *ALDH1A1* or its two

paralogs, *ALDH1A2* and *ALDH1A3* (*ALDH6*), was detected in the mesodiencephalic area during the stages analyzed (Fig. 1D,D' and data not shown). We concluded that *PITX3* together with *LMX1A/B* and *NGN2* are the first genes associated with mdDA production to be expressed in these regions of chick neural tube. *PITX3* is initially confined to the diencephalic p1-3 domain in the rostral cephalic flexure but extends later into the ventral mesencephalon. Our analysis also revealed that in contrast to mouse, *TH* transcription initiates only 4 d after *PITX3* in *PITX3*$^+$ and *NR4A2*$^+$ cells. Later in development, *TH* is co-expressed with *PITX3* and *NR4A2* in the mesodiencephalic (A9/10), pretectal and hypothalamic (A12/14) DA groups, suggesting that *PITX3*$^+$ and *NR4A2*$^+$ precursors in chick ventral di-mesencephalon generate the mdDA neurons, among others. An overexpression of siRNA (pRNAT-siPitx3-GFP) against *PITX3* in midbrain resulted in a reduction of *PITX3, NR4A2, LMX1B and TH* expression in this region (Fig. S2; n=2), suggesting that *PITX3* indeed plays a role for the development of *TH*$^+$ DA neurones in chick mesencephalon.

PITX3 is first expressed in proliferating diencephalic neural progenitors and later in mesencephalic postmitotic neurons of the chicken embryo

Our data suggested that in contrast to mouse, where *Pitx3* expression is confined to postmitotic mdDA precursors and neurons (Maxwell et al., 2005; Smidt et al., 1997; Zhao et al., 2004), in chick *PITX3* is expressed earlier in the proliferating neural progenitors. To verify whether this is indeed the case, we pulse-labeled E3.5 chicken embryos for 2 h with BrdU and determined the number of BrdU$^+$ / *PITX3*$^+$ double-labelled cells versus those labelled with BrdU$^+$ alone in the diencephalic domain at E3.5 (Fig. 2A-B). We found that approximately 20% of all BrdU-labelled cells were also expressing *cPITX3* at E3.5 (Fig. 2C) (% Pitx3$^+$/BrdU$^+$ cells (mean±s.d.): chick #1, 19,07± 8,04 (n=6 sections); chick #2, 21,19± 6,65 (n=7); chick #3, 18,62± 6,07 (n=5); chick #4, 23,68± 4,97 (n=5)). Thus, at E3.5 a fraction of the diencephalic *PITX3*$^+$ cells were neural progenitors in S-phase. Double-labeling for *PITX3* and HuC/D, which labels post-mitotic neurons, confirmed that *PITX3* was expressed

in ventricular (VZ) and intermediate zone (IZ), and not in the HuC/D$^+$ mantle zone (MZ) of rostral diencephalon at HH 26 (E5; Fig. 2D,E). However, at E5 in ventral midbrain, *PITX3* expression was located exclusively in the HuC/D$^+$ MZ (Fig. 2F,G). Hence, chick *PITX3* shows an unexpected dichotomy in its expression. In diencephalic cells it is present in proliferating neural progenitors and early postmitotic precursors, whereas in the mesencephalon it appears in late postmitotic precursors and differentiating neurons.

Divergent expression patterns of *PITX3* in the developing chicken and mouse brain

These results suggested that the spatiotemporal expression pattern of *PITX3* in this region of the developing brain of mouse and chicken may have diverged during evolution. Thus prompted, we re-examined the spatiotemporal expression pattern of *Pitx3* in the developing mouse embryo. *Pitx3* is first detected at E11.5 in mouse brain (Smidt et al., 1997), and we confirmed restricted *Pitx3* expression in ventral midline of the mouse mesencephalon between E11.5 and E14.5 (Fig. S3A,A',C,C',E,E' and data not shown; n=3/stage; (Hoekstra et al., 2013)). However, our expression analysis futher revealed two additional *Pitx3*$^+$ domains in the di- and mesencephalon. We found, *Pitx3* expression was also located at the dorsolateral caudal mesencephalon close to the MHB between E12.5 to E18.5, (Fig. S3B,B',D,D',F,F'; n=3/stage). From E14.5 till E18.5, the initially ventral mesencephalic expression domain of *Pitx3* extended as a narrow stripe in the ventrolateral caudal diencephalon (Fig. S3F-H'). The *Pitx3*$^+$ domain in dorsolateral caudal midbrain did not overlap with *Th* expression (Fig. S1B',G-H''' and data not shown), whereas the *Pitx3*$^+$ domain in the ventrolateral caudal diencephalon clearly overlapped with *Th* expression (Fig. S3G-H'''). Thus, in mouse *Pitx3* expression is initially confined to the mesencephalic mdDA domain and only later extends into a diencephalic mdDA domain. This is the exact opposite of the expression pattern of *PITX3* in chick, which initiates in the diencephalon and then expands into the midbrain. In mouse Pitx2, which is expressed in diencephalon might play a similar role as chick PITX3 in this brain region (Martin et al., 2002; Muccielli et al., 1996). We

also discovered a so far unknown non-dopaminergic *Pitx3* expression domain in the developing mouse mesencephalon.

Divergent expression of *WNT* genes in the developing chicken and mouse brain

Murine *Pitx3* is an indirect target of the WNT1/β-catenin signaling pathway during mouse mdDA development (Chung et al., 2009). To investigate whether *PITX3* in chick is similarly induced by a member of the WNT/β-catenin family, we conducted a detailed literature and RISH screen of chick *WNT* gene expression patterns at E2.5. Our research identified three patterns of *WNT* gene expression in this region. The first is exemplified by *WNT11* and *WNT8C*, which are not expressed in anterior neural tube (see Fig. 3K for *WNT11* and (Hume and Dodd, 1993) for WNT8a). The second is displayed by *WNT1*, *WNT3A*, *WNT4*, *WNT6*, *WNT7B* and *WNT8B,* all of which are either restricted to the dorsal midline or spared the ventral midline of the di- and mesencephalon (Fig. 3F-J, for *WNT7B* and W*NT8B* see (Garda et al., 2002)). Thus, their expression did not overlap with the *PITX3*$^{+}$ mdDA domain in E2.5 brains. The third pattern is that shown by *WNT5A*, *WNT5B*, *WNT7A* and *WNT9A,* which do show expression in the ventral mesodiencephalic *region.* Of these, *WNT9A* overlapped closest with *PITX3* expression in the cephalic flexure of the E2.5, E3.5, and E5 chick brains (Fig. 3L-R), and thus its expression resembled that of mouse *Wnt1*. Interestingly, murine *Wnt9a* expression was mostly restricted to the dorsal midline of the anterior neural tube (fore-, mid- and hindbrain) in midgestational mouse embryos (Fig. S4A-F'), resembling *WNT1* expression pattern in chick brain. In E5 chick brain *WNT9A* expression became confined to the VZ of the cephalic flexure, whereas *PITX3* expression was restricted to the mesencephalic MZ (Fig. 3R and Fig. 2D). Taken together, *WNT9A* and *PITX3* are transcribed in an overlapping pattern in the cephalic flexure of the early chicken brain (E2.5-E3.5, Fig. 3S). Later in development (E5), *WNT9A* is expressed in di-/mesencephalic neural progenitors in the VZ generating the *PITX3*$^{+}$ postmitotic precursors in the MZ. Thus, it would seem that *WNT9A* performs a similar function in chicken as *Wnt1* in mouse.

WNT9A induces a transient ectopic expression of _LMX1A_ and sustained ectopic activation of mdDA-related genes in the chicken mesencephalon

To establish a possible role for *WNT9A* for the induction of *PITX3* expression and the formation of mdDA precursors in the chicken brain we electroporated full-length and bicistronic *WNT9A*/*GFP* (pMES-WNT9A-IRES-eGFP) into the right ventrolateral and/or lateral half of the mesencephalon at E1.5 (HH 10-12). After 1 dpe neither *SHH*, (Fig. 4D), nor *PITX3*, *NR4A2* or *LMX1B* were induced ectopically (data not shown) only *LMX1A* (Fig 4A-C) was detected in the *WNT9A* electroporated side. Two dpe (n=2), *SHH* and *PITX3* expression expanded ectopically towards the dorsal mesencephalon in the electroporated side (Fig. 4E,G,H). *WNT9A* induced *LMX1A* expression had ceased 2 dpe (Fig. 4F I,J). 3 dpe (n=5) ectopic *PITX3*, *NR4A2* and *NGN2* were still present within the *WNT9A* electroporated domain (Fig. 4K,L). *WNT9A* overexpression also resulted in ectop*ic NGN2* expression in ventrolateral mesencephalon (compare bracketed areas in (left) and (right) midbrain in Fig. 4L), and ectopic *PITX3* expression.

Importantly, we found that ectopic *LMX1A* was only found in *WNT9A* expressing cells (Fig. 4C). In contrast, cells ectopically expressing *SHH*, *PITX3* and *NR4A2* were both those electroprated with *WNT9A* as well as adjacent non-electroporated, *WNT9A*-negative cells (eg. compare Fig. 4E with Fig. 4G,H,L). This suggests a non-cell-autonomous induction of the latter genes. Notably, the expansion of ectopic *SHH*, *PITX3* and *NR4A2* respected a ventro-dorsal border in midbrain, with no expression occurring in dorsal mesencepalon even though ectopic *WNT9A*$^{+}$ cells were present dorsally (compare Fig. 4E with Fig. 4G,H, and Fig. 4I with Fig. 4K,L). This may be due to the fact that there are SHHhh and WNT inhibitors expressed in the dorsal midbrain (Ladher et al., 2000; Li et al., 2007; Paxton et al., 2010; Quinlan et al., 2009).

To determine whether the ectopic induction of *LMX1A* and subsequently of *PITX3* after overexpression of *WNT9A* was specific to WNT9A or if it could also be mediated by other *WNTs* expressed in the ventral midline of the chicken di- and mesencephalon, we

electroporated full-length *WNT1*, *WNT5A* and *WNT7A* into ventrolateral di-mesencephalon (Fig. S5). Interestingly, 3 dpe we did not detect any ectopic expression of *PITX3*, *LMX1A*/B, or *SHH* after overexpression of any of those Wnts.

Our findings suggest that the expression of *LMX1A* is directly (cell-autonomously) activated by *WNT9A* mediated signaling, whereas the ectopic induction of *SHH*, *PITX3* and *NR4A2* might be mediated by an intermediate signaling pathway or indirect (non-cell-autonomous) mechanism in the developing chick mesencephalon.

Lmx1a induces ectopic expression of _SHH_, _PITX3_ and mdDA-related genes in chick mesencephalon

To determine whether *LMX1A* is sufficient to induce the subsequent expression of *SHH*, *PITX3*, *NR4A2 and LMX1B* in chick mesencephalon, we electroporated mouse *Lmx1a* (Andersson et al., 2006b) together with *eGFP* as markergene (*pCAX-EGFP*, (Chen et al., 2004)) into the right ventrolateral di-mesencephalon. One dpe of *Lmx1a/GFP* (n=5), *WNT9A*, *SHH* and *PITX3* expression remained restricted to their endogenous domains in the right ventral diencephalon (Fig. 5A-D). Two dpe of *Lmx1a/GFP (*n=4), ectopic expression of *SHH* and *PITX3* was detected in the electroporated ventrolateral mesencephalon in a similar continuous and dorsally extending pattern as after *WNT9A* electroporation, whereas ectopic *Lmx1a* expression was patchy (compare Fig. 5E with G,H). *WNT9A* expression was only weakly induced in mesencephalon 3 dpe of *Lmx1a/GFP* (n=8), (Fig. 5F,I,J). At 3dpe, there was also strong ectopic, continuous and dorsally extending expression of *NR4A2*, *LMX1B* and *NGN2,* and additionally *SHH* and *PITX3* expression appeared in the transfected ventrolateral mesencephalon. Again, the extension of ectopic expression appeared to obey a similar ventro-dorsal limit as observed after *WNT9A* electroporation (Fig. 5 K-O). Thus, *Lmx1a* can induce ectopic expression of mdDA precursor markers with a similar temporal delay of 2 dpe as *WNT9A* in chick mesencephalon (Fig. 5P). Taken together, our findings strongly indicate that ectopic induction of mdDA precursor markers by *WNT9A* and its

putative direct target gene *LMX1A* is in fact mediated by an intermediate signaling pathway (non-cell-autonomous) in the developing chicken mesencephalon. In addition, the indirect activation of *WNT9A* transcription by *Lmx1a* suggests a feedback mechanism between these two genes in the chicken midbrain.

***SHH* is sufficient for ectopic induction of *PITX3* expression in chick mesencephalon**

Since *SHH* expression expanded after ectopic *WNT9A* or *Lmx1a* electroporation, we next asked whether *SHH* was sufficient to induce expression of *PITX3* and other mdDA marker genes in the mesencephalon. Overexpression of *SHH* in ventrolateral di-mesencephalon led to an ectopic induction of *PITX3* in the transfected tissue after 2 dpe (n=3) but not after 1 dpe (n=2) (Fig. 6A,B,D,E). Ectopic *PITX3* persisted 3 dpe (n=5) and was present in non-transfected (*GFP*$^{-}$) cells (compare Fig. 6F,G), with a similar ventro-dorsal midbrain boundary to that observed after *WNT9A* and *Lmx1a* overexpression (see Figs 4 and 5). *SHH* did not induce any ectopic expression of *LMX1A* and *WNT9A* even after 3 dpe. This indicated that both genes act upstream of *SHH* (Fig. 6C,H-J). We concluded that WNT9A-mediated signaling directly activates its putative target gene *LMX1A* and that *LMX1A* in turn induces the expression of *SHH*, which is sufficient for an induction of *PITX3* expression in the chicken mesencephalon.

SHH signaling is necessary for the ectopic induction of *PITX3* expression in the chicken mesencephalon

Our results suggested that the SHH signaling pathway is the most likely candidate for any indirect (non-cell-autonomous) activation of ectopic *PITX3* transcription by WNT9A in chicken mesencephalon. To determine whether SHH signaling is necessary for ectopic induction of *PITX3*, we treated *WNT9A* or *LMX1A* electroporated neural tubes with cyclopamine, a potent inhibitor of SHH signaling pathway (Chen et al., 2002; Incardona et al., 1998), or with DMSO, as control. Two dpe (n≥2 to 5/treatment) of *WNT9A* and treatment with DMSO, *PITX3*

expression was induced and expanded dorsally into the ectopic *WNT9A* area (Fig. 7A,B). The expression of *PTCH1*, the SHH receptor (Chen and Struhl, 1998) appeared also stronger within the electroporated site (Fig.7C). Embryos at 3 dpe (n≥2/treatment) of *LMX1Aa* treated with DMSO showed weakly ectopic *WNT9A* and strong ectopic, dorsally expanded expression of *SHH*, *PITX3* and *NR4A2* in the mesencephalon (Fig. 7G-K). Thus, as already previously described (Fig.4,5) both, ectopic *WNT9A* and *LMX1A* induced ectopic *SHH* and *PITX3*. However, in the cyclopamine-treated embryos no ectopic *PITX3* transcription was induced at 2 dpe (Fig. 7D,E,L-O). Endogenous *PITX3* and *PTCH1* expressions in the electroporated side are both slightly repressed, despite a strong ectopic induction and dorsal expansion of *SHH* transcription (Fig. 7D,E,L-O and data not shown). Interestingly, cyclopamine treatment did not affect endogenous expression of *PITX3*, *SHH*, *WNT9A* and *PTCH1* in the untransfected mesencephalon (Fig. 7D-F,M-P). These results indicated that SHH signaling is required for the ectopic activation but not for the endogenous maintenance of *PITX3* transcription.

Interestingly, the endogenous ventrolateral expression domain of *PTCH1* corresponds to the ventrodorsal extent of ectopic gene induction observed after *WNT9A* electroporation (Fig. 7B,C) and is reduced after cyclopamine treatment. Thus, the reception of the SHH signal via PTCH1 might be essential for ectopic activation of *PITX3* in this region. Blocking the SHH signal transduction pathway by cyclopamine mainly abolished ectopic transcription of *PITX3* without affecting endogenous and/or ectopic transcription of *WNT9A* and *SHH* (Fig. 7H,I). We conclude that signaling mediated by the ectopically induced expression of *SHH* after electroporation of *WNT9A* or *LMX1A* is necessary and sufficient to activate the ectopic transcription of *PITX3* in chick mesencephalon.

Discussion

In this study, we have shown a number of key differences between chicken and mice with respect to the spatiotemporal expression pattern of selected mdDA marker genes, such as *PITX3* and *ALDH1A1* and their regulation by the WNT/β-catenin and SHH signaling pathways. These differences point to evolutionary divergences in the genetic mechanisms that control the generation of proliferating mdDA progenitors and precursors between birds and mammals.

Divergent spatiotemporal expression patterns of selected mdDA marker genes in chicken and mice

In mice, *Pitx3* is crucial for the correct differentiation of mdDA neurons, especially of the SNc DA neurons, and their survival during development and adulthood (Blaess and Ang, 2015; Hegarty et al., 2013; Veenvliet and Smidt, 2014). *Pitx3* expression initiates in the ventral mesencephalon and later extends into the caudal ventral diencephalon and it is restricted to postmitotic mdDA precursors and neurons located in the MZ of mesencephalic tegmentum (this work, (Hoekstra et al., 2013). The expression of this gene initiates only shortly before or just after *Th* expression at E11.5 in mouse (Maxwell et al., 2005; Smidt et al., 1997; Zhao et al., 2004). We have shown here that the murine *Pitx3* expression pattern is only partly conserved in chick embryos: *PITX3* transcription initiates in the ventral chick diencephalon (p1/2) and only later extends into the mesencephalon. In fact, *PITX3* together with *LMX1A*/*B* and *NGN2* are the first mdDA marker genes expressed in the cephalic flexure of chick brain even before *NR4A2* transcription is initiated. In the chick diencephalon, *PITX3* is expressed in proliferating neural progenitors and early postmitotic precursors. Though, in the chick mesencephalon *PITX3* expression is restricted to postmitotic precursors and neurons within the tegmentum. We also detect a notable time gap of 4 d between the initiation of *PITX3* transcription in chick mesodiencephalic region (at E2.5) and the first expression of *TH* (at E6.5 (Puelles and Medina, 1994). This time gap contrasts with that seen during mouse

development but resembles the 4 d delay in TH expression during the histogenesis of chick DA amacrine neurons (Gardino et al., 1993); reviewed by (Smeets and Gonzalez, 2000). *TH* transcription in the mesencephalon of the E6.5 chick embryo is restricted to a lateral tegmental domain that appears to derive from a *PITX3*$^{+}$ and *NR4A2*$^{+}$ neural (mdDA) precursor area. At E9, expression of *TH* overlaps with that of *PITX3* and *NR4A2* in the mesodiencephalic A9/10, pretectal and hypothalamic A12/14 DA cell groups, suggesting that these neurons do indeed derive from *PITX3*$^{+}$ and *NR4A2*$^{+}$ precursors.

We also discovered another notable difference between chicken and mice and that is the lack of *ALDH1A1* expression in the chick ventral mesencephalon. In mouse, *Aldh1a1* expression initiates around E9.5 in proliferating mdDA progenitors and is later confined to a rostrolateral mdDA neuron subset (Jacobs et al., 2007; Smits et al., 2013; Stuebner et al., 2010; Wallen et al., 1999). The complete absence of expression of *ALDH1A1* and its two paralogs, *ALDH1A2* and *ALDH1A3* (*ALDH6*), in the chicken mesencephalon is similar to the lack of *ALDH1A* expression in quail mesencephalon during development (Reijntjes et al., 2005). Although we cannot exclude the expression of these enzymes in the chicken VM later in development, our finding strongly suggests that the RA-synthesizing and DA-metabolizing ALDH1 family members are not involved in early stages of chicken mdDA neuron development and survival.

By contrast, we found that the spatiotemporal expression patterns of *LMX1A/B*, *NR4A2* and *NGN2* were conserved between chick and mice (this report; (Andersson et al., 2006b), suggesting that these TFs are under similar transcriptional control and most likely direct the same developmental pathways (Andersson et al., 2006b). The transcription of endogenous and ectopic *NR4A2* always initiated one day after endogenous and ectopic *PITX3* expression in chick, indicating that the onset of *NR4A2* transcription after *PITX3* was preserved after ectopic overexpression of upstream inducing factors.

Divergent transcriptional regulation of *cPitx3* expression by the SHH or WNT signaling pathways in chicken and mice

The divergent spatiotemporal expression pattern of *PITX3* in chick and mouse suggests different transcriptional regulation between the species. In mice, WNT1-mediated β-catenin signaling controls the correct differentiation of mdDA progenitors/precursors into mature mdDA neurons through the direct activation of *Lmx1a* (Chung et al., 2009; Joksimovic et al., 2009; Prakash et al., 2006; Tang et al., 2009; Tang et al., 2010);(Joksimovic and Awatramani, 2014; Wurst and Prakash, 2014). Murine LMX1A, in turn, binds to and activates the transcription of the *Wnt1*, *Pitx3* and *Nr4a2* promoters (Chung et al., 2009) (Wurst and Prakash, 2014) our own unpublished data). In contrast to the chick (Andersson et al., 2006b), in the murine brain transcription of *Lmx1a*, *Pitx3* or *Nr4a2* is not regulated by the SHH signaling pathway (Chung et al., 2009). Hence mature mouse TH^+ mdDA neurons do not derive from SHH-responsive cells (Mesman et al., 2014). Furthermore, ablation of SHH signaling components in midgestational mdDA precursors does not affect the generation of mdDA neurons in the mouse embryo (Blaess et al., 2006; Zervas et al., 2004). Instead, the expression of *Shh* has to be antagonized by active WNT/β-catenin signaling to enable mdDA neurogenesis in mouse mesencephalon (Joksimovic et al., 2009). Thus, SHH signaling pathway seems to play a minor role during murine mdDA neuron development (Fig. 8B).

We have also investigated the contribution of the WNT/β-catenin pathway to the generation of mdDA precursors in the chick and the epistatic relationships between SHH and WNT signaling pathways and *PITX3* in this species. We confirmed that *WNT1* is not expressed in the chick ventral mesodiencephalic region (Hollyday et al., 1995), but rather found that *WNT9A* exhibits the closest overlap with *PITX3* expression in this region. Although *WNT1* and *WNTt9a* expression in mouse and chick is reversed, both WNT proteins signal via the WNT/β-catenin (“canonical”) pathway (Guo et al., 2004; Megason and McMahon, 2002), and in keeping with this we found that ectopic expression of *WNT9A* activated ectopic

transcription of *LMX1A* in a cell-autonomous manner. Thus, the epistatic relationships and gene regulatory interactions between the WNT signaling pathway and *Lmx1a* seem to be conserved between chicken and mice (Fig. 8A,B). Ectopic and non-cell-autonomous induction of *SHH*, *PITX3*, *NR4A2* and *NGN2* expression with a temporal delay of 2-3 d after electroporation of *WNT9A* or *LMX1A* into the mesodiencephalic area of the chick suggested that these are indirect targets of WNT9A and LMX1A.

In contrast to the mouse, *WNT9a* and *LMX1A* appear to act upstream of the SHH signaling pathway in the chick. We showed that SHH signaling was both, necessary and sufficient to induce ectopic and non-cell-autonomous expression of *PITX3*, but not of *WNT9A* and *LMX1A* in the chicken mesodiencephalic area (Fig. 8A). Our results agree with (Watanabe and Nakamura, 2000) who showed that ectopic expression of *SHH* in the chicken mesencephalon induced non-cell-autonomously ectopic TH^+ DA neurons, and thus strongly supports a crucial role of the SHH pathway for the generation of mdDA neurons in chick but not for the maintenance of PITX3 transcription. In agreement with (Andersson et al., 2006b), we noted that despite a strong expression of transgenes in dorsal mesencephalon no ectopic induction of mdDA marker genes in dorsal midbrain ever occurred. This dorso-ventral expression boundary seems to coincide with expression of *PTCH1*, which is restricted to the ventral midbrain. Thus, only the ventrolateral mesodiencephalic region of the chick embryo appears to be competent to respond to SHH inductive signal for mdDA neuron development in this region. The limited ectopic induction of *SHH* and *PTCH1* to ventral midbrain might be due to the expression of several proteins present in dorsal midbrain like RAB23 and GLI3 that suppress SHH signalling dorsally (Li et al., 2007; Litingtung and Chiang, 2000; Persson et al., 2002). Our observation also further confirm the presence of a dorso-ventral signalling boundary in the chick midbrain (Li et al., 2005)

The ontogeny of chicken mdDA precursors might reflect their phylogenetic history

The clear differences in spatiotemporal appearance of the *PITX3*$^{+}$ mdDA progenitors/precursors and in *PITX3* transcriptional regulation by the WNT/β-catenin and SHH signaling pathways points not only to ontogenetic but also phylogenetic differences in mdDA development between birds and mammals. In fact, the transcriptional regulation of *PITX3* in the early chick embryo exhibits more similarities to zebrafish than to mouse. Zebrafish *pitx3* is expressed in proliferating diencephalic progenitors and postmitotic precursors, but not in TH^{+} DA neurons (Filippi et al., 2007). Moreover, zebrafish *pitx3* is a target of the NODAL (a TGF family member) and HH pathways in neural tissues (Zilinski et al., 2005). We and others showed that both pathways are implicated in chicken mdDA development (Agarwala et al., 2005; Andersson et al., 2006b; Bayly et al., 2007; Farkas et al., 2003; Watanabe and Nakamura, 2000). Sequence comparisons show chick *PITX3* gene being more closely related to *Xenopus* and zebrafish *pitx3* than to the mammalian *Pitx3* genes (Zilinski et al., 2005). The cues directing the expression (and possibly also function) of *Pitx3* during mdDA development thus appear to have diverged considerably between the avian and teleost lineages and the mammalian lineage, and it remains to be investigated whether this is also the case for the amphibian and reptile lineages.

Materials and Methods

Chicken and mouse embryos

Fertilized White-Leghorn chicken eggs (Brüterei Hölzl, Moosburg/Germany) were incubated at 38°C until the desired Hamburger and Hamilton (HH, (Hamburger and Hamilton, 1951)) stage. Outbred CD-1 mouse embryos were collected from timed-pregnant females (Charles River, Kisslegg/Germany), noon of the day of vaginal plug detection was designated as E0.5. Pregnant dams were killed by CO_2 asphyxiation. All expression studies are based on a minimal of 3 embryos per stage. This study was carried out in strict accordance with the

recommendations in the EU Directive 2010/63/EU and the Guide for the Care and Use of Laboratory Animals of the Federal Republic of Germany (TierSchG). The protocol was approved by the Institutional Animal Care and Use Committee (ATV) of the Helmholtz Zentrum München. All efforts were made to minimize suffering.

Radioactive and whole-mount in situ hybridization (RISH/WISH).

8 µm paraffin sections were processed for radioactive RISH as described by (Fischer et al., 2007). WISH using digoxigenin- or fluorescein-labeled riboprobes was performed according to (Henrique et al., 1995). Riboprobes used were: chicken *PITX3* (ChEST246m15), *ALDH1A1* (ChEST396f5), *ALDH1A2* (ChEST650k18), *EN1* (ChEST92p12), *TH* (ChEST1010e8), *WNT3A* (ChEST1005M7) obtained from UK chicken EST Consortium (Boardman et al., 2002); *ALDH6* (*ALDH1A3*; RefSeq NM_204669), *LMX1A* (XM_001236605) and *PTCH1* (NM_204960) (R. Klafke), *LMX1B* (Matsunaga et al., 2002), *NGN2* (*NEUROG2*) (D. Henrique), *SHH* (Nohno et al., 1995), *PAX6* (Goulding et al., 1993), *FGF8* (Crossley et al., 1996), *WNT1* (Bally-Cuif and Wassef, 1994), *WNT4*, *WNT5A* and *WNT5B* (Hartmann and Tabin, 2000), *WNT6* (NM_001007594) and *WNT7A* (NM_204292) (A. Wizenmann), *WNT9A* (Hartmann and Tabin, 2001), *WNT11* (C. Hartmann); mouse *Pitx3*, *Th* and *Nr4a2* (Brodski et al., 2003), *Wnt9a* (NM_139298) (J. Zhang); and *GFP* (R. Koester).

Immunohistochemistry

Whole-mount IHC was performed as described by (Li et al., 2005) after WISH for *PITX3* using mouse anti-HuC/D, a marker for early postmitotic and differentiating neurons (1:600; Molecular Probes/Germany). Post-fixed (4% paraformaldehyde (PFA)), gelatin-albumin-embedded embryos were vibratome-sectioned (40 µm).

5'-bromo-2-deoxyuridine (BrdU) treatment and cell counting.

BrdU (250 µg/ml in 0.9% saline; Sigma/Germany) was injected into anterior neural tube of HH 21 (E3.5) embryos (Belecky-Adams et al., 1996), incubated for 2 h, fixed in 4% PFA,

processed for *cPitx3* WISH, post-fixed in 4% PFA, transferred into 20% sucrose/PBS and cryosectioned (16 µm) and processed with a BrdU Labeling and Detection Kit II (Roche/Germany). *cPitx3*$^{+}$/BrdU^{+} double-labeled and BrdU^{+} single-labeled cells were counted (5-7 sections, 4 different BrdU-treated embryos), and the proportion of double- versus single-labeled BrdU^{+} cells was calculated for each embryo.

Imaging

Images were taken with a LSM 510 META confocal laser scanning microscope, Axioplan2 microscope or StemiSV6 stereomicroscope (Zeiss) and processed with Adobe Photoshop CS3 software (Adobe Systems Inc./USA).

Vector constructs for electroporation

Full-length chicken *WNT9A* (*pMES-WNT9A-IRES-eGFP*), *WNT5A* (*pMES-WNT5A-IRES-eGFP*), *WNT7A* (*pMES-WNT7A-IRES-eGFP*), *SHH* (*pMES-SHH-IRES-eGFP*), and mouse *LMX1A* (*pMES-LMX1A-IRES-eGFP*) and *WNT1* (*pMES-WNT1-IRES-eGFP*) cDNAs were inserted into the *pMES* expression vector that contains an internal ribosomal entry site (*IRES*) followed by an enhanced Green Fluorescent Protein (*eGFP*) (Swartz et al., 2001). The *pECE-mLmx1a* and *pCAX-EGFP* vectors were described by (Andersson et al., 2006b; Chen et al., 2004).

***In ovo* electroporation.**

Chick neural tubes were injected with vector DNA (1-3 µg/µl, 0.005% Fast Green (Sigma)) and electroporated into ventrolateral and/or lateral di-mesencephalon (Huber et al., 2013). After 1-3 d (dpe), embryos were removed from egg, staged, and fixed in 4% PFA at 4°C.

Cyclopamine treatments

Cyclopamine (100 µM in DMSO; Biomol/Germany) or DMSO as control were injected into neural tube 3 and 24 h post-electroporation. Embryos were incubated for another 24-48 h before fixation (4% PFA).

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Author contributions

N.P., A.W.,W.W. conceived the project; R.K., N.P., W.W., and A.W. designed experiments; A.P.A., R.K., N.P., and A.W. performed experiments; R.K., A.P.A., N.P., W.W., and A.W. prepared and edited the manuscript.

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Figures

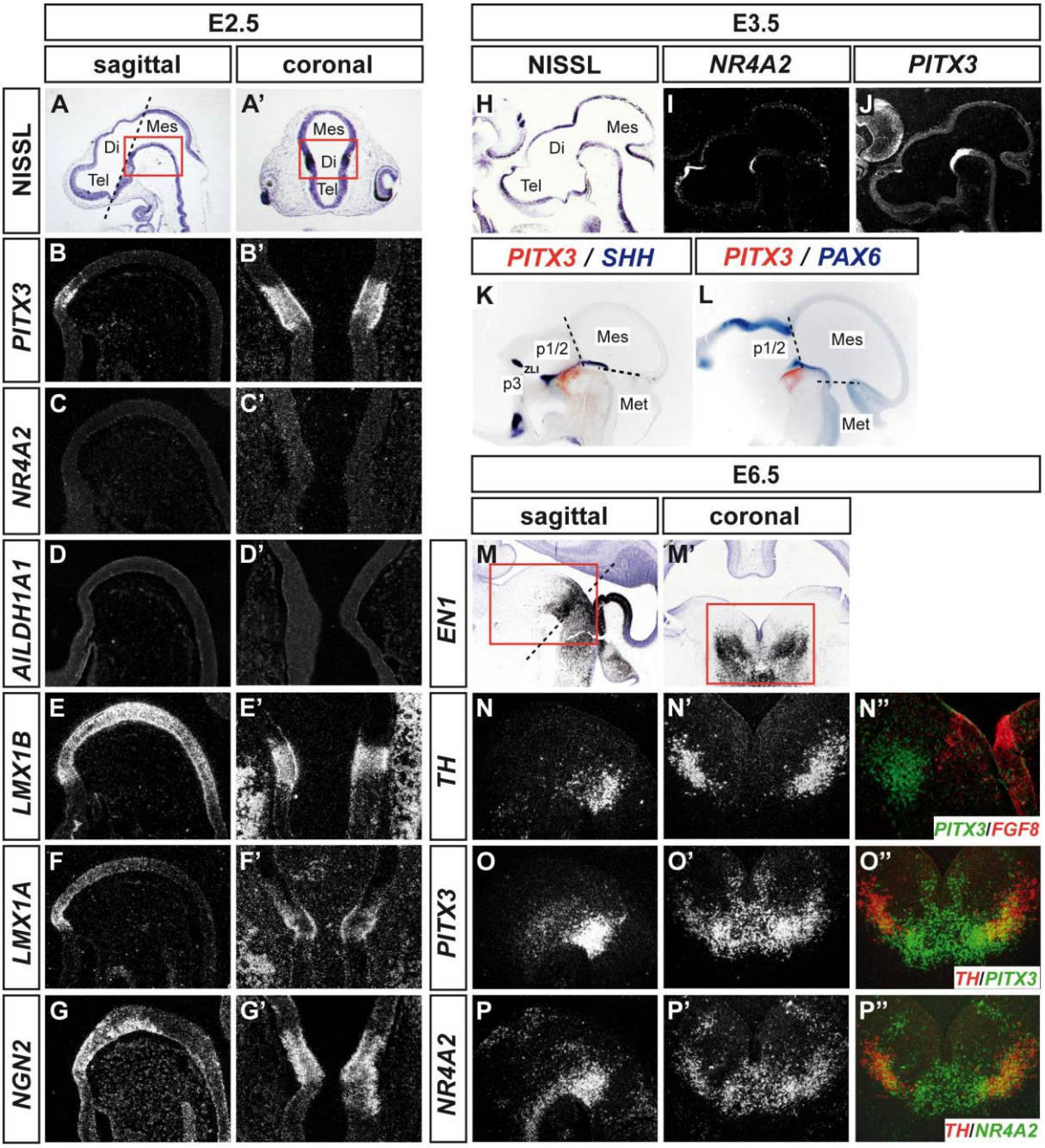

Fig. 1

***PITX3* is one of the first mdDA orthologs expressed in the E2.5 chicken ventral diencephalon.** Midsagittal (A,-G,H-J,M-P,) and coronal (A'-G',M'-P'') paraffin sections from head or brain of E2.5 (HH 16/17) (A-G'), E3.5 (HH 21) (H-J) and E6.5 (HH 29) (M-P'') chick embryos. Red rectangles in Nissl stained (A,A',M,M') depict the sector of (B-G, B'-G', N-P and N'-P'') respectively. Expressions of *PITX3* (B,B',J,N''-O''), *NR4A2* (C,C',I,P-P''), *ALDH1A1* (D,D'), *LMX1B* (E,E'), *LMX1A* (F,F'), *NGN2* (G,G'), *EN1* (M,M'), *TH* (N,N',O'',P'') or *FGF8* (N'') were examined by *RISH*. (N''-P'') are pseudo-colored overlays of consecutive sections hybridized for *PITX3* (green in N'',O''), *FGF8* (red in N'') (N''), *TH* (red in O'',P'') and *NR4A2* (green in P'') with overlapping expression domains in yellow. (K,L) Midsagittal sections of E3.5 (HH 21) chicken heads show expression (*in situ* hybridization*)* of *PITX3* (red) and *SHH* (blue) (K) or *PITX3* (red) and *PAX6* (blue) (L). All expression pattern studies involved n≥3 embryos per stage. Black broken lines delimit the border between the mesencephalon and the p1/2 domain or the metencephalon. Abbreviations: Di, diencephalon; Mes, mesencephalon; Met, metencephalon; p1/2, prosomere 1/2; p3; prosomere 3; Tel, telencephalon; ZLI, zona limitans intrathalamica.

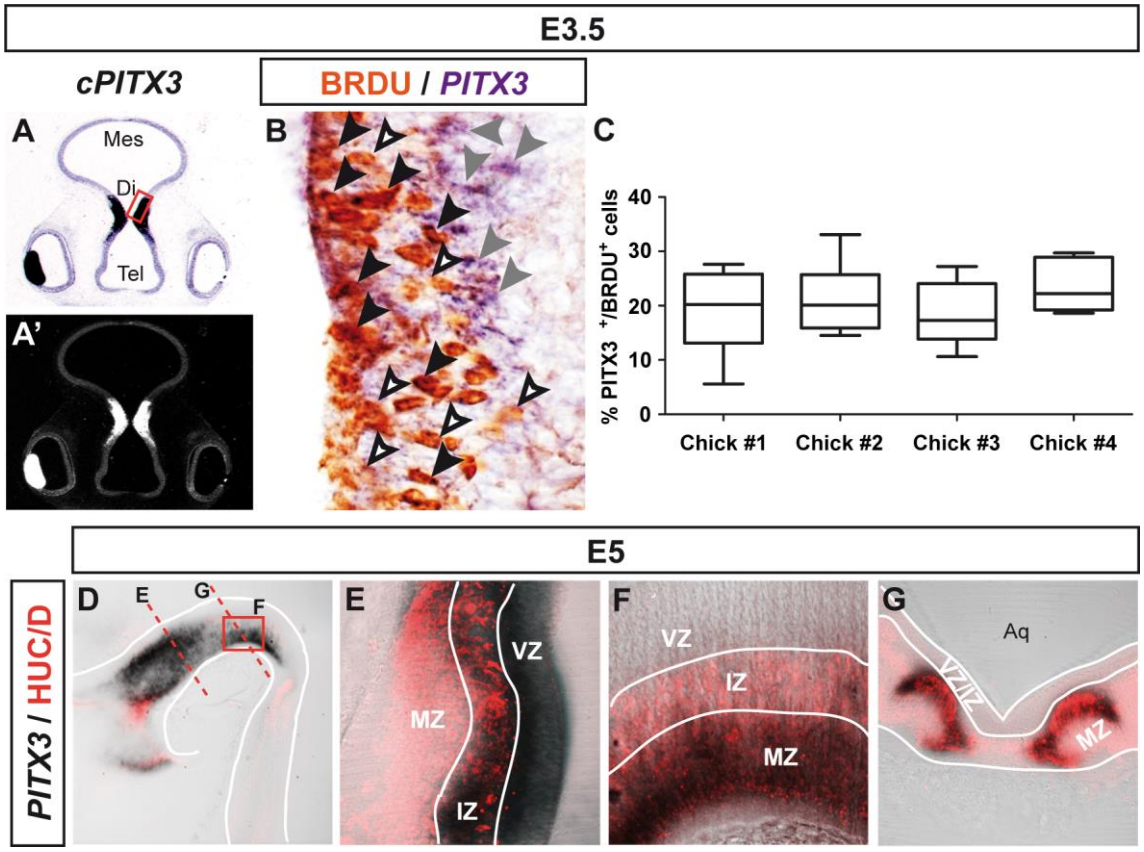

Fig 2

***PITX3* is first expressed in proliferating diencephalic neural progenitors and later in mesencephalic postmitotic neurons of the chicken embryo. (A-A')** Nissl-stained coronal section of E3.5 rostral mesencephalon and caudal diencephalon hybridized for *PITX3* (A brightfield, A' darkfield). (B) Close-up of the boxed area in (A) showing BrdU immunostaining (open arrowheads) and *PITX3* RISH (grey arrowheads). Black arrowheads point at *PITX3*/BrdU double-positive cells. (C) Quantification of *PITX3*/BrdU double-positive cells per total BrdU single-positive cells in the caudal diencephalon/rostral mesencephalon of four different BrdU-treated E3.5 chicken embryos. Upper and lower hinges of the box-and-whiskers plots correspond to the first and third quartiles; whiskers extend to the minimum and maximum values; and lines within boxes represent median. (% $Pitx3^{+}/BrdU^{+}$ cells

(mean±s.d.): chick #1, 19,07± 8,04 (n=6 sections); chick #2, 21,19± 6,65 (n=7); chick #3, 18,62± 6,07 (n=5); chick #4, 23,68± 4,97 (n=5)) (D-G) Midsagittal (D,F) and coronal (E,G) sections from ventral mesencephalon and caudal diencephalon of E5 (HH 26) chick, *RISH* for *PITX3* (black) and immunostained for HuC/D (red). Levels of (E,G) are indicated by red lines and the area of F by the red rectangle in D. White lines delimit neuroepithelium, IZ and VZ/MZ. Abbreviations: Aq, aqueduct; Di, diencephalon; IZ, intermediate zone; Mes, mesencephalon; MZ, mantle zone; Tel, telencephalon; VZ, ventricular zone.

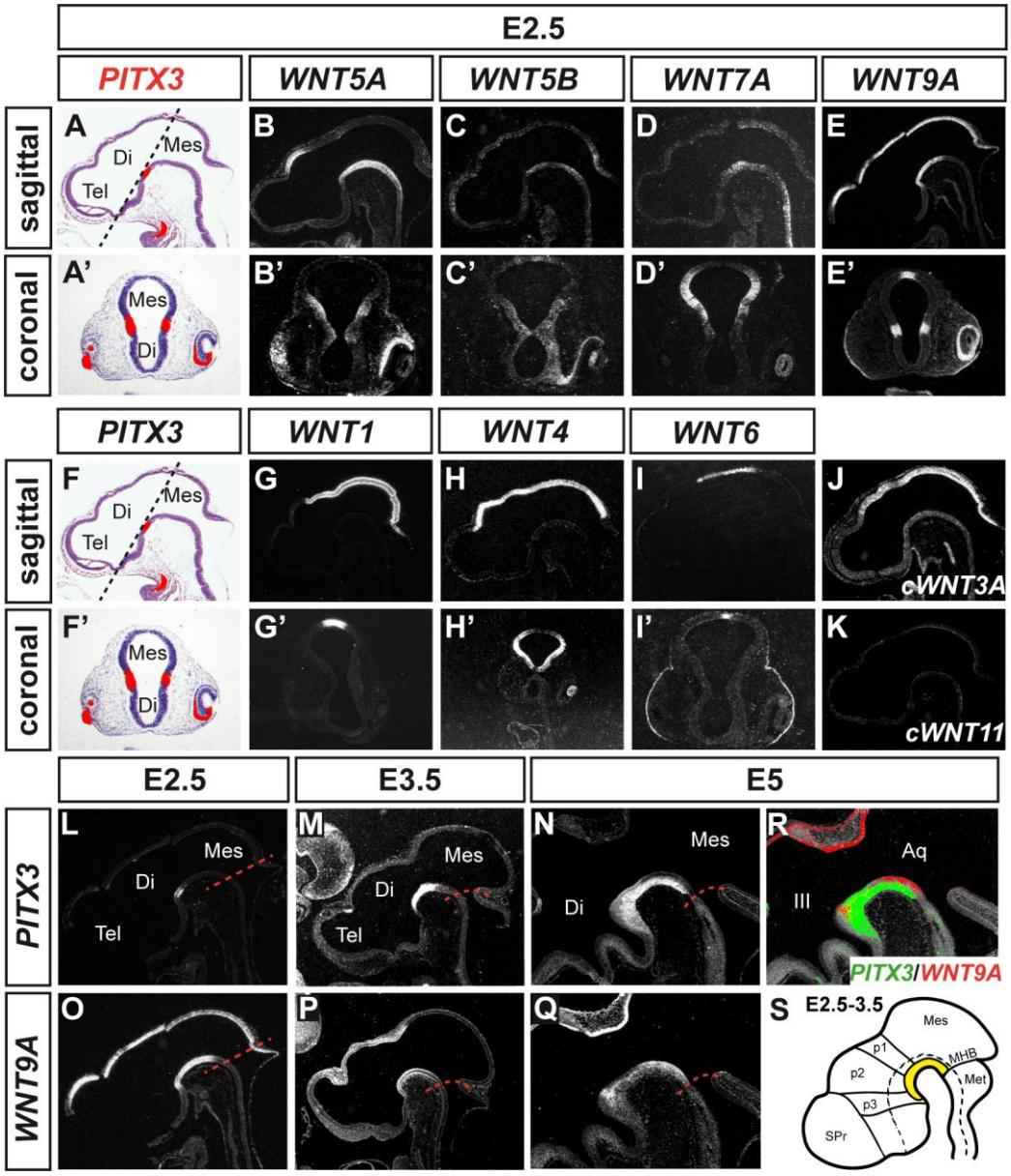

Fig 3

***WNT9A* and *PITX3* expression coincide in embryonic chick ventral diencephalon and mesencephalon. (A-K)** Midsagittal (A-K) and coronal (A'-I') sections of E2.5 (HH 16/17) chicken heads, *in situ* hybridized for *PITX3* (red, A,A',F,F'), *WNT5A* (B,B'), *WNT5B* (C,C'),

WNT7A (D,D'), *WNT9A* (E,E'), *WNT1* (G,G'), *WNT4* (H,H'), *WNT6* (I,I'), *WNT3A* (J) or *WNT11* (K). (B-E',G-K) are darkfield, (A,A',F,F') are brightfield views. . Black lines in A,F indicate level of coronal sections in (A'-E',F'-I'). Note, (K) belongs to (F). (L-Q) Midsagittal sections from E2.5 (L,O), E3.5 (M,P) and E5 (N,Q) chicken heads *in situ* hybridized for *PITX3* (L-N) or *WNT9A* (O-Q). Red lines indicate approximate position of MHB. (R) Overlay of consecutive midsagittal sections from E5 chick, *in situ* hybridized for *PITX3* (green) and *WNT9A* (red) with overlapping expression domains in yellow. (S) Schematic drawing depicting the overlapping expression domain of *WNT9A* and *PITX3* (yellow) in the midline of E2.5-3.5 chick ventral diencephalon (p1-3) and mesencephalon. Each gene expression study included n≥3 embryos per stage. Abbreviations: III, third ventricle; Aq, aqueduct; Di, diencephalon; Mes, mesencephalon; Met, metencephalon; MHB, mid-/hindbrain boundary; p1-3, prosomeres 1-3 (diencephalon); SPr, secondary prosencephalon.

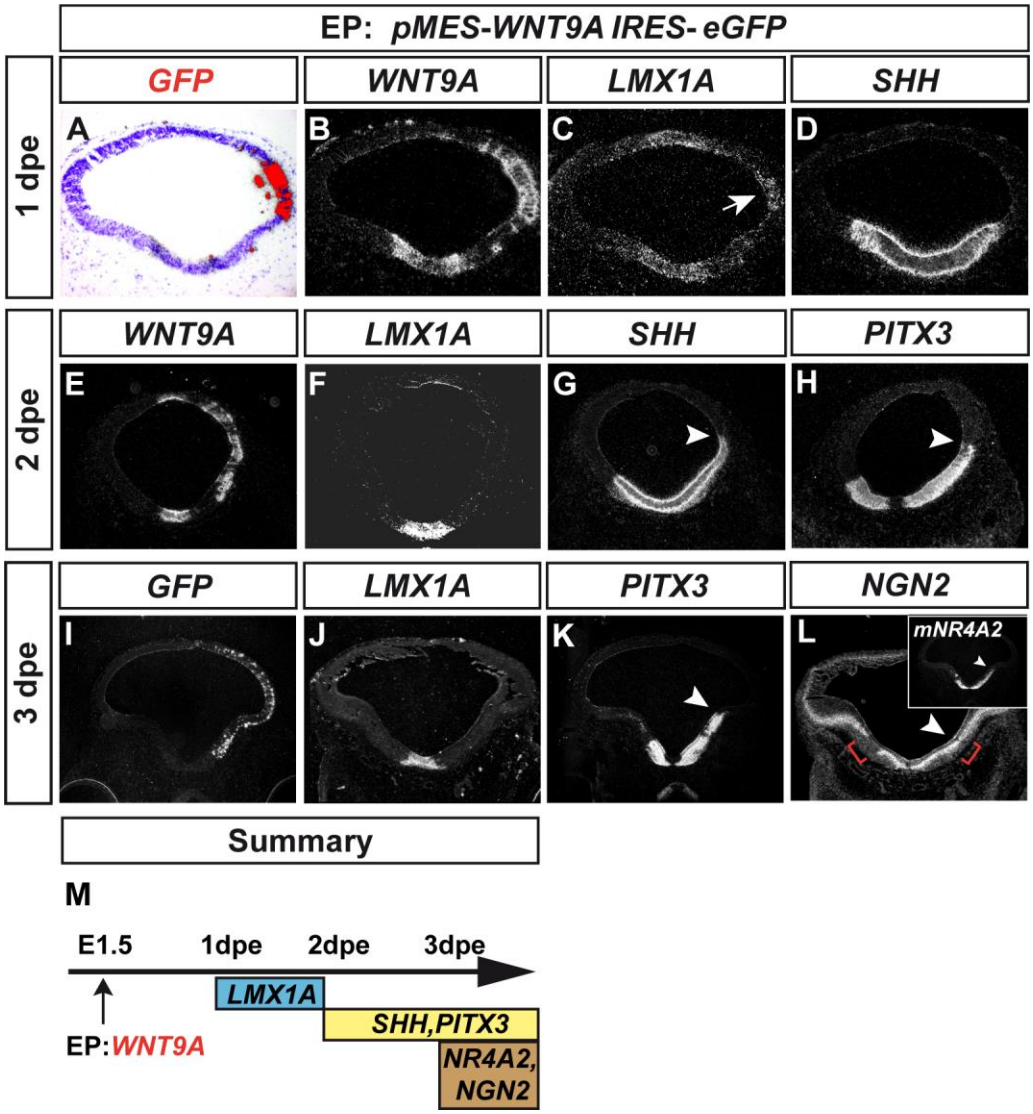

Fig 4

***WNT9A* induces transient ectopic expression of *LMX1A* and sustained ectopic activation of mdDA-related genes in chick mesencephalon. (A-L)** Consecutive coronal sections of chick mesencephalon overexpressing *WNT9A* in the right half of the mesencephalon (A,E,I). Left half serves as control. Brains were electroporated at HH 10-12, fixed after 1 (A-D; n=3), 2 (E-H; n=2) or 3 (I-L; n=5) days post-electroporation (dpe). *RISH* for

GFP (A,I), *WNT9A* (B,E), *LMX1A* (C,F,J), *SHH* (D,G), *PITX3* (H,K), *NGN2* (L) or *NR4A2* (inset in L). White arrow in (C) shows transient ectopic expression of *LMX1A*. White arrowheads in (G,H,K,L) indicate the dorsal-most ectopic induction of the particular gene. Red bracket in (L) delimits ventrolateral midbrain domain devoid of *NGN2* expression in wild type (left side). Ectopic *WNT9A* expression (I) leads to ectopic *PITX3* (K) and ectopic *NGN2* expression (indicated by a red bracket at the right midbrain side in (L)). (M) Summary of the temporal course of ectopic gene induction after electroporation (EP) of *WNT9A*.

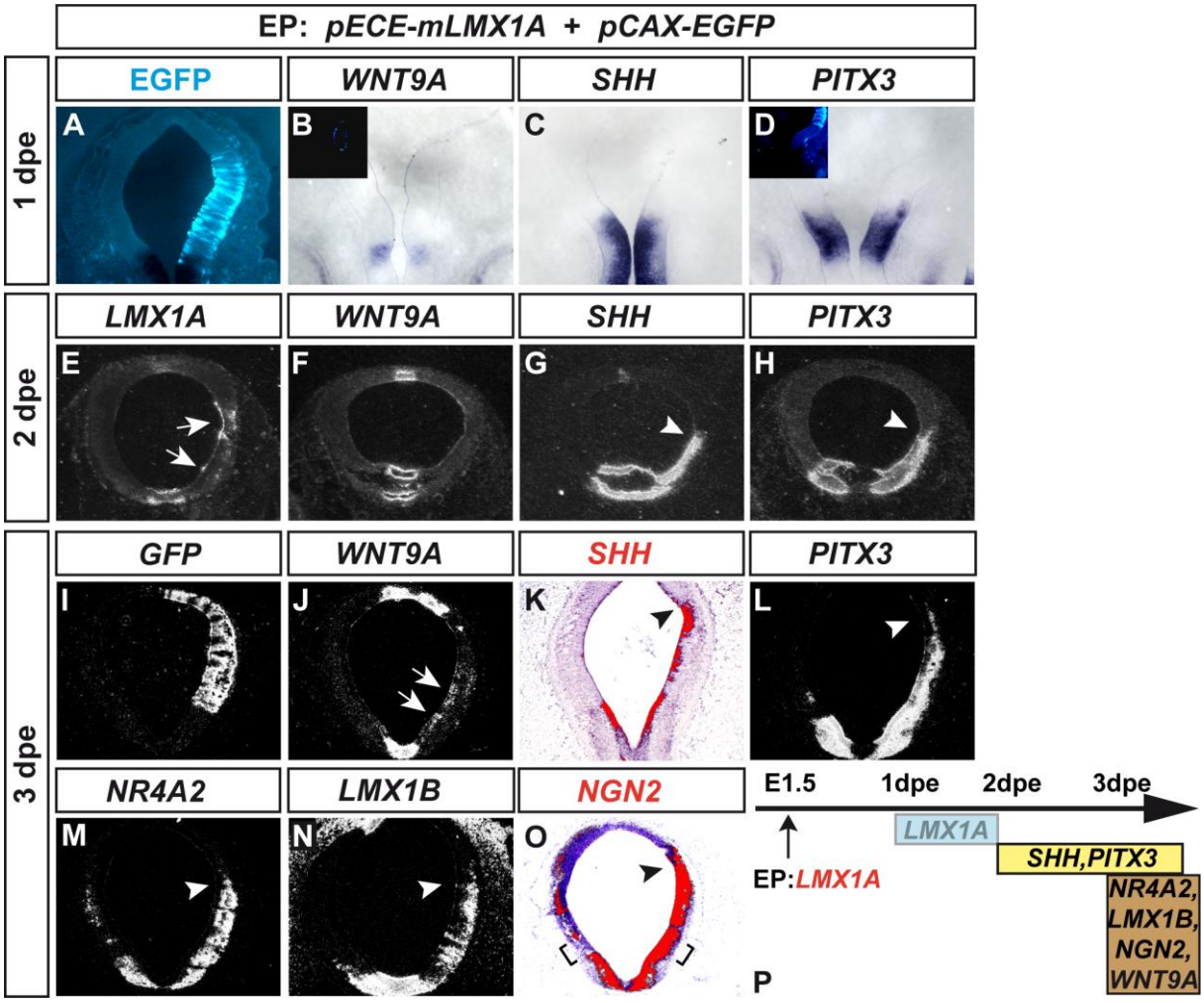

Fig 5

***Lmx1a* induces sustained ectopic expression of *SHH*, *PITX3* and mdDA-related genes in chick mesencephalon. (A-O)** Consecutive coronal sections of caudal chick diencephalon and mesencephalon incubated for 1 (A-D; n=5), 2 (E-H; n=4) or 3 days (I-O; n=8) post-electroporation (dpe) after *Lmx1a* electroporation of the right brain half (A, E, I; HH 10-12). The left brain half serves as non-electroporated control. (A) and insets in (B,D) show GFP immunostaining, (B-O) are *RISH* against *WNT9A* (B,F,J), *SHH* (C,G,K), *PITX3* (D,H,L), *LMX1A* (E), *GFP* (I), *NR4A2* (M), *LMX1B* (N) or *NGN2* (O). White arrows in (E,J) indicate a weak ectopic expression of *LMX1A* (E) and *WNT9A* (J). Arrowheads in (G,H,K,L,M,N,O)

show the dorsal-most extension of ectopically induced genes. Black brackets in (O) delimit the ventrolateral midbrain domain normally devoid of *NGN2* expression (see left control side and Fig. 4L). (P) Summary of the temporal course of ectopic gene induction after electroporation (EP) of *Lmx1a*.

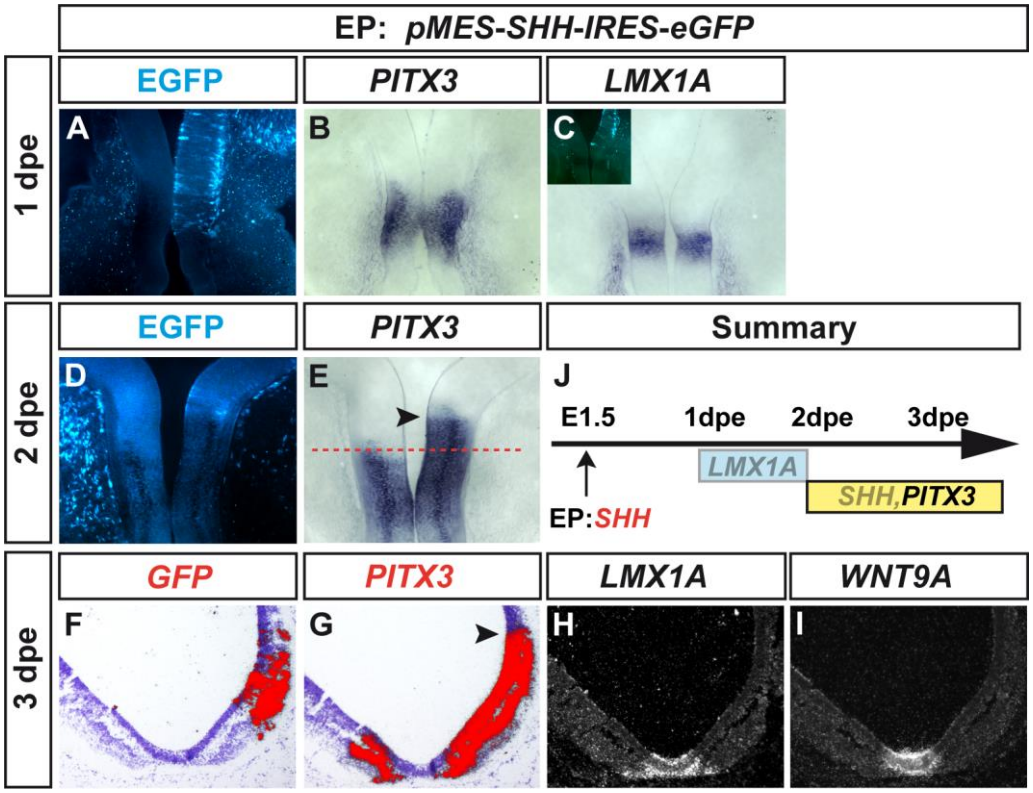

Fig 6

***SHH* is sufficient for the ectopic induction of *PITX3* expression in chick mesencephalon. (A-I)** Consecutive coronal sections of chick caudal diencephalon and mesencephalon overexpressing *SHH* in the right brain half. Embryos were electroporated at HH 10-12 and incubated for 1 (A-C; n=2), 2 (D,E; n=3) or 3 days (dpe) (F-I; n=5). Left side of the brain serves as non-electroporated control. (A,D) and inset in (C) are GFP immunostainings, (B,C,E, F-I)) are *RISHs* for*PITX3* (B,E,G), *LMX1A* (C,H), *GFP* (F) or *WNT9A* (I). Arrowheads in (E,G) indicate the dorsal-most extension of ectopic gene induction (compare extent of expression pattern on left and right side). Lines in (E, G) indicate dorsal

limit of endogenous gene expression. **(J)** Summary of the temporal course of ectopic gene induction after electroporation (EP) of *SHH*.

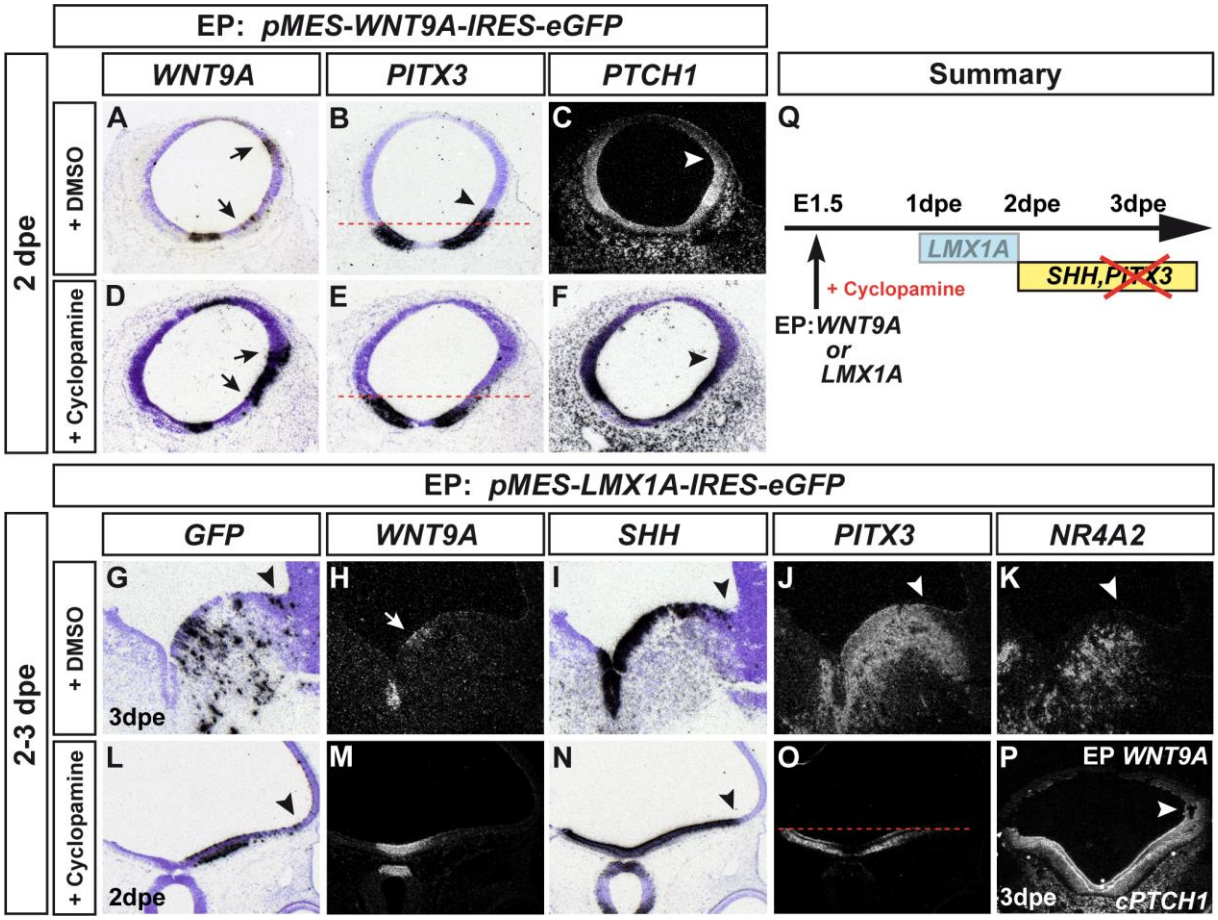

Fig 7

SHH signaling is necessary for ectopic induction of *PITX3* in chick mesencephalon. (A-P) Consecutive coronal sections of chick mesencephalon electroporated with *WNT9A*- (A-F,P) or *Lmx1a* (G-O) at HH 10-12 and incubated for 2 (A-F; n≥5/treatment; L-O; n≥2/treatment) or 3 days post-electroporation (dpe) (G-K,P; n≥2/treatment). Embryos were treated with DMSO (control; A-C,G-K) or cyclopamine (D-F,L-P) 3 and 24 h post-electroporation. Left brain half serves as non-electroporated control. *RISHs* for *WNT9A* (A,D,H,M), *PITX3* (B,E,J,O), *PTCH1* (C,F,P), *GFP* (G,L), *SHH* (I,N) or *NR4A2* (K). Arrows in

(A,D,H) delimit ectopic *WNT9A* domain (A,D, H). Arrowheads in (B,C,F,G,I-L,N,P) indicate dorsal-most extension of ectopic gene induction (B,G,I-L,N) or endogenous gene expression (C,F,P). Red lines in (B,E,O) indicate the dorsal limit of endogenous gene expression in the non-electroporated control side (left). **(Q)** Summary of the temporal course of ectopic gene induction after electroporation (EP) of *WNT9A* or *Lmx1a* and cyclopamine treatment.

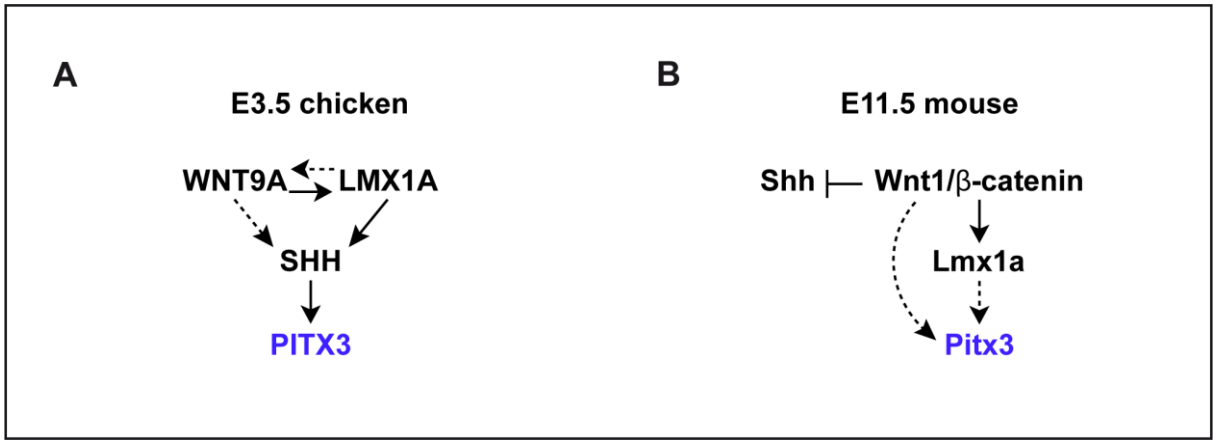

Fig 8

Differences in meso-diencephalic *PITX3* gene regulation between chick and mice.(A,B) (A) Our results suggest the shown epistatic relationships between *WNT9A*, *LMX1A*, *SHH* and *PITX3* in chick mesodiencephalon: WNT9A induces a transient expression of *LMX1A after 24h*, and *LMX1A* induces the expression of *WNT9A* after 72h; *SHH* and SHH signaling pathway act downstream of *LMX1A* and *WNT9A*, and are necessary and sufficient for the induction of *PITX3* expression. (B) In mouse the epistatic relationships between *Wnt1*, *Lmx1a*, *Shh* and *Pitx3* in the mesodiencephalic region looks like: WNT1 represses the expression of *Shh* (Joksimovic et al., 2009) and activates the expression of *Lmx1a*; LMX1A in turn induces the expression of *Wnt1* and *Pitx3* (Chung et al., 2009); NP unpublished observations). Stippled arrows indicate that the genetic interaction might be indirect.